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Environmental antibiotics drives the genetic functions of resistome dynamics

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ABSTRACT

The increasing prevalence of antibiotic-resistant microorganisms imposes a global threat to public health. The over reliant use of antibiotics in the food industry has contributed considerably to the dissemination of antibiotics into various environments, yet the mechanisms by which antibiotic dissemination influences the assembly of the microbial community continues to remain obscure. Here, we examine bacterial and fungal community assemblies in swine manure, compost, compost amended, and unamended agricultural soil in five suburban areas of Beijing, China. Total antibiotic concentration decreased by factors of 10–1000 from manure and compost to soils. The bacterial α-diversity was found to be low in manure and compost samples, while the fungal α-diversity was similar across all samples. We detected significantly ($p < 0.05$) higher relative abundances of well recognized pathogenic microbial taxa, virulence associated genes, and antibiotic resistance genes (ARGs) in manure and compost than those in agricultural soils, revealing the higher microbial capacity of pathogenicity, virulence and antibiotic resistance. Unexpectedly, the relative abundances of both bacterial and fungal taxa did not predict the antibiotic concentration. A possible explanation was that bacterial and fungal communities were mainly shaped by random assemblies. Rather, antibiotic concentration could be well predicted by relative abundances of antibiotic resistance, stress and virulence associated genes. Despite the weak interconnection between ARGs and the microbiome, we demonstrate that microbial genes should be the focal point in tracking the ecological effects of antibiotic dissemination by revealing microbial community patterns along the dissemination chain of antibiotics.

1. Introduction

Livestock is commonly treated with antibiotics to prevent animal disease, making livestock manure a large reservoir for the antibiotic residuals. Composts of aerobic or anaerobic digestion of livestock manure are commonly used as organic fertilizers for agricultural crops worldwide (Marti et al., 2013). Antibiotic resistance genes (ARGs) and their bacterial hosts, which are a part of the microbial resistome (Costa et al., 2006), can be released into the soil environment along with the dissemination of antibiotics from manure or compost (Su et al., 2015).

There are growing concerns about the possible contributions of both manure and compost to increases of ARGs found in the soil resistome and in known pathogenic bacteria. It was shown that the relative abundance of β-lactam resistant bacteria was much higher in manure-amended soil than in unamended soil (Gou et al., 2018). Excessive application of manure with intensive sulfonamide increased soil ARGs (Heuer et al., 2011). Since there is frequent exchange of ARGs between bacteria in animal manure and clinical pathogens (Jiang et al., 2017), an increase of ARGs in the soil resistome has potential consequences for human health.

Ecological processes, including deterministic (e.g. abiotic and biotic selection) and stochastic processes (e.g., birth, death, random immigration and dispersal limitation), are major forces in shaping microbial communities (Zhang et al., 2019; Zhou et al., 2014). Bacterial diversity has been widely documented to decrease with higher antibiotic levels, owing to the antibiotic selection (Raymond et al., 2016).

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However, ARGs can enhance antibiotic resistance of certain bacterial taxa (Larkin et al., 2006), which can use antibiotics as nutrient sources (Dantas et al., 2008; Liao et al., 2017). In the presence of high concentrations of antibiotics, fungal growth might be favored because fungi are not sensitive to antibiotics and are able to degrade complex organic compounds (Deng et al., 2012).

It is still unclear whether ARGs can be predicted by microbial community composition (Forsberg et al., 2014; Su et al., 2015). During a lab-scale composting of sewage sludge, diversity and abundance of ARGs were strongly correlated with bacterial compositions (Su et al., 2015). Similarly, microbial phylogenetic and taxonomic structure are the primary determinants of soil ARG content both across and within soil types in global soil resistome (Forsberg et al., 2014). However, other studies conclude that ARGs may not correlate with the taxonomic signatures of specific microbial communities since ARGs can be highly mobile with the help of genetic elements such as bacteriophages and plasmids (Subirats et al., 2016). Resistance cluster alleles were enriched in swine farms (Johnson et al., 2016), yet this enrichment was independent of the bacterial phylogenetic composition. Due to these discrepancies in resistance allele enrichment being independent of community composition, the response for microbial functionality and assembly to antibiotic dissemination remains controversial.

Here, we examine how bacterial and fungal communities respond to the residual antibiotics found in manure from swine farms, compost, compost-amended agricultural soil, and -unamended agricultural soil (control soil). We hypothesize that: (1) ARG and virulence gene abundance decay from manure to compost to compost-amended soil, which corresponds to changes of antibiotic concentrations; (2) bacterial but not fungal diversity is lower in manure and compost than in soils; (3) due to the functional redundancy inherent in microbial communities, composition of microbial ARGs can be decoupled from microbial taxonomy (Galand et al., 2018; Louca et al., 2018). Therefore, ecological processes underlying microbial taxonomy assembly might differ from those of regulating microbial gene structure along the dissemination chain of antibiotics.

2. Material and methods

2.1. Sample collection and measurements of geochemical factors

In April 2015, we collected samples from five swine farms located in the suburban area of Beijing, China (Fig. S1). In each swine farm, four manure samples and four aerobic compost samples were taken. Four soil samples applied with the compost (i.e., compost-amended soil) were also taken from a nearby agricultural field. Four control soil samples were taken from soils without compost amendment in the agricultural field. Compost-amended soil and control soil samples were mixed by three soil cores and were taken at the 0–15 cm depth.

The temperature was measured on-site for three times by thermometer and averaged for each sample. Similarly, water content was measured three times by hygrometer and averaged. We focused on antibiotics of tetracyclines, sulfonamides, and quinolones since these three categories of antibiotics are commonly used in livestock practices (Yang and Carlson 2003). Concentrations of chlortetracycline, oxytetracycline, tetracycline, norfloxacin, ofloxacin, sulfadiazine, sulfadimidine, sulfamerazine, and sulfamethoxazole were analyzed using a previously published method (Zhao et al., 2010). Briefly, 0.1 g of Na_2EDTA and 10 mL of an extraction solution containing phosphate buffer (pH = 3.0) and acetonitrile (1:1 vol/vol) were used. After sonication for 30 min, the samples were then centrifuged at 7,000 × g for 10 min. The extraction process was performed three times for each sample. The obtained supernatants were combined and then diluted to 500 mL with deionized water, filtered through 0.45 μm filters, and acidified to pH 3.0 before solid-phase extraction. The samples were extracted using 6 mL of Oasis HLB extraction cartridges. Final extracts were transferred to 2 mL amber vials for analysis of liquid chromatography-MS/MS (ABI 3200 Q TRAP, Applied Biosystems, California, USA).

