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Partial replacement of inorganic phosphorus (P) by organic manure reshapes phosphate mobilizing bacterial community and promotes P bioavailability in a paddy soil

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Abstract: The optimization of more sustainable fertilization practice to relieve phosphorus (P) resource scarcity and increase P fertilizer utilization, a better understanding of the regulatory roles of microbes in P mobilization is urgently required to reduce P input. The genes *phoD* and *pqqC* are responsible for regulating organic and inorganic P mobilization, respectively. Using high-throughput sequencing, the corresponding bacterial communities harbored by these genes were determined. We conducted a 4-year rice-rice-crop rotation to investigate the responses of *phoD*- and *pqqC*-harboring bacterial communities to the partial replacement of inorganic P fertilizer by organic manure with reduced P input. The results showed that a combination of organic and inorganic fertilization maintained high rice yield, and also produced a more complex and stable phosphate mobilizing bacterial community, which contributed to phosphatase activities more than their gene abundances in the model analysis. Compared with conventional mineral fertilization, organic-inorganic fertilization with reduced P input slightly increased *pqqC* gene abundance while significantly enhancing the abundance of *phoD*-harboring bacteria, especially the genera *Bradyrhizobium* and *Methylobacterium* known as potential organic P mineralizers which can maintain high rice production. Moreover, the increased pH was the most impactful factor for the *phoD*- and *pqqC*-harboring bacterial communities, by promoting microbial P turnover and greatly increasing bioavailable P pools ($\text{H}_2\text{O-Pi}$ and $\text{NaHCO}_3\text{-Pi}$, NaOH-Pi) in P-deficient paddy soil. Hence, our study demonstrated that the partial replacement of mineral P with organic manure could reshape the phosphate mobilizing and alkaline-phosphomonoesterase encoding bacterial communities towards more resilient and effective to the high P utilization and productivity over intense cultivation, providing insights into the potential of soil microbes in the efficient management of agricultural P fertilization.

Keywords: Organic-inorganic fertilization, reduced phosphorus input, phosphate mobilizing bacteria, paddy soil, network interactions

Graphical abstract

1. Introduction

Phosphorus (P) is a critical nutrient for plant growth, but its availability is mostly limited in agro-ecosystems worldwide (George et al. 2017). Thus, mineral P with high input is continuously applied to the soil to maintain high crop yields. However, P fertilizer is easily fixed in soils rather than absorbed by plants (Kochian 2012), and then an extra P fertilizer beyond the crop demand, is further applied. This can leave the continuous accumulation of soil residual P and potential eutrophication of surface water (Powers et al. 2016). The global reserves of mineral P are estimated to be 100% depleted by 2100 (Penuelas et al. 2013). Thus, the eco-friendly fertilization management for decreasing P usage and improving P use efficiency is urgently needed for both agricultural production and environmental health.

Organic fertilization, which better supports soil fertility and quality, is becoming a feasible practice to maintain high crop yield to meet the ever-increasing food demand for booming global populations (Sun et al. 2015). Long-term organic fertilization (*e.g.*, dry straw, animal manure, among others) is beneficial for plant-available P supplies, soil quality improvement, and P-related enzyme activities (Steiner et al. 2007; Zhu et al. 2010). Additionally, P sourced from manure is more likely to remain available forms than that from chemical fertilizers, causing a better plant uptake (Kahiluoto et al. 2015). Moreover, chemical fertilization over decades may further decrease potential alkaline phosphatase (ALP) activity (Fraser et al. 2015a). The combined application of organic and inorganic fertilizer greatly increases ALP activity, and soil P availability (Chen et al. 2017), and the increased soil organic carbon (C) content and aggregation improve environmental conditions for soil microbial community (Lin et al. 2019). Furthermore, a reduction in chemical fertilization supplemented with organic applications, possibly

enhances soil microbial nutrient cycling (Ding et al. 2018). For example, comparing to the conventional chemical fertilization, reducing nitrogen (N) or chemical fertilizer input coupled with organic amendments can strengthen soil nitrification and N fixation with the increase in N utilization efficiency and crop productivity (Hsu and Buckley 2009; Zhao et al. 2016). Accordingly, reducing mineral P input combined with organic-inorganic fertilization is expected to produce higher efficient soil P mobilization and cycle, which is necessary to enhance P fertilizer utilization and then the environmental sustainability of crop production.

