Effect of *Phanerochaete chrysosporium* inoculation on bacterial community and metal stabilization in lead-contaminated agricultural waste composting

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Abstract

The effects of *Phanerochaete chrysosporium* inoculation on bacterial community and lead (Pb) stabilization in composting of Pb-contaminated agricultural waste were studied. It was found that the bioavailable Pb was transformed to stable Pb after composting with inoculum of *P. chrysosporium*. Pearson correlation analysis revealed that total organic carbon (TOC) and carbon/nitrogen (C/N) ratio significantly ($P < 0.05$) influenced the distribution of Pb fractions. The richness and diversity of bacterial community were reduced under Pb stress and increased after inoculation with *P. chrysosporium*. Redundancy analysis indicated that C/N ratio, total organic matter, temperature and soluble-exchangeable Pb were the significant parameters to affect the bacterial community structure, solely explained 14.7%, 11.1%, 10.4% and 8.3% of the variation in bacterial community composition, respectively. In addition, the main bacterial species, being related to organic matter degradation and Pb stabilization, were found. These findings will provide useful information for composting of heavy metal-contaminated organic wastes.

Keywords: Composting; *Phanerochaete chrysosporium*; Bacterial community; Lead; Redundancy analysis

1. Introduction

Composting is currently considered as an effective strategy to keep sustainable development of agricultural ecosystems (Zeng et al., 2015), which can converts agricultural wastes into valuable organic products for reusing (Jiang et al., 2015). Agricultural wastes are mainly comprised of cellulose, hemicellulose and lignin, among which lignin is a highly insoluble and irregular polymer of phenylpropane units that provides strength and rigidity to wood, protecting most of the cellulose and hemicellulose against enzymatic hydrolysis (Sharma et al., 2004). In consequence, the biodegradation process of agricultural wastes is often slow in nature (Tuomela et al., 2000). Alternatively, considerable attentions have been focused on the development of composting with the inoculation of fungi for the efficient process of agricultural wastes, whereby a variety of fungi aroused great interest for their potential lignin degradation ability (Ferreira et al., 2016; Zeng et al., 2010). *Phanerochaete chrysosporium* (*P. chrysosporium*), the representative species of white-rot fungi that are the most efficient lignin degrading microorganism (Huang et al., 2008), has been widely studied owing to its powerful degradation ability against many kinds of organic substrates (Cheng et al., 2014; Zhao et al., 2015).

Composting has been considered as an alternative to the bioremediation of soils contaminated with polycyclic aromatic hydrocarbons (Tang et al., 2008; Zeng et al., 2013b), petroleum, pesticides (Zeng et al., 2013a), chlorophenols (Gong et al., 2009; Lai et al., 2016) and heavy metals (Xu et al., 2012). However, the efficiency of inoculation composting systems is affected by various factors such as the microbe, processing time, environmental condition and raw material (Xi et al., 2015). It has been reported that the composting system with inoculation do not always exhibit the superior performance, empirically caused by the competition between

53 indigenous microbes and inoculated microbes (Nakasaki et al., 2013). Obviously, understanding
54 the dynamic changes of microbial communities during the composting process becomes the key
55 for successful application of inoculation in composting. To acquire a comprehensive knowledge
56 on microbial communities during the composting, many molecular techniques including
57 single-stranded conformation polymorphism (SSCP), terminal restriction fragment length
58 polymorphism (t-PFLP) and polymerase chain reaction denaturing gradient gel electrophoresis
59 (PCR-DGGE) have been developed (Egert et al., 2004). Among these methods, PCR-DGGE has
60 been widely used in many fields to monitor microbial community dynamics (Cahyani et al., 2003;
61 Huang et al., 2017a). Redundancy analysis (RDA) and canonical correspondence analysis (CCA)
62 based on the PCR-DGGE profiles were widely applied to reveal the correlation between
63 microbial community composition and environmental factors (Aydin et al., 2015; Wang et al.,
64 2015), and to identify the primary factors affecting microbial communities (Chen et al., 2014;
65 Zhang et al., 2014).

66 Heavy metal pollution is a common environmental problem in the world (Abdolali et al., 2017;
67 Hu et al., 2011). Lead (Pb), as a non-essential element, is a toxic heavy metal and widely
68 distributed contaminant in the environment (Huang et al., 2008). The agricultural wastes derived
69 from the Pb-contaminated sites will affect public health through the food chain and thus need
70 proper disposal. Previous study confirmed that biosorption was an effective technology to
71 remove Pb(II) by *P. chrysosporium* in aqueous medium (Yetis et al., 2000). However, there is
72 limited information on the remediation of Pb-contaminated agricultural wastes by composting.
73 Since the growth and activity of the indigenous microorganism and fungal inocula can be
74 affected by heavy metals to some extent, for instance by Pb^{2+} (Cheng et al., 2014). Therefore, the

understanding of agricultural wastes composting with fungal inoculum in the presence of Pb^{2+} is critical and necessary.

This study aimed to evaluate the impact of *P. chrysosporium* inoculation on the indigenous bacterial community and metal toxicity in composting of Pb-contaminated agricultural wastes. The distribution of Pb fractions during composting and the correlation analysis between Pb fractions and the physico-chemical parameters were investigated. The dynamics of bacterial communities were revealed by heatmap and cluster analysis. RDA was performed to clarify the correlation between the physico-chemical parameters and bacterial community compositions. Variation partitioning analysis was used to distinguish the effect of each significant parameter as well.

2. Materials and methods

2.1. Microorganism and inoculant preparation

The inoculant strain *P. chrysosporium* (BKM-F-1767) was purchased from China Center for Type Culture Collection (Wuhan, China). The strain was maintained on potato dextrose agar (PDA) slants at 4 ℃ and transferred to PDA plates at 37 ℃ for 48 h before use. The fungal spores from plates were diluted in sterile distilled water and then adjusted to a concentration of 2.0×10^6 CFU/mL according to our previous work (Huang et al., 2016).

