### Microbial Diversity and Community Structure in Agricultural Soils Suffering from 4-year Pb Contamination

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Microbial Diversity and Community Structure in Agricultural Soils

Suffering from 4-year Pb Contamination

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Abstract

Heavy metal pollution has become a widespread environmental problem due to rapid economic development. The phylogenetic diversity and structure of microbial communities in Pb-contaminated Lou soils were investigated using Illumina MiSeq sequencing of 16S rRNA genes. The presence of Pb$^{2+}$ in soil showed weak impact on the diversity of soil bacteria community, but influenced the abundance of some genera of bacteria, as well as soil physicochemical properties. We found significant differences in the relative abundances of heavy metal resistant bacteria such as *Bacillus*, *Streptococcus* and *Arthrobacter* at the genus level. Available Pb (APb) and total Pb (TPb) negatively correlated with soil organic matter (SOM), but positively affected available phosphorus (AP). The abundance of main bacteria phyla was highly correlated with total Pb (TPb). The relative abundance of *Gemmatismonadetes*, *Nitrospirae* and *Planctomycetes* were negatively correlated with total Pb (TPb). Collectively, Pb influences both the microbial community composition and physicochemical properties of soil.

Key words: 16S rRNA gene; Soil microbial diversity; Pb contamination; Illumina MiSeq sequencing
Introduction

Due to rapid economic development, heavy metal contamination of soil and water resources has become a widespread environmental problem, endangering the health of natural ecosystems and human populations (Chou et al. 2011). Lead (Pb), one of the most frequently encountered heavy metals in polluted areas, is derived from mining and smelting of metalliferous ores, burning of leaded gasoline, disposal of municipal sewage, industrial waste enriched in Pb, and use of Pb-based paint (Gisbert et al. 2003; Ganesh et al. 2015). One of the main pathways for human uptake of Pb is ingestion of crops grown on polluted soils. The threat of heavy metals to human health is aggravated by the long-term persistence of these toxins in the environment. For instance, the estimated soil retention time for Pb, one of the most persistent heavy metals, is 150–5000 years (Szczyglowska et al. 2011).

Soil microorganisms play an important role in energy flow and matter cycling in terrestrial ecosystems (Chodak et al. 2013; Golebiewski et al. 2014; Long et al. 2014; Rincon-Florez et al. 2013). Microbes largely contribute to biogeochemical cycles through their involvement in humification processes, degradation of pollutants, and maintenance of soil structure (Chodak et al. 2013; Preston et al. 2001). The structure of microbial communities is an important index of soil quality. Because of its indisputable importance to terrestrial ecosystems, microbial community structure has been widely studied in various terrestrial ecosystems (Chodak et al. 2013; Wang et al. 2014).

A number of existing studies have shown that short-term or long-term exposure to heavy metals induces changes in microbial community structure (Dheeba et al. 2013; Haque et al. 2008; Li et al.
2013; Lorestani et al. 2011). Heavy metals decreased the diversity of bacteria community, increased the abundance of *Proteobacteria*, but decreased the abundance of *Acidobacteria* and *Actinobacteria* (Gołębiewski et al., 2014). Cd contamination has been reported to have significant impacts on the abundance and community structure of bacteria in soil (Deng et al., 2015; Zhang et al., 2016). Being subjected to heavy metal stress, most microbial populations become extinct, while certain resistant community members survive to form the basis of a new community (Hemme et al. 2010). Collectively, the bacteria communities are vulnerable to heavy metal stress, whereas different groups of bacteria might respond to heavy metal differently. Therefore, more researches are needed for us to predict the succession patterns of bacteria community along with heavy metal levels for long term.