Microbial community diversity and compositions can be greatly altered by agricultural activities, with consequent effects on the biogeochemical cycles mediated by microorganisms (Zhu et al. 2017). One positive feedback is the regulation of plant P acquisition by solubilizing insoluble P with organic protons or mineralizing recalcitrant organic-P with enzymes (Richardson and Simpson 2011). The gene encoding pyrroloquinoline-quinone involved in gluconic acid production for inorganic-P release, a potential bioindicator for inorganic phosphate solubilizing bacteria (iPSB) communities (Rodríguez et al. 2006), was previously used in a long-term fertilized soil (Zheng et al. 2017). The putative Pho regulon genes (*phoA*, *phoD*, and *phoX*) are responsible for alkaline phosphatase expression in soil and aquatic ecosystems (Chen et al. 2017; Hu et al. 2018; Ragot 2016). While *phoD* is dominant in soil and can provide evidence for organic P transformation (Chen et al. 2017; Ragot et al. 2016; Tan et al. 2012). Several studies indicated that different fertilizing strategies alter the *phoD*-harboring bacterial community composition and diversity with the potential effects on organic P mineralization, but responses were inconclusive, which is probably due to the various soils and organic fertilizer types and also the different P statuses (Chen et al. 2017; Fraser et al. 2015b; Hu et al. 2018; Tan et al. 2012). For

example, the *phoD* gene abundance and diversity decreased when chemical fertilizer added with corn straw but increased in combination with swine manure in a red soil (Chen et al. 2017). In a calcareous soil, only organic fertilization combinations with high amounts of organic matter significantly affected the *phoD* gene abundance and community, however, the soil organic mineralization in different treatments all benefited from the abundant *phoD*-harboring taxa (Hu et al. 2018). However, in the mineral P-fertilized paddy soil, rare *phoD*-harboring taxa were found to contribute to the increase in *phoD* gene abundance (Wei et al. 2019). Thus, more studies on the alkaline-phosphomonoesterase encoding bacterial community in different soils or fertilization management are needed. Moreover, after long-term organic-inorganic fertilization, some specific microbial taxa were found to be more effective in the decomposition of complex organic matter and soil C, N, and P transformation (Gu et al. 2017; Li et al. 2017). However, the major studies of P mobilizing bacterial community are based on the phosphomonoester mineralization, little is known about the combined role of the alkaline-phosphomonoesterase encoding and phosphate solubilizing bacterial communities on the P mobilization, especially in paddy soils with the partial replacement of mineral P by organic manure.

In this study, a rice-rice field cropping over 4 years in southern China was conducted to investigate the effects of various fertilizing strategies, including conventional chemical, organic, and combinations of organic-inorganic fertilization with or without reducing mineral P input on *phoD*- and *pqqC*-harboring bacterial communities. We hypothesized that different fertilization regimes could change soil P pool distribution, the abundance and compositions of the alkaline-phosphomonoesterase encoding and phosphate solubilizing bacterial communities, particularly the partial replacement of inorganic fertilizer by organic manure would facilitate the potential phosphatase activity and P availability by establishing a

better phosphate mobilizing bacterial community, even while reducing P input. Due to the complexity of soil microbial interactive webs, network analysis and structural equation models (SEM) are introduced to comprehensively understand the relationships between soil properties, *phoD*- and *pqqC*-harboring communities, and their functionality, to explore how the phosphate mobilizing bacterial community is driven.

2. Materials and methods

2.1. Field design and soil sampling

The field was located in Yichun, Jiangxi, China (28° 15' 1.62" N, 115° 07' 13.34" E). This region has a subtropical monsoon-type climate with an average annual temperature of 17.2 °C and average annual precipitation of 1,680 mm. The red soil had the following basic properties: pH 5.4; total organic C 16.3 g kg⁻¹; total N 1.1 g kg⁻¹; total potassium (K) 21.1 g kg⁻¹; total P 0.6 g kg⁻¹; and available P 23.8 mg kg⁻¹. To accomplish the aim of “Zero Increase Action Plan” in China by 2020 (Liu et al., 2016), we set this paddy field experiment under rice-rice (*Oryza sativa* L.) rotation with different fertilization regimes in 2013, to reduce chemical fertilizer especially P input. The fertilization regimes, including four replicates of six treatments, were set in a random block design, each measuring 72 m². The treatments were (1) control without fertilizer (CK), (2) chemical fertilizers with normal NK doses (NK), (3) chemical fertilizers with normal NPK doses (FP), (4) organic manure (OM) alone with 20% of normal P doses (M(20%P)), (5) OM (20%P) + chemical fertilizers (80%P) with the same total nutrient doses as FP treatment (CM(P)), (6) OM (20%P) + chemical fertilizers (64% P) with the same total NK doses, and 84% P dose as FP treatment (CM(-P)). It was noted that each treatment except the control and M(20%P) treatments was fertilized with the same N and K content levels but differed in mineral P input. Each plot

in the FP and CM(P) treatments received the same levels of N, P, and K from fertilizers applied in the early rice (N: 165 kg ha⁻¹, P₂O₅: 90 kg ha⁻¹ and K₂O: 150 kg ha⁻¹) and late rice (N: 195 kg ha⁻¹, P₂O₅: 90 kg ha⁻¹ and K₂O: 150 kg ha⁻¹). The N fertilizer (urea) was applied as basal and supplementary fertilizer, while the P (calcium magnesium phosphate), K (potassium chloride), and organic manure were only used as basal fertilizers. The organic manure was mainly sourced from chicken manure, which contains 1.86% N, 3.11% P₂O₅, 0.85% K₂O, and 47.8% organic matter. The details regarding the amounts of nutrient inputs are shown in Table S1.

