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# Responses of microbial communities to a gradient of pig manure amendment in red paddy soils

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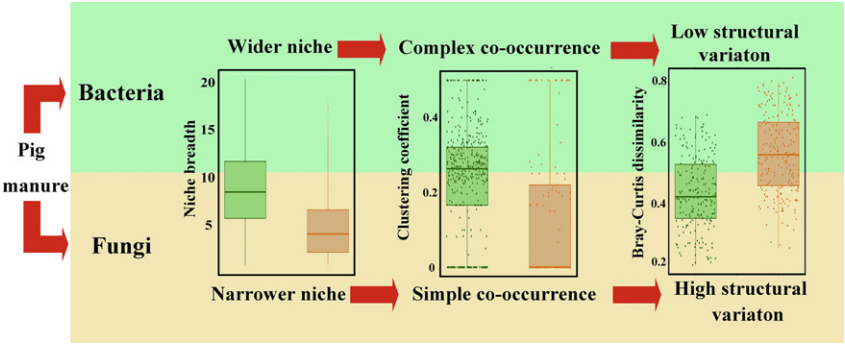
## HIGHLIGHTS

- Bacteria and fungi responded differently to pig manure amendment.
- Bacterial communities are principally governed by heavy metals.
- Fungal communities are primarily controlled by soil physiochemical properties.
- Bacteria had wider niche breadth than that of fungi.
- Bacteria showed closer species co-occurrences than that of fungi.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Microbial communities play a key role in maintaining agroecosystem functioning and sustainability, but their response to excessive animal manure application and relevant mechanisms have not been thoroughly elucidated to date. This study investigated the responses of soil bacterial and fungal communities to pig manure (PM) amendment in red paddy soils. High-throughput sequencing revealed that PM amendment significantly reduced the relative abundance of *Acidobacteria* yet increased that of *Bacteroidetes*, *Ignavibacteriae*, *Firmicutes*, and *Rozellomycota*. The Cu and available phosphorus were the primary impact factors influencing bacterial and fungal diversity, respectively. Bacterial alpha-diversity tended to sharply decrease when the content of soil Cu was >30.70 mg $kg^{-1}$, while fungal alpha-diversity did not continuously increase when the content of soil available phosphorus was >82.84 mg $kg^{-1}$. Bacterial communities with a wider niche breadth showed significantly lower structural variation, whereas fungal communities with a narrower niche breadth showed greater variation in community structure. Soil heavy metals, primarily Cu and Zn, were the primary factors that affected bacterial communities, whereas soil fungal communities were mainly influenced by soil phosphorus. Bacterial and fungal communities showed distinct co-occurrence patterns, with bacterial communities showing a higher degree, a clustering coefficient, and betweenness centrality, but a lower closeness centrality. The findings highlighted that bacteria and fungi responded differently to PM amendment because of their discrepant niche breadth, interspecific relationships, and different tolerance to heavy metal and soil nutrient.



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## 1. Introduction

The fast-growing livestock industry produces a significant amount of livestock waste (Peng et al., 2015; Guo et al., 2018). For example, the livestock waste reached nearly 38 billion tons in China in 2016, and pig manure (PM) accounted for most of it (Shen et al., 2016). PM can effectively improve soil fertility and greatly increase crop yield by imbuing it with a large amount of organic matter, nitrogen (N), and phosphorous (P); hence, the direct application to agricultural fields is a universal method for treating PM (Mandal et al., 2007).

Soil microbial communities play a key role in maintaining agroecosystem functioning and sustainability by influencing biogeochemical cycling and the soil structure (D. Chen et al., 2019; Q.L. Chen et al., 2019; Saleem et al., 2019). Various studies have indicated that manure application significantly affects microbial diversity and composition in different agroecosystems. In a 33-year long-term fertilization experiment in dryland, PM greatly increased microbial biomass and alpha diversity (Luo et al., 2015). Another study on paddy fields got similar results that microbial diversity significantly increased after PM application (Zhang et al., 2012). However, some studies have also indicated that microbial diversity can significantly decline with excessive manure (Sun et al., 2014). This discrepancy calls for further investigation of microbial responses to excessive PM application; more importantly, the ecological mechanisms in mediating these responses need to be investigated.

Until now, several potential mechanisms have explained the microbial responses to PM application. First, PM has been shown to strongly affect microbial communities by shifting oligotrophic organisms toward microbe decomposing complex organic compounds (Hartmann et al., 2015). This occurs because PM is enriched with the types of organic matter and nutrients that benefit microbial growth (Wang et al., 2017). Second, PM can also affect microbiota by changing the soil pH, since soil pH is a fundamental factor that influences microbial communities (Zhou et al., 2017). Third, microorganisms are the first biota that undergoes direct and indirect impacts from heavy metals, which accumulate in arable soils as a result of direct manure application (Ji et al., 2012). For instance, some metals, such as Cu and Zn, are of vital importance for many microbial activities when they occur at low concentrations (Lenart-Boroń and Boroń, 2014). However, high concentrations of heavy metals may have inhibitory or even toxic effects on living organisms (Bruins et al., 2000). Until now, the key environmental factors that shape the community and diversity of soil microbes under direct PM amendment have remained unclear. In particular, as different microbial organisms show distinct heavy metal tolerance abilities (Rajapaksha, 2011), responses of bacterial and fungal communities to heavy metals may differ.

