# The role of long-term mineral and manure fertilization on P species accumulation and phosphate solubilizing microorganisms in paddy red soils

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# Abstract

~~Fertilization managements have important impacts on~~Understanding soil P transformation~~,~~ and turnover~~, and~~ under various fertilization managements is important for evaluating sustainable P fertility and potential bioavailability in agriculture managements. Thus, long-term fertilization experiments (~38 years) with the application of different inorganic and organic fertilizers in paddy red soils were conducted to determine ~~their~~the effect of different fertilizer applications on P pool accumulation and microbial communities, especially for phosphate solubilizing microorganisms (PSM). Long-term inorganic P fertilization increased the concentrations of total P (~479 mg~~/~~·kg$^{-1}$), available P (~417 mg~~/~~·kg$^{-1}$), and inorganic P (~18 mg~~/~~·kg$^{-1}$), but manure fertilization accelerated the accumulation of organic P, especially for orthophosphate monoesters (e.g. myo-IHP, ~12 mg~~/~~·kg$^{-1}$). Long-term mineral fertilization decreased bacterial richness, evenness, and complexation of bacterial networks. In contrast, long-term manure fertilization and rhizosphere accumulated more amounts of total carbon, total nitrogen, and organic carbon, as well

as regulated the soil pH, thus improving the separation of bacterial communities. ~~Unlike bacteria, the responses of fungi to those factors were not sensitive.~~ Furthermore, PSM compositions were greatly influenced by fertilization managements and rhizosphere. For example, inorganic P fertilization increased the abundance of *Thiobacillus* (i.e. the most abundant phosphate solubilizing bacteria (PSB) in this study) and shifted the community structure of PSB. Correspondingly, the concentrations of inorganic and total P were the key factors for the variation of PSB community structure. These findings are beneficial for understanding ~~P accumulation, responses~~the variation of ~~PSB,~~inorganic and ~~soil P sustainable fertility~~organic P pool, and microbial community, especially for PSM under ~~different~~long-term inorganic and/or organic fertilization ~~strategies~~.

**Keywords**: long-term fertilization, P species accumulation, phosphate solubilizing microorganisms, paddy red soils, P-NMR

# 1. Introduction

Phosphorus (P) as an essential nutrient for crop growth has been widely applied to soil through mineral and/or organic fertilization (Grant et al., 2005). Manures have been frequently used as organic fertilizers in agriculture production (Braos et al., 2020). The P from manures exists in forms of various inorganic and organic species, whereas

mineral fertilizers usually only contain highly soluble $Ca(H_2PO_4)_2$ (Sharpley and Moyer, 2000). Fertilization managements are important factors for P species transformation and bioavailability. For example, mineral fertilization results in an initial high P availability but follows a decrease of P concentration over time by adsorption, complexation, and precipitation with soil particles. On the other side, the application of manure usually leads to an accumulation in labile organic P pools with potential supply to plants (Schneider et al., 2016). Additionally, the application of mineral fertilizer and manure brought different changes in soil physical, chemical, and biological attributes such as soil pH, organic carbon, microbial communities, and so on, which also induce different P transformation processes and potential availability (Yue et al., 2016; Tao et al., 2021). Soil microorganisms are usually involved in a wide range of biological processes including the transformation of insoluble soil nutrients (Babalola and Glick, 2012). After long-term fertilization, insoluble or soluble organic matter in soil may increase, thus leading to the increases of microbial biomass and activity (Marschner et al., 2003). Among them, phosphorus solubilizing microorganisms (PSM) could solubilize insoluble inorganic P, mineralize organic P, and play an important role in P transformation and availability (Sharma et al., 2013). The response of PSM in soil is

69 strongly related to the availability of P which is greatly different under various
70 fertilization managements (Sánchez-Esteva et al., 2016; Gómez-Muñoz et al., 2018;
71 Raymond et al., 2021).
72 Currently, the information about how long-term various inorganic and organic
73 fertilization managements affect the evolution characteristics of different P pools
74 remains scarce. Furthermore, the responses of microbial community especially PSM
75 shift in bulk and rhizosphere soils to the different P pool evolution under various
76 fertilization managements are still unclear. This information plays a pivotal role for
77 understanding soil P transformation mechanisms and evaluating sustainable P fertility
78 and potential bioavailability in agriculture managements. The accumulation, turnover,
79 and bioavailability of soil P pool under different fertilization managements could be
80 well evaluated by long-term fertilization experiences. Currently, numerous long-term
81 fertilization experiences have been established to evaluate the impact of different
82 fertilizer amendments on crop production and at the same time provide valuable
83 information on soil fertility by investigating changes in soil process over time (Wen et
84 al., 2019). Thus, in this study, long-term fertilization experiments (~38 years) under
85 inorganic fertilizer and/or manure amendments were conducted to determine their

effects on P pool accumulation, soil microbial communities, and PSM in paddy red soils. We hypothesized that ~~the inputs of~~ (1) long-term ~~mineral fertilizer and~~ input of inorganic fertilizers accumulates more inorganic P but the manure ~~(1) caused P~~ application and rhizosphere may accelerate the accumulation ~~with different species and potential availability~~of organic P and (2) ~~drove the shift of soil microbial community including PSM~~the long-term manure fertilization and rhizosphere could accumulate more organic nutrients, thus driving the separation of bacterial communities compared to the mineral fertilizer application.

# 2.Materials and methods

## 2.1. Field design and sampling

Long-term fertilization experiments were conducted since 1982 in a national observation and research station of farmland ecosystem (26°45′N, 111°52′E), Qiyang, Hunan Province, China. The soil was classified as Ferralic Cambisol according to World Reference Base for soil resources (Wrb, 2014), and classified as red soil according to Chinese soil classification (Baxter, 2007). Rice (*Oryza sativa*) is the major crop in this region. ~~The experimental field was disposed with five different fertilizer treatments: CK (control without fertilizer), NPK (mineral N, P, and K fertilizers), M (cattle manure),~~

NPKM, and NKM (Qaswar et al., 2020).The early rice was transplanted at the end of April and harvested in July, and the late rice was transplanted at the end of July and harvested in October. All straw (except the rice stubble) was removed from the fields after each seasonal rice harvest (Zhang et al., 2017; Yang et al., 2012). The experimental field was disposed with five different fertilizer treatments: CK (control without fertilizer), NPK (mineral N, P, and K fertilizers), M (cattle manure), NPKM, and NKM (Qaswar et al., 2020; Gao et al., 2011). Mineral fertilizers were applied in the forms of urea for N, calcium superphosphate for P, and potassium chloride for K with the amounts of 145 kg $ha^{-1}$ of N, 49 kg $ha^{-1}$ of P, and 56 kg $ha^{-1}$ of K, respectively. Additionally, the manure was added with the average nutrient contents including 18000 kg $ha^{-1}$ of C, 145 kg $ha^{-1}$ of N, 49 kg $ha^{-1}$ of P, and 56 kg $ha^{-1}$ of K. All the mineral fertilizers and manure were applied as basal application. Bulk soil samples collection with five different fertilizer treatments (1-20 cm topsoils) were conducted before the harvest of late rice in October 2020 with field replications. In each field, three soil cores (0-20 cm topsoil) were collected and then pooled to form a composite sample. Besides, before the rhizosphere soil collection, the bulk soil was manually removed, and approximately 1 mm of soil on the rice roots was collected as rhizosphere soil (Shao

et al., 2021). Soil samples used for physical and chemical analyses were four replications (2 field replication×2 replication of each field, n=4) and those for DNA extraction were six replications (2 field replication×3 replication of each field, n=6).