After the 4-year rice-rice cultivation, soil samples were collected in October 2016 (at the same time of late rice harvest). Fresh samples were obtained from the upper 20 cm of the plots, each replicate was a mix of five soil cores that were 5 cm in diameter, then mixed thoroughly. After removing stones, plant roots, and associated debris, subsamples for the enzymatic determination were stored at 4 °C, and DNA extraction was stored at -80 °C. Subsamples for measuring physiochemical properties were air-dried and sieved (<200 µm).

2.2. Soil chemical and biological analyses

Soil pH was determined in a 1:2.5 (soil/water ratio, w: v) suspension using a pH meter. Soil total C (TC) and N (TN) were measured by dry combustion using an elemental analyzer (Vario max CN, Elementar, Hanau, Germany). Soil organic carbon (SOC) was determined by the external-heat potassium dichromate oxidation-colorimetric method (Nelson and Sommers, 1982). Soil total P (Pt) and available P (P_{ols}) were measured by H₂SO₄-HClO₄ digestion (Parkinson and Allen 1975) and NaHCO₃ extraction (Olsen 1954), respectively, followed by the molybdenum-blue colorimetric method (Murphy and Riley 1962). The

phosphatase activities, including alkaline phosphomonoesterase (ALP), acid phosphomonoesterase (ACP), and phosphodiesterase (PDE), were determined using the method described by Tabatabai (Tabatabai 1994). In brief, the soil was incubated in a buffer solution (pH 6.5 and pH 11.0 Tris-maleate buffer and pH 8.0 Tris-HCl buffer for ACP, APase and PDE, respectively) with substrate (*para*-nitrophenyl (*p*NP) for ALP and ACP, bis-*para*-nitrophenyl phosphate for PDE) for 1 h at 37 °C, and the formation of *p*NP measured using a UV-Vis spectrophotometer (Metash UV-754, China) at 410 nm. The phosphatase activity was defined as the amount of *p*NP released by 1 kg of soil per hour. Soil P pools were determined using a modified Hedley method (Hedley et al. 1982; Tiessen and Moir 1993), which has also been introduced in detail previously (Bi et al. 2018). Briefly, through Milli-Q water, 0.5 M NaHCO₃ (pH = 8.5), 0.1 M NaOH and 1 M HCl extraction with the molybdenum-blue colorimetric method of measurement, we could obtain the contents of H₂O-P, NaHCO₃-P, NaOH-P and HCl-P in both Pi (inorganic P) and Po (organic P).

2.3. DNA extraction and gene quantification

DNA was extracted from 0.5 g soil with a FastDNA Spin Kit for soil (MP Biomatrical, Illkirch, France) according to the manufacturer's instructions. The quality of extracted DNA was analyzed with a spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Waltham, MA) and the concentration of DNA was determined using a QuantiFluor® dsDNA system (Promega, Madison, WI) based on fluorometric analysis with a microplate reader (Spectramax M5, Molecular Devices, Sunnyvale, CA). DNA was stored at -20 °C until use.

The *phoD*, *pqqC* and 16S rRNA genes were measured using real-time quantitative PCR (qPCR) with the ALPS-F730/ALPS-R1101(CAGTGGGACGACCACGAGGT/GAGGCCGATCGGCATGTCTG)

(Sakurai et al. 2008), Fw/-Rv (AACCGCTTCTACTACCAG/GCGAACAGCTCGGTCAG) (Zheng et al. 2017) and the F515/R907 (GTGCCAGCMGCCGCGG/CCGTCAATTCMTTTRAGTTT) (Zhou et al. 2011b) primer sets, respectively. For *phoD* gene, the primers ALPS-F730/ALPS-1101 has been argued that could cause biases toward *Alphaproteobacteria* (Ragot et al. 2015). However, due to the multiple previous studies that have employed this set of primers on the *phoD*-harboring bacterial community, it's feasible to compare between different studies. The DNA reaction system for each sample was prepared in triplicate as a mixture of 10 ng of template DNA, 0.3 μ M each primer, 1X SYBR premix *Ex Taq* and 0.1 mg mL⁻¹ bovine serum albumin (BSA). The gene copy number was quantified by a LightCycler 480 (Roche, Basel, Switzerland). The PCR reaction used for 16S rRNA and the *pqqC* gene was: 98 °C for 3 min for 1 cycle, and 40 cycles of 98 °C for 20 s, 62 °C for 30 s, and 72 °C for 30 s. The amplification protocol of the *phoD* gene was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and annealing at 55 °C for 1 min, and finally 1 min at 72 °C. All standard curves were prepared in triplicate with eight serial 10-fold dilutions of standard plasmids carrying *phoD*, *pqqC*, or the 16S rRNA gene, and quantification was calculated based on the concentration of plasmid DNA and the base pairs therein (vector plus primer). The efficiency of each reaction was 90-110%.