Several studies have revealed the impact of heavy metals on soil microbial communities using traditional methods such as plate counting, phospholipid fatty acids (PLFAs), and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Pan and Yu 2011; Shi et al. 2002). However, due to the limited sensitivity and scope of these classical measurement techniques, it is difficult to accurately characterize the quantitative and qualitative changes in soil microbial communities (Maron et al. 2011). Multiple studies have focused on heavy metal contamination in complex systems such as forest ecosystems, groundwater, tailings, and lake sediments (Epelde et al. 2015; Frey et al. 2006; Gough and Stahl 2011; Hemme et al. 2010). High-throughput sequencing of 16S rRNA genes has been successfully used to assess microbial community diversity, but only a few studies have used this technique to analyze the effects of heavy metals on microbial communities in soils (Sheik et al. 2012; Golebiewski et al. 2014; Větrovský and Baldrian 2015; Li et al. 2016). However, the influence of Pb on agricultural soil microbial community structure and physicochemical properties is not clear.
In the present study, high-throughput sequencing of 16S rRNA genes was introduced to assess the structure of microbial communities in Pb-contaminated soils. The objectives were to (1) identify the factors controlling the structure and diversity of microorganisms in soils polluted with different levels of Pb, and (2) reveal relationships between physicochemical soil parameters and microbial structure and diversity.

**Materials and methods**

**Study area**

This study was performed at the “National monitoring base of soil fertility and fertilizer efficiency on Loess soil” (34°17'51" N, 108°00'48" E), which is the test plot of Northwest Agriculture and Forestry University, Yangling city, Shaanxi Province, China (Figure 1). The soil type of this study site is Eum-Orthic Anthrosols (Chinese Soil Taxonomy) with a mean bulk density of approximately 1.25 g/cm$^3$. The experimental area was located in the Guanzhong Plain, which belongs to zone 5 (semihumid) of the drylands of northern China, with the elevation 524.7 m and the annual precipitation 550-650 mm, 60%–80% of which occurs from July to September. The annual mean temperature is about 13°C.

**Experimental design**

The experiment reported in this paper used soil samples from field trials. Each trial plot was added with corresponding levels of exogenous Pb (in the form of Pb(NO$_3$)$_2$ solution) in June 2010. According to the Pb limits of the Chinese Secondary Environment Quality Standards for Soils (GB 15618–1995), three treatments were set up for the experiment: the low concentration treatment (LOW) was half the amount of the standard maximum threshold (175 mg/kg plus the background level); the
high concentration treatment (HIGH) was equal to the standard level (350 mg/kg plus the background level); the control treatment (CK) was no Pb added to the soil, only with the background level. The background content of Pb mainly originates from soil parent material. In this study, the background of Pb was about 23.06 mg/kg, which is within the range of China background of heavy metals (Wei et al, 1991).

The corresponding amount of Pb(NO$_3$)$_2$ based on the soil bulk density and depth (0-20 cm) was added to each plot. The top 20 cm soils of each plot were excavated from the field, and mixed with the prescribed Pb(NO$_3$)$_2$ immediately and thoroughly. The soils with amendment of Pb were backfilled correspondingly. The soils were subjected to aging and equilibration under natural conditions for three months. During the aging period, the soil moisture content was maintained at approximately 75% of the maximum water holding capacity. Meanwhile, the soils were ploughed frequently. After the aging period, all the treated plots were cultivated under a wheat–corn rotation system beginning in 2010. The varieties of wheat and corn were Xiaoyan-22 and Shandan-16, respectively.

Each Pb treatment has 3 replicated plots (2 × 1 m), totaling 3 × 3 = 9 plots. The plastic plates (0.5 m belowground and 0.05 m aboveground) and intervals (0.5 m) were used in separating the plots, to avoid cross diffusion of Pb.

Treated soil samples were collected randomly from the surface layer (0–20 cm) of each plot in August 2014. Approximately 0.5 kg of soil from each site was collected. A standard soil corer device was used to collect samples without disturbing plant roots. Five collected soil cores from each plot were pooled as a replicate. A total of 9 sample replicates were transferred to laboratory. The soil samples were sieved through 2-mm mesh to remove plant residues and stones. A subsample of each soil core was delivered to laboratory and stored at -80 °C before molecular analysis. The remaining
sample was air-dried at room temperature, passed through a 2-mm sieve, and stored until use for soil physicochemical analyses.