## 2.2. Soil physical and chemical properties

Soil pH was measured by pH meter in the mixed solution (the mass ratio of soil and water is 1:2.5). Soil moist content was measured by drying moist soil ~~to~~at 105 °C for 16 h until it became a constant mass ~~at 105 °C~~. Total carbon (TC), organic carbon (OC), and total nitrogen (TN) were determined by CHNS elemental analyzer (Vario EL Cube manufactured by Elementar, Germany) (Schumacher, 2002). ~~The soil extracts with 2 M KCl treatment were determined~~The soil was pretreated by 1M HCl with a soil-liquid ratio of 1:1 before OC determination. 1g soil was extracted with 5mL KCl (2M) to determine for ammonia-N ($NH_4^+$) by indophenol blue colorimetric method (Dorich and Nelson, 1983), and for nitrate-N ($NO_3^-$) by dual-wavelength ultraviolet spectrophotometry (Norman et al., 1985). After potassium persulfate and $H_2SO_4$ pre-digestion (Bowman, 1989), soil samples were determined for total P by a colorimetric method (Murphy and Riley, 1962). ~~The extraction of available phosphorus (AP) was referred to the method described by Olsen (Olsen, 1954)~~The extraction of available

phosphorus (AP) was referred to the method described by Olsen (1954), and the concentration was measured using a colorimetric method (Murphy and Riley, 1962). The extracted P with 0.5 M $NaHCO_3$ before/after 24 h of $CHCl_3$ fumigation was determined using ICP-OES (PerkinElmer, Avio 500, USA). A KEC factor of 0.4 was used for the calculation of soil microbial biomass P. Soil microbial biomass P was measured using a chloroform fumigation-extraction technique (Brookes et al., 1982). ~~Additionally, the activities of acid and alkaline phosphatase~~Additionally, phosphatases could mediate soil P transformation and recycling. The alkaline phosphatase in soil is released by bacteria, whereas acid phosphatase can derive from plants, fungi and bacteria (Nannipieri et al., 2011; Acosta-Martínez and Ali Tabatabai, 2011). The activities of acid and alkaline phosphatase were indicators to reflect the microbial activity and P cycling ability in soil, and were assayed by the method described by Tabatabai and Bremner (1969) using *p*-nitrophenyl phosphate as substrate at 37 °C.

## 2.3 Organic P analyses

Soil organic P was extracted with NaOH-EDTA solution according to the method described by Jiang et al. (2017). In short, 4 g air-dried soil was extracted for 4 h using 40 ml solution containing 0.25 M NaOH and 0.05 M $Na_2EDTA$. After centrifuging at

13,000 × *g* for 20 min, 2 mL aliquot of each supernatant was used to determine Fe, Mn, and P by ICP-OES. The remaining supernatants were freeze-dried and prepared for solution $^{31}$P-NMR spectroscopy. Each freeze-dried extract (~100 mg) was re-dissolved in 0.1 mL of deuterium oxide and 0.9 mL of a solution containing 1.0 M NaOH and 0.1 M $Na_2EDTA$, then immediately determined with solution $^{31}$P-NMR spectra using a Bruker 500-MHz spectrometer. The NMR parameters were: 28 K data points, 0.68 s acquisition time, 90° pulse width, and 8000 scans. The repetition delay time was calculated based on the concentration ratio of P to (Fe+Mn) according to the research by Mcdowell et al. (2006). Peak areas were calculated by integration on spectra processed with 2 and 7 Hz line-broadening using MestReNova software. Phosphorus species were identified based on their chemical shifts, including orthophosphate (6 ppm), pyrophosphate (~ -5 ppm), polyphosphate (-4 to -5, -5 to -50 ppm), orthophosphate monoesters (3 to 6, 6 to 7 ppm), orthophosphate diesters (3 to -4 ppm), and phosphonates (7 to 50 ppm). The orthophosphate peak was standardized to 6 ppm during processing (Cade-Menun et al., 2010; Young et al., 2013). Individual P compounds were identified based on their chemical shifts from the study by (Cade-Menun, 2015) and by spiking selected samples with myo-inositol hexakisohosphate

(myo-IHP), α- and β-glycerophosphates (Fig. S1 and S2).

The concentrations of individual P species were calculated by multiplying $^{31}$P-NMR proportions by the total NaOH-$Na_2$EDTA extractable P concentration. The α- and β-glycerophosphates and mononucleotides were considered as degradation of orthophosphate diesters, though they were detected in the orthophosphate monoester region (Young et al., 2013; Liu et al., 2015).

## 2.4. Soil DNA extraction, PCR amplification, Illumina Miseq sequencing, and bioinformatics analyses

The DNA was extracted from 0.25 g soil using FastDNA® Spin Kit (MP Biomedicals, USA). The purity and concentration of DNA were measured by Nanodrop 2000 (Thermo Fisher Scientific, USA). For bacteria, the V3-V4 region of the 16S rRNA gene was amplified with the primer pair 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012; Dennis et al., 2013). For fungi, the primer pair ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) were used to target the ITS1 region (Blaalid et al., 2013). After sequencing, the raw sequences of each sample were assembled by QIIME 2 according to the unique barcode after removing the adaptors and primer

sequences (Bolyen et al., 2019). Demultiplexed sequences were quality filtered, trimmed, de-noised, and merged, then the QIIME2 dada2 plugin was used to identify and remove chimeric sequences to obtain the feature table of amplicon sequence variant (ASV) (Callahan et al., 2016). ASV sequences were aligned to the GREENGENES database and UNITE database separately to generate the taxonomy table for bacteria and fungi (Bokulich et al., 2018). Besides, phosphorus-solubilizing microbes were collected according to the researches by RodríGuez and Fraga (1999) as well as Alori et al. (2017) ( see Table S1). The raw reads of bacteria and fungi were deposited in the NCBI Sequence Read Archive (SRA) database under accession numbers PRJNA804681 and PRJNA805018, respectively.

## 2.5. Statistical analyses

All statistical analyses were conducted using SPSS 25.0. All indicators between different fertilizer treatments (i.e., CK, NPK, M, NPKM, and NKM) were tested for significant differences (set to $p < 0.05$) by one-way ANOVA. The LSD was used to test significant differences of all indicators between bulk and rhizosphere soils. Alpha (α) diversity indices, such as Chao1 richness estimator and Shannon diversity index, were calculated using the core-diversity plugin within QIIME2. Nonmetric multidimensional

scaling (NMDS) based on Bray Curtis distance was measured by R package "vegan" and visualized via R package "ggplot 2". Co-occurrence network analysis was performed by using R package "psych" to calculate Spearman's rank correlations for taxa among 6 repetitions of each treatment group and then Gephi 0.9.2 software was used to draw networks. Redundancy analysis (RDA) was performed by Monte Carlo analysis using Canoco 5 to reveal the association of microbial communities and soil environmental factors.

# 3. Results

## 3.1 Soil physicochemical properties

In this study, we found that TC, TN, and OC increased significantly after the long-term application of fertilizers, especially for manure fertilization (Table 1). ~~It was expected that the application of fertilizers increased the plant biomass such as plant residues and root exudates (Tong et al., 2019). In addition, the input of manure also brought high C and N contents in soil (Wei et al., 2017).~~ The concentrations of microbial biomass P increased under long-term fertilization (Table 1). Additionally, the activities of acidic phosphatase (ACP) were higher than alkaline phosphatase activities (ALP) for all the treatments (Fig. 1H and I). On the other side, soil pH value, gravimetric moisture, $NO_3^-$

-N, and $NH_4^+$-N contents were not affected by the long-term fertilizer treatments significantly (Table 1). ~~Notably, a previous study of the fields has found that the pH decreased with NPK treatment but increased with organic fertilization (Ahmed et al., 2019), which was inconsistent with this study. The possible reason is that two sampling time was different and continuous heavy rainfall before sampling may also reduce the difference of pH among treatments in this study.~~

## 3.2 Soil P species

Long-term application of inorganic P fertilizer (NPK and NPKM) could significantly increase total P (TP), available P (AP) and inorganic P (IP) concentrations in both bulk and rhizosphere soil (Fig. 1A, B, and C). The concentrations of NaOH-$Na_2$EDTA extracted P in the soils were ~243-739 mg·kg$^{-1}$, accounting for ~38-66% of total P (Table 2). Orthophosphate, pyrophosphate, orthophosphate monoesters (e.g. myo-IHP, scyllo-IHP), and orthophosphate diesters (e.g. DNA) were found in the soils (Table 2). The amounts of soil organic P (i.e. sum of orthophosphate monoesters and diesters) were not much and accounted for 8-30% of total P (data not shown). Generally, the concentrations of organic P were higher with long-term manure fertilization compared to those of CK and NPK (Fig. 1D). Among the OP, the amounts of orthophosphate

monoesters (57-96 mg·kg$^{-1}$) were higher than those of orthophosphate diesters (34-65 mg·kg$^{-1}$) (Table 2). The long-term manure amendments had an obvious effect on the accumulation of orthophosphate monoesters: the concentrations of orthophosphate monoesters and myo-IHP were higher significantly with manure fertilization (i.e. M, NPKM, NKM) than those with other treatments (i.e. CK, NPK) (Fig. 1E and G). ~~Phosphate monoesters were regarded as relatively stable and were the dominant group of organic phosphorus compounds in most soils (Tabatabai, 1989), mainly including inositol phosphates (e.g. myo, scyllo, D-chiro, neo) (Cosgrove and Irving, 1980; Turner et al., 2002).~~ The concentrations of orthophosphate diesters were also higher with manure treatments compared to CK and NPK although the tendency was not significant (Fig. 1F).