2.4. PCR amplification, high-throughput sequencing, and *phoD* and *pqqC* gene community identification

The partial fragments of the bacterial *phoD* and *pqqC* gene were the same as in the qPCR experiment and amplified using the primers ALPS-F730/ALPS-R1101 and Fw/-Rv, respectively. To distinguish samples, the reverse primer was tagged with different 6-mer barcodes at the 5' end. Each 50- μ L PCR reaction mixture contained 10 ng of genomic DNA, 1 μ L of Premix *Ex Taq* Hot Start Version (TAKARA,

Dalian, China), 0.2 μM of each primer and 0.1 mg mL^{-1} BSA. The amplification protocol for the *phoD* gene was: 3 min at 94 °C, followed by 40 cycles at 95 °C for 15 s, 30 s at 56 °C for annealing, and finally 1 min at 72 °C. The amplification protocol for the *pqqC* gene was: initial denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a 5-min extension at 72 °C. The PCR products were purified and quantified using a Universal DNA Purification Kit (TIANGEN, Beijing, China) and the QuantiFluor® dsDNA system, respectively. The purified amplicons were pooled in equimolar volumes and subjected to paired-end sequencing using the Illumina HiSeq2500 platform (Novogene, Beijing, China).

The Quantitative Insights Into Microbial Ecology (QIIME) system was used to trim all raw sequencing data. In our study, a total of 2975113 of raw sequences with a range of 28531 to 339515 in the *phoD* gene community and 3103247 of raw sequences with a range of 28264 to 245238 in the *pqqC* gene community per sample were obtained. For high-quality filtered sequences, a total of 2762173 with a range of 25915 to 315346 in the *phoD* gene community and 2975113 with a range of 27034 to 237657 in the *pqqC* gene community per sample were produced. During the raw data filtering, reads shorter than 200 bp, reads containing any ambiguous bases, reads with an average quality score lower than 30, and any nucleotide mismatches within the barcode or primer were removed before analysis. Chimeric sequences were identified and eliminated using UCHIME. Operational taxonomic units (OTUs) were clustered with UCLUST clustering at a cutoff of 75% similarity, and representative sequences were retrieved and classified with the RDP classifier. Singletons were removed during this clustering process. Subsequently, representative OTUs were taxonomically assigned using Local Blast 2.2.27+ (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.2.27/>), and the potential phosphate mobilizing

bacterial species were annotated with critical criteria ($E < 1 \times 10^{-10}$ and sequence identity $> 99\%$) (Zheng et al. 2017).

2.5. Construction of phylogenetic molecular ecological networks

To decipher the effects of organic-inorganic fertilization on the differences among the soil phosphate mobilizing bacterial communities comparing to the Non-P fertilized soils, the network analysis (based on OTU level) was performed using the random matrix theory (RMT)-based approach in two treatments (Non-P: the control and NK treatments; Organic-inorganic: CM(P) and CM(-P) treatments) according to the Molecular Ecological Network Analyses Pipeline (<http://ieg4.rccc.ou.edu/mena>) (Deng et al. 2012). More information regarding RMT and algorithms, pipeline structure, and procedures was described in detail in previous studies (Deng et al. 2012; Ling et al. 2016; Zhou et al. 2010; Zhou et al. 2011a). The topological properties including average clustering coefficient, network density, modularity and the number of positive or negative correlations edges and nodes reflect how the patterns of the interrelationships among OTUs, and also the nodes with the max degree, max betweenness, max stress centrality, and max eigenvector centrality can provide important microbes performing essential network interactions (Deng et al. 2012; Guimera et al. 2007). Additionally, the topological roles of individual network nodes were shown in a Zi (measuring how well a node was connected to other nodes in its module) –Pi (measuring how well a node was connected to nodes in different modules) plot, which have been proposed to represent keystone taxa (e.g., generalists), which have a potential regulatory function on the target microbial community by regulating the microbe-microbe interactions (Deng et al. 2012). Gephi 0.9.2 software was used to visualize the network graphs.