In this study, we assumed bacterial and fungal communities changed differentially under direct PM amendment to agricultural fields because of their different tolerance to nutrient and heavy metal accumulation, especially under a large amount of PM. Thus, the responses of bacterial and fungal communities to PM amendment were investigated using high-throughput sequencing based on a five-year PM amendment experiment in subtropical red paddy soils. Specifically, the aims of this study were to investigate 1) how bacterial and fungal community diversities respond to PM amendment; 2) the key factors that govern variations in microbial communities; and 3) the possible mechanisms that explain the microbial responses to PM application.

## 2. Materials and methods

### 2.1. Experimental design, samples collection, and characterization of soil properties

The field experimental site in Jiangxi, China (28°13′44″ N, 116°53′52″ E), operated since 2013 with a cropping system of double cropping rice (*Oryza sativa* L.) (i.e. early and late season rice), was selected in the present study. The experimental sites had subtropical monsoon climates, with abundant sunshine and rainfall (mean annual sunshine hours = 1739.4 h, mean annual temperature = 17.6 °C, and mean annual precipitation = 1750 mm). A gradient of composted dry PM was applied to each plot (9 m × 3 m) at the beginning of each cropping season at doses of 0 (PM0), 1400 (PM1), 2800 (PM2), 5600 (PM3), 11,200 (PM4), 22,400 (PM5), and 44,800 (PM6) kg $ha^{-1}$, of which PM6 represented extreme conditions of manure amendment in the region (Ma et al., 2016). The composted manure contained 260.33 g $kg^{-1}$ total organic C (TOC), 20.97 g $kg^{-1}$ total N (TN), 22.47 g $kg^{-1}$ total P (TP), and 9.55 g $kg^{-1}$ total potassium (TK) based on the dry weight; it had a C:N ratio of 14.48 and a slightly alkaline pH of 7.43. Plots with different PM amended amounts were arranged in a completely randomized block design. Each treatment was repeated three times. In addition, rice was cultivated successively in every cropping season (approximately May for early-season rice and August for late-season rice every year).

After 10 seasons of successive rice cultivation, soil samples were collected in December 2017. Within each plot, five 20-cm deep soil cores (6 cm in diameter and free from rice roots) were collected, and then mixed thoroughly to obtain a homogeneous composite sample. The bulk soil samples were subsequently refrigerated at 4 °C using a portable fridge and transported to the laboratory. After subsampling into two parts, subsamples for physical and chemical properties were air-dried, ground, and sieved through a 2-mm mesh. Subsamples for microbial properties were stored at −40 °C.

Soil physiochemical properties were determined using the methods described by Pansu and Gautheyrou (2006). Soil pH was assayed using a pH meter (FE30, Mettler-Toledo, CH) with a 1:2.5 soil:water suspension. Cation exchange capacity (CEC) was qualified by ammonium acetate method. Soil organic carbon (SOC) was determined using the sulfuric acid-potassium dichromate oxidation method. Total nitrogen (TN) and available nitrogen (AN) were measured as Kjeldahl-N; total P (TP) and available P (AP) were assayed using HF–$HClO_4$ digestion and sodium bicarbonate extraction (molybdenum blue method), respectively; total K (TK) and available K (AK) were determined by HF-$HClO_4$ digestion and ammonium acetate extraction (flame emission spectrometry). To determine heavy metals, soil samples were digested with a mixture of $HNO_3$/$HClO_4$ (5/1, v/v), and the total contents of Cu, Zn, Cd, and As were analyzed using an inductively coupled plasma–mass spectrometry analyzer (7500a, Agilent, USA).

### 2.2. Soil DNA extraction, amplification, Illumina sequencing, and sequence processing

Soil DNA was extracted from 0.5 g of soil (fresh weight) using a Fast®DNA SPIN Kit (MP Biomedicals, CA, USA) and then subsequently purified using a PowerClean® DNA Clean-up Kit (MoBio, CA, USA) according to the manufacturers' instructions. The concentration and quality of the extracted DNA were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA).

For the 16S rRNA sequencing, each of twenty-one DNA samples was amplified separately using bacterial PCR primers 515F (5′-GTGCCAGC MGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT- 3′) (Biddle et al., 2008) that target the V4-V5 hypervariable region of 16S rRNA genes. For ITS sequencing, DNA samples were amplified separately using the fungal PCR primers ITS1F (5′-CTTGGTCATTTAGAGG AAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (Gardes and Bruns, 1993) that target the internal transcribed spacer 1 (ITS1) region. The PCR protocols that were used to amplify the 16S rRNA gene and ITS gene were described previously (Mukherjee et al., 2014; Ren et al., 2017). The PCR products were then sequenced on the Illumina MiSeq PE250 platform. Raw sequence data were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (http://www.qiime.org/) (Caporaso et al., 2010). Reads with length < 200 bp or with average quality scores <25 were removed, resulting in 3,476,052 high-quality sequences of the bacterial 16S rRNA gene and 2,139,449

high-quality sequences of the fungal ITS gene. Clustering of sequences into OTUs at a 97% nucleotide similarity level was performed using UCLUST (Edgar, 2010). Generally, the sequences were clustered into 17,147 bacterial OTUs and 2697 OTUs after excluding singletons and rarefying them to even sequencing depth (100,000 sequences per sample for bacteria and 50,000 sequences per sample for fungi). The taxonomic identity of the bacterial and fungal OTU was then respectively determined based on comparisons against the Silva database (https://www.arb-silva.de/) and the UNITE database (https://unite.ut.ee/). Raw 16S rRNA sequences and ITS sequences were submitted to the NCBI Sequence Read Archive (SRA) under the accession numbers SRP 218113 and SRP 218116, respectively.