2.2. Composting materials and experimental set-up

The representative agricultural wastes were obtained from the suburb of Changsha, China. The rice straw was air-dried and cut into a length of about 1 cm, used as the natural substrate rich in lignocellulose. The uncontaminated soil collected from the Yuelu Mountain in Changsha was sieved to pass a 40-mesh screen to remove the coarse plant debris, and was then used to enrich

97 the microbial species and populations and supply nutrients. The vegetables including cabbage,
98 celery leaves and beetroot tops were collected from a local market and used as the easy
99 metabolizing substrate after cutting them into small pieces (about 1 cm × 1 cm). Bran was used
100 to adjust the initial C/N ratio of the composting materials. The chemical characteristics of
101 composting materials are shown in Table 1.

102 The composting materials including rice straw, soil, vegetables and bran were thoroughly
103 homogenized at a ratio of 11: 8: 3: 2 (wet weight). Four experimental composting systems (piles
104 A, B, C and D) with each about 15 kg of composting materials (wet weight) were set up indoors
105 in 90-L open polystyrene boxes with the external and internal dimensions of 0.55 × 0.45 × 0.50
106 m and 0.50 × 0.40 × 0.45 m (length × width × height). The organic matter content of the mixture
107 was about 60% and the initial C/N ratio was about 30: 1. The $Pb(NO_3)_2$ solutions were added to
108 the pile B, C and D with the final Pb^{2+} content of 30, 30 and 400 mg/kg, respectively. The pile A
109 was free from Pb contamination and used the equal amount of sterile water as a replacement. The
110 pile C and D were inoculated with 2% of *P. chrysosporium* spore suspensions, and the pile A and
111 B used as the control without the inoculants. After inoculation, the piles were turned over
112 thoroughly. The experiment was performed in triplicates and conducted for 42 days. Moisture
113 content was kept at about 60% by adding sterile water every 6 days during the first 12 days. To
114 aerate and mix the compost material, the composting piles were turned manually twice a week
115 during the first 2 weeks and once a week afterwards. During the composting, three subsamples
116 from the upper, middle and lower layers of the compost pile were collected on day 0, 3, 6, 9, 12,
117 18, 30 and 42. The subsamples were mixed and divided into two parts. One part was used for
118 physico-chemical parameter determination and Pb analysis, another was kept at −20 ℃ for total

DNA isolation.

2.3. Physico-chemical parameter analysis

The pile temperature in each compost pile was recorded by a thermometer. The moisture content was estimated based on weight loss at 105 ℃. The total organic matter (TOM) was measured by weight loss on ignition at 540 ℃. Total organic carbon (TOC) was determined using a Shimadzu TOC-V analyzer (Shimadzu Corporation, Kyoto, Japan). The total nitrogen (TN) was measured according to the Kjeldahl digestion. After mixing the fresh samples with distilled water at a ratio of 1: 10 (w/v) and mechanically shaking at 150 rpm for 20 min, the suspension was used for pH determination.

2.4. Chemical speciation analysis

About 5 g (wet weight) of sample was dried at 105 ℃ for 6 h and ground in a mortar, then 1 g of dry sample (power) was used for subsequent analysis. The five fractions of Pb were measured by a sequential extraction procedure reported by Tessier et al. (1979). After each extraction, the supernatant extract was obtained from the mixture by centrifuging and used for Pb content analysis by flame atomic absorption spectrometry (AAS700, PerkinElmer, USA). The fraction distributions of Pb were expressed by the ratio of Pb in each fraction to the total content of Pb in compost samples. In this paper, F1 to F5 represents the soluble-exchangeable, carbonates-bound, Fe-Mn oxides-bound, organic matter-bound and residual Pb, respectively.

2.5. DNA extraction and PCR-DGGE analysis

The total genomic DNA was isolated from 0.5 g (wet weight) of each compost sample on day 3, 9, 18, 30 and 42 using the MOBIO PowerSoil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA). The fragment of 16S rDNA gene was amplified with bacterial universal

141 primer 338F/518R with a GC clamp (LaPara et al., 2000). The PCR mixture contained 20 μL 2.5
142 ×HotMaster PCR Mix (Eppendorf), 1 μL each primer (10 μM), and 1 μL template DNA (≈ 20 ng)
143 and diluted to a final volume of 50 μL with sterile Milli-Q water. The PCR program involved an
144 initial denaturation step at 94 ℃ for 5 min, followed by 35 cycles of denaturation at 94 ℃ for 45
145 s, annealing at 55 ℃ for 40 s and elongation at 72 ℃ for 40 s, and a final step of elongation at 72
146 ℃ for 7 min before holding at 4 ℃.

147 DGGE analysis was performed according to Zhang et al. (2011). The PCR samples (30 μL)
148 were loaded onto the 1-mm-thick 8% (w/v) polyacrylamide gels with gradient of 35-65%
149 denaturants. Electrophoresis was performed at 60 ℃ for 12 h at 90 V. After electrophoresis, the
150 DGGE gels were stained with SYBR Green I for 30 min and scanned by a gel imaging system.

151 2.6. Statistical analysis

152 The results were present as the means and standard deviation of three replicates. The
153 differences between the means were analyzed by one-way analysis of variance (ANOVA) and
154 Tukey's multiple-comparison test. Correlation analysis was carried out to clarify the relationship
155 between physico-chemical parameters and Pb fractions. All the above analyses were performed
156 using SPSS (version 18.0, SPSS, Chicago, IL). The significance level of differences was kept at
157 $P < 0.05$.