## 3.3 Long-term fertilization and rhizosphere effect on the composition of microbial community

The dominant bacteria for different treatments at the phylum level were *Proteobacteria, Acidobacteria, Chloroflexi,* and *Nitrospirae* and the dominant fungi were *Ascomycota* and *Basidiomycota* (Fig. 2). As the most abundant phylum of bacteria, *Proteobacteria* were further classified into *Alphaproteobacteria, Betaproteobacteria,*

256 *Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria,* and unclassed
257 groups at the class level. *Gammaproteobacteria* was significantly more abundant for
258 manure treatments than for CK and NPK treatments. The abundance of
259 *Epsilonproteobacteria* increased after mineral fertilization. Both inorganic and organic
260 fertilization could increase the abundance of *Alphaproteobacteria* (Fig. S3). On the
261 other side, certain bacteria and fungi at the phylum level affected by fertilization were
262 different in rhizosphere and non-rhizosphere soils. For example, the long-term manure
263 fertilization accumulated more *Spirochaetes* but less *Actinobacteria* and *TM7* in non-
264 rhizosphere soils (Fig. 2A and C). The relative abundance of *Ascomycota* increased
265 significantly with fertilization in non-rhizosphere soils but not the case in the
266 rhizosphere soils (Fig. 2B and D). These results suggested that both long-term
267 fertilization and rhizosphere affected the microbial community composition together.
268 The relative abundances of PSB were also greatly influenced by fertilization and
269 rhizosphere. The *Thiobacillus* was the most abundant bacterium at genus level and
270 increased with long-term input of inorganic P in both bulk and rhizosphere soils (Fig. 3
271 A and C). Additionally, the long-term manure fertilization increased the abundance of
272 *Flavobacterium* in bulk soil. On the other side, the *Fusarium* was the most abundant

fungus at genus level (Fig. 3 B and D). The influence of fertilization on the phosphorus-solubilizing fungi (PSF) in bulk soil was not obvious. However, manure fertilization increased the abundance of *Aspergillus* and *Trichoderma* in rhizosphere soils.

### 3.4 Microbial community diversity

Soil with long-term mineral fertilization (NPK) presented a lower bacterial richness and evenness (i.e. Chao 1 and Shannon index) than those with manure fertilization (M/NPKM/NKM) and even lower than control soil (CK), indicating that bacterial α-diversity decreased after long-term mineral fertilizer regimes, but was not changed under manure fertilization (Fig. 4). ~~Other long-term field studies have also shown the similar tendency (Li et al., 2015; Francioli et al., 2016; Wang et al., 2018).~~ On the other side, rhizosphere effect was clearly observed on the bacterial diversity: the richness and evenness of bacterial community in rhizosphere soil were significantly higher than those in non-rhizosphere soil ($P<0.001$). It is worth noting that fertilization and rhizosphere effect have no obvious influence on fungal richness and evenness. It suggested that long-term fertilization and rhizosphere affected the richness and evenness of bacterial and fungal communities differently.

The plot of ~~N -MDS~~NMDS identified the variations in microbial β-diversity between

different sites, with the response of bacterial β-diversity being greater than that of fungal β-diversity (Fig. 5). Specially, the profiles of bacterial β-diversity with manure fertilizations (M, NKPM, NKM) were clearly separated from that for CK soil (Fig. 5 A and C). The analysis of similarities (ANOSIM) revealed that R values for rhizosphere soils between different fertilization treatments were higher than those for bulk soils (Table S2). Accordingly, the variations in bacterial β-diversity of rhizosphere soils with manure fertilization were greater than that of bulk soils (Fig. 5 A and C). These results indicated that manure fertilization and rhizosphere effect exacerbated the variation of bacterial β-diversity.

### 3.5 Co-occurrence networks

The co-occurrence network was used to analyze the ecological relationship of both bacterial and fungal communities under five fertilization treatments. After long-term mineral fertilization (NPK), total edges, average degree, positive edges, and positive/negative edges ratio (i.e. P/N ratio) of bacteria and fungi network decreased (Fig. ~~6 and Table S3), indicating that long-term mineral fertilization increased the stability of microbial network (e.g.~~ 6 and Table S3). ~~lower P/N ratio) but decreased the complexity of network (e.g. less total edges and lower average degree) (Tu et al., 2020;~~

~~Olesen et al., 2007; Hernandez et al., 2021). Meanwhile, long-term manure treatments (M, NPKM, NKM) increased the negative connections of microorganisms, and also promoted the stability of network (Zhou et al., 2020).~~ Additionally, the high P input (NPKM vs NKM) brought a larger and more complex but less stable bacterial network (e.g. more total nodes, edges, average degree, average clustering coefficient, average path length, and less modularity). However, the opposite tendency was shown for fungus network (Fig. 6 and Table S3), indicating that the response of bacteria and fungi to the input of inorganic P was different.

**3.6 Factors correlating with microbial community diversity**

Redundancy analysis (RDA) was conducted to determine the correlation of soil properties with microbial community diversity in bulk and rhizosphere soils. The results showed that TC (10.4%, F=4.4, P=0.03), soil pH value (10.3%, F=4.4, P=0.03), TN (10.1%, F=4.3, P=0.03), and OC (9.2%, F=3.9, P=0.03) were significantly correlated with bacterial community diversity (Fig. 7A, Table S4). On the other side, for the fungus, the soil properties had extremely small explanations of <4.4% for the variation for fungi community (Table S4).

The RDA was also performed to establish the linkages of soil properties with

community diversity of PSM. The soil properties together explained more than 55% of the variation in PSB community structure and those correlated with PSB contained TP (27.5%, F=14.4, P=0.03) and IP (26.6%, F=13.7, P=0.03) (Fig. 7C and Table S5). The PSB was well separated by RDA1 (52.60 %) between the samples with inorganic P application (i.e. NPK, NPKM) and without inorganic P application (i.e. CK, M, NKM) (Fig. 7C). The 30.08% of the total variance in the PSF community could be explained by the first and second axes (Fig. 7D).

# 4. Discussion

## 4.1. Long-term fertilization on soil P accumulation

Long-term organic P fertilization increased the utilization of P for crops compared to inorganic P fertilization. The same amount of P was added to soil whatever inorganic or organic fertilization but the total P of soil was significantly higher with mineral fertilization compared to manure treatment, suggesting more P was retained in soil and less P was utilized by crops under long-term mineral fertilization (Fig. 1A). Several researchers have already reported that inorganic P was easily immobilized by clay minerals and was dominantly associated with amorphous Fe/Al oxides compared to crystalline Fe/Al oxides fractions in many soil types such as Sandy soils, Ultisols,

Luvisol, Ferralic Cambisol, and so on (Arai et al., 2005; Rick and Arai, 2011; Jiang et al., 2015; Ahmed et al., 2019). –On the other side, it has been confirmed that the application of manure usually leads to an increase in labile organic P pools, which are protected from the process of adsorption on clay minerals and are readily available to plants (Braos et al., 2020; Kashem et al., 2004). In this study, manure fertilization increased microbial biomass P concentration and alkaline phosphatase activity compared to mineral fertilization (Fig. 1I, Table 1). It was possible that the mineralization of organic P such as orthophosphate diesters from microbes by alkaline phosphatase increased under organic fertilization, thus improving the P availability for crops.

The application of inorganic P fertilizer mainly increased the concentration of inorganic P but manure fertilization accelerated the accumulation of organic P in soil ~~(Fig.~~, which was consistent with our hypotheses (Fig. 1C and D). Phosphorus speciation was usually regulated by the changes in soil mineralogy, mineral and organic P inputs, biological production, and the utilization of various P species (Turner et al., 2007; Jiang et al., 2017). Fertilization especially for manure accelerated the accrual of organic carbon (Table 1) significantly, which also co-accumulated organic P. Generally, the content of

358 organic phosphorus (OP) from manures accounts for a large proportion of total P,
359 among which inositol phosphate (IHP) was the most abundant OP (Maguire et al., 2004).
360 Therefore, long-term manure fertilization also increased the input of OP in the field.
361 The organic P could be effectively mineralized by microorganisms and thus transferred
362 into various inorganic P fractions (Song et al., 2007).
363 The application of manure increased the accumulation of orthophosphate monoesters
364 significantly, especially for myo-inositol phosphates (myo-IHP) (Fig. 1E and G).
365 Normally, phosphate monoesters were the main group of organic P compounds and
366 existed as IHP mainly in most soils (Turner et al., 2005). Those orthophosphate
367 monoesters were commonly stabilized by association with soil minerals such as Fe/Al
368 oxides (Celi and Barberis, 2007; Turner and Engelbrecht, 2011; Jiang et al., 2015).
369 Therefore, the stability and immobilization of orthophosphate monoesters promoted
370 their accumulation in soil no matter by the input of manure or by the P transformation.
371 On the other side, separate manure fertilization (M) also increased the contents of
372 orthophosphate diesters significantly (Fig. 1F). Long-term manure fertilization
373 accumulated more microbial biomass P significantly (Table 1) that were rich in
374 orthophosphate diesters (Turner et al., 2007). The accumulation of orthophosphate

diesters under manure fertilization was probably due to the reduced decomposition of plant residues and manure or increased microbial synthesis under anaerobic paddy-rice management (Jiang et al., 2017).