### 2.3. Statistical analysis

The correlation between the PM-amended amount and soil properties was determined through Pearson's test. Alpha-diversity indices including richness and the Shannon-Wiener index were calculated in QIIME using the script "*alpha_diversity.py*". Statistically significant differences in soil properties, variation in species composition, niche parameters, and network parameters were determined through a one-way analysis of variance (ANOVA) along with the use of Duncan's test for multiple comparisons ($P < 0.05$). If the variances of observations were heterogeneous, the nonparametric Mann-Whitney *U* test was used to determine the statistical significance. Bray-Curtis dissimilarity coupled with non-metric multidimensional scaling (NMDS) was conducted to indicate the community dissimilarities.

Three regression models, including linear regression, multiple linear regression and segmented regression, were used to fit the relationship between each alpha-diversity index and each soil property. The AIC score was used to assess the fitting model with the lowest AIC, indicating the best fitting. However, we could not distinguish which model was better if the difference in AIC (ΔAIC) < 2 (Alzarhani et al., 2019).

To help explain the variation in species composition, Levins' niche breadth index (B) (Levins, 1968) was calculated to indicate the niche breadth using package 'spaa' in R language (https://github.com/helixcn/spaa). Briefly, Levins' niche breadth index was calculated using the following formula:

$$B_j = 1/\sum_{i=1}^{N} P_{ij}^2$$

where $B_j$ represents the habitat niche breadth of $OTU_j$ in a metacommunity; N is the total number of communities in each metacommunity; and $P_{ij}$ is the proportion of OTU j in community i. A high B indicated that the OTU occurred widely and evenly along a wide range of locations, representing a wide habitat niche breadth. We calculated the average B-values from all taxa in a single community (*Bcom*) as an indicator of habitat niche breadth at the community level. Levins' niche overlap index (O) was calculated as follows:

$$O_{jk} = \sum_{i=1}^{N} (P_{ij}P_{kj}) / \sum_{i=1}^{N} (P_{ij})^2$$

where $O_{jk}$ represents the niche overlap between $OTU_j$ and $OTU_k$; N is the total number of communities of each metacommunity; $P_{ij}$ is the proportion of OTU j in community i; and $P_{kj}$ is the proportion of OTU k in community i. A high O indicated that the species had more niches overlapping.

To determine the relative importance of soil physiochemical properties (model formula: pH + SOC + TN + TP + TK + AN + AP + AK + CEC) and heavy metals (model formula: Cu + Zn + Cd + As) to microbial community structure, we modeled bacterial and fungal community composition using multivariate negative binomial GLMs (Wang et al., 2012).The number of sequences in each OTU was treated as its abundance. The fit of soil physiochemical property model and heavy metal model was compared using OTU-specific AIC scores. A model was considered to have support over the other model, if the difference in AIC (ΔAIC) > 2 (Alzarhani et al., 2019).The total AIC score across all OTUs (ΣAIC) for each model was then calculated to make comparisons at the community level. Partial Mantel tests were subsequently conducted to determine the potential effects of each soil property on microbial composition.

A network analysis was performed to explore the microbial co-occurrence patterns using the plugin CoNet in Cytoscape 3.5.1 (Shannon et al., 2003). To avoid the bias introduced by different microbial OTU numbers, we selected the relative abundances of the 500 most abundant OTUs for each microbial group. Soil properties were also included to indicate the potential effects of soil properties on microbial co-occurrence patterns. Robust correlations between two OTUs (or between soil properties and OTUs) were defined as those with Spearman's correlation coefficients >0.85 and false discovery rate-corrected *P*-values <0.01. With these, we formed a correlation network in which each node represented one OTU or one soil property, and each edge represented a strong and significant correlation between two nodes. The topology parameters of each network were determined in Cytoscape 3.5.1 using NetworkAnalyzer (Shannon et al., 2003).

## 3. Results and discussion

### 3.1. Soil properties

Animal manure is enriched with organic matter and nutrients including nitrogen, phosphorus, and potassium (Li et al., 2012); thus soil fertility was greatly improved by PM in this research. For instance, in agreement with previous findings (Hati et al., 2008; Lin et al., 2019), soil pH, SOC, TN, TP, AP, AK, and CEC showed significant positive relationships with the amount of PM amendment (Fig. S1, Tables S1, and S2). CEC also showed a significant increase after PM amendment, due to the fact that soil organic matter build-up also increases humic acid colloids and thus the CEC of the soil (Ndayegamiye and Cote, 1989). Soil Cu and Zn increased significantly with the PM amendment amount (Fig. S1, Tables S1, and S2), since heavy metal-additives (Cu, Zn, and others) are frequently added to animal feed in breeding farms (Lu et al., 2014). The additives result in excessive amounts of heavy metal excreted with manure, and these heavy metals subsequently accumulate in soils when animal manure is applied in significant amounts (J. Li et al., 2015; Y.X. Li et al., 2015).