158 DGGE banding profiles were detected and digitized after average background subtraction for
159 each lane using Quantity One software (version 4.5, Bio-Rad Laboratories, USA). The position
160 and relative intensity data of DNA bands for each sample were recorded according to our
161 previous researches (Huang et al., 2017a; Huang et al., 2017b). Shannon-Wiener diversity index
162 (H) for the bacterial community was calculated using Quantity One V4.5. As the longest gradient

from detrended correspondence analysis (DCA) based on bacterial DGGE profiles was 2.307, redundancy analysis (RDA) was performed to compare species–environment correlations using CANOCO version 4.5 and the significance was evaluated by Monte Carlo permutation test (499 permutations). Prior to analysis, all environmental variables were centered with unit variance. Manual forward selection was performed to test whether the species composition was independent of the environmental variables. Variation partitioning analysis was carried out to distinguish the effect of each significant variable.

3. Results and discussion

3.1. Changes of physico-chemical characteristics during composting

The changes of physico-chemical parameters during the composting process are presented in Fig. 1. Temperature is considered as an important indicator of the composting process performance. As shown in Fig. 1a, the ambient temperature varied from 25–29 ℃ during the whole composting process. The pile temperature increased rapidly in the first 3 days (the mesophilic phase, day 0 to day 3) and maintained above 50 ℃ for 9 days (the thermophilic phase, day 3 to day 12), then gradually decreased to about 40 ℃ (the cooling phase, day 12 to day 18) and finally maintained around 30 ℃ (the maturation phase, day 18 to day 42). The thermophilic phase with the temperature above 55 ℃ for more than 3 days is considered to be sufficient to produce sanitary compost by destroying many pathogens (Rashad et al., 2010). In this study, this phase lasted for more than 3 days for all piles and longer for pile A (control) and C (treatment with 30 mg/kg Pb^{2+} and *P. chrysosporium*) during composting process. The significant difference was found between the temperature of pile A and B (treatment with 30 mg/kg Pb^{2+}) from day 3 to day 30, between pile A and C on day 3, between pile A and D (treatment with 400 mg/kg Pb^{2+}

and *P. chrysosporium*) from day 3 to day 18, indicating the inhibition effect of Pb on microbial activities which mostly associated with the consumption of nutrient. However, there was no significant difference between that of pile A and C from day 6 to day 42 except on day 18, it was suggested that inoculation with *P. chrysosporium* could protect microorganisms against Pb^{2+} toxicity. This is likely the result of the defense mechanisms of *P. chrysosporium* in response to Pb^{2+}, including extracellular chelation and intracellular uptake (Petr, 2003). However, in high concentration of Pb^{2+}, the pile temperature was significantly lower ($P < 0.05$) than the control without Pb^{2+} (pile A) during the first 18 days of composting and no significant difference was observed after 18 days. This may be attributed to the inhibition of high Pb concentration to *P. chrysosporium* in the early exposure period which delayed the stabilization of toxic Pb^{2+} ions (Huang et al., 2010b).

pH is an important parameter in composting process for its role in microbial growth and metabolism. As shown in Fig. 1b, the pH in all piles increased fast in the first 9 days and maintained about 8.0–8.5 during the last stage of composting process. pH in pile A was significantly higher ($P < 0.05$) than pile B and C from day 3 to day 12, higher than pile D from day 3 to day 6. No significant difference was found between the pH in pile B and C, between in pile C and D. The previously study confirmed that the rise of pH at the initial stage lay in the production of ammonia matters (such as NH_4^+) during organic matter catabolism by microorganism (Hattori et al., 1999). Under the stress of Pb^{2+}, microorganism can secrete low molecular weight organic acids like oxalic and citric acid to chelate with Pb^{2+}, which may be account for the lower pH in Pb^{2+} treated piles than that without Pb^{2+} addition (Li et al., 2011). The slightly alkaline pH (about 8.0) at the end of composting for all piles indicated that the

compost was mature (Lin, 2008).

The variations in TOM and TOC were presented in Fig. 1c and Fig. 1d. Composting of agricultural waste is an aerobic process to decomposition of organic matter into humus which used as a good fertilizer for plants (Cahyani et al., 2003). In this study, the rapidly decrease of the organic matter content was observed for all piles in mesophilic and thermophilic phases (the first 12 days) and slowly for cooling and maturation phases (day 18 to day 42). The TOM content was significantly lower ($P < 0.05$) in pile C than the others during the whole process except on day 0, which is 24.6%, 26.6%, 16.8% and 22.6% for pile A–D after 42 days of decomposition, suggesting that the organic matter was effectively degraded by *P. chrysosporium* under Pb^{2+} concentration of 30 mg/kg. The decrease of TOM for pile B and D was restrained (although insignificant) when compared to pile A in the first 9 days, whereas it was reactivated for pile D after 9 days. This implied that high concentration of Pb^{2+} could delay the decomposition of organic matter and the effect would disappear with time shift, the probable reason may be the limited bioavailable fraction of Pb during the later phase due to nonspecific binding (Petr, 2003). As presented in Fig. 1d, the tendency of TOC was similar with TOM for all piles, the initial TOC content was about 270 g/kg and the final was 159–176 g/kg with the degradation ratio range from 35.5%–41.8%. The TOC is usually used as the energy source for the microorganisms (Chan et al., 2016), and the mineralization of TOC will cause the main loss of TOM which results in a similar trend.