**Physicochemical analysis**

The physical and chemical properties of the soil were analyzed in the laboratory. Soil pH (soil: water 1:2.5, weight: volume) was determined with a PHS-3C pH Meter (Shanghai Lida Instrument Factory, China). Soil moisture content was determined gravimetrically by drying at 105°C for 12 h. For determination of soil organic matter (SOM), available phosphorous (AP), available potassium (AK), available nitrogen (AN), total Pb (TPb), and available Pb (APb) concentrations, air-dried soil samples were ground and passed through a 0.25-mm sieve. SOM was determined by dichromate oxidation, and AN was analyzed using the Kjeldahl method. Concentrations of AP and AK were determined using the Egner–Riehm method (Knudsen et al. 1980). Total concentration of Pb in the soils were determined by standard soil testing procedures (Bao, 2000). 5 g soil samples were digested by HNO₃:HCl:HClO₄ (1:2:2) to extract the total Pb. The concentration of available Pb in the soil was extracted with 0.05 M EDTA (pH 6.0) using the method of Smith (1996). Total and available Pb concentrations were measured with ICP-MS (Thermo, model Xseries II, USA).

**DNA extraction, PCR amplification, and sequencing analysis**

Nine soil samples (3 controls, 3 LOW and 3 HIGH treatments) were taken for molecular analysis. Total genomic DNA was extracted directly from these samples using the FastDNA® spin kit (MP bio, Santa Ana, USA) following the manufacturer’s instruction. DNA concentrations were then determined using a NanoDrop ND 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). DNA was stored at -80 °C for subsequent analyses. Total genomic DNA was amplified using a 515f/806r
primer set to amplify the V4 region of the 16S rDNA gene (Bergmann et al. 2011). The forward primer contains a 6-bp error-correcting barcode unique to each sample. DNA was amplified following a protocol described previously (Caporaso et al. 2012). The 16S rRNA tag-encoded high-throughput sequencing was carried out using the Illumina MiSeq platform at the Beijing Genomics Institute (BGI; Shenzhen, China). Reads with an average length of 253 bp were deposited into the NCBI short reads archive database with accession number SRP075183. Pairs of reads were overlapped based on a method described previously (Magoc and Salzberg 2011). Sequencing reads were assigned to each sample according to the individual unique barcode. Sequences were analyzed with the FLASH (Fast Length Adjustment of Short reads, v1.2.11) and UPARSE pipeline (Caporaso 2010). The reads were first filtered by FLASH quality filters. Then, the UPARSE pipeline was used to detect operational taxonomic units (OTUs) at 97 % similarity. For each OTU, a representative sequence was selected and used to assign taxonomic composition using the RDP classifier (Wang et al. 2007a). Estimated species richness was determined with rarefaction analysis; the Chao and Shannon indices for nine libraries were determined following the protocol of Schloss et al. (2009).

Venn diagrams were used to identify and evaluate the number of shared species among soil samples from the three treatments. A total of 2907 OTU were observed in all samples, with 1883 in common among all three treatments (Figure 2).

**Statistical analysis**

Similarity among microbial communities in different samples was determined using UniFrac analysis. QIIME was used to calculate weighted and unweighted UniFrac, determine Bray-Curtis distance, and for principal coordinate analysis (PCoA). The Unweighted pair group method with
arithmetic mean (UPGMA) was conducted based on unweighted and weighted UniFrac following a previously published protocol (Kuczynski et al. 2011).

SPSS 22.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used to determine significant differences in species abundance between groups at the genus level. Redundancy analysis (RDA) was executed in CANOCO 4.5 for Windows (Biometris, Wageningen, Netherlands) to determine chemical properties with significant influences on microbial communities (Braak et al. 2002; Jiang et al. 2015). Figures were generated with CanoDraw 4.0 (Biometrics Wageningen, Netherlands).

Spearman's rank-order correlation and one-way ANOVA were conducted with SPSS 22.0 software for Windows (SPSS Inc., Chicago, IL, USA).

**Results**

**Environmental parameters**

We measured both APb and TPb, as well as other soil physicochemical characteristics. Soil pH, SOM, AP, AK, AN, TPb, APb, and percent moisture content are shown in Table 1. The pH of soil samples ranged from 7.92 to 8.12. Soil pH, AK, and AN did not significantly differ among the three treatments (P > 0.05). Moisture content for the LOW treatment was significantly lower than that HIGH and control (CK) (P < 0.05). The SOM concentration of the CK treatment was significantly higher than that of the two Pb treatment groups (P < 0.05). The AP content for HIGH treatment was significantly higher than that of the LOW and CK groups (P < 0.05).