### 3.2. Species composition

For bacterial responses, the 16S amplicon sequencing yielded 165,526 high-quality sequences for each sample on average; *Proteobacteria* (28.63%), *Chloroflexi* (18.24%), *Acidobacteria* (14.43%), *Bacteroidetes* (6.81%), *Ignavibacteriae* (3.90%), *Planctomycetes* (3.47%), *Actinobacteria* (3.02%), *Firmicutes* (2.19%), and *Cyanobacteria* (1.18%) dominated all the samples (Fig. S2). The relative abundance of *Acidobacteria* was significantly negatively correlated with the PM amended amount, whereas the relative abundance of *Bacteroidetes*, *Ignavibacteriae*, and *Firmicutes* showed an opposite trend (Table S3). Various studies have already indicated that biomass and the relative abundance of *Acidobacteria* (most subgroups) in soil are greatly influenced by soil pH, with the highest abundances present in the lowest pH soils (Lauber et al., 2009; Shen et al., 2013; D. Chen et al., 2019; Q.L. Chen et al., 2019). In this research, the PM amendment significantly increased soil pH; thus, the soil condition became more unfavorable for the survival of *Acidobacteria*, resulting in a significant decrease in them. *Bacteroidetes* are considered a typical *copiotrophic* bacteria (Fierer et al., 2007) that can convert cellulose and hemicellulose into smaller polysaccharides (Ren et al., 2014; Wu et al., 2019). Thus, *Bacteroidetes* increased with the amendment of abundant PM enriched with cellulose and hemicellulose (Ren et al., 2014). *Firmicutes* are also *copiotrophic* bacteria (Fierer et al., 2007), but they are important members in PM (Zhou et al.,

2016; Wang et al., 2017). Therefore, the relative abundance of *Firmicutes* was also significantly increased with PM amendment, not surprisingly.

For the fungi analysis, ITS amplicon sequencing yielded 101,879 high quality sequences for each sample on average, with *Ascomycota* (37.95%), *Mortierellomycota* (34.94%), *Basidiomycota* (6.19%), *Chytridiomycota* (4.64%), and *Rozellomycota* (1.41%) dominating all the samples (Fig. S2). The relative abundance of *Rozellomycota*, which is frequently detected in animal guts (Grossart et al., 2016), was significantly positively correlated with the PM amendment amount and soil properties including pH, TP, AP, and AK (Table S4). Similar to *Rozellomycota*, the relative abundance of the anaerobic fungal phylum *Neocallimastigomycota*, which can also be frequently detected in animal guts (Kameshwar and Qin, 2018), was significantly correlated with PM and some other soil properties (Table S4). Apart from the above two phyla, almost all the other fungal phyla did not show a significant positive or negative tendency in relative abundance (Table S4).

### 3.3. Alpha diversities

Bacterial alpha-diversity indexes, including richness and the Shannon-Wiener index, did not change significantly under low a PM amended amount, whereas they decreased sharply when the PM amount was higher than 11,200 kg ha$^{-1}$ (Fig. 1a). Unlike bacterial alpha-diversity, fungal alpha-diversity tended to gradually increase and reach a plateau with a PM amended amount of 5600 kg ha$^{-1}$ (Fig. 1b). Moreover, AIC scores for regression models showed that Cu content best fit the bacterial alpha-diversity indexes using a segmented regression model (Fig. 1c–d, Tables S5, and S6), suggesting that 30.70 mg kg$^{-1}$ and 30.81 mg kg$^{-1}$ of Cu are the thresholds for bacterial richness and the Shannon-Wiener index, respectively. Beyond these thresholds, bacterial alpha diversities decreased significantly (Fig. 1c–d). The AIC scores of the regression models showed that the AP content best fit both with the fungal alpha-diversity indexes and with richness using a segmented regression model (Fig. 1e, Table S7). In addition, this result was also found using the Shannon-Wiener index using a linear regression model and a multiple regression model (Table S8). Specifically, fungal richness stopped increasing when AP content was >82.84 mg kg$^{-1}$. Yet in the fungal Shannon-Wiener index, both solutions of the multiple regression model were invalid in the study ($x_1 = -7.014$ mg kg$^{-1}$, $x_2 = 267.44$ mg kg$^{-1}$), suggesting that the fungal Shannon-Wiener index may not be influenced by AP content or other tested soil properties under PM amendment. These results agree with the results of the random forest model, which also demonstrated that heavy Cu was a better predictor of bacterial alpha-diversity, whereas fungal alpha-diversity was better predicted by the AP content (Table S9).