Fig. 1e presents the dynamic changes of TN concentrations, a loss of nitrogen was observed during the first 9 days for all piles. After 9 days, the TN concentration in pile C (8.96 g/kg) was significantly lower than that in pile A (9.05 g/kg), pile B (9.00 g/kg) and pile D (8.95 g/kg) (P =

0.045, 0.017, 0.045, respectively). However, a greater increase of TN concentration was observed after 12 days for pile C than the others. The NH_4^+-N is accumulated by the degradation and mineralization of organic nitrogen through ammonification and can be volatilized as NH_3 thus resulting in nitrogen loss in the initial stage of composting (Sommer, 2001). Whereas the little loss of TN during the later stage may result from the less emission of NH_3 due to low temperature and the decomposition of biomass to organic nitrogen (Zhang et al., 2017). The ratio of C/N is usually used to evaluate the degree of compost maturity (Mathur et al., 1993). In this work, the C/N ratio presented in Fig. 1f showed an obvious decline from initial value of about 30 to final values of 18.8, 19.6, 17.4 and 18.4 for pile A–D, respectively. The final values for pile A–D all met the criteria (< 20) of mature compost (Huang et al., 2010a). The C/N ratio was significantly lower ($P < 0.05$) in pile C and D than in pile B, indicating extensive decomposition of organic matter under Pb stress by inoculation with *P. chrysosporium*, which was in accordance with our results in Fig. 1c.

3.2. Transformation of Pb fractions

As shown in Fig. 2, Pb was mainly existed in the fraction of soluble-exchangeable at the beginning of composting process, which was 76.4%, 76.3% and 82.0% for pile B–D, respectively. The fraction distribution of Pb transformed from soluble-exchangeable to organic-bound and residual with the composting time, while the fraction of carbonate-bound and Fe-Mn oxides-bound did not exhibit an obvious change compared with the other fractions. The Pb fraction of soluble-exchangeable and residual did not show obvious changes until day 9, which might be ascribed to the acidic or neutral condition (Fig. 1b) that was unfavorable to the stabilization of Pb. It was suggested by Singh and Kalamdhad (2012) that weakly alkaline

condition (pH < 11) was advantageous to passivate Pb by forming Pb-organic matter complex. In this study, the pH was 8.0–8.5 after 9 days and the Pb stabilization was observed at the same time. After 42 days of composting, the Pb-fraction of soluble-exchangeable in pile B–D was in the order: pile B (24.0%) > pile D (7.2%) > pile C (1.5%), and that of residual was in the order: pile C (29.1%) > pile D (25.8%) > pile B (16.6%). The results suggested that the bioavailable Pb had been transformed to stable Pb through composting, especially in pile C and D, which indicated that Pb toxicity was reduced after inoculation with *P. chrysosporium*. It might be because *P. chrysosporium* could secrete extracellular metal chelators to immobilize Pb^{2+} ions and reduce the bioavailability of Pb. Li et al. (2011) reported that the accumulation of oxalate was induced in *P. chrysosporium* under Pb^{2+} stress to reduce Pb^{2+} toxicity by chelation. In addition, *P. chrysosporium* could promote the production of humic substances by enhancing the degradation of organic matter, and the formation of organo-metallic complexes contributed to the passivation of Pb (Zeng et al., 2007).

3.3. Influence of the compost properties on Pb distribution

The distribution and bioavailability of metal mainly rely on the metal itself and the properties of the medium during the composting of metal-contaminated agricultural wastes (Zeng et al., 2007). Correlation matrices of Pb fractions with pile temperature, pH, TOM, TOC, TN, C/N ratio and Shannon-Wiener index (H) of bacteria were performed to investigate the influence of these selected variables on Pb distribution during composting (Table 2). Interestingly, both TOC and C/N ratio had a significant positive correlation with F1 ($P < 0.01$), and negative correlation with the other fractions ($P < 0.05$). TOM and TN were significantly positive correlated with F1 ($P < 0.01$), and negative correlated with F3, F4 and F5 ($P < 0.01$). A similar result was found in

sewage sludge and swine manure composting that the evolution of heavy metal distributions and bioavailability depended on both the total metal concentrations and the other properties such as pH and the degradation of organic matter (Miaomiao et al., 2009). The results suggested that the stabilization of Pb was accompanied by the decomposition of agricultural waste, and composting is an alternative method for the remediation of Pb pollution. However, no significant correlation was found between the Pb distribution and diversity of bacteria.

3.4. Bacterial community variation

Samples from pile A, B, C and D on day 3, 9, 18, 30 and 42 (represent the different stages of composting) were used to evaluate the dynamics of bacterial community by PCR-DGGE in the present study. As shown in Fig. 3, most bands were distributed in the thermophilic phase (day 3 to day 9), which could be attributed to the fast degradation of organic matter in this period (Fig. 1c). There were 17, 13, 17, 13 dominant bands detected in samples from pile A, B, C and D during the whole composting process, respectively. The decline in band numbers for pile B and D could be ascribed to the toxicity of Pb. Unlike pile B and D, the band numbers in pile C did not decrease. Band 7 and 11 appeared in pile A, C and D other than in pile B during the mesophilic and thermophilic phase, band 17 was mainly detected during the last phases in pile A, C and D but not detected in pile B, in addition, band 2, 15 and 16 only exist during the thermophilic phase in pile A and C, band 13 and 14 appeared in all trials except the in pile D. The results suggested that band 7, 11, 17 species were sensitive to Pb stress but could survive under Pb stress with *P. chrysosporium* inoculation, whereas band 2, 15, 16 species were more sensitive to Pb stress and could not survive under 400 mg/kg Pb stress even in treatment with *P. chrysosporium*. It was suggested that the richness of bacteria would be reduced by Pb toxicity

and *P. chrysosporium* inoculation could increase the amounts and types of dominant bacteria species during composting under low Pb stress (30 mg/kg). However, band 9 disappeared during the cooling and maturation phase after exposure to Pb, regardless the inoculation or not. Band 5 appeared during the whole composting process for all groups, indicating its strong adaptability to Pb stress and *P. chrysosporium* inoculation. Interestingly, band 12 emerged only in piles with the treatment of Pb, it was suggested that this species might be Pb dependent and had resistance to Pb.