**Microbial community composition**

After quality filtering of the raw reads, we obtained 153125 16S rRNA gene sequences
(16388–17608 sequences per sample, average of 17014). The number of different bacterial OTUs at
the 97% similarity level ranged from 1938 to 2047 per sample, with an average of 1994 OTUs (Table
S1). The rarefaction curves indicated similar profiles for all samples (Figure 3). OTU richness
estimators (ACE and Chao) and diversity indices (Shannon and Simpson) were introduced to evaluate
the bacteria diversity. Exposure to the low concentration treatment (175 mg/kg of soil) resulted in
increased species richness as detected via both the ACE and Chao estimation (Figure 4) as well as
increased microbial diversity as estimated by the Shannon index (Figure 4).

The microbial taxonomical composition at the phylum level is presented in Figure 5.

*Proteobacteria* was the most abundant phylum in all samples, accounting for 25.91%–30.87% of total
valid reads, with an average relative abundance of 27.89%. *Acidobacteria* was the second most
abundant phylum in all samples, with an average relative abundance of 20.72% (16.54%–24.46%).
Other dominant phyla included *Actinobacteria* (10.34%–13.92%, average of 11.88%), *Bacteroidetes*
(7.87%–11.58%, average of 9.30%), *Planctomycetes* (5.56%–7.36%, average of 6.50%), *Firmicutes*
(5.56%–6.78%, average of 6.02%), *Chloroflexi* (5.43%–6.87%, average of 6.01%), and
*Gemmatimonadetes* (3.05%–4.50%, average of 3.54%). *Actinobacteria*, *Bacteroidetes* and
*Cyanobacteria* were more abundant in the high concentration treatment than in the control or low
concentration treatment.

Abundant classes included *Acidobacteria-6*, *Chloracidobacteria*, *Alphaproteobacteria*,
*Deltaproteobacteria*, *Betaproteobacteria*, *Bacilli*, *Gammaproteobacteria*, *Saprospirae*, *Actinobacteria*,
and *Thermoleophilia* (Figure 6A). Taxonomical classification at the order level indicated that 27 orders
predominate in all soil samples (relative abundance, > 1%). Based on average relative abundance,
*iili-15* (8.41%) was the most abundant order followed by *RB4I* (8.33%), *Saprospirales* (4.49%),
Actinomycetales (4.32%), Cytophagales (3.62%), Lactobacillales (3.59%), Xanthomonadales (3.05%), and Sphingomonadales (3.01%) (Figure 6B). At the family level, Chitinophagaceae, Cytophagaceae, Streptococcaceae, Sphingomonadaceae, Syntrophobacteraceae, Pirellulaceae, Sinobacteraceae, Rhodospirillaceae, Rubrobacteraceae, Gaiellaceae, Comamonadaceae, mb2424, and Ellin6075 were the major groups (> 1%) in all soils (Figure 6C).

**Significant differences between treatments**

There were 7 bacteria genera showing significant difference among the three treatments at the genus level as shown in Figure 7. Generally, the relative abundance of Bacillus, Lactobacillus and Truepera decreased in response to increasing Pb levels. The relative abundance of Streptococcus and Arthrobacter exerted a unimodal pattern with the increased Pb gradients. However, the Pb treatment increased the relative abundance of Bosea and Aquicella.

The relative abundance of Streptococcus, Arthrobacter, Bosea, Aquicella and Truepera significantly varied between the LOW treatment and CK (P < 0.05); the relative abundances of Lactobacillus, Streptococcus and Truepera differed significantly between the HIGH treatment and CK (P < 0.05). Besides, between the treatment of LOW and HIGH, the relative abundances of Bacillus and Arthrobacter varied significantly (P < 0.05).

In addition, Streptococcus, Arthrobacter, Bosea and Aquicella were more abundant in the LOW and HIGH treatment than in the CK, and Arthrobacter was only found in the Pb treatment. Besides, the relative abundance of Bacillus, Streptococcus, Arthrobacter and Aquicella were higher in the LOW treatment than in the HIGH.
Correlations between environmental parameters and microbial community structure

Possible linkages among environmental parameters and microbial community structure were discerned based on RDA (Figure 8). Each environmental variable in the RDA biplot is represented by an arrow, and the length of an individual arrow indicates how much variance is explained by that variable. The eigenvalues (indicating the strength of the model) for the first two multivariate axes were 0.334 and 0.249, respectively. The sum of all canonical eigenvalues was 1.000. The first canonical axis was positively correlated with all investigated environmental parameters except pH and SOM.