## 4.2 Long-term fertilization and rhizosphere effect on soil microbial communities

Our results indicated that long-term mineral fertilization decreased bacterial richness, evenness, and the decrease of complexation of bacterial networks, indicating that long-term mineral fertilization increased the stability of microbial network (e.g. lower P/N ratio) but decreased the complexity of network (e.g. less total edges and lower average degree) (Tu et al., 2020; Olesen et al., 2007; Hernandez et al., 2021). On the other side, long-term organic fertilization did not change the bacterial richness and evenness, and even promoted the separation of bacterial communities. This conclusion was also expected in our hypothesis. Meanwhile, long-term manure treatments (M, NPKM, NKM) increased the negative connections of microorganisms, and also promoted the stability of network (Zhou et al., 2020). Previous studies have reported that long-term mineral fertilization changed soil properties and these perturbations may have an adverse effect on soil microbes (Marschner et al., 2003; Geisseler and Scow, 2014;

Liang et al., 2020). In contrast, organic fertilizer contained a large amount of organic matter which could be utilized by soil bacteria (Wu et al., 2020; Wu et al., 2021). Additionally, there were significant increases for diversity of bacterial communities in rhizosphere soil compared to bulk soil. Generally, microbes concentrated in the rhizosphere where organic compounds were released by plant roots (Achat et al., 2010), and plants tend to recruit bacteria as symbiotic microbes by releasing phenolic compounds (Gkarmiri et al., 2017; Badri et al., 2013).

Accordingly, redundancy analysis showed that the key factors related to the shift of bacterial communities included pH, TC, TN, and OC. The previous study showed that the soil ~~bacteria~~bacterial community was indirectly impacted by pH via the alteration of metals and nutrient availability (Xiao et al., 2021), and directly modulated by the abundance and mineralization of carbon in soil (Chen et al., 2019) as well as soil nitrogen deposition (Zeng et al., 2016). In this study, the long-term organic fertilization and rhizosphere soil accumulated more TC, TN, and OC, which provided more nutrients, changed the soil pH, and thus drove the shift of bacterial communities (Ingwersen et al., 2008; Liu et al., 2019).

Additionally, the application of both mineral and organic fertilizers increased the

409 stability of bacterial networks (i.e., increasing negative correlations). Compared to CK,
410 long-term fertilization provided more nutrient elements, stimulated the growth and
411 competition of bacteria, and finally facilitated the stability of ecological network (Faust
412 and Raes, 2012; Simard et al., 2012).
413 It was worth noting that fertilization and rhizosphere effect had no obvious influence
414 on fungal community structure. Redundancy analysis showed that the explanations of
415 soil properties were extremely small for the variation for fungi community. It has been
416 found that fungi were less sensitive to soil substrates and environmental conditions
417 whereas bacteria were more sensitive (Dong et al., 2014). The high TOC provided by
418 the long-term fertilization and rhizosphere soil gave an advantage for bacteria to
419 compete with fungi for resources, thus decreasing influences of long-term fertilization
420 and rhizosphere on fungi (Zelezniak et al., 2015).

421 ## 4.3 Response of PSM

422 *Thiobacillus* was the most abundant PSB at genus level and increased with the input of
423 inorganic P fertilizers in bulk and rhizosphere soil (Fig. 3 a and c). It was involved in
424 sulfur oxidation, and acidity resulted from sulfur oxidation could solubilize mineral P
425 (Aria et al., 2010). Acidic and anaerobic conditions provided by paddy-rice

management of red soil in this study were beneficial for the growth of *Thiobacillus* considering that it belongs to acidophilic bacterium (Monachon et al., 2019; Kumar et al., 2020). The applied calcium superphosphate as inorganic P fertilizer in this study contained a certain amount of $CaSO_4$, therefore the input of inorganic P fertilizer also provided S source for the growth of *Thiobacillus.* On the other side, *Fusarium* was the most abundant PSF at genus level (Fig. 3 B and D) and was proven to produce organic acid to solute the mineral P (Elias et al., 2016). It was known that *Fusarium* was widely distributed in soil around the world and acted as a saprophyte (Deacon, 1997), among which many species were also found as phytopathogens (Suga and Hyakumachi, 2004). Besides, the long-term organic fertilization increased the abundance of *Flavobacterium, Aspergillus*, and *Trichoderma. Flavobacterium* was associated with the degradation of phosphotriester (Brown, 1980) and was proven to grow in a nutrient-rich condition (Kraut-Cohen et al., 2021). *Aspergillus*, as a saprophytic fungus, could produce organic acid to dissolve mineral phosphorus (Li et al., 2016) and also preferred to the nutrient-rich condition (Martins et al., 2014). Additionally, *Trichoderma* as a biological control fungi (Zin and Badaluddin, 2020) was colonized in the root epidermis and outer cortical layers (Harman, 2006). Long-term organic fertilization provided more organic matter

443 for these microbes.

444 PSM could solubilize mineral P and mineralize organic P (Sharma et al., 2013). The
445 PSB of samples with inorganic P input (i.e. NPK, NPKM) and none mineral P
446 application (i.e. CK, M, NKM) could be well separated, indicating mineral P had a
447 strong effect on community diversity of PSB. Correspondingly, TP and IP were key
448 factors driving the diversity of soil PSB community and those indicators were all higher
449 significantly with inorganic P amendments (Fig. 1A, and C). As discussed before,
450 *Thiobacillus* as the most abundant PSB at genus level in this study increased with the
451 input of mineral P. It is because that mineral P could provide additional S source for the
452 growth of *Thiobacillus*. Furthermore, the availability of P in soil was considered as a
453 key condition for PSM to express P-solubilization traits. Low availability of P in soil is
454 widely considered as a favorable condition for PSM whereas recent studies suggested
455 that a minimum P threshold is required to achieve a response by plants (Sánchez-Esteva
456 et al., 2016; Gómez-Muñoz et al., 2018; Raymond et al., 2021).

457 # 5. Conclusion

458 Long-term inorganic and organic fertilization managements brought different effects on
459 P accumulation, microbial community, and PSB. Long-term mineral fertilization

increased inorganic and available P concentrations~~. In contrast,~~, while manure fertilization increased soil organic P concentrations, microbial biomass P contents, and ~~alkaline phosphatase activity, which is beneficial for the~~potential organic P mineralization ~~of organic P, especially for orthophosphate diesters.~~. The turnover of P by bacteria seems strong under long-term organic fertilization and rhizosphere soil considering that more organic nutrient was provided for bacteria and the bacterial community diversity increased. Furthermore, ~~the responses of PSM to different fertilization managements were also different. For example,~~ inorganic P fertilization increased the abundance of *Thiobacillus* ~~(i.e. the most abundant PSB in studied soil)~~ whereas organic fertilization increased the abundance of *Flavobacterium, Aspergillus*, and *Trichoderma*. The concentrations of TP and IP strongly influenced by inorganic P fertilization were key factors driving the diversity of soil PSB community. These findings provide useful insights into P accumulation, turnover, and soil P sustainable fertility under different fertilization strategies.

**Acknowledgments:**

This study was financially supported by the National Natural Science Foundation of

China (No.41907063) and -Special Project of Hunan Innovative Province Construction (2022JJ30648).

