Influence of Engineering Bacteria Quantitative Inspection on Diversity of Anpeng Alkali Mine Resources Exploitation

Yu Tao

School of Economics and Management
China University of Geosciences
Wuhan, 388 Lumo Road, Hongshan District
Wuhan, Hubei, 430074, China
E-mail: taoyutyy@163.com

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Abstract: *Cadmium (Cd) is a heavy metal pollutant seriously threatening creatures, and highly concentrated Cd in soil severely inhibits the activity of microbial populations. Soil in Anpeng Alkali Mine area in Nanyang city (Henan province) is seriously polluted by heavy metal. Both copper (Cu) and Cd content are found to be over standard, in which, Cu belongs to mild contamination while Cd is a serious contamination. To detect diversity of microbial communities in soil in the process of bioremediation, Cd polluted soil samples are collected from orefield for pot experiment, Biolog micro-plate technology is used to study the influence of applying low, medium and high amount of rice straw (5.3 t/ha, 10.2 t/ha and 23.4 t/ha) in polluted soil and combining low, medium and high amount of rice straw with surface displayed engineering bacteria (X4/pCIM) on microbial community. In the meantime, X4/pCIM is quantitatively measured by real-time polymerase chain reaction (PCR). Biolog experimental results indicate that the combination of rice straw and engineering bacteria is able to change the composition of soil microbial community, and has a difference in influencing rhizosphere and non-rhizosphere microorganisms. Through real-time PCR, it is found that the number of engineering bacteria falls to 103 after 120 days of bioremediation. Therefore, it can be concluded that combining rice straw with engineering bacteria can change the composition of soil microbial community and have diverse influences as application rate changes, without obvious rules to follow.*

Keywords: *Biolog micro-plate technology, Real-time PCR, X4/pCIM, Cu-Cd, McIntosh index, Shannon index.*

Introduction

Soil, a complex environment composed of solid, liquid and gas, includes diversified microbial communities, mainly involving bacteria, actinomyces and fungi. These microorganisms participating in decomposition and transformation of organic matters, formation of mycorrhiza, mutualism with plants, biological diversity and ecosystem function play a crucial role in the ecosystem [18]. More pollutants enter into soil as economy develops and people's living standard is improved, in which, cadmium (Cd) becomes one of the important pollutants. To date, Cd has been considered as the first kind of carcinogen by International Agency for Research on Cancer (IARC) [15].

The diversity of microbial populations depends on ecological conditions in soil. Soil heavy metal pollution will have a great influence on microbial physiological groups [4, 9]. External harmful substances impact microorganisms when entering into soil, thereby changing microbial communities [16]. Therefore, it is quite necessary to further explore the composition of microbial community in soil, so as to provide effective measures for monitoring and governing environmental pollution.

In-depth studies on the diversity of microbial community are limited by backward research techniques, which has been a fact without controversy, although soil microbial researches have received extensive attention from the scientists worldwide [19]. At present, microbial diversity study is in the stage of accumulating practical experiences and constructing a theoretical framework [2, 17]. But, an increasing number of methods applicable to soil microbial diversity research appear with the development of molecular biology, for instance, traditional micro-plate culture, Biolog micro-plate analysis, fatty acid analysis and molecular biological method, etc. This study discusses the influence of engineering bacteria quantitative inspection on diversity of Anpeng Alkali Mine resources exploitation based on Biolog micro-plate analysis and real-time polymerase chain reaction (PCR) methods.

Materials and methods

Tested materials

Two kinds of repairing adhesives are involved in this experiment, namely, organic material and engineering bacteria. Organic material adopts rice straw smashed to 1~2 cm, and engineering bacteria is constructed by displaying monkey metallothionein on the surface of pseudomonas putida through genetic engineering to acquire the recombined engineering bacterium X4/pCIM with high resistance and adsorptivity to Cd. Table 1 shows the heavy metal content in soil and rice straw.