The distinct response of bacterial and fungal alpha diversities to soil nutrient status and heavy metal contents could be explained using the following mechanisms. First, bacteria and fungi have discrepant heavy-metal tolerance abilities (Rajapaksha, 2011). Generally, soil fungi are more tolerant to heavy metals than bacteria. Rajapaksha

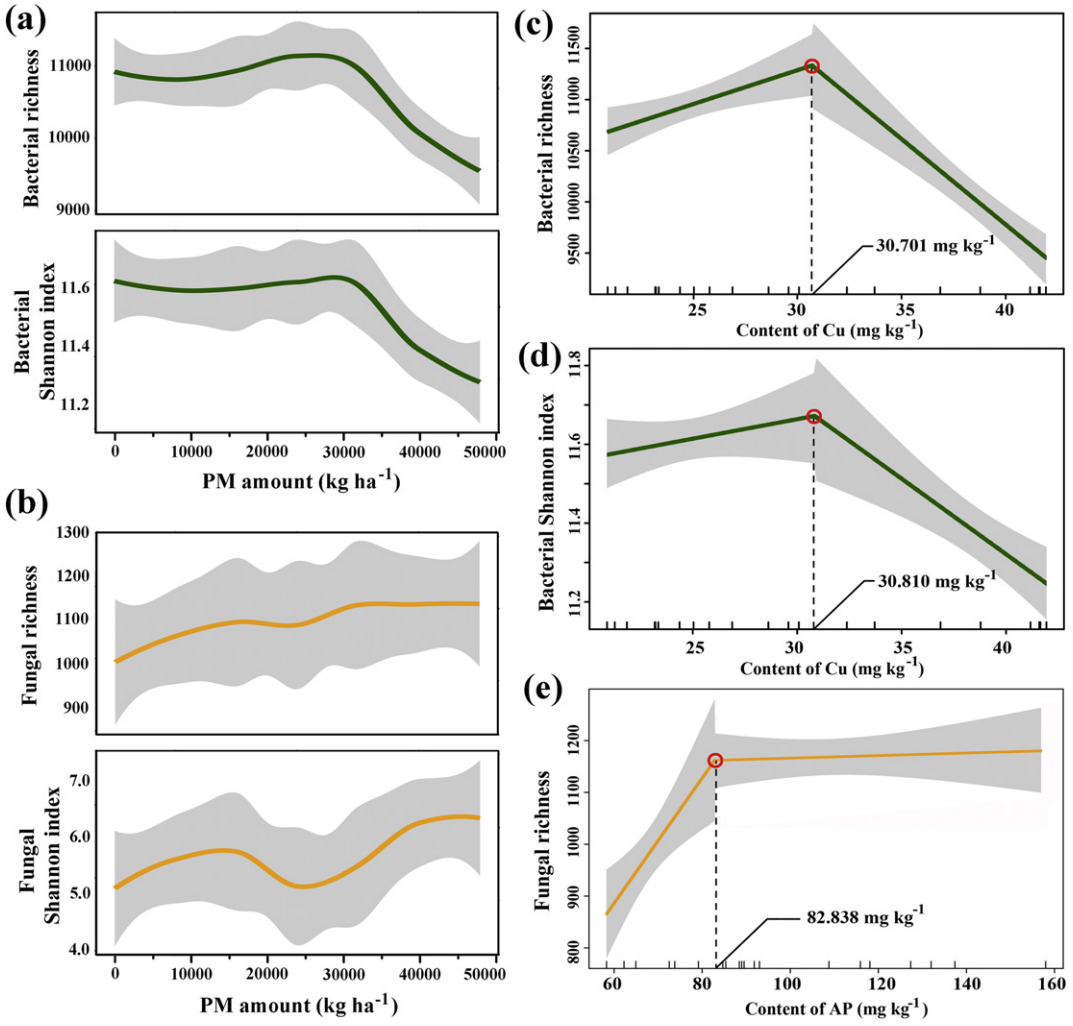


**Fig. 1.** (a–b) Alpha-diversity of bacterial and fungal communities. (c–d) Segmented regression models showing the relationships between Cu content, bacterial richness, and Shannon-Wiener index. (e) Segmented regression models showing the relationship between the content of AP and fungal richness. The horizontal axis of a-b represents the PM amounts of treatment PM0 to PM6.