2.6. Statistical analyses

Normal distribution and homoscedasticity were tested with the Shapiro-Wilk test and Levene test in R. One-way ANOVA (analysis of variance) with post-hoc Tukey tests were performed using SPSS (V20.0, IBM, USA)) software with the criterion for statistical significance set at $P < 0.05$. The ADONIS test based on Bray-Curtis distances, was used to evaluate the effects of different fertilization regimes on bacterial communities, which was performed in R using the vegan package (Oksanen J 2016). Ternary plots and differential analyses were performed using the edge package in R to determine the differential expressions of OTUs among different fertilization regimes according to a $\log_2$ fold change ≥ 2 and a Q value > 0.05 (Robinson M D 2010). These figures were generated using OriginPro 2018. Heat maps were conducted in R with the package “pheatmap”. Redundancy analysis (RDA) analysis was performed using Canoco version 5.0 software. The structural equation modeling (SEM) framework was applied to test for the significant relationships among soil properties, gene abundance, diversity, and composition of the *phoD*- and *pqqC*-harboring communities and phosphatase activities using AMOS software (IBM SPSS AMOS 21). The best-fit model was conducted using a maximum likelihood estimation, and the chi-square test (χ^2) ($P > 0.05$), goodness-of-fit index (CFI) (> 0.90), root mean square residual (RMR < 0.08), and root mean square errors of approximation (RMSEA < 0.08) were used to test the overall goodness of the model fit.

2.7. Sequence accession numbers

Sequences are available in the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA543033, the accession numbers of per sample are shown in table S2.

3. Results

3.1. Soil properties and P status

The selected biological and chemical characteristics of soil samples are presented in Table 1. Soil SOC, pH, Pt, NO_3^- , *Posl* contents, and ACP, ALP, and PDE activities were significantly increased after 4-year conventional (FP treatment) and organic-inorganic fertilization (CMP and CM(-P) treatments) compared with the other treatments ($P < 0.05$). Moreover, in comparison with conventional fertilization, organic-inorganic fertilization significantly enhanced the *Posl*, NH_4^+ contents, and ACP activity ($P < 0.05$). However, no significant differences in the selected soil properties were observed between the CMP and CM(-P) treatments. In addition, the highest rice yield was observed in both the conventional and organic-inorganic fertilization ($P < 0.05$). For soil Hedley P pools, both organic-inorganic and the conventional fertilization significantly increased the concentrations of H_2O -, NaHCO_3^- and NaOH-Pi pools (Table S3).

3.2. Abundances of *phoD* and *pqqC* genes and community diversity

The *phoD* and *pqqC* gene abundances were significantly altered by the different fertilization regimes (Fig. 1a and 1b, $P < 0.001$). For the *phoD* gene abundance, organic-inorganic fertilization showed the greatest change, with increases of 1.3- and 1.4-fold in the CM(P) and CM(-P) treatments compared with the conventional (FP) treatment. The CM(P) treatment increased *pqqC* gene abundance by 1.6-fold compared with the conventional treatment, but a similar level was observed between CM(-P) and FP treatments. Additionally, conventional and organic fertilization significantly enhanced *phoD*-harboring community diversity (Fig. 1c, $P < 0.05$). However, no significant difference was found in the *pqqC*-harboring community diversity among different treatments (Fig. 1d). Moreover, a significant positive relationship was observed between the abundance of the *phoD* and *pqqC* genes (Fig. S1).

3.3. Effects of fertilization regimes on the compositions of *phoD*- and *pqqC*-harboring bacterial communities.

Based on the composition of all soil samples, the dominant *phoD*- and *pqqC*-harboring phylum was *Proteobacteria*, reaching up to 96.5% and 72.3%, respectively (Fig. S2). *Actinobacteria*, with average percentages of 17.1%, was also abundant in the *pqqC*-harboring bacterial community. The *phoD* and *pqqC* gene community compositions of different treatments were well-separated from each other at the genus level (Fig. 2a and c). For the *phoD*-harboring community composition, sixteen genera (>1%) were aligned, and the most dominant genera belonged to *Methylobacter* (4.6–50.5%), *Pelomonas* (12.1–30.5%), *Methylobacterium* (4.3–29.0%), and *Bradyrhizobium* (10.0–27.9%) (Fig. 2b). The relative abundances of *Methylobacterium* and *Bradyrhizobium* were the highest in the CM(-P) treatment, accounting for approximately 50% of the total *phoD*-harboring community. The control treatment showed the highest abundance of *Methylobacter* ($47.5 \pm 3.6\%$). For the *pqqC*-harboring community, the dominant genus in each treatment was *Oxalobacteraceae* (20.3–38.6%), which was the highest in the control and M(20%P) treatments (Fig. 2d). Moreover, the CM(-P) treatment showed the highest abundance in *Solirubrobacterales* compared with other treatments ($P < 0.05$).

The Adonis test showed that in contrast to the control treatment, the *phoD*- and *pqqC*-harboring communities were significantly shaped by CM(-P) treatments ($P < 0.05$, 999 permutations, Table 2). The phosphate mobilizing bacterial communities following CM(-P) treatment were distinct from those under the CM(P) and FP treatments. The significant difference in these three fertilization regimes was further confirmed by the striking differences of enrichment OTUs assemblages in both the *phoD*- and *pqqC*-

harboring communities (Fig. 3). Whereas the taxonomic affiliations of the enriched OTUs were represented largely by *Al*, *Beta*, *Delta*, *Gamma-Proteobacteria*, and *Bacilli*.