Metals including zinc, copper, lead, arsenic, and mercury were measured for each sample. 0.1 g of air-dried and milled samples were weighed into 50 mL polypropylene digest tubes. Then, 2 mL of concentrated HNO_3, 3 mL of HCl, and 1 mL of $HClO_4$ were added sequentially. Tubes were capped and stood overnight. Tubes were randomized and heated in a microwave-assisted digestion system (CEM Corporation, Matthews, NC). The temperature was raised to 120 °C within 15 min with a holding time of 20 min. Temperature was then raised to 170 °C within 15 min with a holding time of 30 min. After cooling to room temperature, liquid in tubes was transferred to 50 mL volumetric flasks. The liquid was stored and passed through a 0.45 μm × 13 m nylon filter (Membrana, Corp., and Gelman Sciences) before analysis. For quality control, reference materials of GBW-07401 and GBW-07405 (IGGE IRMA, China, 590 ± 80 ng g^{-1}) were included in the analysis. The average recovery rate was 90%. In addition, concentrations of metals were measured by 7500cx inductively coupled plasma mass spectrometry (Agilent, Forrest Hill, Victoria, Australia).

2.2. DNA extraction

DNA was extracted from 1.5 g of each sample by a freeze-grinding and SDS-based lysis as described previously (Zhou et al., 1996), and purified with a MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. The DNA quality was assessed based on 260/280 nm and 260/230 nm absorbance ratios using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). PicoGreen was used in quantifying the final DNA concentrations with a FLUOstar Optima fluorescence plant reader (BMG Labtech, Jena, Germany). DNA was stored at −80 °C until further analysis.

2.3. Amplicon sequencing experiments and raw data analyses

The V4 hypervariable regions of 16S rRNA genes were amplified with the primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′ GGACTACHVGGGTWTCTAAT-3′). Fungal ITSs between 5.8S and 28S rRNA genes were amplified with the primer pair ITS7F (5′-GTGARTCATCGARTCTTTG-3′) and ITS4R (5′-TCCTCCGCTTATTGA TATGC-3′). The sequencing experiment was carried out on a MiSeq equipment (Illumina, San Diego, CA, USA) using 2 × 250 pair-end format. Raw sequences were submitted to our open-source Galaxy sequence analysis pipeline (http://zhoulab5.rccc.ou.edu:8080) (Guo et al., 2019). Firstly, each sequence was assigned to its corresponding sample according to the barcode. Before combining the forward and reverse reads, the primer sequences at the end of each read were trimmed by the Btrim program, then a quality control threshold > 30 over a 5 base pair (bp) window size was used to filter the reads (Kong 2011). Secondly, paired-end reads with at least a 50 bp overlap and < 5% mismatches were joined using FLASH (Magoč and Salzberg 2011). Any joined sequences with an ambiguous base, or a length of < 245 bp for the 16S rRNA gene or < 220 bp for the ITS were discarded. Thirdly, sequences were clustered into OTUs using UPARSE7 at 97% identity (Edgar 2013), and singleton OTUs (with only one read) were removed. All sequences were randomly resampled to the depth of 31,109 sequences per sample for the 16S rRNA gene and 10,269 sequences per sample for ITS. Representative sequences from each OTU were taxonomically annotated by the Ribosomal Database Project (RDP) Classifier on the website (https://rdp.cme.msu.edu/classifier/classifier.jsp) with 50% confidence estimates (Wang et al., 2007). The version of "16S rRNA training set 14" on the website was used for 16S rRNA gene classification, and the version of "UNITE fungal ITS trainset 07-04-2014" was used for ITS sequence classification.

2.4. GeoChip hybridization and raw data processing

The functional gene array GeoChip 5.0 was used for DNA microarray hybridization. Scanning of GeoChip hybridization and data processing were performed as described previously (Liu et al., 2015). In short, DNA samples were labeled with fluorescent dye Cy-3 dUTP and hybridized with the slides with GeoChip 5.0 M in a rotator/incubator at 67 °C plus 10% formamide and rotated at 20 rpm for 24 h. After hybridization, the slides were scanned with a NimbleGen MS200 Microarray Scanner (Roche, South San Francisco, CA, USA), and the scanned images were digitally extracted using the Agilent Feature Extraction software v11.5. Raw data were submitted to the Microarray Data Manager on the website (http://ieg.ou.edu/microarray). We removed spots with the signal-to-noise ratio below 2, considered as poor quality. Both the universal standard and functional gene spot intensities were used to normalize the signals among arrays.

2.5. Statistical analyses

Differences in antibiotic and metal concentrations in different samples were determined by Principal Component Analysis (PCA) based on Euclidean distance. Mantel test was used to evaluate correlations between dissimilarity matrices of microbial genes (based on GeoChip data), bacterial community, fungal community and dissimilarity matrices of environmental factors including antibiotics, metals and soil geochemical factors. In the Mantel test, Bray-Curtis distance was used to obtain dissimilarity matrices of microbial genes as well as bacterial and fungal OTUs. Euclidean distance was used to obtain dissimilarity matrices of environmental factors. We also used a Mantel test to examine correlations between dissimilarity matrices of ARGs (Bray-Curtis distance) and dissimilarity matrices of bacterial or fungal communities (Bray-Curtis distance). Comparisons of bacterial and fungal diversities between different farms were examined by ANOVA test. All analyses were performed in R.

The response ratios of treatment to control were used to compare relative abundances of microbial genes in different sample types. ARGs, virulence, metal resistance, organic remediation, secondary metabolism, carbon degradation, and virus-associated genes covered by GeoChip were examined. Since there are multiple gene probes for each individual gene on the GeoChip microarray, relative abundances of all probes for an individual gene were summed. Then, the response ratio was calculated for each gene. A source-tracking program (Knights et al., 2011) was performed to compare the community composition in compost-amended soil to that in manure, compost, and control soil to track sources of microbial OTUs detected in compost-amended soil.

