Rare microbial taxa as the major drivers of ecosystem multifunctionality in long-term fertilized soils

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Abstract

Soil microbial communities play an essential role in driving multiple functions (i.e., multifunctionality) that are central to the global biogeochemical cycles. Long-term fertilization has been reported to reduce the soil microbial diversity, however, the impact of fertilization on multifunctionality and its relationship with soil microbial diversity remains poorly understood. We used amplicon sequencing and high-throughput quantitative-PCR array to characterize the microbial community compositions and 70 functional genes in a long-term experimental field station with multiple inorganic and organic fertilization treatments. Compared with inorganic fertilization, the application of organic fertilizer improved the soil multifunctionality, which positively correlated with the both bacterial and fungal diversity. Random Forest regression analysis indicated that rare microbial taxa (e.g. Cyanobacteria and Glomeromycota) rather than the dominant taxa (e.g. Proteobacteria and Ascomycota) were the major drivers of multifunctionality, suggesting that rare taxa had an over-proportional role in biological processes. Therefore, preserving the diversity of soil microbial communities especially the rare microbial taxa could be crucial to the sustainable provision of ecosystem functions in the future.

Keywords: biological processes; microbial diversity; rare taxa; ecosystem functions; biogeochemical cycling

1. Introduction

Soil microbes represent the most abundant and diverse organisms on Earth (Locey and Lennon, 2016). It is estimated that 1 cm^3 of soil contains 0.4 - 2 billion prokaryotic microbes, tens of thousands of taxa and up to ~200 m fungal hyphae, which play key roles in maintaining multiple ecosystem functions simultaneously (i.e. ecosystem multifunctionality) that are critical to the biogeochemical nutrient cycling, primary production, litter decomposition and climate regulation (Bardgett and van der Putten, 2014; Wagg et al., 2014; Bender et al., 2016). Recent studies provide evidence that global environmental drivers, such as land use changes, nitrogen deposition, and climate change, can severely impact multifunctionality in terrestrial ecosystems through manipulating the belowground soil biodiversity (Garcia-Pichel et al., 2013; Maestre et al., 2015; Delgado-Baquerizo et al., 2016; Delgado-Baquerizo et al., 2017a; Luo et al., 2018). Common agricultural practices, such as soil tillage, fertilization, pesticide application, and monoculture, can have adverse effects on the maintenance of soil microbial diversity and interactions (de Vries et al., 2012; Tsiafouli et al., 2015), with unkown consequences for soil multifunctionality. Given that farming intensity is projected to constantly increase on a global scale (Bender et al., 2016) to feed a growing human population (Ort et al., 2015), it is imperative to understand the consequence of agricultural practices on belowground biodiversity and multifunctionality.

It is estimated that ~23% of the world soil faces degradation and the area of degraded land increases at an annual rate of 5 - 10 million ha, which may affect the food

42 security for approximate 1.5 billion people globally (Stavi and Lal, 2015).
43 Fertilization as an important agricultural practice accelerates the rate of land
44 degradation, as long-term inorganic fertilization may result in soil acidification (Guo
45 et al., 2010). Before the innovation of industrial ammonia synthesis, the biological
46 nitrogen fixation has sustained life on Earth for thousands of years. Modern
47 agricultural practices are based predominantly on industrially produced mineral
48 fertilizers, and have directly caused several environmental problems, such as surface
49 and ground water eutrophication through excessive discharge of nutrients including
50 Nitrogen and Phosphorus into water (Smith and Schindler, 2009), and global warming
51 through conversion of ammonium to nitrogen oxides (Foley et al., 2005). A growing
52 body of evidence indicated that intensive fertilization also indirectly influences a wide
53 range of crucial ecosystem functions via altering the diversity of soil microorganisms
54 (Hartmann et al., 2015; Ling et al., 2016), but we know little about how fertilization
55 will impact the ecosystem multifunctionality. To the best of our knowledge, few
56 studies have explicitly addressed the impact of fertilization on the ecosystem
57 multifunctionality and the relationships between soil biodiversity and ecosystem
58 functioning (Luo et al., 2018). Such knowledge is essential to the development of
59 management frameworks to protect soil biodiversity involved in multifunctionality
60 and reduce impacts of intensive fertilization on terrestrial ecosystems.
61 Herein, we hypothesized that the positive relationship between ecosystem
62 multifunctionality (especially the functional traits related to nutrient element cycles)
63 and microbial diversity is maintained in the agroecological system, as it has been

widely demonstrated in the natural ecosystem (Mori et al., 2016; Delgado-Baquerizo et al., 2017a). We characterized the bacterial and fungal communities in soil samples collected from a long-term fertilization experimental field, using amplicon sequencing of bacterial 16S rRNA genes and fungal internal transcribed spacer 2 (ITS2) region, respectively. Given that the relationships between biodiversity and multifunctionality were reported to be dependent on the identity and number of measured functions (Meyer et al., 2018), we measured multiple ecosystem functions from functional gene level to enzyme level and specific biological processes: (i) We used quantitative microbial element cycling (QMEC) for high-throughput quantitative assessment of 70 functional genes related to Carbon (C), Nitrogen (N), Phosphorus (P), and Sulphur (S) biogeochemical cycling (Zheng et al., 2018); (ii) We measured four enzyme activities including β-glucosidase, N-acetyl-β-glucosaminidase, urease and phosphatase; (iii) We also determined soil basal respiration, potential ammonia oxidation and denitrification enzyme activity.

2. Materials and Methods

2.1. Study sites and sample collection

We collected soil samples in September of 2017 (the standing crop was maize) from a long-term experimental station of the Chinese Academy of Agricultural Sciences, in Shandong Province, China (37°20' N, 116°38' E). The experimental station was established in 2006 to investigate the impact of land application of sewage sludge, chicken manure and inorganic fertilizers on the N and P input-output balances and soil

P accumulation. A total of eight treatments with three replicates were set up, including one control, two inorganic urea treatments (N), four sewage sludge treatments (S), and one chicken manure treatment (CM) (i.e. CK, 0.5N, 1N, 0.5S, 1S, 2S, 4S, 1CM). Before wheat sowing every year (mid of October), urea, air-dried sewage sludge and chicken manure were applied as the basic fertilizer to the soil. We collected top bulk soil samples (0-15 cm) from five random sites in each plot, and about two kg soils were mixed together for each plot. The collected soil samples were sieved (~2mm). A small part of soil (~20 g) for DNA extraction was stored at -20 °C and the rest soil was stored at 4 °C. The detailed information of the field experimental design is shown in Table S1.