As shown in Figure 8, all sites were generally separated on the two ordination axes. There was a marked correlation between AK and microbial composition based on OTU level. The species composition in the CK showed a clear positive correlation with SOM concentration. High metal treatments (Pb23S) showed a clear positive correlation with Pb concentration, and low metal treatments (Pb13S) showed a clear positive correlation with AK. Besides, APb/TPb negatively correlated with SOM, but significantly and positively affected AP.

Spearman's rank correlation coefficient was calculated to reveal the relationship between environmental factors and soil microbial diversity at the phylum level (relative abundance above 1%; Table 2). TPb negatively and significantly correlated with the relative abundance of Planctomycetes (P=0.03). Besides, The relative abundance of Gemmatimonadetes (P=0.094) and Nitrospirae (P=0.065) also negatively correlated with TPb, as the significance were close to P=0.05 after FDR correlation (Table 2). The concentration of available Pb (APb) was in close association with the content of TPb (P<0.001).
**Discussion**

Heavy metal pollution from agriculture and industry is a major environmental concern that can reduce plant growth and affect soil microbial community structure (Chen et al. 2014; Gómez-Sagasti et al. 2012; Gomez-Balderas et al. 2014; Martínez-Alcalá et al. 2016; Nayak et al. 2015; Poirel et al. 2013). Given that the control soil had some Pb, its content is in the normal range (Wei et al., 1991). The Pb level gradient was thus effective in the experimental setup. Heavy metals can decrease the diversity and activity of soil microbial community (Wang et al. 2010; Sandaa et al. 1999).

Overall, our results show that Pb had slight effect on bacteria community diversity in soil. This is unexpected but not difficult to understand. The microbial community is shaped by a number of environmental factors such as soil organic matter, pH and moisture, with heavy metal being a potential factor. The weaker effect of high heavy metal content on the soil microbial properties was reported before, in which the effect of other edaphic factors might overwhelm the negative effect of heavy metal (Chodak et al. 2013). Meanwhile, relatively small effect of heavy metal pollution on soil microbial community structure has also been report for short-term and long-term experiments (Pennanen et al. 1998; Niklinska et al. 2005). Therefore, when predicting the microbial community succession, more factors including heavy metal content should be taken into consideration.

However, Pb significantly altered the abundance of particular microbial populations at the OTU level. Soil microbial species richness and diversity tended to increase as a result of low lead (175 mg/kg) exposure (Figure 4), which corroborates the findings presented in other studies. Golebiewski et al. 2014 reported that species richness, diversity, and bacterial communities in soils with low levels of Pb contamination were higher than in highly polluted soils. The diversity of soil bacteria has been
shown to be negatively affected by high levels of heavy metal pollution (Chodak et al. 2013; Větrovský and Baldrian 2015).

At the phylum level, microbial communities were roughly similar in all soil samples, regardless of heavy metal concentration. *Proteobacteria* was the most abundant phylum in this study, similar to communities found in grassland soils, forest soils, and so on (Barnard et al. 2013; Orr et al. 2015; Park et al. 2015; Peiffer et al. 2013; Rojas et al. 2016; Zhao et al. 2014). *Proteobacteria* encompass an enormous level of morphological, physiological and metabolic diversity, and are of great importance to global carbon, nitrogen and sulphur cycling (Harichová et al. 2012). Kuppusamy et al. (2016) found *Actinobacteria* to be predominant in soils suffering from long-term contamination of PAHs and heavy metals such as Pb, Cu, and Zn. In this study, *Actinobacteria* and *Acidobacteria* were present in high abundance in all samples, which is in accordance with the findings of Kuppusamy et al. (2016). *Cyanobacteria’s* abundance was higher in HIGH treatment than in CK or LOW treatment, owing to that the phylum has the capacity for adsorbing high amounts of solubilized metals through its cell wall (El-Enany et al. 2000). El-Enany et al. (2000) reported that *Cyanobacteria* can serve as a biosorbent of heavy metals in sewage water by adsorption onto pellets (cell surface) and/or through sequestration via metal-binding proteins. Golebiewski et al. (2014) mentioned that the phylum and class levels are insufficient for evaluating variations in bacterial communities in soils polluted by heavy metals. Thus, lower taxonomic levels should be explored to determine the effects of heavy metals on microbial community structure.