3.4. Network structure and key taxa

Distinct network structures of the *phoD*- and *pqqC*-harboring communities were observed between Non-P (CK and NK treatments) and organic-inorganic (CM(P) and CM(-P) treatments) (Fig. 4). The connectivity was well-fitted by Random Matrix Theory, with R^2 values of 0.92 and 0.94 for the *phoD* and *pqqC* gene networks, respectively ($P < 0.05$). For the *phoD*-harboring community, the organic-inorganic treatment presented higher modularity, greater numbers of nodes, and positive links in the network. Whereas, for the *pqqC*-harboring community, more nodes and links of the network were found in the Non-P treatment. In addition, 94% of these links were positive in the Non-P treatment, while only 82% were positive in the organic-inorganic treatment. Moreover, the Non-P network exhibited higher complexity and connectivity than that in the organic-inorganic treatment, reflecting the larger average degree and connectedness, the shorter average path length, and the higher clustering coefficient and density (Table S4).

The topological roles of individual network nodes were shown in a Zi-Pi plot (Fig. 4c and f). In this study for both Non-P and organic-inorganic treatments, microbial key species (generalists) mainly belong to the genus *Bradyrhizobium* of the *phoD* gene network, and the order *Oxalobacteraceae* of the *pqqC* gene network (Table S5). Among all network hubs and connectors, *Bradyrhizobium* accounted for 37.5% and 61.5% in the Non-P and organic-inorganic treatments, respectively. The proportions of *Oxalobacteraceae* in the Non-P and organic-inorganic treatments were 42.9% and 37.5%, respectively. Particularly, the hub

members of max eigenvector centrality were identified as *Bradyrhizobium* in the *phoD* gene networks, shifted from *Glucosotrophus* in the Non-P to *Oxalobacteraceae*, *Herbaspirillum*, and *Candidatus Portiera* in the organic-inorganic soils of *pqqC* gene networks (Table S6).

3.5. The main driver of the *phoD*- and *pqqC*-harboring communities under different fertilization regimes

After the strong positive correlations of Hedley P pools and rice yield with soil pH, PDE and ALP activities ($P < 0.05$, Table S7), the RDA analysis also revealed that pH, PDE, Pt, *Posl* and Hedley P pools were the significant predictors for the *phoD*- and *pqqC*-harboring bacterial communities across different fertilization regimes ($P < 0.01$, Fig. S4). The total explained the proportion of RDA1 and RDA2 were 20.9 and 7.9%, 16.6 and 8.5% for the variance in the *phoD*- and *pqqC*-harboring communities, respectively. Moreover, except for the P-related index, the soil pH was the main factor influencing phosphatase activities by reshaping the phosphate mobilizing the bacterial communities in the SEM model (Fig. 5). Additionally, the abundances of the *phoD* and *pqqC* gene were also increased with soil pH ($P < 0.01$, Fig. S3). Linear fitting also showed significant and positive correlations between gene abundance and *Posl*, PDE activity ($P < 0.01$, Fig. S3).

4. Discussion

In the acidic paddy soil, low P availability is the major limitation of crop production. Thus, fertilization is the most common agricultural management practice for improving soil P fertility, mainly through the amendment of mineral P or organic manure (Ai et al. 2018; Hu et al. 2018; Zhao et al. 2016). In the current study, compared with the single organic manure fertilized soil (M(20%P) treatment) and the Non-

P fertilized soils (the control and NK treatments), the conventional chemical fertilization (FP treatment) and organic-inorganic fertilization (CM(P) and CM(-P) treatments) greatly increased phosphatase activities (sum of ACP, PDE and ALP activities), and soil available P by 2- and 4-fold, respectively. Additionally, the sizes of H₂O-Pi, NaHCO₃-Pi, and NaOH-Pi pools were also mainly enhanced. No significant difference was observed in the storage of P (TP) between conventional and organic-inorganic fertilization. Increasing evidence has shown that receiving organic manure can largely enhance whole soil bacterial abundance (Chen et al. 2016; Ding et al. 2018), which was also found in organic-inorganic fertilized soils in comparison with the conventional fertilization. After the 4-year input of organic manure, the storage of SOC was greatly increased, and then probably more substrate availability and enzyme stabilization through interactions between inorganic nutrient and manure components in the soil might explain the increased P availability (Chen et al. 2017; Liu et al. 2010). Even though C and N are necessary to microorganisms and crops, the increased phosphatase activity suggested that the large demand of energy for microbes to mobilize organic P (Fujita et al. 2017), this might indicate that a more important role of P for bacteria and rice growth in the P-deficient paddy soil (Long et al. 2018; Wei et al. 2019). Thus, we speculate that the added organic matter at seeding might greatly stimulate the growth of the phosphate mobilizing bacteria, potentially triggering organic P mineralization or inorganic P solubilization, consequently contributing to plant P acquisition and high rice yields.