The Shannon-Wiener index (H), an indicator of species richness as well as equitability in ecology (Dilly et al., 2004), was calculated to evaluate the diversity of bacterial community composition during the composting process. As shown in Table 3, H of bacteria was significantly lower in pile D than pile A during the whole process, lower in pile B than pile A except on day 9, indicating the negative impact of Pb on the diversity of bacterial community. Whereas the diversity of bacteria in pile C maintained stable on day 3, increased on day 9, decreased on day 18 and showed no significant difference thereafter when compared with in pile A. The results revealed that there was an obvious shift in the composition of the bacterial community due to Pb toxicity and *P. chrysosporium* inoculation could increase the bacterial diversity.

Heatmap of the bacterial community composition based on the DGGE bands and relative band intensities (Chen et al., 2016), as well as the cluster analysis of DGGE profiles, are shown in Fig. 4. As reported by Marschner et al. (2001), the variations of the relative intensity of a specific band in different lanes indicated the changes in the abundance of this species. It was obvious that the bacterial communities changed sharply with the composting time. Cluster analysis (UPGMA) separated the DGGE fingerprints of bacterial community into two major clusters based on the

317 time of day 3 and the others with the similarity of 27%. The homology coefficient of four piles
318 on day 9 and 18 was 0.38 and 0.41, respectively, which showed that both the Pb stress and
319 inoculation with *P. chrysosporium* had an impact on the bacterial community composition. With
320 the exception of a few samples, the patterns of different samples at the same sampling period
321 tend to cluster together (Fig. 4), such as the group of A3, D3, C3 and B3 (the mesophilic phase)
322 with the similarity of 52%, the group of D42, D30, B42 and B30 (the maturation phase) with the
323 similarity of 72%, suggesting that the pile temperature was another important factor in bacterial
324 community succession.

325 3.5. Correlation between bacterial community with environmental parameters

326 The physico-chemical parameters will influence or be influenced by the changes and
327 distribution of bacteria community during agricultural waste composting (Wang et al., 2015). To
328 identify the degree of influence of the environmental parameters on the bacterial community
329 composition and which parameters were most influential, RDA was performed to clarify the
330 correction between bacterial community composition and environmental variables. The results
331 were shown in Table 4. The Monte-Carlo test with 499 permutations showed that the correction
332 between the bacterial species data and environmental variables was statistically significant for
333 both the first canonical axis (P = 0.006, F = 1.361) and all canonical axes (P = 0.008, F =
334 2.550). The first two canonical axes explained 40.5% and 14.9% of the variation in the species
335 data, respectively, and 93.9% of the variation was explained by all selected environmental
336 variables. The manual forward selection using a Monte Carlo permutation test ($P < 0.05$)
337 indicated C/N ratio was the primary parameters driving the succession of the bacterial species,
338 followed by TOM, temperature and F1 (soluble-exchange Pb). According to the variation

partitioning analysis shown in Table 5, C/N ratio solely explained 14.7% (P = 0.004) of the variance in bacterial community composition, TOM 11.1% (P = 0.006), temperature 10.4% (P = 0.010) and F1 8.3% (P = 0.020), respectively. The variance shared by C/N, TOM, temperature and F1 was 11.2%. Four variables together explained up to 55.7% of the variance (P = 0.002). These results suggested that above four parameters and the interactions among them played an important role in bacterial community dynamics. TOM and C/N ratio represent the process of organic matter degradation and compost maturity, are also associated with the carbon and nitrogen sources for microbes, and are considered as the important parameters in composting (Huang et al., 2016). The pile temperature and substrates are reported to be the main two factors driving the bacterial community (Cahyani et al., 2003). Microbial metabolism is highly dependent on temperature and the dynamics of microbial community composition are greatly affected by temperature (Zhang et al., 2011). As the most bioavailable and toxic fraction of Pb, F1 greatly influences microorganism growth and metabolism due to its high mobility (Huang et al., 2010b). However, it does not imply that the other parameters do not have impacts although the significance level was low (P > 0.05) in our study. Each species is influenced by different levels of physico-chemical parameters during the composting process (Wang et al., 2015).

To assess the relationship between bacterial community and physico-chemical parameters, ordination triplot of RDA was performed to display the distribution of bacterial species to environmental variables (Fig. 5). Since the arrow length represented the importance of the variable, and its orientation reflected the correlation with the axis, it was evident that F1 had a more important influence on the distribution of bacterial community when compared to the other fractions of Pb and showed positive correlation with both axis 1 and 2. Moreover, among five

factions of Pb, F1 was the only significant environmental variable, indicating the propable toxicity of bioavailable Pb to bacterial community. F1 was positive correlated to pile temperature, moisture, C/N, TOC and TOM, while the other Pb fractions (F2 to F5) were positive correlated to pH and highly negative correlated to the other parameters. It is reasonable to speculate that inoculation with *P. chrysosporium* resulting in a lower C/N, TOC and TOM (Fig. 1) may contribute to the transformation of mobile and toxic fraction of Pb (F1) to insoluble and non-toxic fraction of residual Pb (F5), which is consistent with our results in Fig. 2. Band 15 and 16 bacteria, that only appeared in pile A and C on day 9 (thermophilic phase) as presented in Fig. 3, were significantly affected by F1 and pile temperature, indicating both species were sensitive to Pb toxicity and adapted to high temperature conditions. Band 1, 2, 4, 6, 8, 9, 12, 14, 15 and 16 bacteria showed positive correlation to TOM, TOC and C/N, suggested their potential role in decomposition of organic matter. Band 3, 5 and 17 bacteria had a highly correlation to F4 and F5, which might contribute to the stabilization of Pb.