Random forest was used for the regression between predictors (i.e., the abundance of individual microbial genes or bacterial/fungal OTUs) and total antibiotic concentration. Random forest models were constructed using 'randomForest' function in the R package by following steps (Liaw and Wiener 2002): (i) drawing n bootstrap samples from the dataset; (ii) for each bootstrap sample, growing regression trees (n = 500) by randomly sampling m_{try} (number of predictors randomly sampled as candidates at each split) of the predictors and choosing the best split from these predictors at each tree node. By default, $m_{try} = p/3$, where p is the total number of predictors; (iii) predicting new data by aggregating the predictions of n bootstrap samples, i.e., majority votes for averages of regressions. Accordingly, the parsimonious random forest model is the outcome of an ensemble of decision trees based on bootstrapped samples from a dataset (Breiman 2001). The Pseudo-R^2, which represents how much of the variation in antibiotic concentration is explained by the random forest model, was reported. The predictors were selected based on mean decrease accuracy values. The mean decrease accuracy value represents the decreasing prediction accuracy if one predictor is replaced by others. Therefore, predictors with higher mean decrease accuracy values were selected. The squared correlation coefficient (r^2) for the correlation between the relative abundance of each predictor and total antibiotic concentration was also calculated. We also used partial dependence plots to show how the relative abundance of a given gene category changed in association with total antibiotic concentration (Hastie et al., 2009).

We calculated Raup–Crick (RC) values to determine the assembly processes of microbial communities (Stegen et al., 2013). We used Bray-Curtis distance to examine whether the observed degree of community assembly deviated from that expected if the community was assembled by stochastic processes. $|RC| > 0.95$ indicates that turnover in community composition is governed primarily by a deterministic process such as selection, dispersal limitation or homogenizing dispersal; $|RC| < 0.95$ is indicative of stochastic assembly (Chase et al., 2011). In addition, we calculated the standard effective size (SES) (Kraft et al., 2011), which calculated β deviation as observed β diversity minus the mean of the null distribution of β diversity values, divided by the standard deviation of this distribution. We took a set of simulations (n = 999) and applied the null model randomization to calculate β deviation. Therefore, β deviation represents a standard effect size, with + 2 and −2 representing 95% confidence intervals for the null hypothesis that average SES equals zero. $|SES| > 2$ indicates that community assembly is significantly different from the null hypothesis, revealing deterministic processes. On the contrary, $|SES| < 2$ indicates that community assembly is not different from null expectations, revealing stochastic processes.

3. Results

3.1. Antibiotics, heavy metals, and soil variables

All target antibiotics were detected in every sample (Table S1). Tetracycline antibiotics, which can be naturally produced by Streptomyces (Aminov 2017; Jukes 1985), showed an average concentration of 96,204 $\mu g \cdot kg^{-1}$ in manure, 136,850 $\mu g \cdot kg^{-1}$ in compost, 3,042 $\mu g \cdot kg^{-1}$ in compost-amended soil, and 291 $\mu g \cdot kg^{-1}$ in control soil. The concentration of quinolones, which are typically synthesized antibiotics against animal infections (Aminov 2017), was high in manure (6,727 $\mu g \cdot kg^{-1}$) and compost (737 $\mu g \cdot kg^{-1}$) but was low in compost-amended soil (21 $\mu g \cdot kg^{-1}$) and control soil (8 $\mu g \cdot kg^{-1}$). Sulfonamides, which are also fully synthetic antibiotics (Aminov 2017), showed the lowest concentrations for most of the samples, averagely 350 $\mu g \cdot kg^{-1}$ in manure, 661 $\mu g \cdot kg^{-1}$ in compost, 141 $\mu g \cdot kg^{-1}$ in compost-amended agricultural soil and 65 $\mu g \cdot kg^{-1}$ in control soil. Among sulfonamides, sulfamethoxazole had the highest concentrations, ranging from 60 $\mu g \cdot kg^{-1}$ to 294 $\mu g \cdot kg^{-1}$. We also examined the variation in antibiotic concentration between farms. The concentration of six categories of antibiotics (chlortetracycline, oxytetracycline, tetracycline, norfloxacin, sulfamerazine and sulfamethoxazole) and total concentration of all antibiotics did not vary significantly between farms (Fig. S2).

Principal Component Analysis (PCA) was used to assess differences between the total antibiotic concentrations of different samples. The samples were divided into two distinct clusters according to the total antibiotic concentrations: the cluster constituted by manure and compost samples and the cluster constituted by compost-amended soil and control soil samples (Fig. 1a). Relatively high antibiotic concentrations in manure and compost decayed to low levels (near 0%) in compost-amended soil for most antibiotics (Fig. 1c). However, sulfamethoxazole and sulfadimidine showed lower decay rates than others.

Metals of lead and arsenic, often used as feed additives in livestock farms, showed higher ($F = 6.07$, $p < 0.05$, ANOVA) concentrations in manure and compost than in control soil (Table S1). The highest average concentrations of lead and arsenic were 9,212 $\mu g \cdot kg^{-1}$ and 320 $\mu g \cdot kg^{-1}$, respectively. By contrast, zinc and copper showed higher concentration in soils than in manure and compost (Table S1). Zinc and copper are both strongly associated with soil organic matter and bind as organic complexes (Han et al., 2001; Harrison et al., 1981). Animal