At the genus level, microbial communities suggested significant variation among the three treatments. Some genera were found to be more abundant in Pb treatments than in CK of this study (e.g., *Bacillus*, *Streptococcus* and *Arthrobacter*). Several studies have reported *Bacillus*, *Streptococcus*
and *Arthrobacter* to be heavy metal resistant bacteria (Olukoya et al. 1997; Ranjard et al. 2000; Harichová et al. 2012; ZampieriBdel et al. 2016). Olukoya et al. (1997) reported *Bacillus sp.* and *Streptococcus sp.* can resist to many types of heavy metal, such as Pb, Zn, Cr, Co and Cu. ZampieriBdel et al. (2016) identified *Bacillus sp.* as the dominant genus in areas polluted with heavy metals. Moreover, *Arthrobacter* and *Bacillus* were identified not only as heavy metal resistant bacteria (Ranjard et al. 2000; Harichová et al. 2012) but also as plant growth promoting rhizobacteria. *Arthrobacter* and *Bacillus* were phosphate-solubilizing bacteria which can dissolve inorganic phosphate and mineralize organic phosphate, improving phosphorus availability to plants (Rodríguez et al. 1999). Heavy metal stress can severely limit the sensitive bacteria genus, allowing more tolerant bacteria to adapt and proliferate. The presence of Pb\(^{2+}\) in soil supported the growth of metal resistant species which may be used for bioremediation in the future.

In our study, the relative abundance of bacteria phyla was highly correlated with TPb. Here, the relative abundance of *Planctomycetes* negatively and significantly correlated with TPb (P < 0.05). *Planctomycetes* have been found both in aquatic and terrestrial habitats, indicating their physiological adaption to diverse environments (Barnard et al. 2013). They play a clear role in biomass remineralization, which might be associated with the SOM dynamic in this research (Lage et al. 2012). Our results showed that Pb negatively affected the abundance of *Planctomycetes*, which can be explained by the findings of Lage et al. (2012) that lead was the most toxic to *Planctomycetes*. TPb also negatively correlated with the abundance of *Gemmatimonadetes* (P=0.094) and *Nitrospirae* (P=0.065). *Gemmatimonadetes* was characterized as one of the top phyla in soil bacterial community (DeBruyn et al. 2011), and also negatively responded to increasing heavy metal concentrations (Zhang et al. 2012). As to the phylum of *Nitrospirae*, they are closely involved in nitrogen cycling, mainly
contributing to conversion from nitrites to nitrates (Lücker et al. 2010). Thus, the mediation of the abundance of *Nitrospirae* might affect the content of nitrates in soil.

Heavy metals can influence microbial community composition and thereby affect nutrient (especially available phosphorous and potassium) and soil organic matter concentrations. Soil microbial abundance and diversity play a key role in determining soil properties and soil ecosystem sustainability (Xiong et al. 2015). In the present study, Available Pb (APb) and total Pb (TPb) were negatively correlated with soil organic matter (SOM), but positively affected available phosphorus (AP). The reason for these results may be that Pb stimulates phosphate-solubilizing bacteria (e.g. *Bacillus*, and *Arthrobacter*) but inhibits potassium-solubilizing microorganisms. Another reason could be chemical effect of Pb on binding of phosphate to soil. SOM concentration was strongly negatively correlated with total and available Pb contents, reflecting the affinity of Pb for SOM (Hooda et al. 1998; Mcbridea et al.1997; Šípková et al. 2013; Strawn et al.2000). The SOM are mainly contributed by the root exudates and litters. Previous study showed that the Pb stress had negative impact on crop growth (Lamhamdi et al. 2013), which might cause decline of root exudates. Moreover, the decreased litter from crops is another reason for the reduced SOM in Pb treated plots.

**Conclusions**

Illumina MiSeq sequencing of 16S rRNA genes effectively assessed the microbial community structure in Pb-contaminated Lou soils. Pb showed weak impact on the diversity of soil bacteria community, but influenced the abundance of some genera of bacteria. The presence of Pb$^{2+}$ also affects the physicochemical properties of soil by altering concentrations of nutrients (i.e., available phosphorous) and SOM. APb and TPb negatively affected SOM, and positively affected AP. The
abundance of major phyla was highly correlated with soil TPb. The relative abundance of
_Planctomycetes_, _Nitrospira_ and _Gemmatimonadetes_ negatively correlated with TPb. Future studies
are required to illuminate the functional genes involved in metal resistance and provide insight into
monitoring changes in microbial communities.