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**Competing interests:**

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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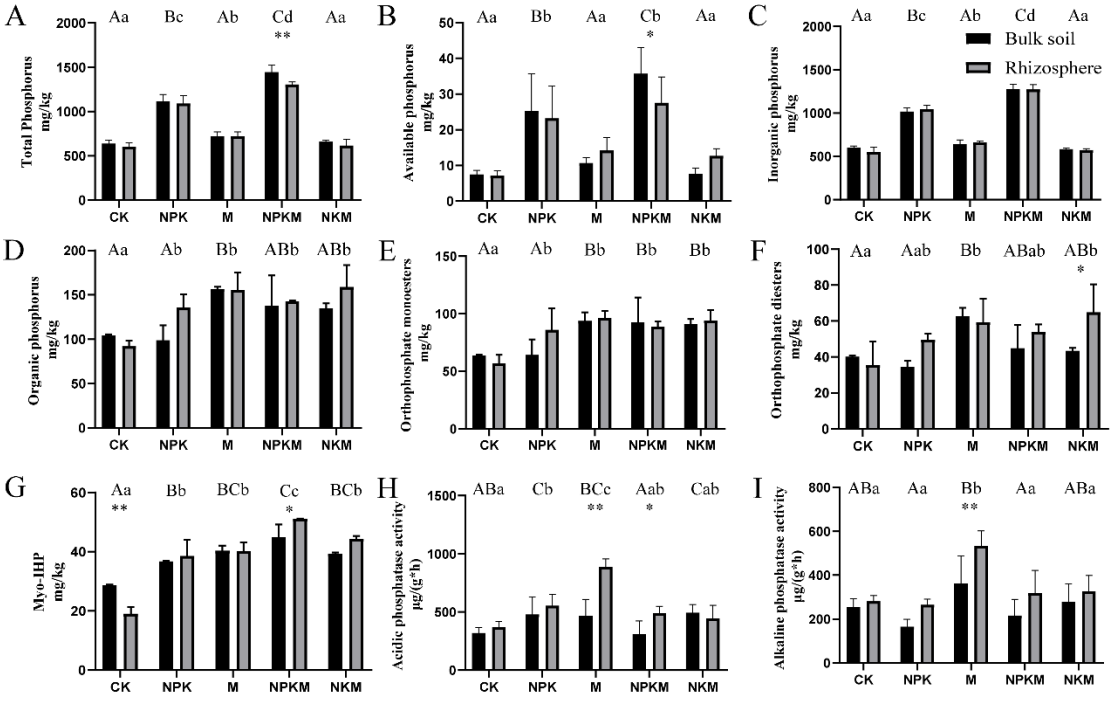



Fig. 1 Different phosphorus forms and phosphatase activities in five treatments (CK, NPK, M, NPKM, NKM) and two sample types (rhizosphere and bulk soil), where A: Total phosphorus, B: Available phosphorus, C: Inorganic phosphorus, D: Organic phosphorus, E: Orthophosphate monoesters, F: Orthophosphate diesters, G: Myo-IHP, H: Acidic phosphatase activity, I: Alkaline phosphatase activity. Significant differences between treatments in bulk soil are indicated by capital letters ($p<0.05$, $n = 4$). Significant differences between treatments in rhizosphere are indicated by lowercase letters ($p<0.05$, $n = 4$). Significant differences between rhizosphere and bulk soil are indicated by

asterisks, where * p < 0.05, ** p < 0.01 (Duncan's test, n=4)

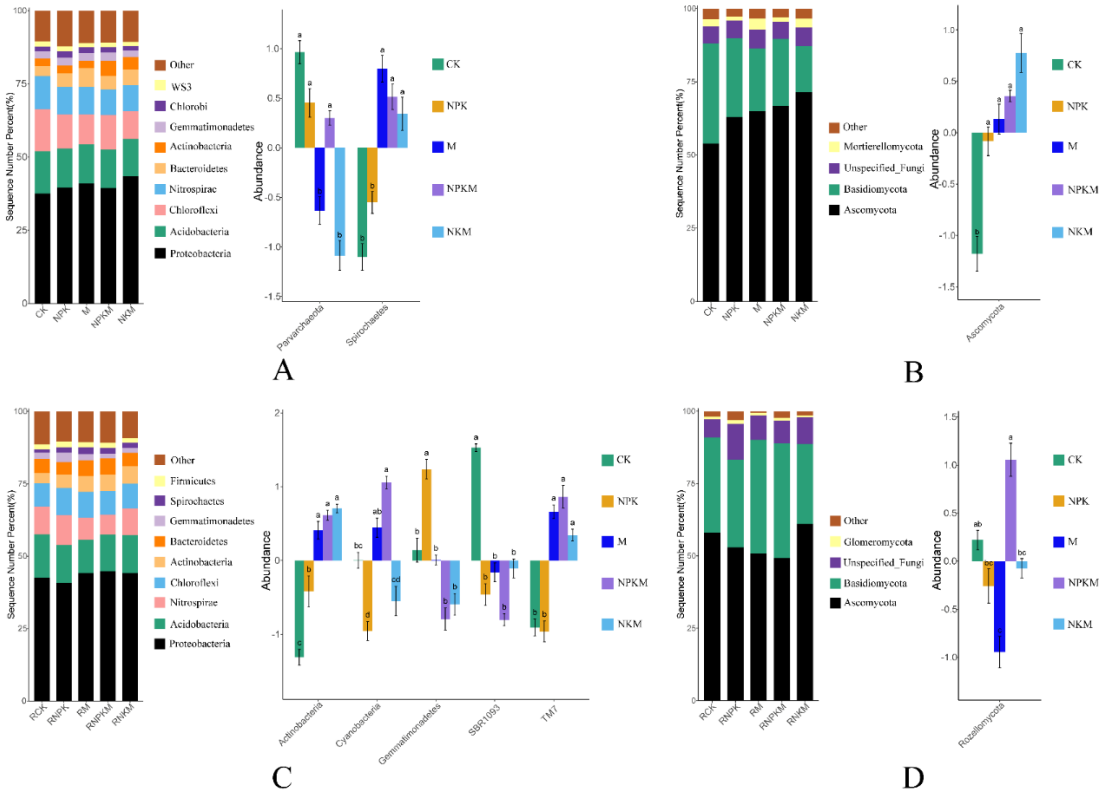



Fig. 2 The microbial relative abundance (left) and the features with significant differences (Anova + Duncan, p<0.05, n=6) between groups (right) at the phylum level in five treatments (CK, NPK, M, NPKM, NKM). Capital letters means different classification (A: bacteria in bulk soil, B: fungi in bulk soil, C: bacteria in rhizosphere soil, D: fungi in rhizosphere soil)