Microorganisms are the drivers for the biodegradation of agricultural wastes during composting. Composting process could be accelerated by inoculating with functional microbes (Zhang & Sun, 2014). Since heavy metals such as Pb can influence the microbial growth and activity thus leading to inhibit the biodegradation process, *P. chrysosporium* was inoculated in this study due to its tolerance and assistance to Pb as reported previously (Huang et al., 2010b). Previous work indicated that bacterial community was mainly influenced by environmental parameters (Zhang et al., 2011). In this work, the main environmental parameters affecting bacterial community under Pb stress were identified (Fig. 5). Based on the redundancy analysis of the bacterial community data and environmental variables, a clearly correlation between

bacterial species and parameters associated to composting efficiency, as well as the main species related to the distribution of Pb fractions, were also presented in Fig. 5. However, further studies are needed to explore the detailed microbial community composition by high-throughput sequencing and the response of specific microbial species in heavy metal-contaminated agricultural waste composting. Then it will be possible to enhance the agricultural waste degradation and metal stabilization by regulating the corresponding environmental factors or by isolating the functional microbes and putting them into application.

4. Conclusion

Pb^{2+} significantly influenced the richness and diversity of bacterial community, and the inoculation with *P. chrysosporium* could contribute to the transformation of bioavailable Pb to inactive Pb thus reducing its toxicity to microorganisms. The distribution of Pb fractions was significantly related to TOC and C/N ratio ($P < 0.05$) according to Pearson correlation analysis. Based on RDA analysis, the bacterial species compositions were significantly affected by C/N ratio, TOM, temperature and soluble-exchangeable Pb. Moreover, the main species related to each composting factor was different, thus might supply a strategy to improve the composting process by adjusting relevant composting parameters.

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References

1. Abdolali, A., Ngo, H.H., Guo, W.S., Zhou, J.L., Zhang, J., Liang, S., Chang, S.W., Nguyen, D.D., Liu, Y., 2017. Application of a breakthrough biosorbent for removing heavy metals from synthetic and real wastewaters in a lab-scale continuous fixed-bed column. Bioresour. Technol. 229, 78-87.
2. Aydin, S., Shahi, A., Ozbayram, E.G., Ince, B., Ince, O., 2015. Use of PCR-DGGE based molecular methods to assessment of microbial diversity during anaerobic treatment of antibiotic combinations. Bioresour. Technol. 192, 735-740.
3. Cahyani, V.R., Matsuya, K., Asakawa, S., Kimura, M., 2003. Succession and phylogenetic composition of bacterial communities responsible for the composting process of rice straw estimated by PCR-DGGE analysis. Soil Sci. Plant Nutr. 49, 619-630.
4. Chan, M.T., Selvam, A., Wong, J.W.C., 2016. Reducing nitrogen loss and salinity during 'struvite' food waste composting by zeolite amendment. Bioresour. Technol. 200, 838-844.
5. Chen, Y.N., Ma, S., Li, Y.P., Yan, M., Zeng, G.M., Zhang, J.C., Zhang, J., Tan, X.B., 2016. Microbiological study on bioremediation of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) contaminated soil by agricultural waste composting. Appl. Microbiol. Biotechnol. 100, 9709-9718.
6. Chen, Y.N., Zhou, W., Li, Y.P., Zhang, J.C., Zeng, G.M., Huang, A.Z., Huang, J.X., 2014. Nitrite reductase genes as functional markers to investigate diversity of denitrifying bacteria during agricultural waste composting. Appl. Microbiol. Biotechnol. 98, 4233-4243.

7. Cheng, M., Zeng, G.M., Huang, D.L., Liu, L., Zhao, M.H., Lai, C., Huang, C., Wei, Z., Li, N.J., Xu, P., 2014. Effect of Pb^{2+} on the production of hydroxyl radical during solid-state fermentation of straw with *Phanerochaete chrysosporium*. Biochem. Eng. J. 84, 9-15.
8. Dilly, O., Bloem, J., Vos, A., Munch, J.C., 2004. Bacterial diversity in agricultural soils during litter decomposition. Appl. Environ. Microb. 70, 468-474.
9. Egert, M., Marhan, S., Wagner, B., Scheu, S., Friedrich, M.W., 2004. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L.(Oligochaeta: Lumbricidae). FEMS Microbiol. Ecol. 48, 187-197.
10. Ferreira, J.A., Mahboubi, A., Lennartsson, P.R., Taherzadeh, M.J., 2016. Waste biorefineries using filamentous ascomycetes fungi: Present status and future prospects. Bioresour. Technol. 215, 334-345.
11. Gong, J.L., Wang, B., Zeng, G.M., Yang, C.P., Niu, C.G., Niu, Q.Y., Zhou, W.J., Liang, Y., 2009. Removal of cationic dyes from aqueous solution using magnetic multi-wall carbon nanotube nanocomposite as adsorbent. J. Hazard. Mater. 164, 1517-1522.
12. Hattori, T., Nishiyama, A., Shimada, M., 1999. Induction of L-phenylalanine ammonia-lyase and suppression of veratryl alcohol biosynthesis by exogenously added L-phenylalanine in a white-rot fungus *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. 179, 305-309.
13. He, M.M., Li, W.H., Liang, X.Q., Wu, D.L., Tian, G.M., 2009. Effect of composting process on phytotoxicity and speciation of copper, zinc and lead in sewage sludge and swine manure. Waste Manage. 29, 590-597.
14. Hu, X.J., Wang, J.S., Liu, Y.G., Li, X., Zeng, G.M., Bao, Z.L., Zeng, X.X., Chen, A.W., Long,

F., 2011. Adsorption of chromium (VI) by ethylenediamine-modified cross-linked magnetic chitosan resin: Isotherms, kinetics and thermodynamics. J. Hazard. Mater. 185, 306-314.