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Figure Legends

Figure 1. Location of sampling sites contaminated with Pb. (Experimental sites at the “National monitoring base of soil fertility and fertilizer efficiency on Loess soil”, the test plot of Northwest Agriculture and Forestry University, Yangling city, Shaanxi Province, China)

Figure 2. Venn diagram showing shared bacterial OTUs between three treatments. (CK = control; LOW = low concentration treatment; HIGH = high concentration treatment.)

Figure 3. Rarefaction curves of partial sequences of 16S rRNA gene. (CK1S, CK2S, CK3S indicate CK; Pb11S, Pb12S, Pb13S indicate LOW; Pb21S, Pb22S, Pb23S indicate HIGH)

Figure 4. Estimated OTU richness and diversity indices for 16S rRNA gene libraries of soil samples. (CK = control; LOW = low concentration treatment; HIGH = high concentration treatment.)

Figure 5. Taxonomic classification of bacterial reads retrieved from different samples at the phylum level. (CK1S, CK2S, CK3S indicate CK; Pb11S, Pb12S, Pb13S indicate LOW; Pb21S, Pb22S, Pb23S indicate HIGH)
Fig 6. The relative abundances of the top bacterial from samples at the different classification levels. (A: Class levels; B: Order levels; C: Family levels)

Fig 7. Significant differences genera among three treatments at the genus level.
(CK = control; LOW = low concentration treatment; HIGH = high concentration treatment.)