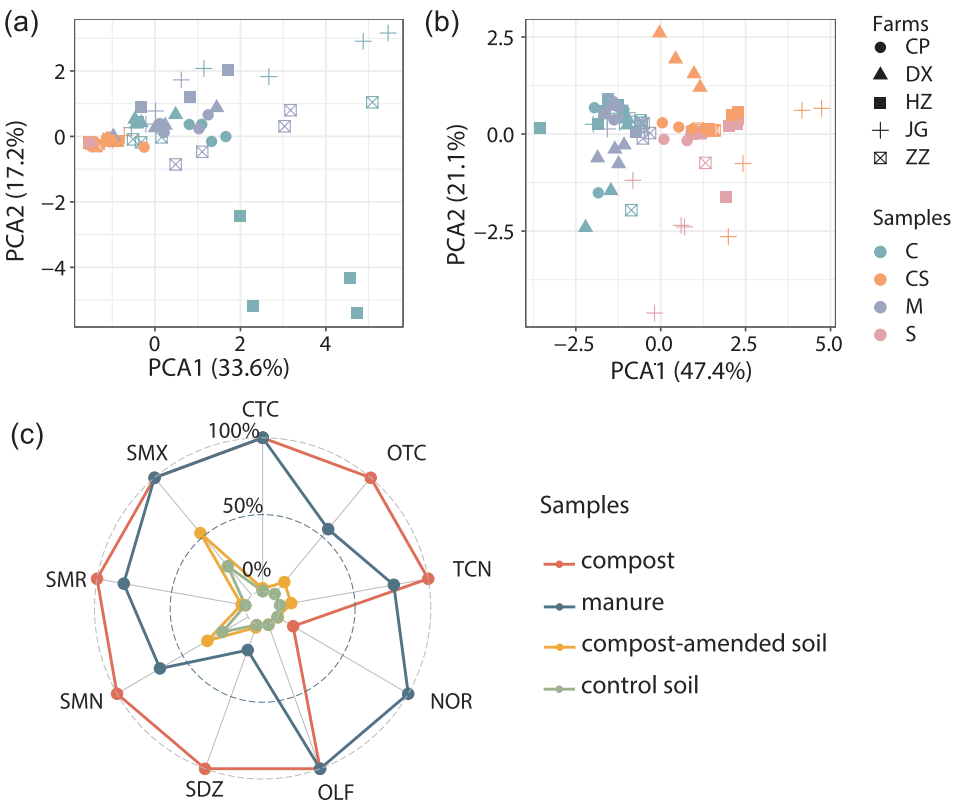

Fig. 1. Principal Component Analysis (PCA) of the total concentration of (a) antibiotics and (b) metals in different samples. (c) The radar plot for nine categories of antibiotics in manure (M), compost (C), compost-amended soil (CS) and control soil (S). Abbreviations: CTC, chlortetracycline; OTC, oxytetracycline; TCN, tetracycline; NOR, norfloxacin; OFL, ofloxacin; SDZ, sulfadiazine; SMN, sulfadimidine; SMR, sulfamerazine; SMX, sulfamethoxazole.

manure and compost tend to have high dissolved organic carbon levels in the percolating solution, which may increase the runoff of copper and zinc complexes (Brock et al., 2006; Japenga and Harmsen 1990). The highest average concentration of zinc was 533,223 $\mu g \cdot kg^{-1}$, and that of copper was 46,215 $\mu g \cdot kg^{-1}$. The concentration of mercury was the lowest among all detected metals, ranging from 13 to 23 $\mu g \cdot kg^{-1}$. PCA analysis showed that samples were also partitioned into two clusters based on metal concentrations, with one cluster constituted by manure and compost samples and the other constituted by compost-amended soil and control soil samples (Fig. 1b). The concentration of different metals did not vary significantly between farms (Fig. S2).

Mean soil temperature was the highest in compost samples (28 °C), followed by manure (25 °C), control soil (24 °C), and compost-amended soil (22 °C) (Table S1). Mean soil water contents were much higher in manure (95%) and compost (63%), compared to those in compost-amended soil (28%) and control soil (17%) (Table S1).

3.2. Bacterial and fungal taxa

We identified 74,676 bacterial OTUs and 6,359 fungal OTUs among 80 samples. Rarefaction curves showed that both bacterial and fungal sequencing had reached saturation (Fig. S3). A total of 31,109 bacterial sequences and 10,269 fungal sequences were subsampled based on their rarefaction curves. There was a dominance of bacterial phyla *Firmicutes* (73%) and *Bacteroidetes* (11%) in manure and compost, while there was a dominance of bacterial phyla *Proteobacteria* (47%) and *Actinobacteria* (23%) in compost-amended soil and control soil (Fig. S4a). *Clostridiales* constituted 53% among the 229 orders in manure and compost. Other major orders included *Lactobacillales* (14%), *Bacteroidales* (7%), and *Pseudomonadales* (4%). At the genus level, *Clostridium*, *Streptococcus* and *Lactobacillus* were highly abundant in manure and compost (Fig. 2), while *Ohtaekwangia* and *Serpens* were the most abundant genera in compost-amended soil. *Streptomyces* was the most abundant genus in the control soil.

Ascomycota (53%) and *Basidiomycota* (16%), which include many opportunistic human pathogens, were the most abundant fungal phyla (Fig. S4b). *Saccharomycetales* (20%), *Eurotiales* (9%), *Xylariales* (8%), *Neocallimastigales* (7%), and *Diversisporales* (5%) were the most abundant orders in manure and compost. By contrast, *Saccharomycetales* (10%), *Hypocreales* (7%), *Chytridiomycota* (6%), *Sporidiobolales* (5%), and *Ascomycota* (5%) were the most abundant orders in compost-amended soil and control soil. *Kazachstania* (27%) was the most abundant genus in manure, followed by *Hyponectriaceae* (13%) and *Piromyces* (10%) (Fig. 2). *Aspergillus* (15%) was the most abundant genus in compost, followed by *Diversisporaceae* (10%) and *Mortierella* (8%). *Bionectria*, *Basidiomycota*, *Sporormiella*, *Archaeorhizomyces,* and *Chytridiomycota* were the most abundant genera in compost-amended soil. *Ascomycota* was the most abundant genus in the control soil.

Bacterial community composition in compost-amended soil was similar to that in the control soil (Fig. S5a). This was consistent with the finding that there were 49.3% of OTUs overlapped (based on the presence or absence of OTUs) between compost-amended soil and control soil (Table S2), much higher than the percentage of overlapped OTUs between compost-amended soil and manure (11.4%) and between compost-amended soil and compost (10.6%). In contrast, 26–29% fungal OTUs in manure, compost, and control soil overlapped with fungal OTUs in compost-amended soil (Fig. S5b and Table S2).

3.3. Microbial genes encoding diverse functions

Relative abundances of ARGs were higher in manure and compost compared to compost-amended soil and control soil (the response ratio analysis, Fig. 3a). Specifically, relative abundances of chromosomally encoded β-*lactamase* genes, *tet* genes, and *mfs* genes were higher in manure and compost compared to that in compost-amended soil and control soil. Relative abundances of mobile ARGs encoded episomally, such as plasmid-borne *smr* genes associated with multidrug resistance efflux and *abc* genes associated with ATP-binding cassette transporters, were also more abundant in manure and compost. According to our analysis using the Mantel test, 8 out of 27 ARGs were significantly