The evaluation of the microbial potentials for organic P hydrolysis and inorganic P solubilization is based on the *phoD* and *pqqC* genes. Although we hypothesized an improved effect of organic-inorganic fertilization with reduced P input on phosphate mobilizing bacterial abundance would be observed in contrast to the conventional fertilization, a significant enhancement was only found in the *phoD* rather

than the *pqqC* gene abundance. We propose that the higher bacterial abundance might be responsible for the increase of *phoD* gene abundance and enzyme activities (Hu et al. 2018). Additionally, organic-inorganic fertilization showed higher diversity in the *phoD*-harboring community than the Non-P treatments, but remained at the same significant level following conventional fertilization. A possible reason for this finding might be attributed to the low adaptability of exogenous phosphate mobilizing bacteria derived from organic manure, but indigenous phosphate mobilizing bacteria may be stimulated by organic matter input through the provision of vast quantities of utilizable energy sources (Hu et al. 2018; Li et al. 2015; Sun et al. 2015).

The organic-inorganic fertilization significantly shaped the *phoD*- and *pqqC*-harboring community compositions. By employing the RMT-based network approach, the phosphate mobilizing bacterial community in the organic-inorganic fertilized soils was found to have a more complex network, which contains more interspecific competition and potentially operational community with a greater number of organic P hydrolysis interrelated bacterial taxa (Chagnon et al. 2012). The abundant organic matter and nutrients in the organic-inorganic fertilized soils might increase the diversity and abundance of organic P mineralizers, which greatly enhanced phosphatase activity that could generate more competition with other organic P mineralizers (Ling et al., 2016). The complex network observed following the organic-inorganic fertilization could accelerate microbial potentials in ALP and gluconic acid secretion by the *phoD*- and *pqqC*-harboring community, respectively. Moreover, compared to the networks in the Non-P treatment, the lower average path distance in the organic-inorganic fertilized soils is considered to have the capacity to quickly respond to environmental perturbations (Zhou et al. 2010). Thus, the 4-year organic-inorganic fertilization, by shaping a more complex and stable community, could promote

phosphate mobilizing bacterial community resilience to exogenous interferences that helped to relieve P deficiency and maximize crop yield over intense cultivation.

In agreement with these previous studies, the dominant *phoD*- and *pqqC*-harboring bacteria were *Proteobacteria* and *Actinobacteria* (Fraser et al. 2015b; Hu et al. 2018; Lagos et al. 2016; Tan et al. 2012; Zheng et al. 2017). With abundant nutrient supplies, both of the conventional and organic-inorganic fertilization enriched the copiotrophic bacteria, including *Alpha*-, *Beta*-, *Delta*-, and *Gamma*-*Proteobacteria* and *Bacilli* in the *phoD*- and *pqqC*-harboring communities. *Bacilli* have been commonly identified to possess phosphate-solubilizing capacity (Sharma et al. 2013), and carrying β -propeller phytase genes (Jorquera et al. 2011). For the *phoD*-harboring community, *Rhizobiales* including the enriched *Bradyrhizobium* and *Methylobacterium* in the CM(-P) treatment, is generally dominant in P-rich soil (Long et al. 2018; Ragot et al. 2016) and a beneficial participant in plant-microbe interactions (Erlacher et al. 2015). Specifically, *Bradyrhizobium*, an N-fixing symbiotic bacteria that requires a phosphate transport system (Bardin et al. 1996), can produce phosphatase or organic acid to increase soil available P (Ikoyi et al. 2018; Jorquera et al. 2011; Mander et al. 2012; Sharma et al. 2013). *Methylobacterium* contains non-specific acid phosphatase proteins and thereby can dephosphorylate diverse organophosphates (Neal et al. 2017). For the *pqqC*-harboring community, *Oxalobacteraceae* accounted for 20.3–38.6% of the total community, and are known to metabolize oxalate as a C source (Chapelle et al. 2016). Furthermore, with organic-inorganic fertilization, *Bradyrhizobium*, and *Oxalobacteraceae* (in the *phoD*- and *pqqC*-harboring networks, respectively) were also identified as the keystone taxa in modulating the compositions and functions of the *phoD*- and *pqqC*-harboring communities. Both of them also had the highest connections with the other central nodes (*i.e.*, max

eigenvector centrality), and probably play important roles in maintaining ecosystem stability (Olesen et al. 2007). Taken together, the 4-year organic-inorganic fertilization with reduced P input facilitated the formation of stable and efficient key phosphate mobilizing bacterial populations with higher *Bradyrhizobium* and *Methylobacterium* abundances, suggesting a strong potential in inorganic P solubilization and organic P mineralization in paddy soil.