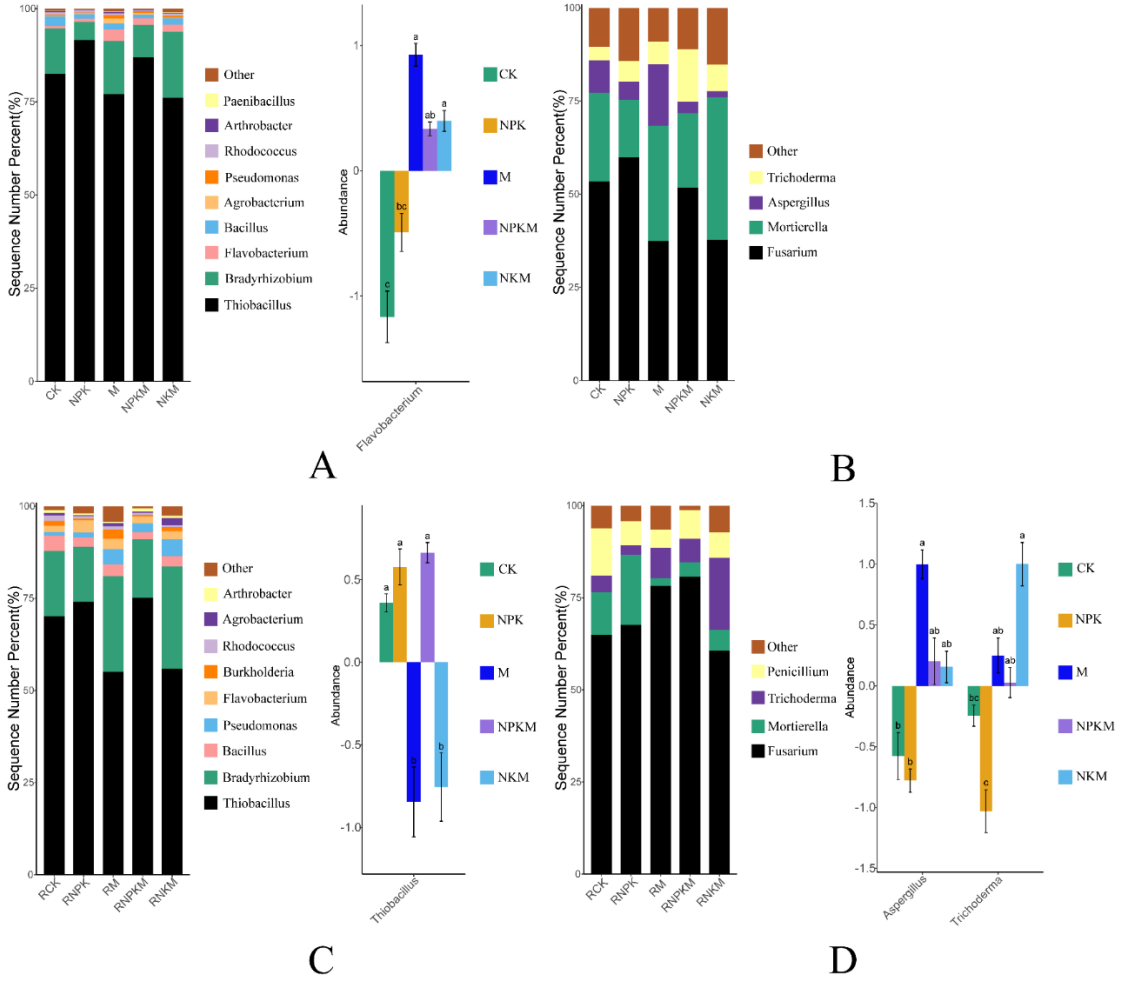



Fig. 3 The relative abundance of phosphorus-solubilizing microbe (left) and features with significant differences (Anova + Duncan, $p<0.05$, $n=6$) between groups (right) at the genus level in five treatments (CK, NPK, M, NPKM, NKM). Capital letters means different classification (A: bacteria in bulk soil, B: fungi in bulk soil, C: bacteria in rhizosphere soil, D: fungi in rhizosphere soil)

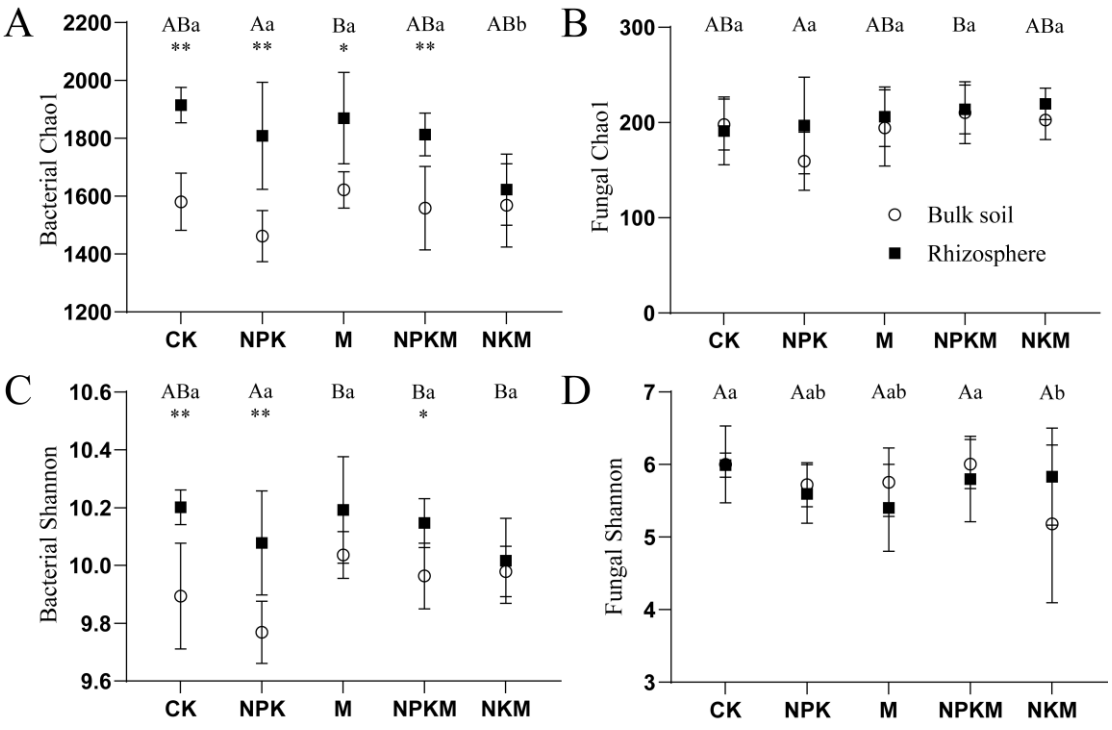
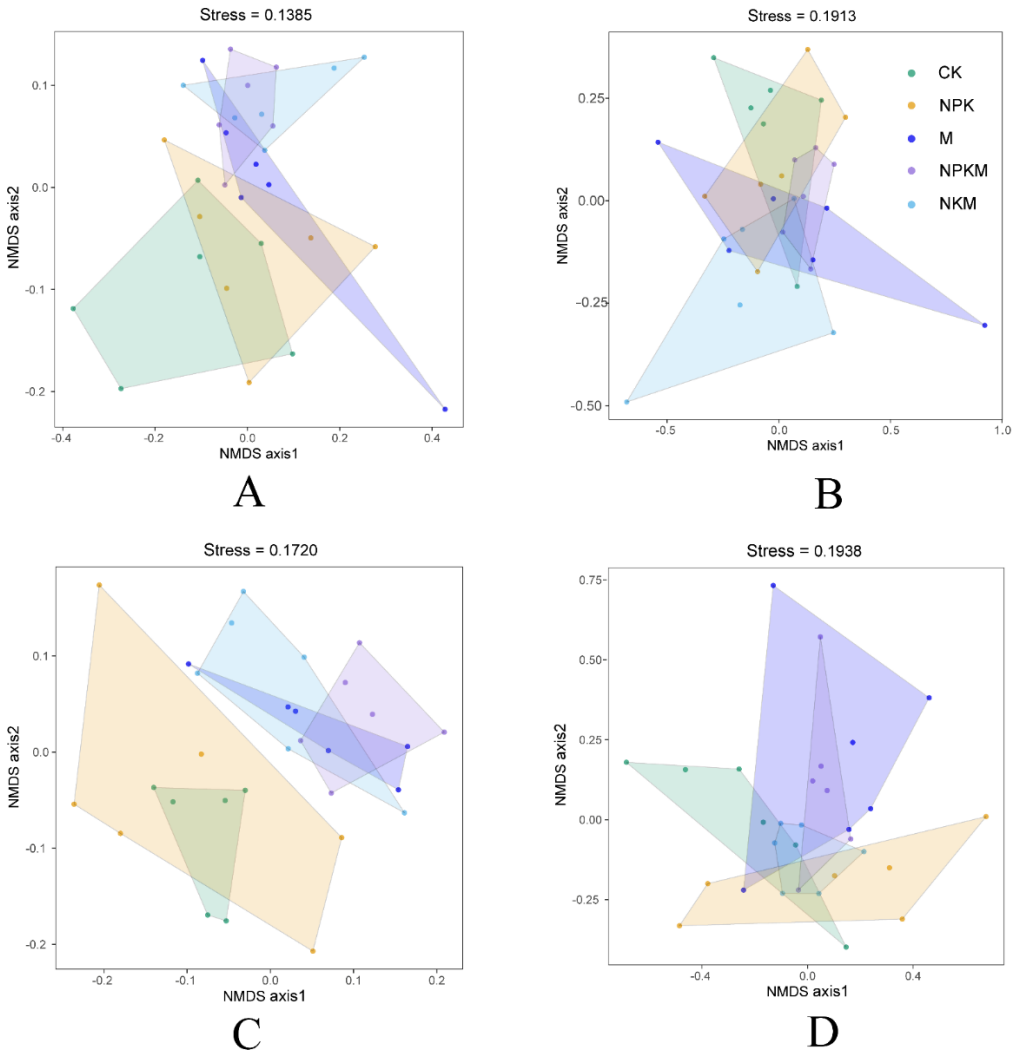



Fig. 4 Mean ± SE values for microbial α-diversity (A: Bacterial Chao1 index, B: Fungal Chao1 index, C: Bacterial Shannon index, D: Fungal Shannon index) in five treatments (CK, NPK, M, NPKM, NKM) and two sample types (rhizosphere and bulk soil). Significant differences between treatments in bulk soil are indicated by capital letters ($p<0.05$, $n = 6$). Significant differences between treatments in rhizosphere are indicated by lowercase letters ($p<0.05$, $n = 6$). Significant differences between rhizosphere and bulk soils are indicated by asterisks, where * $p < 0.05$, ** $p < 0.01$ (Duncan's test, n=6)