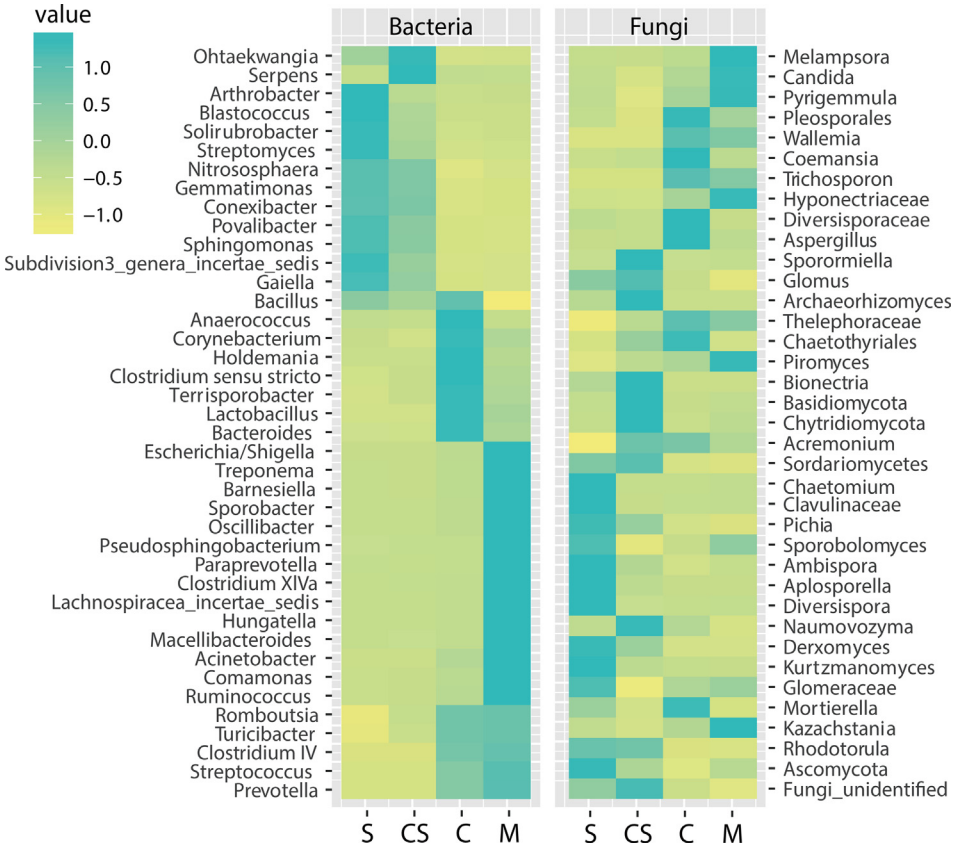

Fig. 2. Heat map representing bacterial and fungal genera that constitute > 1% of the community in manure (M), compost (C), compost-amended soil (CS) and control soil (S).

correlated with bacterial OTUs. Only one ARG was correlated with fungal OTUs ($r = 0.10$, $p < 0.01$, Table S3), suggesting that ARGs were weakly related to bacterial and fungal phylogeny. In addition, we detected more abundant virus associated genes responsible for the eukaryotic and prokaryotic structure and replication in manure and compost than in soils (Fig. 3b), and more abundant virulence associated genes responsible for secretion, toxin, and antiphagocytosis (Fig. 3c).

For metal resistance associated genes, arsenite transporter genes *arsB*, copper transporter genes *ycnJ*, lead uptake genes *pbrT*, mercury resistant genes *merT*, and zinc transporter genes *zrc1* and *zrt3* were more abundant in manure and compost than in compost-amended soil and control soil (Fig. S6a). Relative abundances of most of the carbon degradation associated genes were higher in manure and compost (Fig. S6b), suggestive of higher microbial carbon degradation capacities. Consistently, *nfsa*, *nifb* and *nbac* genes associated with nitroaromatics degradation, *hcaacd* genes associated with aromatic carboxylic acid degradation, *pchcf* and *tutfdg* genes associated with benzene compounds degradation as well as *cbaa* genes associated with chlorinated aromatics degradation were more abundant in manure and compost.

To examine the effect of antibiotics on microbial genes encoding different functions, we compared the relative abundances of microbial genes in compost-amended soil to those in control soil. ARGs, virus-associated genes, metal resistance-associated genes, and carbon degradation associated genes were more abundant in compost-amended soil than in control soil (Fig. S7).

3.4. Linkages between antibiotics and microbial communities

Bacterial α-diversity was the highest in control soil but was significantly ($F = 9.05$, $p < 0.05$, ANOVA) lower in compost-amended soil (Fig. S8a). However, fungal α-diversity was similar between two soil types (Fig. S8b). Notably, the antibiotic concentration was negatively correlated with bacterial α-diversity within soil samples (compost-amended soil and control soil) ($r = -0.39$, $p = 0.02$) and manure samples ($r = -0.45$, $p = 0.04$) but not in compost (Table 1). No correlation was observed between the antibiotic concentration and fungal α-diversity. Mantel test results showed that the bacterial community had the strongest association with sulfamethoxazole concentration, while microbial genes had the strongest associations with chlortetracycline concentration (Fig. 4). The fungal community had few associations with antibiotics, metals, and soil geochemical factors.

We carried out random forest modeling to further explore correlations between individual microbial genes or OTUs with antibiotic concentrations. Random forest model based on microbial genes had an overall high (Pseudo-$R^2 = 0.88$) prediction accuracy for variance of the total antibiotic concentration. The best predictors were genes that belonged to functional categories of virulence (e.g., *hrpB2*, *algK*), stress (e.g., *baeR*, *kata*, *soxR*), organic remediation (e.g., *adpb*, *pcpb*) and metal resistance (e.g. *pbrA*) (Fig. 5a and Table S4). In addition, there was a significant ($r^2 = 0.42–0.63$, $p < 0.05$) correlation between the relative abundance of each most predictive gene and total antibiotic concentration (Fig. 5a). Although individual ARGs were not among the most predictive genes for antibiotics, total abundance of all individual ARGs significantly increased in response to increasing antibiotic concentration, as shown by partial dependence plots (Fig. S9). In sharp contrast, the random forest model based on bacterial (Pseudo-$R^2 = 0.54$) and fungal (Pseudo-$R^2 = 0.20$) OTUs had much lower prediction accuracy for antibiotics (Table S5). The predictive bacterial OTUs were mainly from phylum *Actinobacteria*, and the predictive fungal OTUs were mainly from phylum *Ascomycota* (Table S5). Collectively, the results suggest that functional traits are more useful than phylogenetic markers in predicting antibiotic concentrations.