2.2. Soil physicochemical characterization

Total soil carbon and nitrogen were determined by combustion on a C/N instrument using 200 mg air-dried soil (Vario MAX C/N; Elementar, Germany). Soil pH and electrical conductivity (EC) were measured in a 1:2.5 mass/volume soil-water suspension (shaking for 30 min at 200 rpm) with a compound electrode on a pH meter (Accumet Excel XL60, Fisher Scientific Inc., Waltham, USA).

2.3. DNA extraction and assessment of microbial diversity

Nucleic acids from each soil sample were extracted from 0.5 g of soil using a FastDNA Spin kit (MP Biomedical, Santa Ana, California, USA) according to the instructions provided by the manufacturer. The DNA quality was assessed on a spectrophotometer (NanoDrop Technology, Wilmington, USA). The DNA concentration was determined with a Qubit™ dsDNA HS Assay kit on a Qubit™ 3.0

fluorometer (Thermo Fisher Scientific Inc., Waltham, USA).

To assess the bacterial and fungal communities, we amplified the V4 - V5 hypervariable regions of the bacterial 16S rRNA gene and fungal ITS2 region using the primers F515/R907 (Jing et al., 2015) and gITS7/ITS4 (Ihrmark et al., 2012), respectively. For both bacteria and fungi, we conducted PCR amplification with 25 µl of 2×premix (TaKaRa, Kusatsu, Japan), one µl of each forward and reverse primer (10 mM), and one µl of DNA template, and the volume was adjusted to 50 µl with PCR-grade water. Each sample was amplified in three technical replicates with the 50 µl reaction under the following condition. For bacteria: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 10 min. For fungi: 95 °C for 15 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. Negative controls (template DNA replaced with water) were included to detect any contamination during PCR preparation. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (TIANGEN Biotech, Beijing, China). The purified PCR products were quantified and pooled at the same concentration and then sequenced using an Illumina Hiseq2500 platform (Novogene, Beijing, China).

Raw sequences were processed using the QIIME pipeline (Caporaso et al., 2010). To guarantee the quality of downstream analyses low-quality regions ($Q < 20$) were trimmed from the 5′ end of sequences, and paired ends were joined for 16S rRNA sequences and ITS reads with FLASH (Magoc and Salzberg, 2011). A further quality control was conducted to remove sequences containing ambiguous bases (N), reads

129 containing bases with a quality score < 25. Chimeric sequence was checked using the
130 UCHIME algorithm from the USEARCH package (Edgar et al., 2011). The remaining
131 high-quality chimera-free sequences were used for downstream analysis. After quality
132 filtering, a total of 1,144,015 and 1,327,383 high-quality sequences were obtained for
133 bacteria and fungi, respectively. Operational taxonomic units (OTUs) were identified
134 using the UCLUST algorithm with a 97% sequence similarity (Edgar, 2010).
135 Taxonomy classification was assigned using UCLUST against Ribosomal Database
136 Project (RDP) database (Version 11.5) for bacterial OTUs (Cole et al., 2009). For
137 fungal ITS2 sequences, taxonomy classification was assigned by using BLAST
138 (Altschul et al., 1990) against the UNITE database (Version 7.2) (Nilsson et al., 2018).
139 The resultant OTU abundance tables were filtered to remove singletons, in addition,
140 chloroplast and mitochondrial sequences were discarded from the final OTU data set
141 for 16S rRNA gene primer set. We rarefied to an even number of sequences per
142 sample to ensure an equal sampling depth (17,860 and 19,000 for 16S rRNA gene and
143 ITS, respectively). All the raw sequencing data were deposited in the National Center
144 for Biotechnology Information Sequence Read Archive under the accession number
145 SRP140546 for bacteria and SRP201641 for fungi.

146 2.4. Assessing multifunctionality

147 The multifunctionality includes multiple ecosystem functions such as nutrient cycling,
148 litter decomposition, climate regulation, soil fertility and food production. In the
149 present study, we assessed 18 ecosystem functions related to C, N, P, and S cycling
150 including (1) total carbon (TC), (2) total nitrogen (TN), (3) ratio of C/N, (4) soil pH,

(5) soil EC, (6) soil basal respiration (SBR), (7) potential ammonia oxidation (PAO), (8) denitrification enzyme activity (DEA), (9) β-glucosidase, (10) N-acetyl-β-glucosaminidase, (11) urease (12) phosphatase), 70 functional genes classified into six subtypes (13) C-fixation, (14) C-degradation, (15) denitrification, (16) nitrification, (17) P-cycling, (18) S-cycling. SBR was measured using 10 g soil in a 100 ml sealed serum bottle and incubated with three negative controls (without soil) for 48 h at 25 °C. Carbon dioxide (CO_2) was analyzed using a gas chromatograph (7890A; Agilent Technologies, Santa Clara, CA, USA) (Liu et al., 2018). DEA was determined using a short-term anaerobic assay protocol by using 10 g soil amended with 10 ml substrate (1.0 mM KNO_3 and 1.0 mM glucose) and 10 ml acetylene in a 100 ml evacuated serum bottle. Nitrous oxide (N_2O) in the headspace was analyzed after 1 and 5 h using a gas chromatograph (7890A; Agilent Technologies, Santa Clara, CA, USA). PAO was measured using the chlorate inhibition method (Deng et al., 2009) by using 5 g soil amended with 20 ml phosphate-buffering solution (pH = 7.1) in a 50 ml centrifuge tube. The soil suspension was incubated on a rotary shaker (180 rpm) at 25 °C in the dark for 24 h. Nitrite-N was extracted by 2M KCl, filtered (0.45 μm syringe cellulose acetate filter) and analyzed with flow injection analyzer (QC8500, Lachat). Extracellular soil enzyme activities (β-glucosidase, N-acetyl-b-glucosaminidase, urease and phosphatase) were measured from 5 g of soil by fluorometry with a microplate fluorometer (Thermo Multiskan GO) as described in previous studies (Bell et al., 2013). Overall, the variables selected in this study constitute good proxies of nutrient cycling processes, biological productivity and

greenhouse gas emission. Details of measurements and the primer information are provided in Zheng et al. (2018).