Fig 8. Ordination triplot based on RDA of relationships between soil microbial community structure (relative abundances of OTUs) and environmental variables.
(CK1S, CK2S, CK3S indicate CK; Pb11S, Pb12S, Pb13S indicate LOW; Pb21S, Pb22S, Pb23S indicate HIGH)
### Table 1 Characteristics of soil samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>Moisture (%</th>
<th>SOM (g/kg)</th>
<th>AP (mg/kg)</th>
<th>AK (mg/kg)</th>
<th>AN (mg/kg)</th>
<th>APb (mg/kg)</th>
<th>TPb (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>8.01 ± 0.09a</td>
<td>1.51 ± 0.02a</td>
<td>14.91 ± 0.24b</td>
<td>9.55 ± 0.48b</td>
<td>145.34 ± 6.48a</td>
<td>58.31 ± 2.69a</td>
<td>3.97 ± 0.08c</td>
<td>23.06 ± 0.49c</td>
</tr>
<tr>
<td>LOW</td>
<td>8.01 ± 0.06a</td>
<td>1.44 ± 0.03b</td>
<td>12.76 ± 0.40b</td>
<td>8.84 ± 0.27b</td>
<td>149.04 ± 15.77a</td>
<td>55.29 ± 2.70a</td>
<td>126.61 ± 4.98b</td>
<td>188.65 ± 1.79b</td>
</tr>
<tr>
<td>HIGH</td>
<td>8.01 ± 0.10a</td>
<td>1.52 ± 0.01a</td>
<td>12.93 ± 0.06b</td>
<td>12.30 ± 0.67a</td>
<td>148.15 ± 8.01a</td>
<td>56.48 ± 3.54a</td>
<td>254.46 ± 7.13a</td>
<td>361.42 ± 3.36a</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation (n=3). Means followed by the same letter for a given factor are not significantly different (P<0.05; Turkey’s HSD test).
Table 2 Correlation analysis of environmental variables and microbial species richness at the phylum level (relative abundance > 0.01).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>pH</th>
<th>Moisture</th>
<th>SOM</th>
<th>AP</th>
<th>AK</th>
<th>AN</th>
<th>APb</th>
<th>TPb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidobacteria</td>
<td>-0.201(0.983)</td>
<td>-0.607(1)</td>
<td>0.317(0.586)</td>
<td>-0.008(0.983)</td>
<td>-0.300(0.9)</td>
<td>-0.059(0.954)</td>
<td>-0.067(0.937)</td>
<td>-0.183(0.69)</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.251(0.983)</td>
<td>0.250(1)</td>
<td>-0.233(0.624)</td>
<td>0.117(0.863)</td>
<td>0.217(0.9)</td>
<td>0.176(0.954)</td>
<td>0.100(0.937)</td>
<td>0.25(0.61)</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>-0.092(0.983)</td>
<td>-0.033(1)</td>
<td>-0.317(0.586)</td>
<td>0.184(0.863)</td>
<td>0.117(0.9)</td>
<td>0.184(0.954)</td>
<td>0.433(0.397)</td>
<td>0.517(0.276)</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>-0.385(0.983)</td>
<td>-0.250(1)</td>
<td>0.117(0.765)</td>
<td>-0.100(0.863)</td>
<td>-0.417(0.9)</td>
<td>-0.569(0.954)</td>
<td>0.017(0.966)</td>
<td>0.017(0.966)</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>-0.025(0.983)</td>
<td>-0.483(1)</td>
<td>-0.550(0.361)</td>
<td>-0.209(0.863)</td>
<td>-0.250(0.9)</td>
<td>-0.276(0.954)</td>
<td>0.400(0.413)</td>
<td>0.500(0.276)</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>0.134(0.983)</td>
<td>0.083(1)</td>
<td>-0.467(0.444)</td>
<td>0.326(0.847)</td>
<td>0.567(0.9)</td>
<td>0.084(0.954)</td>
<td>0.467(0.381)</td>
<td>0.433(0.352)</td>
</tr>
<tr>
<td>Gemmatimonadetes</td>
<td>0.008(0.983)</td>
<td>-0.250(1)</td>
<td>0.217(0.624)</td>
<td>-0.377(0.847)</td>
<td>0.183(0.9)</td>
<td>-0.167(0.954)</td>
<td>-0.683(0.109)</td>
<td><strong>-0.700(0.0094)</strong></td>
</tr>
<tr>
<td>Nitrospirae</td>
<td>0.276(0.983)</td>
<td>-0.200(1)</td>
<td>0.233(0.624)</td>
<td>-0.636(0.563)</td>
<td>-0.317(0.9)</td>
<td>-0.301(0.954)</td>
<td>-0.733(0.108)</td>
<td><strong>-0.750(0.065)</strong></td>
</tr>
<tr>
<td>Planctomycetes</td>
<td>-0.075(0.983)</td>
<td>-0.100(1)</td>
<td>0.583(0.361)</td>
<td>-0.360(0.847)</td>
<td>-0.083(0.9)</td>
<td>1.134(0.954)</td>
<td>-0.700(0.109)</td>
<td><strong>-0.750(0.03)</strong></td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>0.025(0.983)</td>
<td>0.000(1)</td>
<td>-0.350(0.586)</td>
<td>0.117(0.863)</td>
<td>0.400(0.9)</td>
<td>0.008(0.983)</td>
<td>0.233(0.71)</td>
<td>0.300(0.563)</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>0.092(0.983)</td>
<td>0.583(1)</td>
<td>0.650(0.361)</td>
<td>0.167(0.863)</td>
<td>-0.133(0.9)</td>
<td>0.092(0.954)</td>
<td>-0.533(0.301)</td>
<td>-0.533(0.276)</td>
</tr>
<tr>
<td>APb</td>
<td>-0.092(0.983)</td>
<td>0.067(1)</td>
<td>-0.583(0.361)</td>
<td>0.544(0.563)</td>
<td>0.200(0.9)</td>
<td>-0.151(0.954)</td>
<td>1.000(0)</td>
<td>0.950(0)</td>
</tr>
<tr>
<td>TPb</td>
<td>-0.134(0.983)</td>
<td>0.067(1)</td>
<td>-0.533(0.361)</td>
<td>0.552(0.563)</td>
<td>-0.017(0.966)</td>
<td>-0.126(0.954)</td>
<td><strong>0.950(0)</strong></td>
<td>1.000(0)</td>
</tr>
</tbody>
</table>

Each value represents $r$ with the $P$-value in parenthesis. *Correlations significant at $P<0.05$, **Correlations significant at $P<0.01$. Significance was corrected for multiple comparisons with a false discovery rate (FDR) = 0.05 (Benjamini and Hochberg, 1995).
80x65mm (300 x 300 DPI)
84x178mm (300 x 300 DPI)