Table 1. Heavy metal content in soil and rice straw

	Total Cd, mg/kg	**Available Cd, mg/kg**	**Total Cu, mg/kg**	**Available Cu, mg/kg**
Soil	5.34	4.26	440.39	175.47
Rice straw	0.11	-	11.41	-

Laboratory research materials

Primer sequence applied this study is displayed in Table 2. Primers are synthesized by Oko Biological Technology Co., Ltd (Beijing, China).

Table 2. Primers

Primers	**Sequences**	**Application**
U968GC	5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC 3'	V6-V8 area with GC clip general upstream primer
L1401r	5' CGG TGT GTA CAA GAC CC 3'	V6-V8 area general downstream primer
F	5' GAC ACC GCC GAC ATC ATC 3'	Quantitative PCR upstream primer
R	5' AAC TCC GCA GCA ATC ACG 3'	Quantitative PCR downstream primer

Luria-Bertani (LB) culture medium (pH 7.0-7.2) containing 1% peptone, 0.5% yeast powder and 1% NaCl is prepared, and steamed and sterilized for 30 min under high pressure (121 °C).

Phosphate buffer solution (PBS, KH_2PO_4 2 mmol/L; Na_2HPO_4 10 mmol/L; KCl 2.7 mmol/L; NaCl 137 mmol/L), lysozyme (100 mg/mL), NaAc solution (3 mol/L), color-substrate solution

(1.5% NaOH, 0.5% HCHO), TIANGEN PCR relevant reagents, AXYGEN clean up kit, OMEGA E.Z.N.A Soil DNA Kit, TAKARA PMD 18T Vector, TAKARA loading buffer, TAKARA QPCR Mix DRR091S and Biolog ECO Microplate are used in this study.

Instruments are applied, including Thermo refrigerated centrifuger, Roche 480 II fluorescent quantitative PCR, HITACHI CP80WX centrifuge, constant temperature incubator and Biotek ELX800 ELIASA.

Experimental methods

(1) Pot experiment

Soil samples are collected from Anpeng Alkali Mine orefield (Nanyang city, Henan) for pot experiment, and basic physicochemical properties of soil are shown in Table 3. This experiment sets control, pure application of rice straw, combination of rice straw and engineering bacteria and other treatments (Table 4). After being mixed with bacterial suspension, smashed rice straws are applied in treated soil samples and mixed with topsoil (0~20 cm).

Table 3. Basic properties of soil and rice straw

	Organic matters, g/kg	**Total phosphorus, g/kg**	**Rapidly available phosphorus, mg/kg**	**Total nitrogen, g/kg**	**Available nitrogen, mg/kg**	**Total potassium, g/kg**	**Rapidly available potassium, g/kg**
Soil	45.90	0.81	13.39	1.63	142.72	5.96	162.35
Rice straw	800.26	2.69	-	7.72	-	-	-

Table 4. Application methods of rice straw and engineering bacteria

Processing	**CK**	**RS_1**	**RS_2**	**RS_3**	**RS_{1+B}**	**RS_{2+B}**	**RS_{3+B}**
Rice straw, (t/ha)	-	5.3	10.2	23.4	5.3	10.2	23.4
Engineering bacteria, (CFU/gsoil)	-	-	-	-	108	108	108

Note: CK refers to no amendments; RS refers to rice straw; 1, 2 and 3 express gradient application amount of rice straw; B expresses engineered bacteria, similarly hereinafter.

(2) Biolog micro-plate experiment

First of all, 1 g dry fresh soil is weighed and added into 9 ml sterile PBS tube after moisture content in each sample is calculated. Ice-bath is performed on the tube for 1 min after it is vibrated for 10 min, which is repeated three times. After the tube is stood for 2 min, 3 ml supernatant is taken and put in 27 ml sterile PBS tube. And then, the tube is vibrated slightly (1:100 extracting solution). The steps above are carried out repeatedly until extracting solution is diluted to 1:1000. Afterwards, Biolog ECO micro-plate is taken out of refrigerator (–20 °C) and preheated to 20 °C, the 1000 times diluted bacterium solution is added in the micro-plate (125 μl every well). Inoculated Biolog plate is cultured at 20 °C to perform data test once every 12 h, with the wavelength of 595 nm (color + turbidity). Statistical analysis on data is displayed below.