2.5. Data analysis

Several methods are available to estimate the relationship between biodiversity and multifunctionality including single functions approach, turnover approach, averaging approach and threshold approach (including single-threshold and multiple-threshold) (Byrnes et al., 2014). Single functions approach cannot provide qualitative information while the turnover approach does not measure multifunctionality directly (Byrnes et al., 2014), and therefore averaging and threshold approaches have been commonly used (Maestre et al., 2012; Bradford et al., 2014; Wagg et al., 2014; Delgado-Baquerizo et al., 2016). To allow for a comparison with previous findings, we constructed three metrics of multifunctionality: average, single-threshold, and multiple-threshold multifunctionality, since each metric provides unique information of multifunctionality. Average multifunctionality determines the average level of multiple functions by standardizing each function to a common scale and averaging standardized values into a single index (Maestre et al., 2012; Delgado-Baquerizo et al., 2016; Delgado-Baquerizo et al., 2017b). Z-score transformation (overall mean of 0 and standard deviation of 1) was used to standardize the soil microbial community and ecosystem functions data, because it has advantages over other standardization procedures for the linear model-based statistics (Maestre et al., 2012). The single-threshold approach, to determine if multiple functions are simultaneously performing at high levels, was performed to test whether they exceed a specified

195 threshold percentage of maximum functioning (Bradford et al., 2014; Mori et al.,
196 2016). This approach defines the maximum value of each function across all plots and
197 counts the number of functions performing at or exceeding a threshold of this
198 maximum value in each plot. To define maximum functioning, we calculated the
199 mean of the five highest values for each function across all treatments. We defined
200 single-thresholds of 20%, 40%, 60% and 80% and then fitted a generalized linear
201 model to estimate a linear relationship predicting the number of functions performing
202 at or above their threshold as a function of soil microbial species richness. Compared
203 with the single-threshold approach, the multiple-thresholds approach does not require
204 to choose a threshold value and instead uses a continuous gradient of thresholds. This
205 approach examines the change of the shape of the fitted curve at different thresholds,
206 rather than evaluating the statistical evidence for any single curve. The three metrics
207 of multifunctionality were calculated using the "*multifunc*" package (Byrnes et al.,
208 2014) in R (R Core Team, 2016). We conducted a classification Random Forest
209 analysis with the "*randomForest*" package (Liaw and Wiener, 2002) to identify the
210 major statistically significant microbial predictors for the multifunctionality. A total of
211 29 microbial phyla including all the 21 classified bacterial phyla and all the 8 fungal
212 phyla were selected in the Random Forest modeling. The "*vegan*" package (Oksanen
213 et al., 2017) was used to calculate the microbial alpha diversity (inverse Simpson
214 index) and richness and eveness. The permutational multivariate analysis of variance
215 (PERMANOVA) was performed using the *adonis* function in "*vegan*". The "*labdsv*"
216 package (Roberts, 2016) was used to perform the principal coordinates analysis

(PCoA) with the OTU level data matrix. The "*ggplot2*" package (Wickham, 2009) was used for data visualization.

3. Results

3.1. Impacts of fertilization on bacterial and fungal community composition

After quality filtering, a total of 1,144,015 and 1,327,383 high-quality sequences were obtained for bacteria and fungi, respectively, which could be classified into 39,194 and 4333 operational taxonomic units (OTUs) at a 97% sequence similarity. Proteobacteria (~29%), Actinobacteria (~17%) and Acidobacteria (~14%) were the three most dominant bacterial phyla. At the phylum level, the bacterial community compositions remained relatively stable among different treatments and neither inorganic nor organic fertilization caused any significant shifts ($P > 0.05$) (Figure S1). Ascomycota (~51%) and Basidiomycota (~10%) were the major fungal phyla (~31% fungal sequences could not be reliably assigned to a fungal phylum). The relative abundance of Basidiomycota decreased in all treatments of organic fertilization, for example, its relative abundance was decreased by 63% after application of chicken manure.

The results of alpha-diversity (based on the Inverse Simpson index) showed that application of inorganic fertilizer (0.5N and 1N) induced no significant changes in bacteria or fungi (Figure S2). By contrast, application of organic fertilizers, especially sludge (4S) and chicken manure, significantly increased the alpha-diversity of both bacteria and fungi (Figure S2). A significantly higher OTUs richness of bacteria and

238 fungi compared to 0.5N was observed in 4S and 1CM, while the bacterial OTU
239 evenness remained relatively stable across treatments (Table S2). The Adonis test
240 indicated that both inorganic and organic fertilization significantly altered bacterial (P
241 = 0.02) and fungal ($P < 0.01$) community structure. The principal coordinates analysis
242 (PCoA) based on the Bray-Curtis distance also showed that fertilization (regardless of
243 inorganic or organic fertilizer) altered the overall patterns of both bacterial and fungal
244 communities (Figure S3). For bacteria, samples from treatment with high fertilizer
245 (1N, 2S 4S, CM) clustered separately from control samples. For fungi, however, the
246 samples from organic fertilizer treatments clustered separately from control and
247 inorganic fertilizer treatments.

248 3.2. Impacts of fertilization on soil ecosystem multifunctionality

249 The results indicated that both inorganic and organic fertilization shifted the soil
250 ecosystem multifunctionality (Figure 1). The application of inorganic fertilizer (the
251 0.5N and 1.0N treatments) significantly decreased ecosystem multifunctionality, but a
252 significant increase in multifunctionality was observed after application of organic
253 fertilizers. On average, the highest and lowest multifunctionality indexes (Z-score)
254 were found after fertilization with sewage sludge (4S) and urea (1N). Soil
255 multifunctionality increased along with the increasing doses of sewage sludge
256 application (Figure 1). Compared with sludge application, chicken manure application
257 had a weaker effect on soil ecosystem multifunctionality.

258 3.3. Relationships between microbial diversity and ecosystem multifunctionality

259 We examined the relationship between soil microbial diversity (inverse Simpson

diversity index) and ecosystem multifunctionality using three metrics of multifunctionality: average, single-threshold, and multiple-threshold multifunctionality considering each approach has strengths and weaknesses.