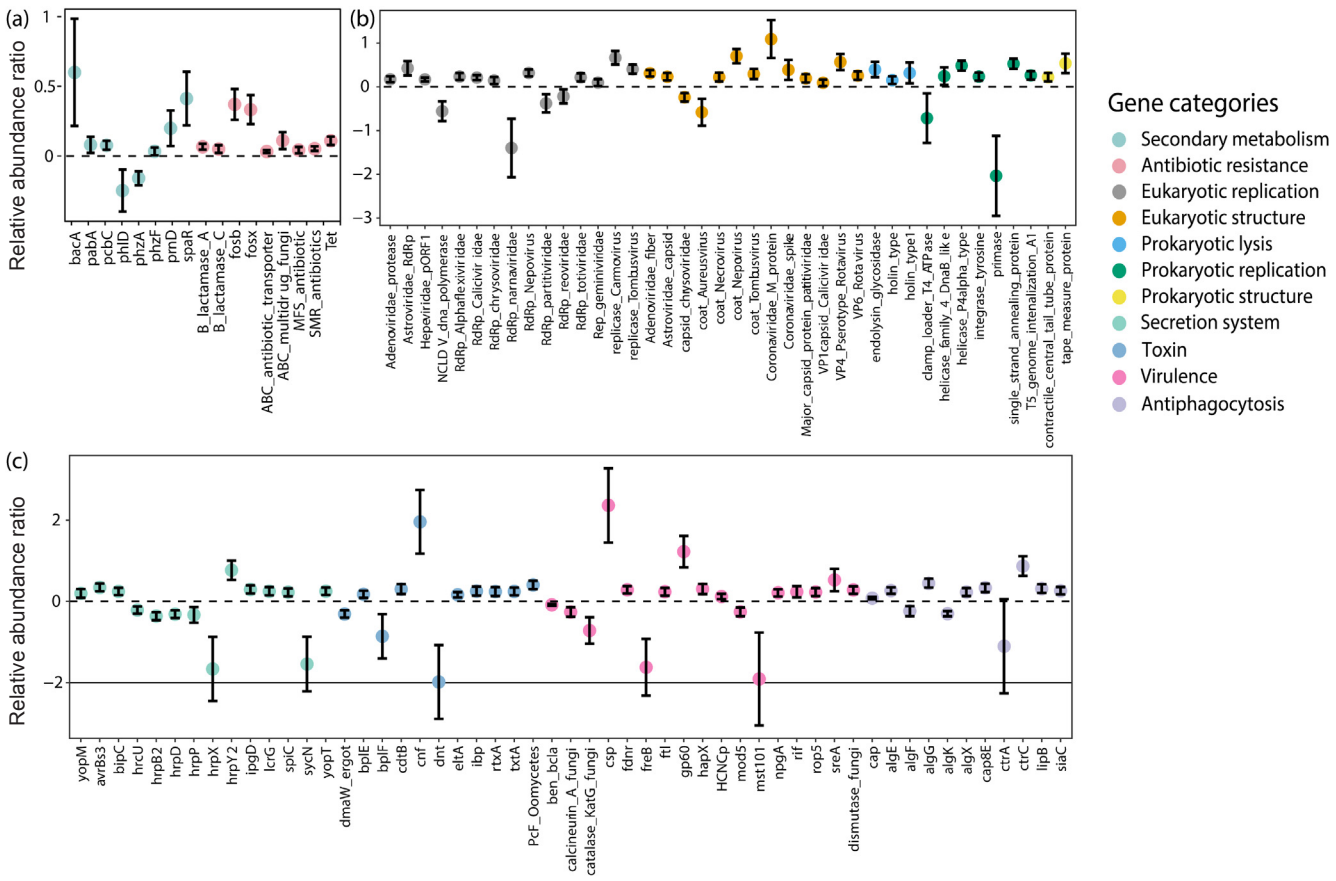

Fig. 3. The response ratio analyses to compare relative abundances of (a) antibiotic resistance genes, (b) virus associated genes and (c) virulence associated genes in manure/compost to those in compost-amended soil/control soil. For each gene (see Table S6 for details), the response ratio was calculated as the summed relative abundances of all gene probes in manure and compost samples relative to the summed relative abundances of all gene probes in compost-amended soil and control soil samples. Only significant changes at the confidence level of 0.95 are shown.

Table 1
Correlations between microbial communities and environmental factors[d].

		M[a]	C	CS	S	M&C	CS&S
Bacteria	antibiotics[b]	[c]-0.454*	0.000	0.124	−0.028	−0.141	−0.386*
	metals	0.341	−0.554**	0.154	0.033	−0.512**	−0.042
	temperature	0.096	0.743**	−0.559**	−0.153	0.407**	−0.246
	water content	−0.078	−0.165	0.295	0.432	−0.042	0.161
Fungi	antibiotics	0.270	0.198	0.580**	0.261	0.210	−0.276
	metals	−0.266	−0.100	0.155	−0.172	−0.248	−0.343*
	temperature	−0.385	0.229	−0.315	0.243	0.070	0.183
	water content	0.121	0.039	0.418	−0.346	0.112	−0.117

[a] M represents manure; C represents compost; CS represents compost-amended soil and S represents control soil.
[b] Antibiotics represent the total concentration of all measured antibiotics. Metals represent the total concentration of all measured heavy metals.
[c] Correlation coefficient (r) of spearman correlation.
[d] Significance of the correlation is labeled as * when $p < 0.05$ and ** when $p < 0.01$.

3.5. Deterministic assembly of microbial functional communities versus stochastic assembly of taxonomic communities

Overall microbial functional community (based on all genes in GeoChip assays) showed $|RC| > 0.95$ and $|SES| > 2$ across samples except for compost-amended soil (Fig. 5b and c), revealing a largely deterministic assembly of microbial functions (Kraft et al., 2011). By contrast, bacterial and fungal communities showed $|RC| < 0.95$ and $|SES| < 2$ in manure and compost, which was suggestive of stochastic assembly for microbial communities in high-antibiotic concentration settings. In compost-amended soil and control soil, both bacterial and fungal communities showed $|RC| > 0.95$ and $|SES| > 2$ (Fig. S10), revealing deterministic assembly under low antibiotic concentration settings.