Average well color development (*AWCD*) of microbial metabolic activity well is calculated as follows:

$$AWCD = \sum (C - R)/n , \quad (1)$$

where *C* expresses optical density differences of two wave bands in each carbon source well, *R* refers to optical density of control well and *n* expresses types of carbon source in culture medium (*n* = 31).

Shannon index (*H'*) and McIntosh index (*U*) of diversity of microbial communities are calculated using data of various samples acquired after 96 h of culture:

$$H' = -\sum P_i \ln P_i , \quad (2)$$

$$U = \sqrt{(\sum n_i^2)} , \quad (3)$$

where P_i refers o the ratio of the sum of relative absorbencies of the *i*-th well and the whole plate; n_i expresses the relative absorbance of the *i*-th well.

(3) Real-time PCR test
Quantitative PCR primers are designed based on insertion element InaX-MT4a sequence of engineering bacteria X4/pCIM.

Quantitative determination of engineering bacteria X4/pCIM and standard curve drawing

Engineering bacteria X4/pCIM is extracted from glycerin tube and cultured in the LB plate containing 100 μg/mL Amp, thereby obtaining single colony. Single colony is taken on 100 mL LB using inoculating loop and cultured for 24 h at 28 °C. Bacterium liquid (100 μL) is added into 0.3 g control soil without containing engineering bacteria and mixed. One hour later, quantitative standard soil is acquired. Afterwards, bacterium liquid (100 μL) is taken for gradient dilution, and each dilutability is repeated for three times. Result (5.2×10^8 CFU) is obtained after 3 days of culture. E.Z.N.A.®Soil DNA Kit is used for extracting total DNA of microorganism in quantitative standard soil and samples to be tested, and DNA is dissolved with Elution Buffer. Standard DNA extracted from quantitative standard soil is diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}). Thus, it can be seen that 1.73×10^7 CFU is contained in 1 μL undiluted sample. Under the same reaction condition, prepared primer is used for amplifying standard substances and samples (RS_{l+B}, RS_{2+B}, RS_{3+B}, RS_{1+BR}, RS_{2+BR}, RS_{3+BR}, where R refers to rhizosphere) for three times.

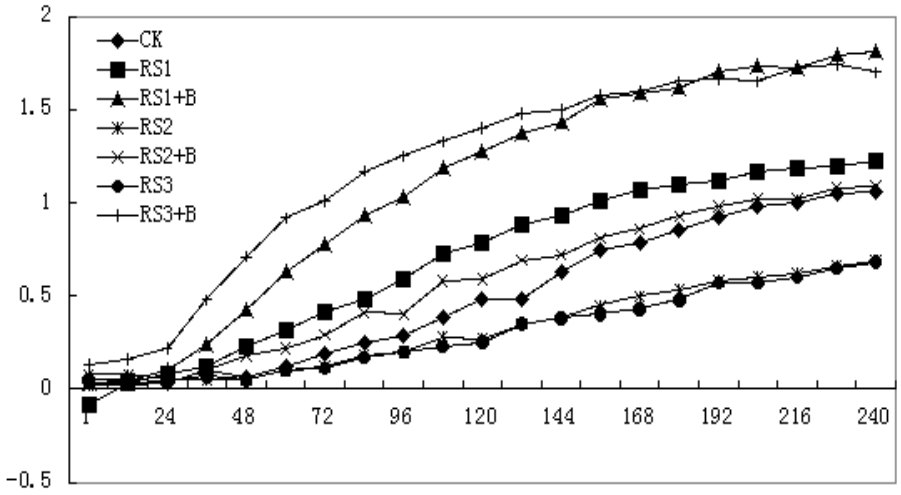
Statistical methods

Data are treated with One-way ANOVA and principle component analysis (PCA). Two-tailed equal variance T-test is performed on sample data, and the difference is considered to be statistically significant if $p < 0.05$.