The average approach was used to evaluate ecosystem multifunctionality using the standardized average of 18 variables. The ordinary least-squares (OLS) regression models revealed a positive linear correlation between ecosystem multifunctionality and soil microbial diversity for both bacteria (R^2=0.486, P < 0.0001) and fungi (R^2=0.373, P = 0.0009) (Figure 2). We also observed a positive correlation between soil microbial diversity and most of the individual functions (Figures 3 and S4). For example, the bacterial diversity was positively correlated with potential ammonium oxidation (R^2=0.205, P = 0.0261) and denitrification enzyme activity (R^2=0.210, P = 0.0250). However, some individual functions, such as soil basal respiration, and functional genes related to P-cycling and S-cycling, had no significant correlations with either bacterial or fungal diversity (P > 0.05).

We employed the threshold approach to evaluate whether multiple functions were simultaneously performing at high levels. When analyzed with the single threshold approach, a significant relationship was found between the bacterial diversity and ecosystem multifunctionality at thresholds of 60% and 80% (Figure S5), while a significant correlation was observed between the fungal diversity and multifunctionality at thresholds of 40%, 60% and 80% (Figure S6). By comparison, the multiple-thresholds approach did not require to set a threshold value and instead investigated a continuous gradient of thresholds (Figure 4). The minimum threshold

(T_{min}), i.e. the lowest threshold where diversity begins to have a positive effect on multifunctionality, were 45% and 23% for bacteria and fungi, respectively. The realized maximum effect of diversity (R_{mde}), i.e. the strength of the relationship where diversity has its strongest positive effects, was 1.143 for bacteria at the threshold of 69%, indicating that addition of one bacterial species can increase 1.143 functions. While the R_{mde} for fungi was 1.245 at the threshold of 67%, indicating that adding one fungal species can increase 1.245 functions.

3.4. Microbial predictors for ecosystem multifunctionality

Random Forest regression modelling was performed to identify the most important microbial taxa (i.e. a large value of Increase in MSE) in predicting the ecosystem multifunctionality. A total of 29 bacterial and fungal phyla were incorporated in this model (Figure 5). The results indicated that the top three most important microbial taxa were all bacterial phyla, including Cyanobacteria (Spearman, $P < 0.0001$), Armatimonadetes (Spearman, $P = 0.0041$), and Fibrobacteres (Spearman, $P = 0.0136$) (Table S3). These bacterial phyla were relatively rare bacterial taxa, accounting for less than 3% of the total reads. For fungi, the phylum, Glomeromycota (Spearman, $P = 0.0035$), as a minor phylum (less than 1%), was the most important driver in shaping ecosystem multifunctionality. In contrast, some of the dominant phyla, such as Proteobacteria (Spearman, $P = 0.7467$) and Planctomycetes (Spearman, $P = 0.9037$), however, played less important roles in ecosystem multifunctionality.

4. Discussion

4.1. Organic fertilization enhances soil ecosystem multifunctionality

By determining multiple soil functions, we explored the effect of long-term fertilization on ecosystem multifunctionality and its relationship with microbial diversity (bacteria and fungi). Our results provide evidence that fertilization significantly altered multifunctionality, consistent with previous findings that land management practices influenced both soil biological traits and ecological functioning (Rodrigues et al., 2013). More importantly, organic fertilization (application of sludge and chicken manure) promoted the multifunctionality while inorganic fertilizer (urea) decreased the multifunctionality. A major implication of this result is that organic fertilizer could potentially buffer some of the negative effects of agricultural practices, such as physical disturbance on microbial composition and reduction in functional redundancy (Laliberte et al., 2010), by adding not only nutrients but also microorganisms into soils. However, it is hard to causally differentiate between bioaugmentation (i.e. adding organisms) and biostimulation (i.e. adding growth substrates for indigenous organisms) as the mechanisms responsible for increased multifunctionality. It has been reported that most of the introduced microbes may not adapt well to the soil environment (Heuer et al., 2011), but some of them may nevertheless survive in the new environment. A recent study suggested that if it is still possible to add a species to an ecosystem, it would likely increase some ecosystem functions (more than it would decrease others) (Meyer et al., 2018). In this respect, organic fertilization is recommended in agricultural practices to maintain or improve

the multifunctionality. However, it should be kept in mind that organic fertilizers (e.g. manure, sewage sludge, compost) can contribute to the distribution and spread of antibiotics, antibiotic resistant bacteria, antibiotic resistance genes, pathogens as well as the accumulation of heavy metals (Chen et al., 2016; Sims and Kline, 1991). Therefore, a balance may be needed between the application of the organic and the inorganic fertilizers in agriculture.

4.2. Response of multifunctionality and single ecosystem processes to microbial diversity

Long-term fertilization significantly shifted the profiles of both bacterial and fungal communities (Figure S3). Soils under organic fertilization had a higher microbial diversity than those from control and inorganic fertilization. The fertilization induced changes in the bacterial and fungal diversity could further impact the soil multifunctionality, because a large body of studies found positive correlations between microbial diversity and multifunctionality in the natural environment (Jing et al., 2015; Delgado-Baquerizo et al., 2016; Delgado-Baquerizo et al., 2017a). Farming systems are reported to usually have a lower level of biodiversity compared to less intensively used or natural ecosystems (Tuck et al., 2014; Tsiafouli et al., 2015). We calculated the multifunctionality by using three metrics including average, single-threshold, and multiple-threshold as each approach has its own strengths and weaknesses (Byrnes et al., 2014), and then evaluated the relationship between multifunctionality and microbial diversity. We found a positive relationship between microbial diversity and multifunctionality in agricultural systems. Consequently, any