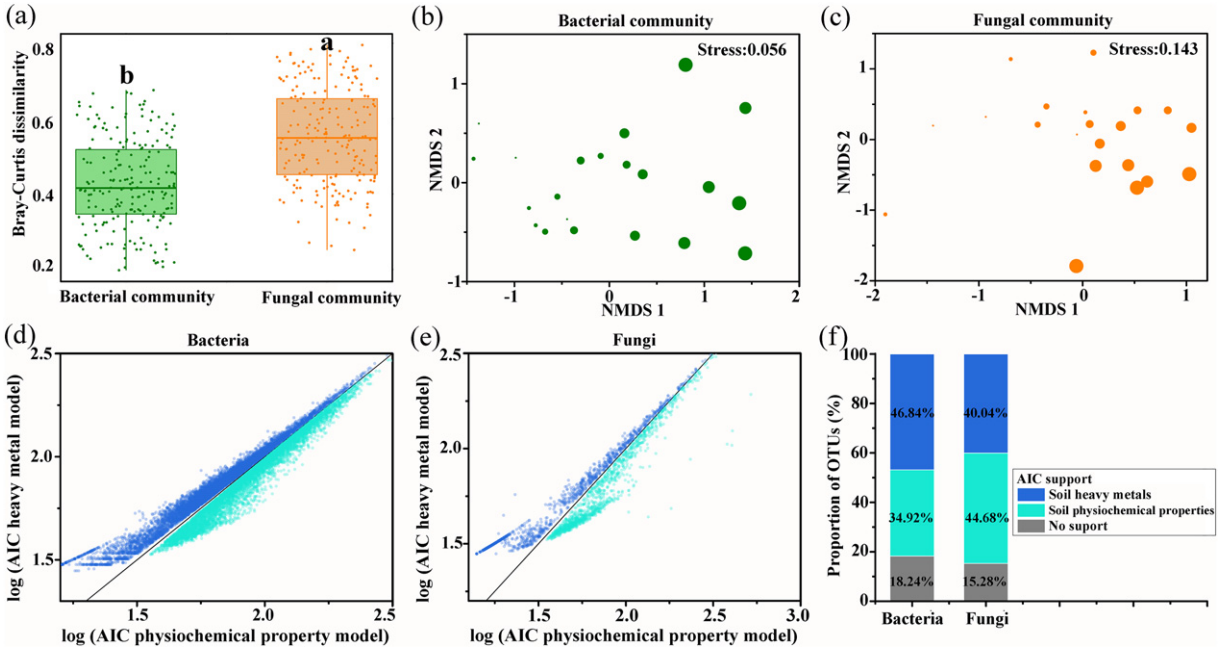


**Fig. 2.** (a) Bray-Curtis dissimilarity of microbial communities. Lowercase letters above the boxes indicate significant difference at $P < 0.05$. (b–c) NMDS based on Bray-Curtis dissimilarity of bacterial and fungal communities. Larger sizes indicate a higher PM amended amount. (d–e) The AIC scores of the soil physiochemical property model and soil heavy metal model for bacterial and fungal community composition. A lower AIC score represents a superior fit (relative to the number of variables in each model). Solid lines indicate equal AIC scores for the two models. For visual clarity, OTUs with an AIC difference of <2 have been removed. (f) The proportion of bacterial and fungal OTUs whose abundance was better modeled with either soil physiochemical or heavy metal variables. As above, "No support" indicates OTUs for which the difference in AIC between the two models was <2, indicating that neither model was sufficiently better than the other.

et al. (2004) demonstrated that bacterial activities decreased sharply (by 90%) under heavy metal (Cu and Zn) contamination, whereas fungal activities turned very strong (three to seven times higher than the control), even under very high levels of metal contamination. In addition, Huysman et al. (1994) found that fungi were approximately twice, and even thousands of times, more tolerant to Cu than bacteria in natural and experimental conditions. Second, fungi are more sensitive than bacteria to soil fertility and P addition (He et al., 2008; J. Li et al., 2015; Y.X. Li et al., 2015). Usually, fungal hyphae can extend into soil and increase the surface area for water and nutrient absorption. Therefore, easy accessibility to soil availability and even immobile P may contribute to the sensitivity of fungi to P addition. Additionally, the possible antibiotics in PM may also drive an opposite tendency in bacterial and fungal diversities as a result of differentiation in antibiotics tolerance abilities between bacteria and fungi (Ding and He, 2010; Qian et al., 2018). Although soil acidification also influences bacterial and fungal diversities (D. Chen et al., 2019; Q.L. Chen et al., 2019), it had no effect in our study. The possible reason was that the soil pH was close to neutral in our study (Table S1), which might have caused the effects of other soil properties to override the influences of pH.

### 3.4. Structure of microbial communities and potential influencing factors

The Bray-Curtis dissimilarity among fungal communities was significantly higher than those of bacterial communities (Fig. 2a), reflecting that fungal communities showed a higher variation in species composition. This suggested that soil fungi are more sensitive than bacteria to PM amendment. An NMDS analysis showed that both the bacterial and fungal communities clustered into different groups according to the PM amended amount (Fig. 2b–c). A multivariate negative binomial GLMs analysis showed that more bacterial OTUs could be better predicted using heavy-metal variables (46.84%) than soil physiochemical variables (34.92%), whereas more fungal OTUs could be better predicted using soil physiochemical variables (44.68%) than heavy metal variables (40.04%) (Fig. 2d–f). The ΣAICs for each model also supported that bacterial communities can better fit a heavy-metal model, and fungal communities can better fit a soil physiochemical model (Table S10). The partial Mantel tests further confirmed that Cu and Zn were largely correlated with bacterial communities, whereas soil TP and pH were largely correlated with fungal communities (Table 1). These results agreed with the documented conclusion that soil bacteria were more sensitive to heavy metals than fungi under PM amendment (Rajapaksha et al., 2004; Rajapaksha, 2011; Lenart-Boroń and Boroń, 2014). Although pH has been suggested as the most important predictor of bacterial composition (Lauber et al., 2009; Ren et al., 2018), it was less effective in shaping microbial communities than other soil characteristics in the present study. This might have been because the soil pH was close to neutral and was less variable in our samples (Table S1).