Results and analysis

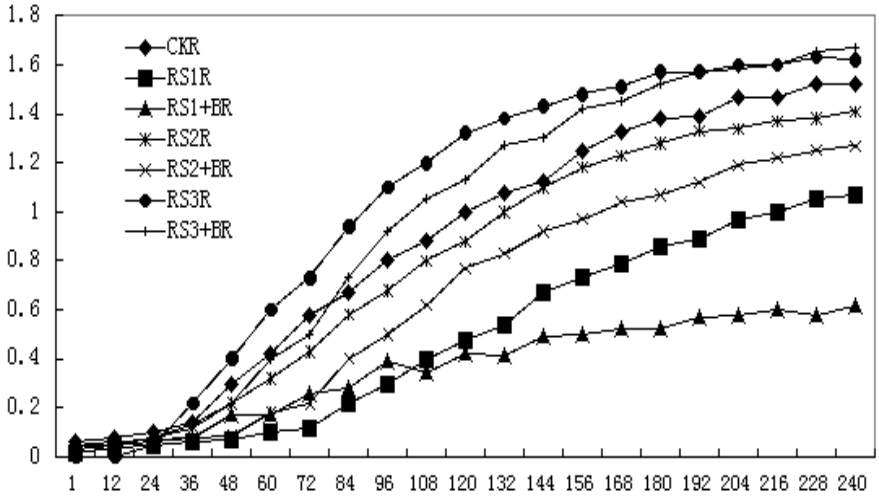
Biolog micro-plate experiment

AWCD reflects the metabolic activity of microorganism in soil, that is to say, using important indicator of single carbon source. *AWCD* computation of each carbon source is shown in Figs. 1 and 2, which indicate that *AWCD* of each soil has no obvious difference before 24 h, but it changes obviously at 96th h.



Culture time (h)

Fig. 1 *AWCD* of non-rhizosphere sample



Culture time (h)

Fig. 2 *AWCD* of rhizosphere sample

Analysis of microbial diversity indexes

Shannon index refers to an aggregative indicator for studying species richness, and McIntosh index mainly reflects species evenness in community. Table 5 displays results of diversity index analysis, which reveals that applying low amount of rice straw not only improves the species richness and evenness of non-rhizosphere sample, but also controls the species richness and evenness of rhizosphere sample. Different from this, applying rice straw in a high amount controls the species richness and evenness of non-rhizosphere sample and improves the species richness and evenness of rhizosphere sample. Applying median amount of rice straw has an

effect on inhibiting the species richness and evenness of both non-rhizosphere and rhizosphere samples. With the combination of applying low and median amount of rice straw and engineering bacteria, the species richness and evenness of non-rhizosphere sample are improved and the species richness and evenness of rhizosphere sample are also inhibited. Using high amount of rice straw with engineering bacteria, the species richness and evenness of non-rhizosphere and rhizosphere samples are improved to some extent.

Table 5. Diversity indexes of non-rhizosphere and rhizosphere samples (mean ± SD)

Processing	Non-rhizosphere		Rhizosphere	
	Shannon index	McIntosh index	Shannon index	McIntosh index
CK	2.65 ± 0.06 c	2.86 ± 0.31 e	3.11 ± 0.05 b	5.55 ± 0.34 bc
RS_1	2.85 ± 0.03 bc	4.90 ± 0.23 c	2088 ± 0.03 d	2.67 ± 0.33 f
RS_2	2.66 ± 0.13 c	2.24 ± 0.39 ef	3.08 ± 0.05 b	4.78 ± 0.22 c
RS_3	2.57 ± 0.17 c	1.89 ± 0.11 f	3.26 ± 0.02 a	7.12 ± 0.12 a
RS_{1+B}	3.15 ± 0.08 ab	7.40 ± 0.28 b	2.94 ± 0.03 cd	3.34 ± 0.32 de
RS_{2+B}	2.80 ± 0.05 bc	3.59 ± 0.17 d	3.01 ± 0.05 bc	3.92 ± 0.24 d
RS_{3+B}	3.13 ± 0.03 a	8.53 ± 0.08 a	3.22 ± 0.07 a	6.02 ± 0.23 b

Note: different letters behind the data express that the differences are significant ($p < 0.05$).