346 changes in microbial diversity resulting from agricultural practices (Govaerts et al.,
347 2007; Chen et al., 2014) such as tillage, fertilization, pesticide application, irrigation,
348 residue management, and crop rotation, may affect the soil multifunctionality.
349 However, fertilization especially organic fertilizer may affect both soil abiotic and
350 biotic factors at the same time making it difficult to derive causal relationships
351 between microbial diversity and multifunctionality. Hence, further studies to deduce
352 the causal relationships between the microbial diversity and multifunctionality are
353 needed in the agricultural ecosystems.
354 We also found positive correlations between soil microbial diversity with some single
355 functions such as denitrification enzyme activity, potential ammonium oxidation, total
356 carbon and nitrogen, and functional genes related to nitrification and denitrification
357 (Figure 3). Of particular interest is the fact that compared with above single functions,
358 soil microbial diversity had the least influence on the soil basal respiration, enzyme
359 activities (except for β-glucosidase related to cellulose degradation) and functional
360 genes related to P and S cycling. This finding may be attributed to the different
361 degrees of dependence of ecosystem functions (functional redundancy) on
362 biodiversity. For example, microbial communities often exhibit high redundancy for a
363 general function, e.g. microbial respiration and biomass production in terrestrial
364 systems (Langenheder et al., 2006; Reich et al., 2012; Miki et al., 2014), which may
365 explain why microbial diversity exerts a weaker influence on these variables. In
366 addition, extracellular enzymes are produced by soil microorganisms, and have widely
367 been used as indicators of soil quality, nutrient demand by plants and soil

368 microorganisms (Bell et al., 2013). Conversely, we found that most of the measured
369 enzyme activities had no significant relationship with either bacterial or fungal
370 diversity, which is inconsistent with previous findings (Delgado-Baquerizo et al.,
371 2017a; Luo et al., 2018). It should be noted that, unlike previous studies, a small
372 number of enzyme activities were measured in the present study and most of the
373 enzyme activities remained relatively stable across different treatments of fertilization.
374 Thus, we believe that the selection of different types of enzymes might partially
375 explain the differences between Luo et al. (2018) and our results. Nevertheless, we
376 still found a significant positive correlation between β-glucosidase activities and the
377 diversity of bacteria (OLS, P= 0.0400) and fungi (OLS, P = 0.0005).
378 The discontinuity in the responses of single ecosystem processes and
379 multifunctionality to altered soil microbial diversity (Bradford et al., 2014) suggests
380 that the biodiversity-multifunctionality relationships may depend on the number of
381 measured functions and their combinations (Meyer et al., 2018). Recently, the use of
382 multifunctionality stimulated a large body of investigations to explore the role that
383 microbial biodiversity plays in providing desired rates of multiple ecosystem
384 processes. However, the use of multifunctionality may not be impeccable, since the
385 data aggregation especially when using the average approach, might provide
386 misleading information by obscuring the true relationships between explanatory and
387 response variables. Therefore, application of the multifunctionality should at the same
388 time emphasize looking at the relationship between communities and individual
389 ecosystem processes.

390 4.3. The disproportional role of rare microbial taxa in multifunctionality

391 An increasing body of studies have demonstrated the key roles of microbial
392 communities in regulating soil belowground ecosystem multifunctionality (Bradford
393 et al., 2014; Delgado-Baquerizo et al., 2016). However, most of our knowledge is still
394 based on dominant species, and less attention has been paid to rare microbial taxa.
395 Our Random Forest regression analysis allowed us to identify particular microbial
396 taxa as the major predictors of multifunctionality. An interesting finding was that
397 bacterial and fungal taxa with a low relative abundance (~1-3%) were identified as the
398 major drivers of multifunctionality. In contrast, the dominant microbial taxa such as
399 Proteobacteria, Acidobacteria, Actinobacteria and Planctomycetes showed little
400 controls on multifunctionality (Table S3). Our results suggest that the rare taxa may
401 have an over-proportional role in soil multifunctionality, which to some extent could
402 explain why fertilization significantly altered the soil multifunctionality (Figure 1),
403 while the relative abundance of both bacterial and fungal dominant phyla remained
404 relatively stable (Figure S1). In the past, most of studies were focused on the
405 dominant taxa, and many rare microbial taxa are routinely removed from data sets.
406 For example, potential sequencing artefacts (e.g. OTUs containing few reads) were
407 usually removed during data processing (Martinson et al., 2017), and this filtration
408 has negligible impacts on the profile of dominant taxa but has a strong impact on the
409 rare taxa. Recently, rare species as the vulnerable components on Earth are
410 increasingly recognized as drivers of key functions in terrestrial and aquatic
411 ecosystems, and even in host-associated microbiomes (Jousset et al., 2017).

412 The disproportionately role of rare taxa in multifunctionality seems counter-intuitive
413 given the expected high functional redundancy in microbial communities. In other
414 words, many taxa share similar functions, rare species might not be necessary for
415 maintaining function. The functional importance of the rare taxa may be due to the
416 'insurance effects' (Jousset et al., 2017). The rarity of taxa may be not permanent,
417 which is influenced by both the external abiotic and biotic factors. It has been
418 suggested that ~1.5 - 28% of all microbes are 'conditionally rare taxa', which are rare
419 in most conditions but may become dominant occasionally (Shade et al., 2014).
420 Microbial taxa that are considered functionally less relevant under a given
421 environmental condition could become more important in providing unique functional
422 traits under a favorable condition. Thus, the rare microbial taxa could be a reservoir of
423 genetic resources that may be activated under appropriate conditions, e.g. in long-term
424 fertilized soils in this study. Our knowledge of the ecological role of the rare
425 microbial taxa is still in its infancy, therefore, future studies related to biodiversity and
426 ecosystem multifunctionality should pay more attention to the rare microbial taxa.
427 In summary, our results provide experimental evidence that long-term fertilization
428 altered the soil microbial compositions and related ecosystem multifunctionality.
429 Compared with inorganic fertilizer, organic fertilization promoted the
430 multifunctionality. The significant correlation between microbial diversity and
431 multifunctionality together with the relatively stable composition of dominant
432 microbial taxa, indicates that fertilization-induced shifts in the rare microbial taxa are
433 the major drivers in shaping the multifunctionality. Further studies regarding

investigation and protection of the rare species is therefore of paramount importance for buffering human impacts on microbially driven ecosystem multifunctionality in agricultural ecosystems.

Conflict of interest

The authors declare no conflict of interest.

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Legends

Figure 1 Average multifunctionality index in response to fertilization. *P < 0.05, **P < 0.01, ***P < 0.001 (ANOVA, LSD). Abbreviations: CK, control; N, N fertilizer treatment; S, sewage sludge fertilizer treatment; CM, chicken manure fertilizer treatment. The multifunctionality index was calculated using the data from quantitative microbial element cycling (QMEC), enzyme activities, soil basal respiration, denitrification activity and potential ammonia oxidation. All organic fertilized treatments received a basic level of urea (similar concentration as 0.5N treatment), thus, the 0.5N is the control for the sewage sludge and chicken manure treatments.