### 3.5. Niche breadth and co-occurrence of microbial communities

A niche analysis was conducted to explain the distinct responses in the species composition of the bacterial and fungal communities. According to the niche analysis, bacteria had a higher niche breadth,

**Table 1**
Partial Mantel tests showing the effects of each soil property on composition of microbial communities. Values in bold font represent significant correlations at $P < 0.05$.

| Variable | Bacterial communities | | Fungal communities | |
|---|---|---|---|---|
| | r | *P* | r | *P* |
| **pH** | **0.800** | **< 0.001** | **0.447** | **< 0.001** |
| **SOC** | **0.476** | **< 0.001** | **0.226** | **0.046** |
| **TN** | **0.452** | **< 0.001** | **0.289** | **0.027** |
| **TP** | **0.725** | **< 0.001** | **0.455** | **< 0.001** |
| TK | 0.115 | 0.168 | 0.102 | 0.236 |
| AN | 0.004 | 0.461 | 0.038 | 0.345 |
| **AP** | **0.648** | **< 0.001** | **0.432** | **< 0.001** |
| **AK** | **0.503** | **< 0.001** | **0.352** | **0.037** |
| **CEC** | **0.680** | **< 0.001** | **0.358** | **< 0.001** |
| **Cu** | **0.836** | **< 0.001** | **0.412** | **< 0.001** |
| **Zn** | **0.821** | **< 0.001** | **0.413** | **< 0.001** |
| Cd | 0.136 | 0.912 | 0.104 | 0.201 |
| As | 0.040 | 0.526 | 0.069 | 0.619 |

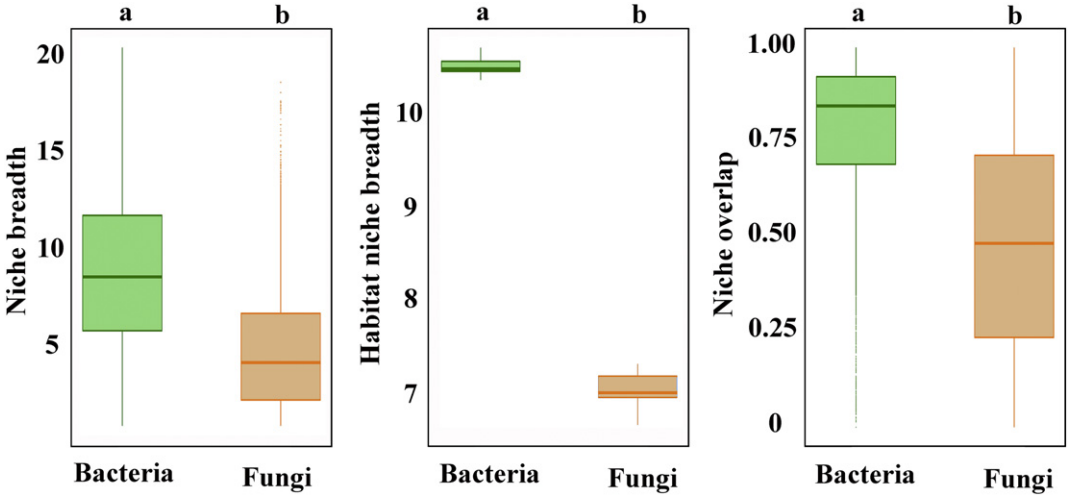


**Fig. 3.** Niche analysis showing the (a) niche breadth, (b) habitat niche breadth (*Bcom*), and (c) niche overlap of microbial communities. Lowercase letters above the boxes indicate a significant difference at $P < 0.05$.

*B*com, and niche overlap than fungi (Fig. 3). Species with a wider niche breadth are distributed more widely and evenly, and consequently are more able to keep stable when suffering environmental disturbance (Pandit et al., 2009; Li et al., 2019). This can partially explain why the variation in species composition was much lower in the bacterial than the fungal communities with large amounts of PM amendment (Fig. 2a).

A co-occurrence network analysis showed that the bacterial network was more complex than the fungal network (Fig. 4a–b), with 397 nodes and 4449 edges in the bacterial network and 113 nodes and 189 edges