Analysis of functional diversity in microbial community

PCA Biolog data shows that the first and second principle components account for 42.263% and 10.545% of the total variation, and the first principle component dominates. Of 7 non-rhizosphere samples, there is an obvious spatial difference in principal component coordinate system constructed based on various carbon sources between samples (RS_{1+B}, RS_{3+B}) with the combination of applying low and high amount of rice straw and engineering bacteria and the control (Fig. 3). The difference of selecting carbon source reflects the transformation of non-rhizosphere samples using carbon source. Using rice straw in different amounts has no apparent impact on carbon source utilization in non-rhizosphere microbial community in soil polluted by Cu and Cd. Using low and high amount of rice straw as well as engineering bacteria changes the utilization efficiency of different carbon sources in non-rhizosphere microbial community. Of 7 rhizosphere samples, there is an obvious spatial difference in principal component coordinate system constructed based on 31 carbon sources comparing samples (RS_{1R}, RS_{3R}) separately applying low and high amount of rice straw and sample (RS_{1+BR}) combining low amount of rice straw with engineering bacteria with the control (Fig. 4). This suggests that separately applying low and high amount of rice straw and combining low amount of rice straw with engineering bacteria changes the utilization efficiency of rhizosphere soil microbial community to different carbon sources, and the species of rhizosphere soil microorganism using carbon source transform.

Real-time PCR

Fig. 5 shows the amplification curve of standard samples by fluorescent quantitative PCR. The template concentrations of standard samples are between 1.73×10^6 and 1.73×10^2 CPU/mL from left to right. It can be seen that these curves are smooth and slant greatly, each cycle threshold is evenly spaced, and bottom-right curve is considered as the negative control which has no positive amplification, with the reaction amplification efficiency of 1.923, conforming to the standard.

A standard curve is drawn based on standard samples provided by amplification curve and cycle thresholds obtained from dilution in different gradients, and the equation of standard curve is $y = -3.394x + 43.94$. The correlation is considered to be good when $r = 0.9968$ (Fig. 6).

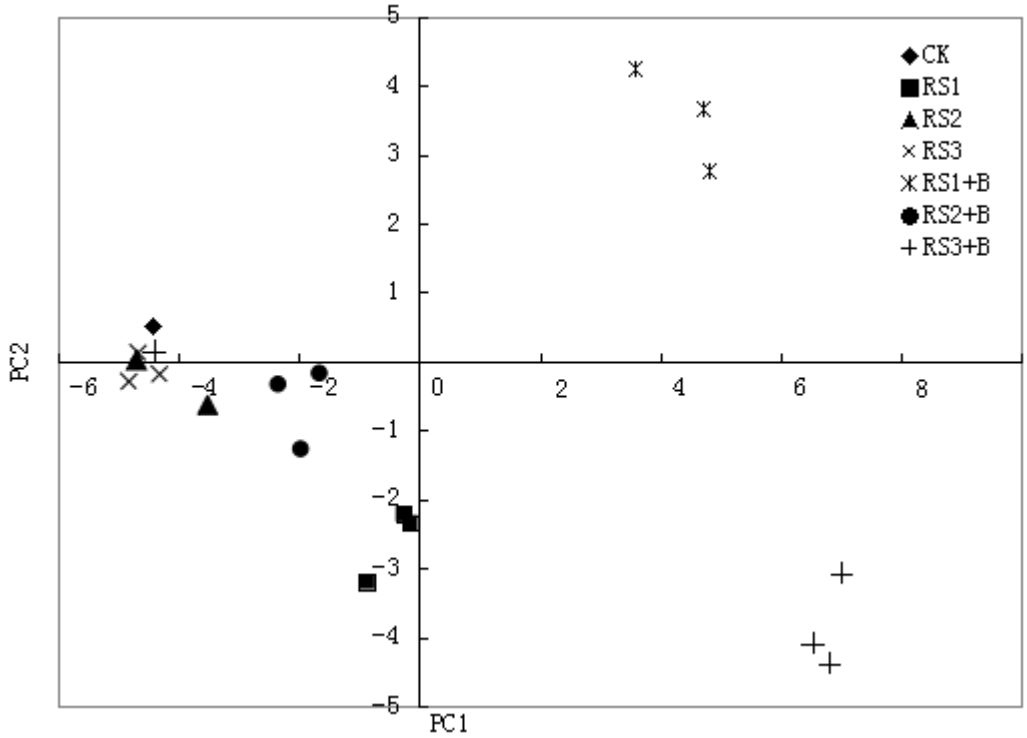


Fig. 3 Principle component analysis of functional diversity in non-rhizosphere sample