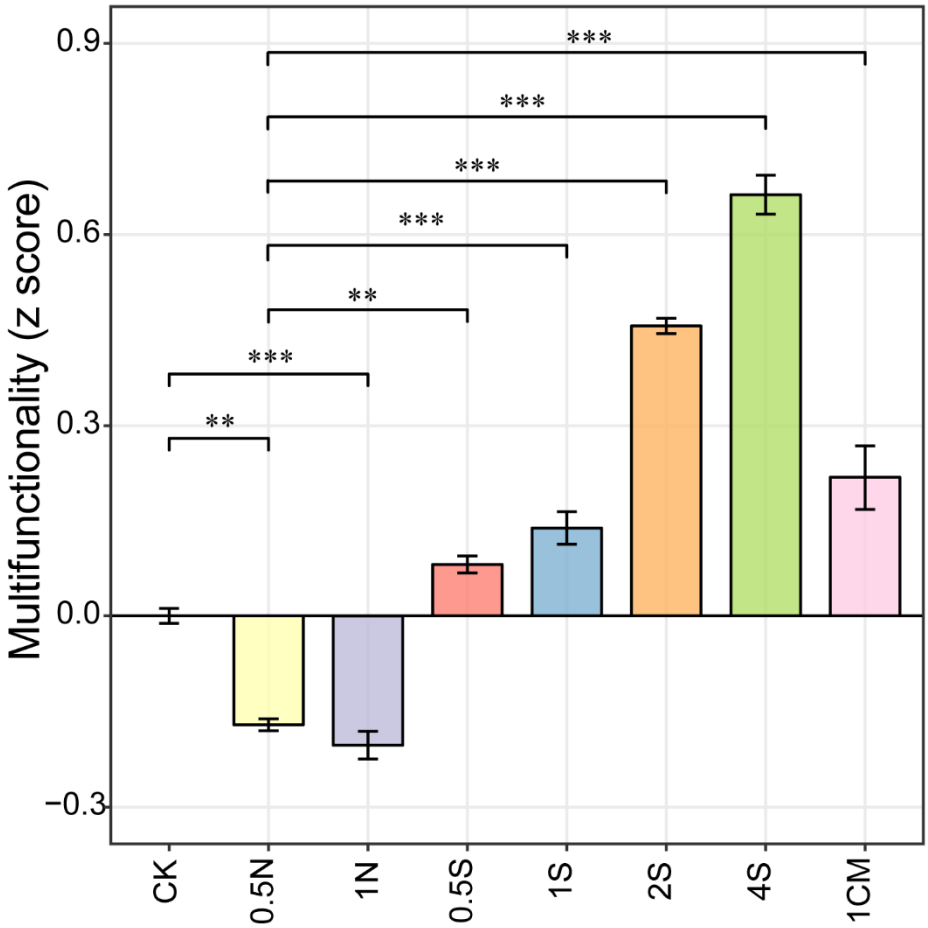
Figure 2 Relationships between ecosystem multifunctionality index and the diversity of bacteria and fungi. Solid dark line represents a significant linear relationship that fitted the ordinary least-squares (OLS) regression model, and the light green and purple shaded area shows the 95% and 99% confidence interval of the fit, respectively.

Figure 3 Relationships between various soil attributes and the diversity of bacteria. Solid dark line represents a significant linear relationship that fitted the ordinary least-squares (OLS) regression model, and the light green and purple shaded area shows the 95% and 99% confidence interval of the fit, respectively. PAO, potential ammonium oxidation; DEA, denitrification enzyme activity; SBR, Soil basal respiration; βG, β-glucosidase; NAG, N-acetyl-b-glucosaminidase; TN, total nitrogen;

637 TC, total carbon; Nitrific, nitrification related genes; Denitrific, denitrification related
638 genes; C-Degra, Carbon degradation related genes; C-Fix, Carbon fixation related
639 genes; P-Cycling, Phosphorus cycling related genes; and S-Cycling, Sulphur cycling
640 related genes.

641 **Figure 4** Diversity effects for a range of ecosystem multifunctionality thresholds.
642 Effects of bacterial (a) and fungal (b) diversity on the number of functions above
643 thresholds. Lines represent the slope between soil microbial diversity and the number
644 of functions greater than or equal to a threshold value ranging from 5 to 99% of
645 maximum for each function. The dotted curves indicate the changes in number of
646 functions per unit increment of diversity of bacteria (c) and fungi (d). T_{min}, is the
647 minimum threshold that multifunctionality becomes influenced by changes in
648 microbial diversity, and R_{med}, is the realized maximum effect of diversity on
649 multifunctionality.

650 **Figure 5** Random Forest regression model shows the main microbial drivers of
651 ecosystem multifunctionality. MSE, is the mean square error. *P < 0.05, **P < 0.01on
652 the bar indicated that the associated taxa had a significant effect on multifunctionality.