4. Discussion

In this study, we detected more abundant virulence and virus associated genes in manure and compost than in soils (Fig. 3b, response ratio), consistent with the previous finding that in-feed antibiotics significantly induced phages from swine gut bacteria (Allen et al., 2011). ARGs transferred from phages to hosts could accelerate the evolution of resistance in the microbiome (Allen et al., 2011). Bacterial pathogens increase their susceptibility to hosts by promoting the expression of virulence factors that facilitate the colonization and invasion of the host (Tang et al., 2009). The potentially more abundant pathogens in

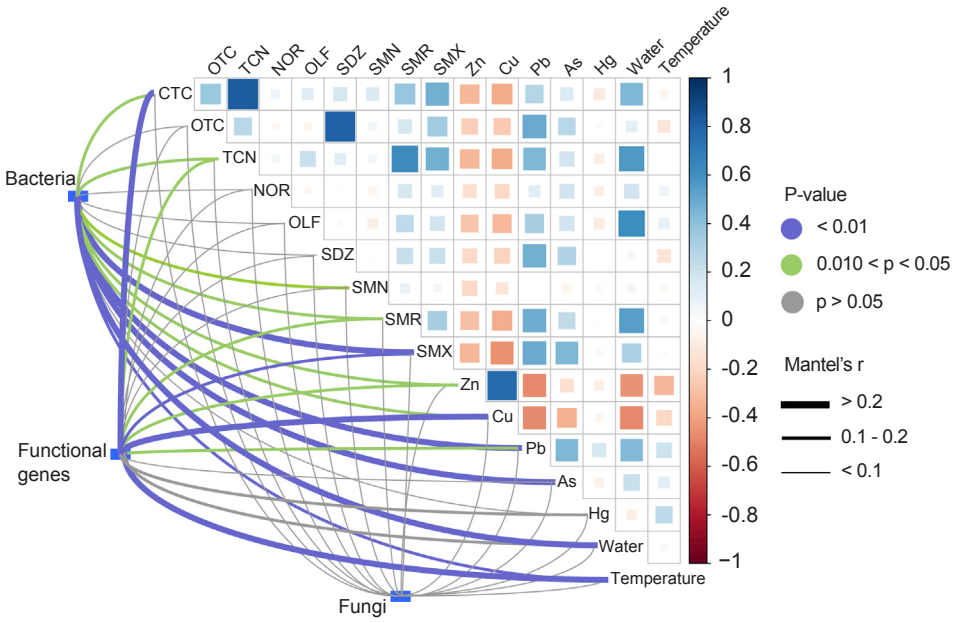

Fig. 4. Relationships of soil microbial communities and environmental factors by partial Mantel tests. Pairwise comparisons of environmental factors with a color gradient denoting Pearson's correlation coefficient. Edge width corresponds to the Mantel's r statistic for the corresponding distance correlations, and edge color denotes the statistical significance.

manure and compost might contribute to the higher abundance of virulence-associated genes.

As one of the major orders of the bacterial community in manure and compost, *Pseudomonadales* have typical large genome sizes (approximately 6 to 10 megabases) with high metabolic versatility that helps degrade and resist the toxicity of antibiotics (Projan 2007). Considering that microorganisms may replicate and mutate rapidly in carbon-rich samples of manure and compost, there could be a wider range of acquired resistance among microbial populations. In accordance, we observed that ARGs were more abundant in manure and compost than in soils (Fig. 3a), likely owing to rich ARGs in gut bacteria. In gut of mice, streptomycin treatment increased enteric oxidized sugars which *S.* Typhimurium used as food resources to grow rapidly (Faber et al., 2016). In another study, antibiotic-induced disruption of the microbial food web gave rise to microbiota-released sugars in the gut that promoted the growth of *S.* Typhimurium (Ng et al., 2013).

There is a significantly ($r = -0.46, p < 0.05$) negative correlation between antibiotic concentration and bacterial α-diversity in manure samples but not in compost (Table 1), suggesting that high antibiotic concentration inhibited some of the bacterial taxa while provided a selective advantage only to those well-adapted species in manure samples. Continuous perturbation from antibiotics could reset microbial community composition to an alternative stable, beneficial state (Sommer et al., 2017). Similarly, microbial community composition

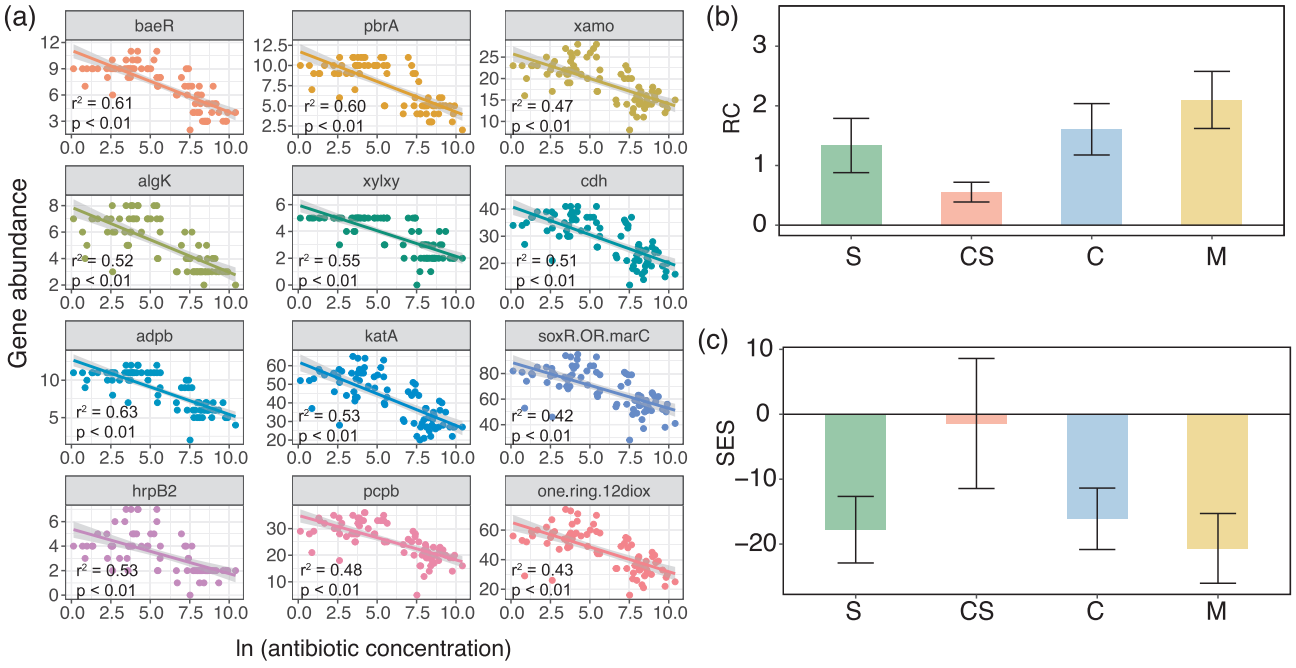

Fig. 5. (a) The most predictive microbial genes for the total antibiotic concentration based on random forest modeling (see detailed information of these genes in Table S4). Correlations between the relative abundance of each functional gene and the natural logarithm transformed antibiotic concentration (the total concentration of the nine categories of antibiotics) are shown. (b) The ecological processes of microbial genes by the Raup–Crick (RC) index. (c) The ecological processes by standardized effect size (SES).