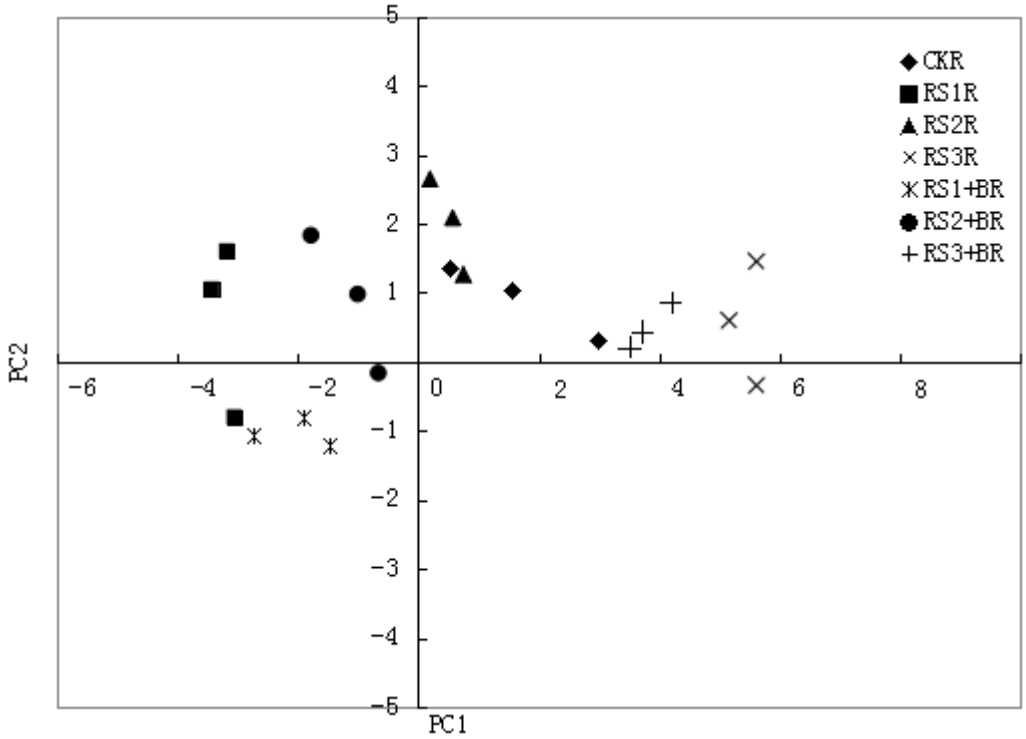


Fig. 4 Principle component analysis of functional diversity in rhizosphere sample