15. Huang, C., Xu, P., Zeng, G.M., Huang, D.L., Lai, C., Cheng, M., Deng, L., Zhang, C., Wan, J., Liu, L., 2017a. The rapid degradation of bisphenol A induced by the response of indigenous bacterial communities in sediment. Appl. Microbiol. Biotechnol. 101, 3919–3928.
16. Huang, D.L., Liu, L.S., Zeng, G.M., Xu, P., Huang, C., Deng, L.J., Wang, R.Z., Wan, J., 2017b. The effects of rice straw biochar on indigenous microbial community and enzymes activity in heavy metal-contaminated sediment. Chemosphere 174, 545-553.
17. Huang, D.L., Qin, X.M., Xu, P., Zeng, G.M., Peng, Z.W., Wang, R.Z., Wan, J., Gong, X.M., Xue, W.J., 2016. Composting of 4-nonylphenol-contaminated river sediment with inocula of *Phanerochaete chrysosporium*. Bioresour. Technol. 221, 47-54.
18. Huang, D.L., Zeng, G.M., Feng, C.L., Hu, S., Jiang, X.Y., Tang, L., Su, F.F., Zhang, Y., Zeng, W., Liu, H.L., 2008. Degradation of lead-contaminated lignocellulosic waste by *Phanerochaete chrysosporium* and the reduction of lead toxicity. Environ. Sci. Technol. 42, 4946-4951.
19. Huang, D.L., Zeng, G.M., Feng, C.L., Hu, S., Lai, C., Zhao, M.H., Su, F.F., Tang, L., Liu, H.L., 2010a. Changes of microbial population structure related to lignin degradation during lignocellulosic waste composting. Bioresour. Technol. 101, 4062-4067.
20. Huang, D.L., Zeng, G.M., Feng, C.L., Hu, S.A., Zhao, M.H., Lai, C., Zhang, Y., Jiang, X.Y., Liu, H.L., 2010b. Mycelial growth and solid-state fermentation of lignocellulosic waste by white-rot fungus *Phanerochaete chrysosporium* under lead stress. Chemosphere 81,

1091-1097.

21. Jiang, J.S., Liu, X.L., Huang, Y.M., Huang, H., 2015. Inoculation with nitrogen turnover bacterial agent appropriately increasing nitrogen and promoting maturity in pig manure composting. Waste Manage. 39, 78-85.

22. Lai, C., Wang, M.M., Zeng, G.M., Liu, Y.G., Huang, D.L., Zhang, C., Wang, R.Z., Xu, P., Cheng, M., Huang, C., Wu, H.P., Qin, L., 2016. Synthesis of surface molecular imprinted TiO2/graphene photocatalyst and its highly efficient photocatalytic degradation of target pollutant under visible light irradiation. Appl. Surf. Sci. 390, 368-376.

23. LaPara, T.M., Nakatsu, C.H., Pantea, L., Alleman, J.E., 2000. Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. Appl. Environ. Microb. 66, 3951-3959.

24. Li, N.J., Zeng, G.M., Huang, D.L., Hu, S., Feng, C.L., Zhao, M.H., Lai, C., Huang, C., Wei, Z., Xie, G.X., 2011. Oxalate production at different initial Pb^{2+} concentrations and the influence of oxalate during solid-state fermentation of straw with *Phanerochaete chrysosporium*. Bioresour. Technol. 102, 8137-8142.

25. Lin, C., 2008. A negative-pressure aeration system for composting food wastes. Bioresour. Technol. 99, 7651-7656.

26. Marschner, P., Yang, C.H., Lieberei, R., Crowley, D.E., 2001. Soil and plant specific effects on bacterial community composition in the rhizosphere. Soil Biol. and Biochem. 33, 1437-1445.

27. Mathur, S.P., Owen, G., Dinel, H., Schnitzer, M., 1993. Determination of compost biomaturity. I. Literature Review. Biol. Agric. Hortic. 10, 65-85.

493 28. Nakasaki, K., Araya, S., Mimoto, H., 2013. Inoculation of *Pichia kudriavzevii* RB1 degrades
494 the organic acids present in raw compost material and accelerates composting. Bioresour.
495 Technol. 144, 521-528.
496 29. Petr, B., 2003. Interactions of heavy metals with white-rot fungi. Enzyme. Microb. Tech. 32,
497 78-91.
498 30. Rashad, F.M., Saleh, W.D., Moselhy, M.A., 2010. Bioconversion of rice straw and certain
499 agro-industrial wastes to amendments for organic farming systems: 1. Composting, quality,
500 stability and maturity indices. Bioresour. Technol. 101, 5952-5960.
501 31. Sharma, R.K., Wooten, J.B., Baliga, V.L., Lin, X., Geoffrey Chan, W., Hajaligol, M.R., 2004.
502 Characterization of chars from pyrolysis of lignin. Fuel 83, 1469-1482.
503 32. Singh, J., Kalamdhad, A.S., 2012. Concentration and speciation of heavy metals during
504 water hyacinth composting. Bioresour. Technol. 124, 169-179.
505 33. Sommer, S.G., 2001. Effect of composting on nutrient loss and nitrogen availability of cattle
506 deep litter. Eur. J. Agron. 14, 123-133.
507 34. Tang, L., Zeng, G.M., Shen, G.L., Li, Y.P., Zhang, Y., Huang, D.L., 2008. Rapid detection of
508 picloram in agricultural field samples using a disposable immunomembrane-based
509 electrochemical sensor. Environ. Sci. Technol. 42, 1207-1212.
510 35. Tessier, A., Campbell, P.G.C., Bisson, M., 1979. Sequential extraction procedure for the
511 speciation of particulate trace metals. Anal. Chem., 51, 844-851.
512 36. Tuomela, M., Vikman, M., Hatakka, A., Itävaara, M., 2000. Biodegradation of lignin in a
513 compost environment: a review. Bioresour. Technol. 72, 169-183.
514 37. Wang, X.Q., Cui, H.Y., Shi, J.H., Zhao, X.Y., Zhao, Y., Wei, Z.M., 2015. Relationship

between bacterial diversity and environmental parameters during composting of different raw materials. Bioresour. Technol. 198, 395-402.