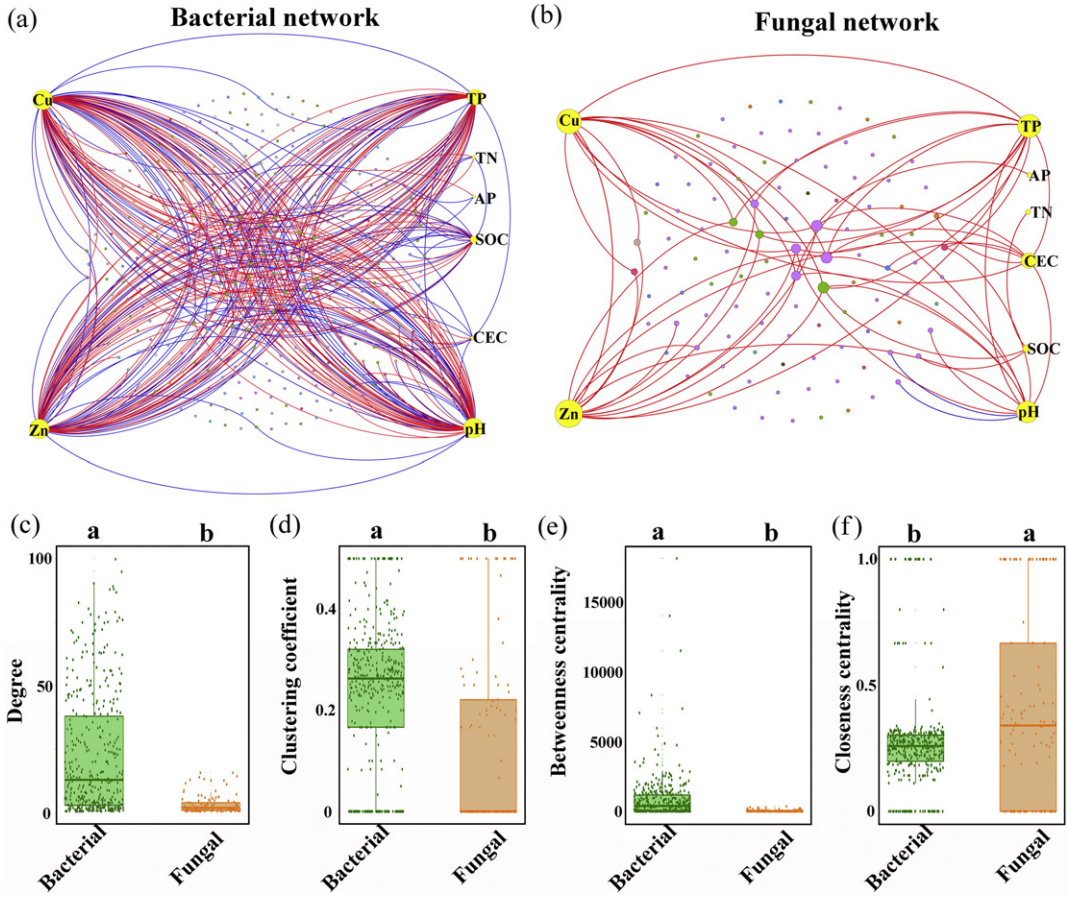


**Fig. 4.** Network analysis showing the co-occurrence of (a) bacterial communities and (b) fungal communities. The co-occurring networks are colored by microbial taxonomic information at the phylum level. Here, only edges that are directly related to soil properties are shown to better visualize the potential effects of soil properties on microbial co-occurrence patterns. The red lines represent positive interrelationships, and the blue lines represent negative interrelationships. The size of the node represents the connecting degree. The width of the lines represents the strength of the correlation. Topology parameters of networks, including (c) degree, (d) clustering coefficient, (e) betweenness centrality, and (f) closeness centrality, were calculated based on bacterial and fungal networks. Lowercase letters above the boxes indicate a significant difference at $P < 0.05$ according to a nonparametric Mann-Whitney *U* test.

in the fungal network (Table S11). The bacterial network had a higher average degree, a more complex network, and more coupling among the OTUs (Fig. 4a–c). A similar trend was observed in the average clustering coefficient (*avgCC*), as bacterial (0.239) > fungal (0.131) ($P < .05$, Fig. 4d). The *avgCC* value was used to evaluate the quality of the connections among the nodes (Zhou et al., 2011), which is another efficient parameter to measure microworld properties (Steele et al., 2011). The higher *avgCC* values in the bacterial network than the fungal network suggested that the application of PM may remit competition between bacteria. Betweenness centrality is a measure of the possible influence of an individual node on other nodes (Jiao et al., 2017). The higher betweenness centrality of the bacterial network indicated that bacteria occupied wider core niches than the fungal taxa did (Fig. 4e), confirming the results of the niche analysis (Fig. 3). Closeness centrality looks for the node that is closest to all other nodes, indicating how close a node is to all other nodes in the network and reflecting the speed of information transmission between nodes to a certain extent (Jiao et al., 2017). The higher closeness centrality of the fungal network implied that information transmission between fungi was higher than that between bacteria (Fig. 4f); thus, fungi can make a response to environmental disturbance more quickly. In the bacterial network, 64.60% of the edges were positive and 35.40% of the edges were negative. In the fungal network, 98.94% of edges were positive and only 1.06e% of the edges were negative (Table S10). Positive correlations dominated both the bacterial and fungal networks (Fig. 4a–b), implying that mutual cooperation rather than competitive exclusion played a more important role in microbial assembly. Negative correlations in the bacterial network were much higher than in the fungal network (Fig. 4a–b), implying that inter-competition occurred more frequently in bacterial communities (Feng et al., 2017). In addition, the niche overlap was much higher in bacteria (Fig. 3), supporting that bacteria competed more strongly. Such a co-occurrence pattern visualized the scenarios of biotic interactions and implied that bacteria were more tightened and closely related to each other than fungi under PM amendment. This suggested that bacterial communities possibly keep a relatively stable state through corporation or competition. This can explain why the variation in fungal communities was much higher than that in the bacterial communities under PM amendment (Fig. 2).

Although positive correlations dominated the bacterial network, the proportion of the negative edges that directly correlated with Cu (53.93%) and Zn (53.41%) was slightly higher than those of the positive edges (Fig. 4a). In contrast, in the fungal network, none of the edges that directly correlated with Cu and Zn were negative (Fig. 4b). In addition, the number of edges directly correlated with Cu and Zn was much higher in the bacterial network. This implied that Cu and Zn may exclude some bacterial taxa while maintaining some fungal taxa. The niche breadth and co-occurrence networks supported the opinion that bacteria and fungi have different tolerance abilities to heavy metals and different sensitivities to soil nutrient status (Rajapaksha, 2011).

## 4. Conclusions

This study demonstrated that soil bacteria and fungi responded differently to PM application in red paddy fields. It indicated that under PM amendment, soil Cu content can best predict the change in bacterial community diversity, whereas phosphorus can best predict fungal community diversity. Bacterial communities had more complex interspecific relationships and a broader niche breadth, therefore showed lower structural variation. While fungal communities had simpler interspecific relationships and a narrower niche breadth, they exhibited greater structural variation under PM amendment.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135884.

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