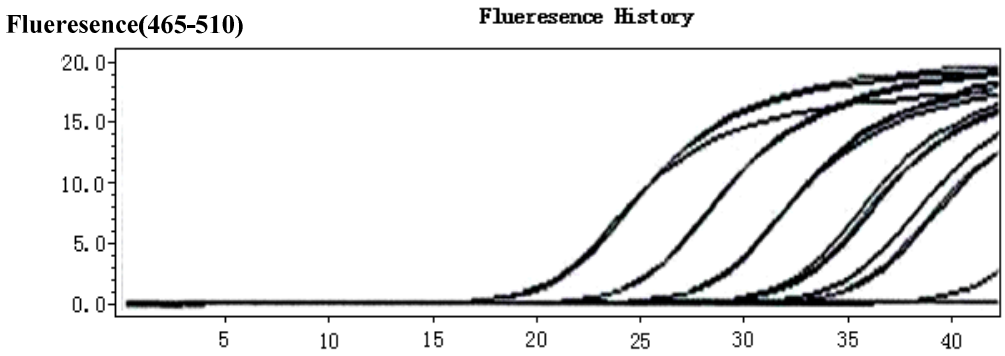


Fig. 5 Amplification curve of standard samples by fluorescent quantitative PCR

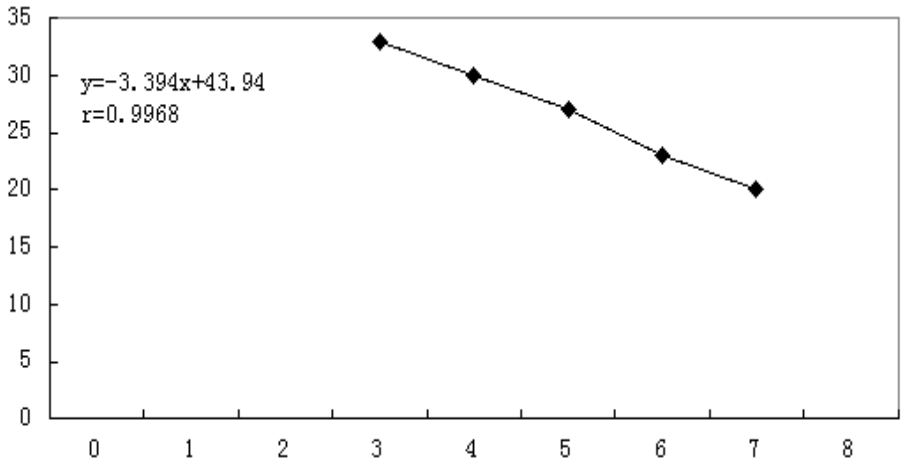


Fig. 6 Standard curve of fluorescent quantitative PCR

Detection results of engineering bacteria X4/pCIM

As shown in Fig. 7, the number of engineering bacteria decreases to 103 after 120 days of repair. Of non-rhizosphere samples, X4/pCIM is observed with the best colonization ability (2798 ± 174 CFU/g) in sample RS_{2+B} applied with median amount of rice straw and engineering bacteria, while 2484 ± 208 CFU/g and 1871 ± 110 CFU/g in sample RS_{1+B} and RS_{3+B} applied with low and high amount of rice straw and engineering bacteria. The differences of the number of X4/pCIM in non-rhizosphere samples are significant ($p < 0.05$). Thus, it can be known that using median amount of rice straw significantly improves the colonization ability of X4/pCIM in non-rhizosphere soil, while using high amount of rice straw inhibits its colonization ability in non-rhizosphere soil. Of rhizosphere samples, X4/pCIM has the best colonization ability (3966 ± 245 CFU/g) in sample RS_{3+BR} applied with high amount of rice straw and engineering bacteria, while 1638 ± 189 CFU/g and 751 ± 55 CFU/g in sample RS_{1+BR} and RS_{2+BR} applied with low and median amount of rice straw and engineering bacteria. The differences of the number of X4/pCIM in rhizosphere samples are significant ($p < 0.05$), suggesting that using low and high amount of rice straw helps X4/pCIM to colonize in rhizosphere soil, while using median amount of rice straw inhibits its colonization.