38. Xi, B.D., He, X.S., Dang, Q.L., Yang, T.X., Li, M.X., Wang, X.W., Li, D., Tang, J., 2015. Effect of multi-stage inoculation on the bacterial and fungal community structure during organic municipal solid wastes composting. Bioresour. Technol. 196, 399-405.

39. Xu, P., Zeng, G.M., Huang, D.L., Feng, C.L., Hu, S., Zhao, M.H., Lai, C., Wei, Z., Huang, C., Xie, G.X., Liu, Z.F., 2012. Use of iron oxide nanomaterials in wastewater treatment: A review. Sci. Total. Environ. 424, 1-10.

40. Yetis, U., Dolek, A., Dilek, F.B., Ozcengiz, G., 2000. The removal of Pb(II) by *Phanerochaete chrysosporium*. Water Res. 34, 4090-4100.

41. Zeng, G.M., Cheng, M., Huang, D.L., Lai, C., Xu, P., Wei, Z., Li, N.J., Zhang, C., He, X.X., He, Y., 2015. Study of the degradation of methylene blue by semi-solid-state fermentation of agricultural residues with *Phanerochaete chrysosporium* and reutilization of fermented residues. Waste Manage. 38, 424-430.

42. Zeng, G.M., Huang, D.L., Huang, G.H., Hu, T.J., Jiang, X.Y., Feng, C.L., Chen, Y.N., Tang, L., Liu, H.L., 2007. Composting of lead-contaminated solid waste with inocula of white-rot fungus. Bioresour. Technol. 98, 320-326.

43. Zeng, G.M., Yu, M., Chen, Y.N., Huang, D.L., Zhang, J.C., Huang, H.L., Jiang, R.Q., Yu, Z., 2010. Effects of inoculation with *Phanerochaete chrysosporium* at various time points on enzyme activities during agricultural waste composting. Bioresour. Technol. 101, 222-227.

44. Zeng, G.M., Chen, M., Zeng, Z.T., 2013a. Risks of Neonicotinoid Pesticides. Science 340, 1403-1403.

45. Zeng, G.M., Chen, M., Zeng, Z.T., 2013b. Shale gas: surface water also at risk. Nature 499, 154-154.

46. Zhang, J.C., Zeng, G.M., Chen, Y.N., Liang, J., Zhang, C., Huang, B.B., Sun, W.M., Chen, M., Yu, M., Huang, H.L., 2014. *Phanerochaete chrysosporium* inoculation shapes the indigenous fungal communities during agricultural waste composting. Biodegradation 25, 669-680.

47. Zhang, J.C., Zeng, G.M., Chen, Y.N., Yu, M., Yu, Z., Li, H., Yu, Y., Huang, H.L., 2011. Effects of physico-chemical parameters on the bacterial and fungal communities during agricultural waste composting. Bioresour. Technol. 102, 2950-2956.

48. Zhang, L., Sun, X.Y., 2014. Changes in physical, chemical, and microbiological properties during the two-stage co-composting of green waste with spent mushroom compost and biochar. Bioresour. Technol. 171, 274-284.

49. Zhang, L.H., Zeng, G.M., Dong, H.R., Chen, Y.N., Zhang, J.C., Yan, M., Zhu, Y., Yuan, Y.J., Xie, Y.K., Huang, Z.Z., 2017. The impact of silver nanoparticles on the co-composting of sewage sludge and agricultural waste: Evolutions of organic matter and nitrogen. Bioresour. Technol. 230, 132-139.

50. Zhao, M.H., Zhang, C., Zeng, G.M., Huang, D.L., Xu, P., Cheng, M., 2015. Growth, metabolism of *Phanerochaete chrysosporium* and route of lignin degradation in response to cadmium stress in solid-state fermentation. Chemosphere 138, 560-567.

Figure captions

Fig. 1. Changes of physico-chemical parameters during the composting process. Error bars indicate standard deviation (n = 3). For each day, the means on the four columns followed by different letters are significant different at $P < 0.05$ according to Tukey's multiple-comparison test.

Fig. 2. The distribution of Pb in different fractions after sequential extraction of the compost samples.

Fig. 3. The diagram of dynamic changes in bacterial community by DGGE fingerprint during the composting process. The color bar above represents the temperature dynamics from day 3 to day 42.

Fig. 4. Heatmap and dendrogram of DGGE profiles of the bacterial community. The cluster analysis was based on the unweighted pair-group (UPGMA) method with arithmetic averages by Quantity One. The labels above the dendrogram denote samples from pile A, B, C and D on day 3, 9, 18, 30 and 42.

Fig. 5. Redundancy analysis of the correlation between bacterial community and environmental variables. F1 to F5 represents the soluble-exchangeable, carbonates-bound, Fe-Mn oxides-bound, organic matter-bound and residual Pb, respectively. Significant environmental variables are indicated by *solid lines with filled arrows* while supplementary variables by *dotted line with unfilled arrows*. Bacterial species are indicated by *empty triangles* and the *italic numbers* refer to the band number. Samples are represented by *empty circles* (pile B), *gray circles* (pile C), and *black circles* (pile D). *Sample numbers* refer to the sampling days.