shifted to enriched microorganisms that were capable of hydrocarbon-degradation after oil spill in the Gulf of Mexico (Hazen et al., 2010). Composting could significantly increase the abundance and diversity of bacterial communities, owing to increased dissolved organic carbon and nitrogen (Xi et al., 2016). Bacterial diversity could undergo changes during composting, possibly due to variations in temperature and the availability of new metabolic substrates at different composting stages (Bhatia et al., 2013).

Fungal communities are highly capable in degrading complex organic compounds (Deng et al., 2012). *Ascomycota*, the most abundant phylum in manure and compost (Fig. S4b), was reported to have high nutrient degrading abilities (Mannan et al., 2005). In addition, *Piromyces*, one of the most abundant fungus in manure (Fig. 2), played key roles in the digestion of recalcitrant carbon substrates to make them available for hosts and other symbiotic species (Haitjema et al., 2017).

Considering the spread of antibiotics in the environment are complex and hard to be controlled, the establishment of potential indicators for antibiotic pollution is required. Overall, community resistance is a potential predictor for antibiotics, as shown by the partial dependence plot (Fig. S9). It is consistent with another study showing that manure of swine fed with antibiotics harbored several to tens of thousands-fold more ARGs than antibiotic-free manure (Zhu et al., 2013). Another metagenomic study showed 30 times higher ARG abundance in animal feces with anthropogenic antibiotic input than those in a pristine region Tibet (Chen et al., 2016). In contrast, a manure fertilization experiment in Finland farm showed that β-Lactamase and multidrug resistance genes were not abundant in manure but were prevalent in unfertilized soils (Muurinen et al., 2017), since those genes were more likely belong to the intrinsic environmental resistome. Farmland soils could harbor multiple resistance genes even years after the last manure application (Schmitt et al., 2006). Therefore, the genetic context of ARGs and the persistence of ARGs after application could also have important impacts on ARG compositions.

Individual ARGs were not among the most predictive genes for predicting antibiotic concentration, as shown by random forest (Table S4). The result might be because the presence of individual ARGs was sometimes relatively independent of their antibiotic inducer (Ji et al., 2012). A previous study had shown weak positive correlations between sulfonamide/tetracycline concentrations and their corresponding ARGs in manure and agricultural soils (Ji et al., 2012). No clear associations were detected between *tet* genes and tetracycline concentrations (Gao et al., 2012) and between sulfonamides and *sul* genes (Xu et al., 2015) in sewage treatment plants.

The total abundance of stress and virulence associated genes also positively corresponded to increasing antibiotic concentration (Fig. S9). There is increasing evidence showing that stress responses of bacteria are likely to act as determinants of bacterial resistance to antibiotics (Grant and Hung 2013; Poole 2012). For example, bacterial envelope stress was found to enhance antibiotic resistance formation (Poole 2012). Our results are in line with another study showing that changes in microbial gene abundance could predict uranium contamination (He et al., 2018), indicating that microbial functional traits could serve as bio-indicators of environmental perturbations. By contrast, antibiotics could not be predicted by relative abundances of bacterial and fungal taxa (Fig. S10), probably owing to high functional redundancy in antibiotic resistance among microbial taxa (Anantharaman et al., 2016; Louca et al., 2016). Consistently, we show that ARGs only weakly correlate with bacterial phylogeny (Table S3). A possible reason was that the vast majority of ARGs are acquired through horizontal gene transfer from other taxonomically distant bacteria (Aminov 2009).

Diverse microbial genes had higher relative abundance in manure and compost than in soils (Fig. 3a and Fig. S6), suggesting that microbial communities still sustain functional diversity at relatively high antibiotic concentrations. The stochastic assembly of bacterial and fungal communities would have little consequences for microbial functions, reinforcing the uncoupled relationships between microbial phylogeny and functions.

Notably, as antibiotics can be natural products of microorganisms in the environment, it is possible that the detected antibiotics in compost, manure, and soils contained both the residuals of the synthesized compounds and natural products. In most cases, antibiotic concentrations occurring in natural environments may be too low to exert any lethal effects on the microbes; instead, they may play signaling and regulatory roles in microbiome (Aminov 2009). Furthermore, the bioavailability of antibiotics strongly depends on soil properties such as pH and organic matter content (Wegst-Uhrich et al., 2014). Antibiotics become largely inactive when being adsorbed to soil (Wang and Wang 2015), resulting in a lower antimicrobial effect from antibiotics. Therefore, the bioavailability of antibiotics might also affect the actual activity of antibiotics in the environment.

5. Conclusion

Here we examine microbial assemblage at both taxonomic and functional trait levels along with the spread of antibiotics in the environment. Microbial taxonomic composition varied stochastically under high antibiotic settings, while microbial functional traits undergone deterministic changes. This observation reflects the environmental constraints on microbial functional traits to maintain community stability, which holds the promise to better understand and predict microbial community changes undergoing antibiotic stress. As a result, functional traits could serve as potential bio-indicators of environmental conditions. We thus highlight the importance of focusing on community functional traits in a stressful environmental setting by showing that microbial functions are highly connected with environmental changes.

CRediT authorship contribution statement

Qun Gao: Investigation, Data curation, Software, Formal analysis, Writing - original draft. **Qiang Dong:** Investigation, Resources. **Linwei Wu:** Methodology, Validation. **Yunfeng Yang:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Lauren Hale:** Writing - review & editing. **Ziyan Qin:** Writing - review & editing. **Changyi Xie:** Writing - review & editing. **Qiuting Zhang:** Writing - review & editing. **Joy D. Van Nostrand:** Data curation, Writing - review & editing. **Jizhong Zhou:** Supervision, Writing - review & editing, Funding acquisition.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

DNA sequences of 16S rRNA gene and ITS amplicons are available in the NCBI Sequence Read Archive under project no. PRJNA516026. GeoChip data are available in the NCBI GEO database under project no. GSE132839.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105398.

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