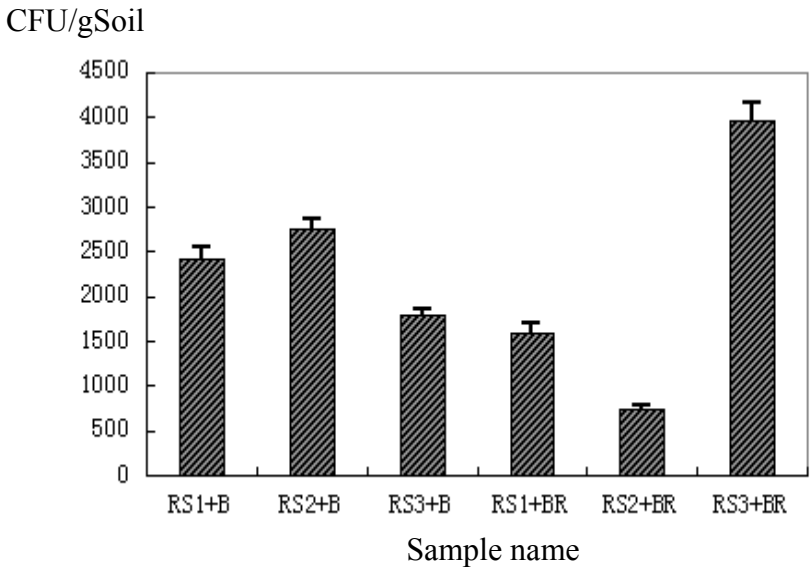


Fig. 7 Detection results of fluorescent quantitative PCR

Discussion

The interaction of Cu and Cd in rice straw and soil is affected by a variety of factors, such as soil organic matter, soil dissolved organic matter (DOM) and pH, etc. [21]. Those factors influence the solubility and mobility of Cu, Cd in soil through affecting the chemical form of Cu and Cd in soil. Rice straw in soil is first degraded into particle organic matter (POM) which has larger specific surface area containing various functional groups and large electric density combining a large number of metal ions [10]. Afterwards, POM is further degraded into macromolecular humus that is combined with Cu^{2+} and Cd^{2+} in soil by functional groups, thereby forming stable compounds [1, 3, 12]. After 120 days of bio-remediation, the number of X4/pCIM in the contaminated soil decreases, which may be because that X4/pCIM is a heavy metal-resistant strain and has a certain growth advantage in the environment polluted by heavy metal compared with indigenous microorganism. The biological effectiveness of Cu and Cd is reduced as the bioremediation carries on, directly resulting in the change of X4/pCIM living environment and destroying the dominant position of engineering bacteria in soil. Thus, the indigenous microorganism loses its advantage and the number of engineering bacteria decreases to some extent. Differently applied amount of rice straw has an effect on Cu and Cd polluting microbial community. The reason is likely to be related to the change of the number of actinomycetes in soil [5, 7, 20, 22]. After applying rice straw in a low amount for 120 days, humus is combined with Cu and Cd, decreasing the survival pressure of microorganism. Thus, various microbes including actinomycetes proliferate. The existence of actinomycetes has obvious inhibition and antagonism effects on rhizosphere bacteria [8, 13]. Therefore, using low amount of rice straw improves the biological activity, richness and evenness of non-rhizosphere microbial community and inhibits the biological activity, richness and evenness of rhizosphere microbial community [11]. Rice straw is incompletely decayed after 120 days of high amount of application. Using median amount of rice straw has a complicated influence on microbial community, which may be associated with the degree of maturity. The degree results in large data fluctuation and the content of microbe in rhizosphere soil is much higher than in non-rhizosphere soil [6, 14]. Results obtained from Biolog micro-plate experiment indicate that using low and median amount of rice straw will decrease the biological activity, richness and evenness of rhizosphere microbial community. Considering that this processing improves the biological activity, richness and evenness of non-rhizosphere and rhizosphere microbial community and is beneficial to X4/pCIM colonization in soil, this

processing is more suitable for the bioremediation of heavy metal contaminated soil although the combination of low amount of rice straw and engineering bacteria decreases the metabolic activity of rhizosphere microbial community.

Conclusion

Combining Biolog micro-plate technology with fluorescent quantitative PCR, the changes of microbial community in soil are comprehensively analyzed. However, the defects of techniques themselves, errors in operations and external interferences impact the final results. Gene chip technology, whole genome sequencing and other novel techniques appear as science develops, providing a new thought and direction for the study of microbial diversity. Therefore, it is very necessary to apply various research methods in the future and combine them organically, so as to acquire more comprehensive information on changes in microbial diversity and perform further researches on microbial diversity in soil.

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Yu Tao, Ph.D. Student
E-mail: taoyutyy@163.com

Yu Tao was born in Wuhan, Hubei Province in December 1986. He is now a Ph.D. Student in School of Economics and Management, China University of Geosciences, Wuhan, Hubei, China.