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799 Fig. 5 Nonmetric multi-dimensional scaling (NMDS) ordination of the microbial community by comparing with
800 Bray-Curtis distance similarities based on the abundance of OTUs. Capital letters means different classification (A:
801 bacteria in bulk soil, B: fungi in bulk soil, C: bacteria in rhizosphere soil, D: fungi in rhizosphere soil)

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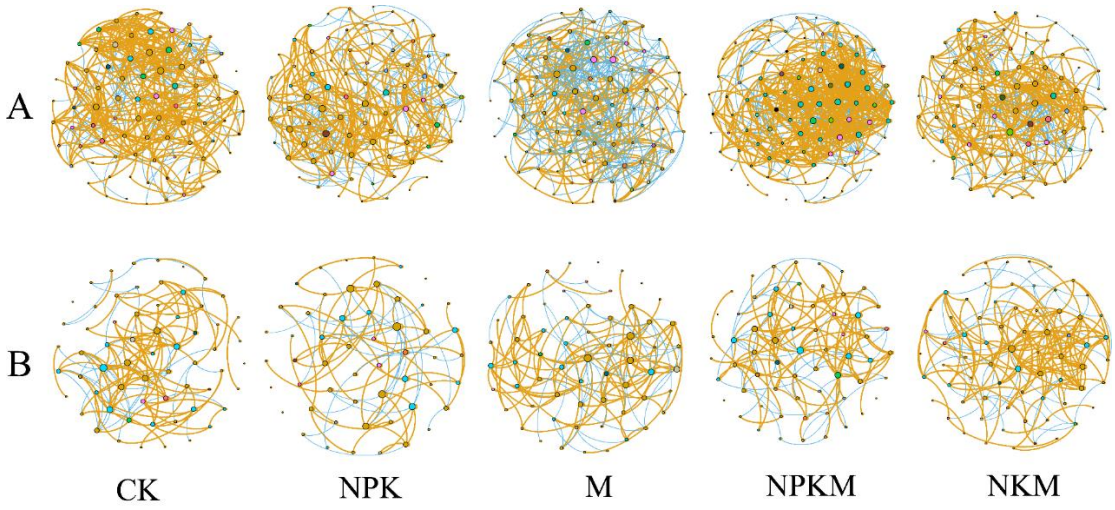


Fig. 6 Network of co-occurring bacterial (A) and fungal (B) OTUs across five fertilizer treatments. Only Spearman's correlation coefficient $r > 0.6$ or $r < -0.6$ significant at $P < 0.01$ is shown. The nodes are colored according to phylum. orange edges represent positive correlations and blue edges represent negative correlations. Node size presents the connecting numbers of each OUT.

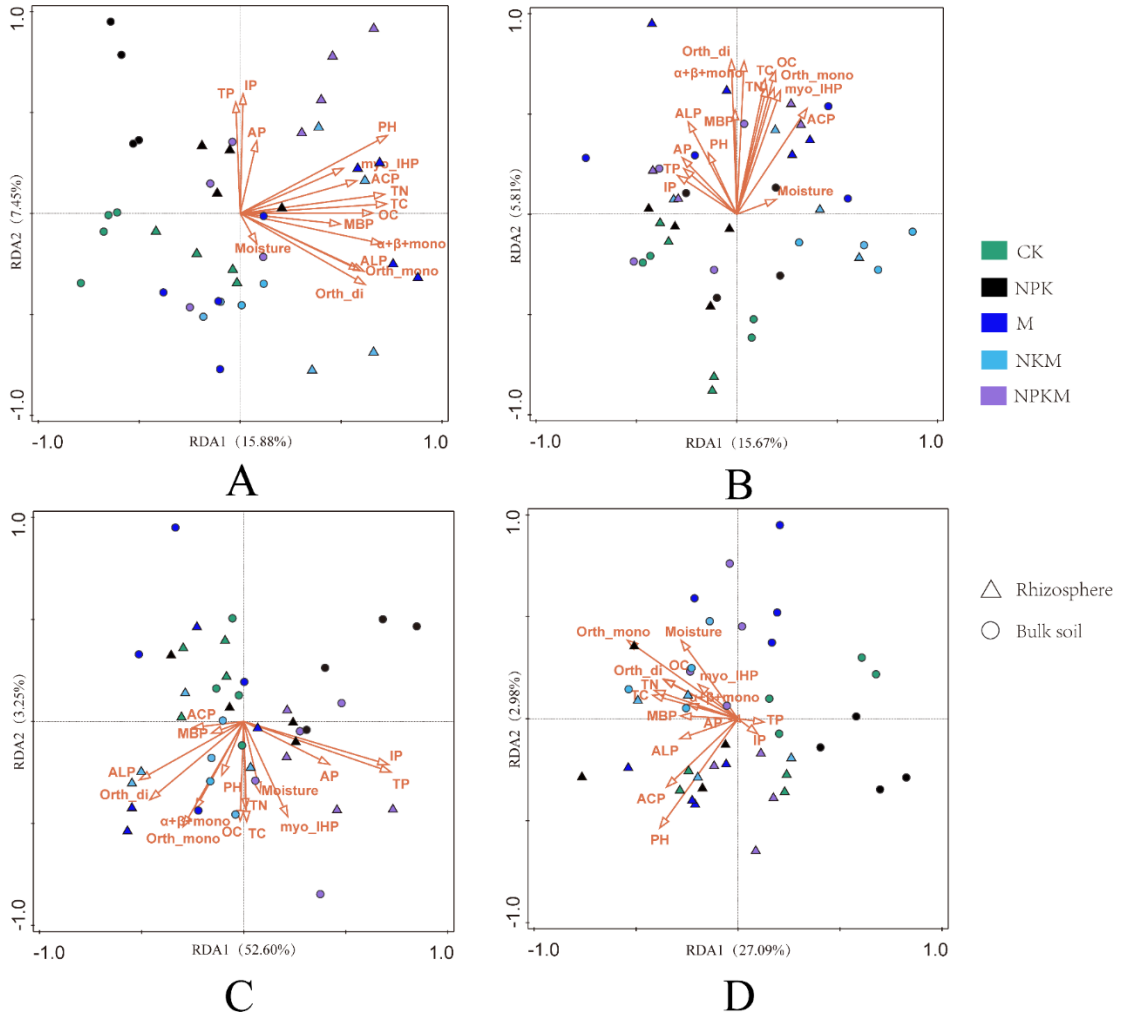


Fig. 7 Correlations between soil properties and the community structure of total bacteria (A), total fungi (B), phosphorus-solubilizing bacteria (C), and phosphorus-solubilizing fungi (D) as determined by redundancy analysis (RDA). MBP, microbial biomass phosphorus; TP, total phosphorus; IP, inorganic phosphorus; AP, available phosphorus; Orth-mono, orthophosphate monoester; Orth-di, orthophosphate diesters; Myo-IHP, myo-Inositol hexakisphosphate; α+β+mono, α- and β-glycerophosphates and mononucleotides; ACP, activity of acidic phosphatase; ALP, activity of alkaline phosphatase.

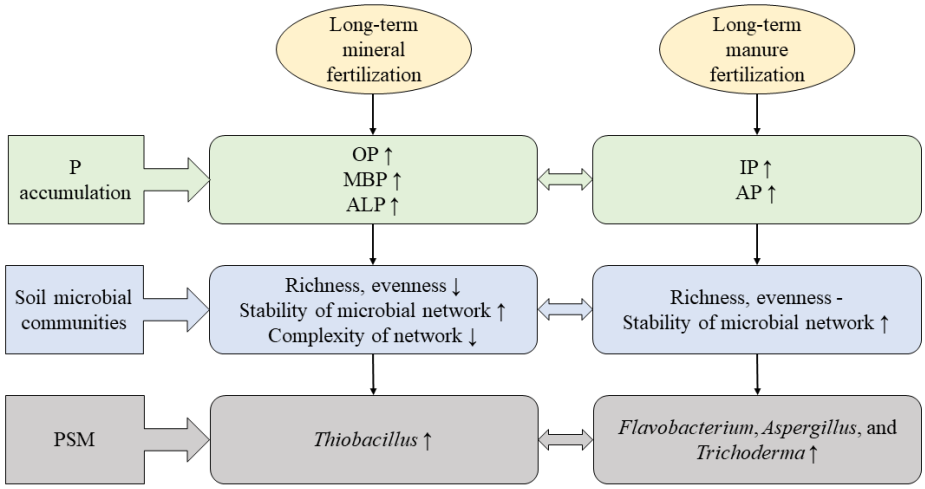


Fig. 8 A diagrammatic sketch showing different responses of P accumulation, soil microbial communities and the PSM after long-term mineral or manure fertilization. ↑, increase; -, no effect; ↓, decrease.