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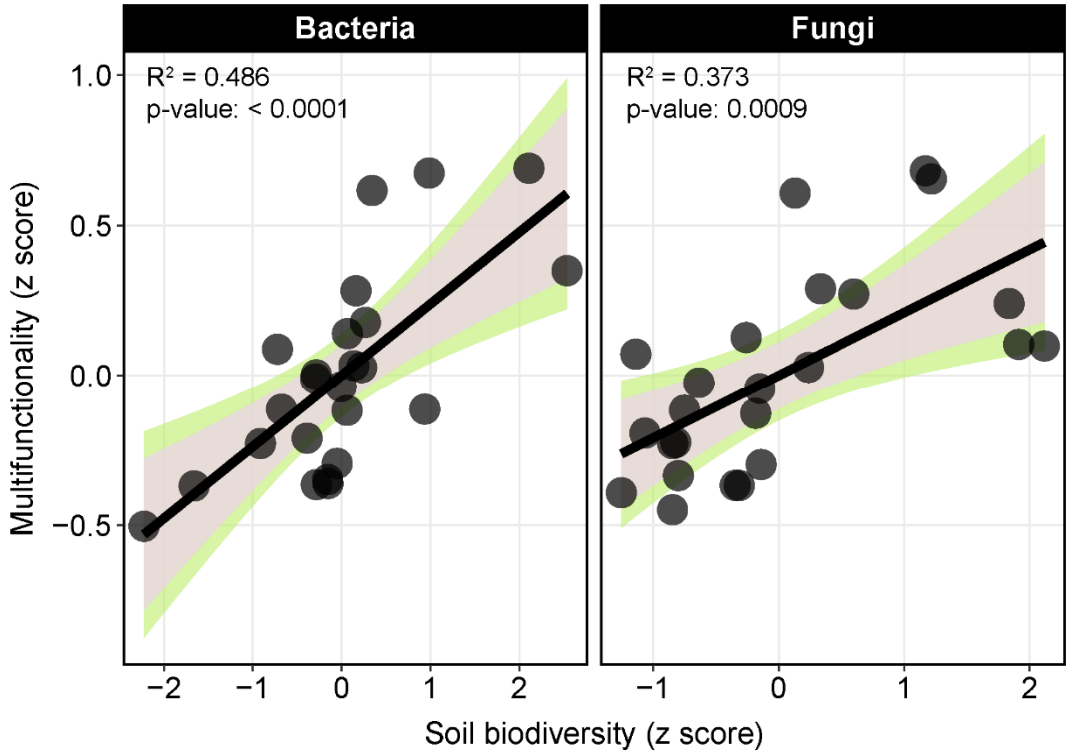

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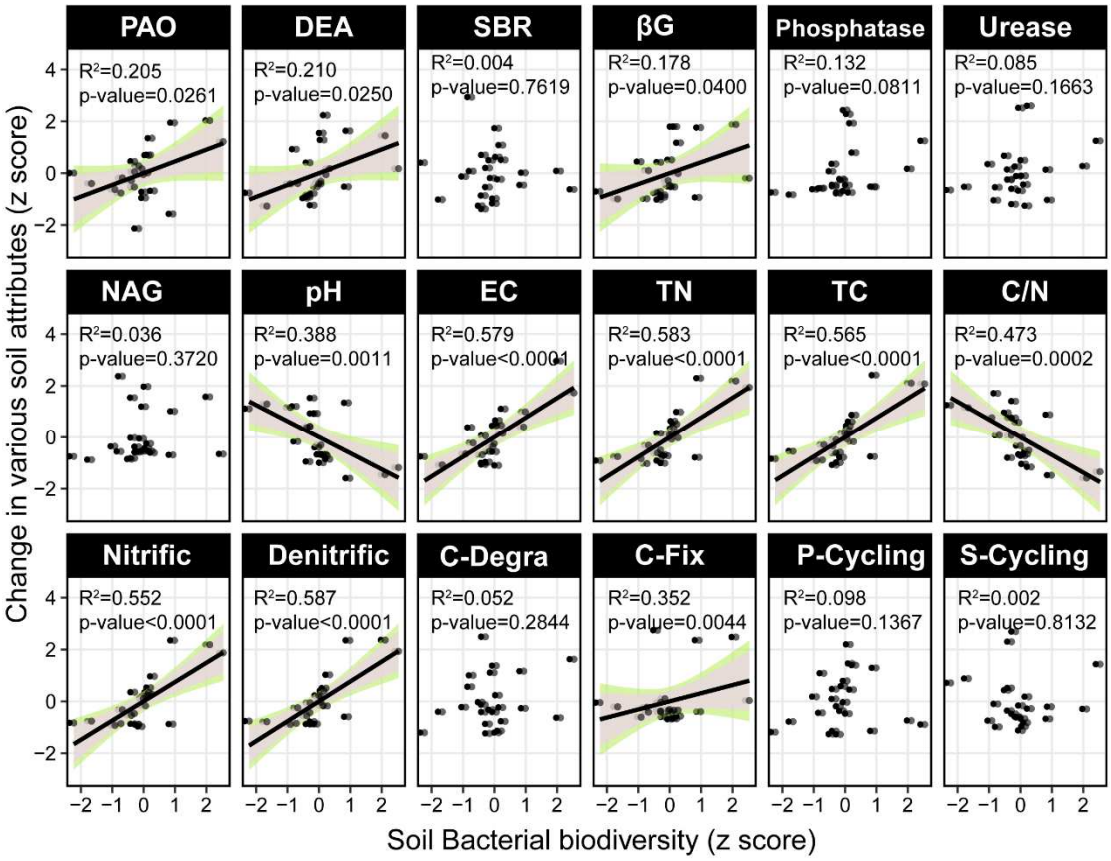

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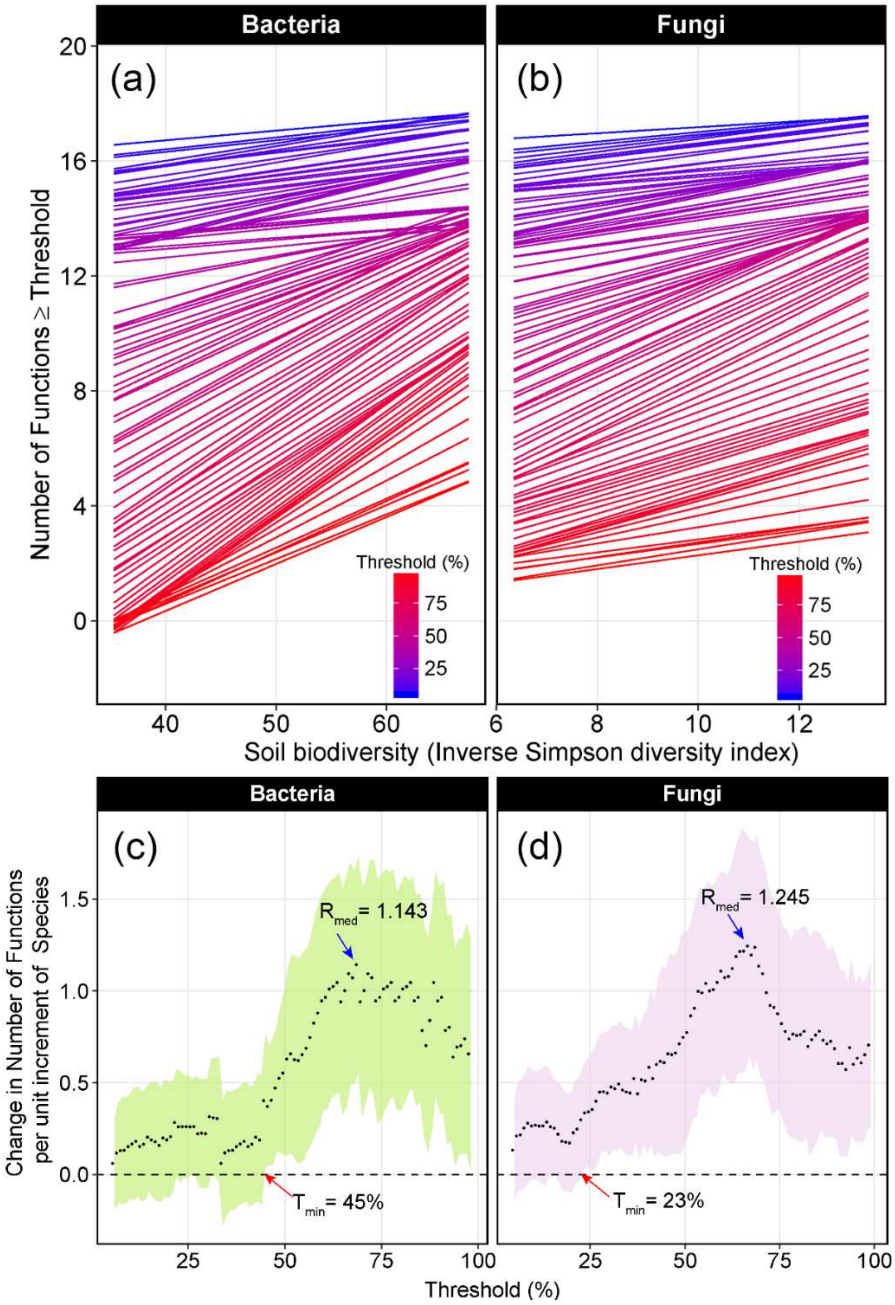

Figure 4 Diversity effects for a range of ecosystem multifunctionality thresholds. Effects of bacterial (a) and fungal (b) diversity on the number of functions above thresholds. Lines represent the slope between soil microbial diversity and the number of functions greater than or equal to a threshold value ranging from 5 to 99% of maximum for each function. The dotted curves indicate the changes in number of functions per unit increment of diversity of bacteria (c) and fungi (d). T_{min}, is the minimum threshold that multifunctionality becomes influenced by changes in microbial diversity, and R_{med}, is the realized maximum effect of diversity on multifunctionality.

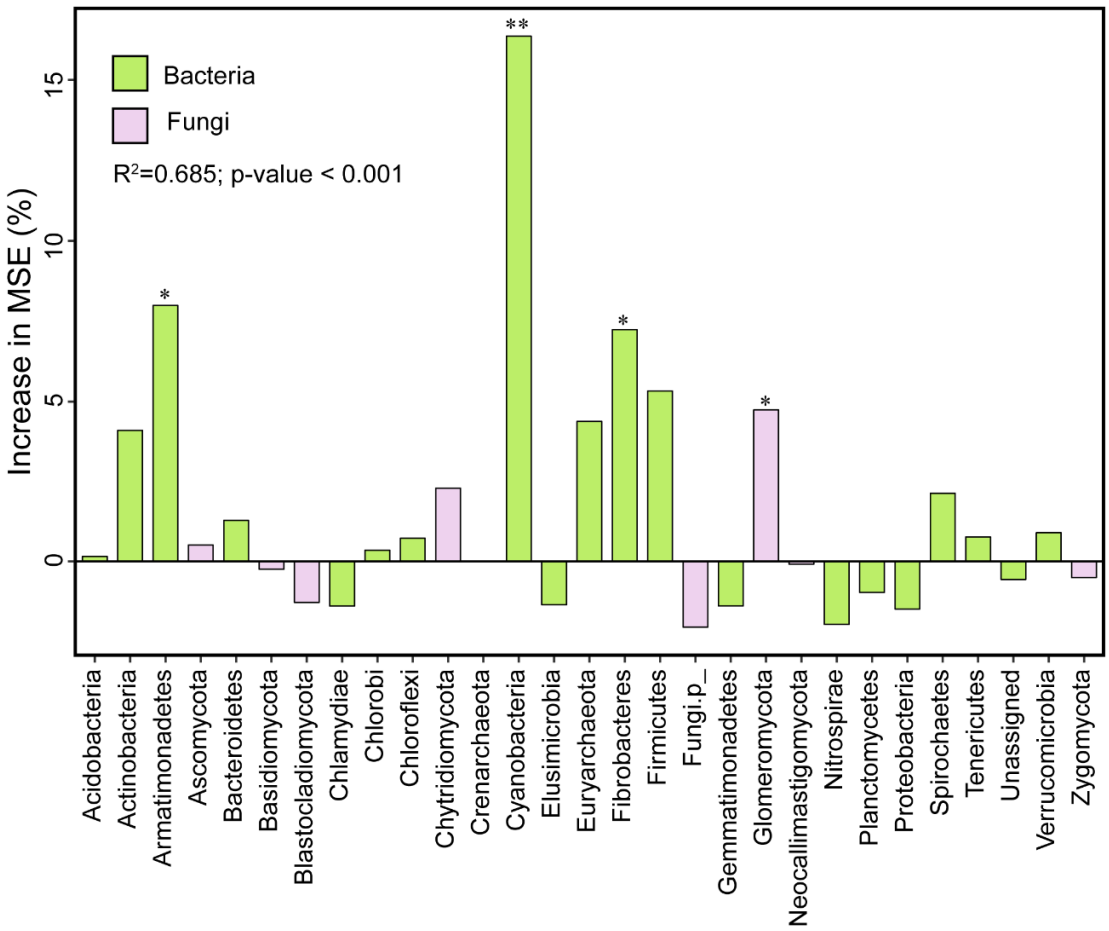

Figure 5 Random Forest regression model shows the main microbial drivers of ecosystem multifunctionality. MSE, is the mean square error. *P < 0.05, **P < 0.01on the bar indicated that the associated taxa had a significant effect on multifunctionality.

Graphical abstract