Table 1

The soil properties in five treatments (CK, NPK, M, NPKM, NKM) and two sample types (Bulk and Rhizosphere soil).

| Soil properties | Sample type | CK | NPK | M | NPKM | NKM |
|---|---|---|---|---|---|---|
| Total C (g/kg) | Bulk soil | 19.08±0.26 a** | 23.38±0.56 b** | 30.30±0.23 d** | 34.08±0.22 e | 28.80±0.87 c |
| | Rhizosphere | 20.93±0.56 a | 26.38±1.59 b | 33.63±0.81 d | 34.48±0.15 d | 30.18±0.29 c |
| Organic C (g/kg) | Bulk soil | 15.13±0.30 a | 17.88±1.16 b | 23.43±1.42 c | 26.18±0.68 d | 22.35±0.37 c |
| | Rhizosphere | 16.38±0.66 a | 18.90±1.00 b | 25.33±1.69 d | 25.88±1.14 d | 22.23±0.52 c |
| Total N (g/kg) | Bulk soil | 2.25±0.06 a | 2.58±0.05 b** | 3.28±0.10 c* | 3.53±0.10 d | 3.00±0.12 e* |
| | Rhizosphere | 2.35±0.06 a | 2.93±0.15 b | 3.45±0.06 d | 3.58±0.05 d | 3.25±0.06 c |
| C/N | Bulk soil | 8.48±0.10 a* | 9.08±0.35 b | 9.26±0.29 b | 9.67±0.23 c | 9.60±0.08 c |
| | Rhizosphere | 8.90±0.06 a | 9.02±0.19 ab | 9.75±0.09 c | 9.64±0.11 c | 9.29±0.25 b |
| pH | Bulk soil | 5.84±0.08 ab | 5.85±0.02 ab | 5.89±0.17 ab | 5.89±0.01 b | 5.76±0.05 a |
| | Rhizosphere | 5.95±0.06 a | 6.13±0.02 b | 6.15±0.09 b | 6.19±0.15 b | 6.07±0.04 b |
| Gravimetric Moisture | Bulk soil | 0.41±0.05 a | 0.43±0.00 a | 0.45±0.03 ab | 0.49±0.03 b | 0.48±0.02 b |
| | Rhizosphere | 0.39±0.03 a | 0.41±0.03 ab | 0.43±0.02 ab | 0.44±0.03 b | 0.44±0.03 b |
| Nitrate-N (mg/kg) | Bulk soil | 0.60±0.05 a | 0.66±0.23 a** | 1.30±0.90 a | 0.99±0.26 a | 1.45±1.13 a |
| | Rhizosphere | 0.95±0.36 a | 0.80±0.34 b | 1.42±0.62 a | 1.26±0.61 a | 1.34±0.62 a |
| Ammonia-N (mg/kg) | Bulk soil | 12.15±2.92 a | 11.08±2.27 a | 10.40±2.32 a | 8.66±1.46 a | 11.82±3.24 a |
| | Rhizosphere | 10.07±2.59 a | 17.23±1.02 a | 11.79±1.60 a | 11.38±2.21 a | 11.66±3.90 a |
| Microbial biomass P (mg/kg) | Bulk soil | 3.70±3.49 a | 7.95±5.70 ab | 15.56±8.42 ab | 12.39±9.60 b | 10.45±3.83 ab |
| | Rhizosphere | 5.53±2.71 a | 12.59±8.06 ab | 17.90±4.27 b | 21.40±8.59 b | 17.77±11.14 b |

Values are means ± standard error.

Significant differences between treatments are indicated by lowercase letters ($p<0.05$, $n = 4$).

Significant differences between rhizosphere and bulk soil are indicated by asterisks, where * $p < 0.05$, ** $p < 0.01$ (Duncan's test, n=4)

Table 2

The phosphorus species in five treatments (CK, NPK, M, NPKM, NKM) and two sample types (Bulk and Rhizosphere soil).

| P Form or Compound Class | Sample type | CK | NPK | M | NPKM | NKM |
|---|---|---|---|---|---|---|
| NaOH-EDTA extracted phosphorus (mg/kg) | Bulk soil | 253.86±34.05 a | 560.13±22.78 b | 361.80±2.00 a | 738.70±40.05 c | 304.31±1.66 a |
| | Rhizosphere | 243.29±38.26 a | 546.67±101.12 b | 345.77±38.23 a | 685.59±36.28 c | 348.40±52.34 a |
| Orthophosphate (mg/kg) | Bulk soil | 146.84±32.01 a | 459.07±2.71 b | 202.19±5.91 a | 598.10±1.61 c | 167.24±2.98 a |
| | Rhizosphere | 148.23±33.88 a | 409.37±88.42 b | 187.37±20.00 a | 540.29±40.60 c | 186.66±25.94 a |
| Pyrophosphate (mg/kg) | Bulk soil | 2.94±0.64 a | 2.30±3.26 a | 3.01±1.34 a | 3.00±4.24 a | 2.52±1.23 a |
| | Rhizosphere | 2.73±1.42 a | 1.73±2.45 a | 2.74±1.02 a | 2.56±3.62 a | 2.89±1.71 a |
| Orthophosphate monoesters (mg/kg) | Bulk soil | 63.87±0.75 a | 64.31±13.36 a | 93.87±7.26 b | 92.73±21.40 b | 91.09±4.29 b |
| | Rhizosphere | 56.90±7.41 a | 85.97±18.57 b | 96.28±6.33 b | 88.72±4.76 b | 93.99±9.14 b |
| Myo-IHP (mg/kg) | Bulk soil | 28.69±0.17 a** | 36.73±0.22 b | 40.40±1.68 bc | 44.86±4.35 c* | 39.29±0.48 bc |
| | Rhizosphere | 19.03±2.31 a | 38.58±5.51 b | 40.21±2.98 b | 51.18±0.04 c | 44.43±0.95 b |
| Scyllo-IHP (mg/kg) | Bulk soil | 5.03±0.08 a | 6.90±3.29 a | 10.15±3.16 a | 8.98±4.25 a | 7.52±1.05 a |
| | Rhizosphere | 4.45±1.02 a | 8.19±1.77 a | 9.37±1.00 a | 7.96±3.21 a | 8.12±2.79 a |
| Other monoesters (mg/kg) | Bulk soil | 30.16±0.49 ab | 20.69±9.86 a | 43.33±8.74 b | 38.89±12.79 ab | 44.29±2.76 b |
| | Rhizosphere | 33.45±10.73 a | 39.20±11.29 a | 46.70±2.35 a | 29.57±1.59 a | 41.43±10.99 a |
| Orthophosphate diesters (mg/kg) | Bulk soil | 40.21±0.66 a | 34.44±3.45 a | 62.72±4.69 b | 44.87±12.81 ab | 43.46±1.59 ab* |
| | Rhizosphere | 35.43±13.20 a | 49.61±3.42 ab | 59.38±12.93 b | 54.03±4.06 a | 64.86±15.55 b |
| DNA (mg/kg) | Bulk soil | 15.31±2.32 ab | 6.90±3.29 a* | 22.20±2.21 b | 11.97±8.49 ab | 13.38±0.24 ab |
| | Rhizosphere | 12.34±6.90 a | 21.58±3.82 a | 18.15±8.52 a | 13.65±4.84 a | 20.06±9.32 a |
| α+β+mono (mg/kg) | Bulk soil | 24.90±1.67 a | 27.54±0.16 a | 40.52±6.90 b | 32.90±4.32 a | 30.08±1.83 a** |
| | Rhizosphere | 23.10±6.30 a | 28.03±0.40 a | 41.22±4.40 b | 40.38±0.78 b | 44.80±6.23 b |

Myo-IHP: myo-Inositol hexakisphosphate; Scyllo-IHP: Scyllo-Inositol hexakisphosphate; α+β+mono, α- and β-glycerophosphates and mononucleotides; Values are means ± standard error.

Significant differences between treatments are indicated by lowercase letters ($p<0.05$, $n = 2$). Significant differences between rhizosphere and bulk soil are indicated by asterisks, where * $p < 0.05$, ** $p < 0.01$ (Duncan's test, n=2)