According to the SEM, phosphatase activities were directly regulated by both *phoD*- and *pqqC*-harboring bacterial communities. It is well-established that soil P level, rather than C, directly influenced the P availability and ALP activity (Hu et al. 2018). The effect of the C amendment on soil organic P availability was found to be decreased in oxygen-deficient rather than oxygen-rich soil (Sun et al. 2018), whereas the P additions significantly shaped the *phoD*-harboring community (Lagos et al. 2016). Furthermore, the increased soil pH was the only environmental variable that was significantly and positively correlated with the abundances of the *phoD* and *pqqC* genes, and also had strong influences on phosphatase activities, which was possibly owing to the changes of the *phoD*- and *pqqC*-harboring community compositions. Similarly, previous studies have also indicated that soil pH mainly drives soil bacterial abundance and composition (Lauber et al. 2009), and is a strong determinant of *phoD*-harboring bacteria (Ragot et al. 2015; Wang et al. 2012) and *pqqC*-harboring communities (Zheng et al. 2017). The strong effects of soil pH on the phosphate mobilizing bacterial communities, may be caused by the increased nutrient availability, altered microbial respiration rates, and the sum of whole microbial activities under abundant NPK inputs (Lagos et al. 2016). Moreover, we also found that the *phoD*- and *pqqC*-harboring community compositions became a more important regulator of soil P mobilization than the increased phosphate mobilizing bacterial abundance. Combined with the positive correlations among

pH, Hedley P pools, and rice yield, our results imply that the P-deficient paddy soil with organic-inorganic fertilization was altered to be more neutral, thereby stimulating soil microbial P transformation and mobilization by reshaping *phoD*- and *pqqC*-harboring communities and increasing their abundances.

5. Conclusions

In this study, a 4-year field experiment demonstrated that organic-inorganic fertilization with reduced P input significantly enhanced soil P availability and maintained high rice yield in comparison with conventional fertilization. Different fertilization regimes notably reshaped the abundance and composition of *phoD*- and *pqqC*-harboring bacterial communities, which was of higher network complexity and stability with the 4-year organic-inorganic fertilization. Moreover, the continued reduction of P fertilizer under organic-inorganic fertilization largely increased the abundances of *Bradyrhizobium* and *Methylobacterium* that are known as organic P mineralizers. In this P-deficient paddy soil, the *phoD*- and *pqqC*-harboring bacterial communities were primarily driven by pH, based on RDA and SEM analysis. We also found that the phosphate mobilizing bacterial community was more important as an up-regulator of phosphatase expression than the gene abundance. Overall, for sustainable agricultural management, our results implied that applying a P-reducing strategy with partial replacement of organic manure for chemical fertilizer could improve phosphate mobilizing bacterial community and promote P mobilization and utilization.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Author contributions

All authors contributed the intellectual input and assistance to this study and manuscript preparation. XYL conceived and designed the study. QFB and KJL collected the samples. QFB did the experiments with help from XPL and BJJ. QFB performed data analysis, with help from KD and XRY. QFB wrote the paper. YGZ, XYL and BXZ revised the manuscript. All authors read and approved the final manuscript.

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Table 1. Soil chemical parameters and rice yield. Mean $\pm$ Standard Deviation (n=4). Different letters within rows followed by indicate significance at $P<0.05$. TC: total carbon, TN: total nitrogen, Pt: total phosphorus, SOC: soil organic carbon, NH_4^+ : ammonium nitrogen, NO_3^- : nitrate nitrogen, P_{os} : available P, ALP: alkaline phosphomonoesterase, ACP: acid phosphomonoesterase, PDE: phosphodiesterase.

Treatment	CK ^a	NK	FP	M(20%P)	CM(P)	CM(-P)
pH (H ₂ O)	4.92 $\pm$ 0.14c	5.00 $\pm$ 0.11bc	5.39 $\pm$ 0.04a	5.01 $\pm$ 0.11bc	5.42 $\pm$ 0.1a	5.31 $\pm$ 0.17a
TC (mg g ⁻¹)	19.78 $\pm$ 1.54a	19.84 $\pm$ 1.4a	20.54 $\pm$ 3a	21.83 $\pm$ 1.89a	21.2 $\pm$ 1.51a	21.5 $\pm$ 1.22a
TN (mg g ⁻¹)	2.21 $\pm$ 0.38a	2.21 $\pm$ 0.13a	2.08 $\pm$ 0.54a	2.12 $\pm$ 0.03a	2.12 $\pm$ 0.23a	1.94 $\pm$ 0.13a
Pt (mg kg ⁻¹)	151.43 $\pm$ 12.89bc	113.52 $\pm$ 19.28c	220.10 $\pm$ 32.47a	158.84 $\pm$ 18.5bc	202.43 $\pm$ 7.57ab	177.20 $\pm$ 4.94ab
SOC (g kg ⁻¹)	28.15 $\pm$ 0.24c	25.73 $\pm$ 0.56d	28.20 $\pm$ 0.39c	26.00 $\pm$ 0.14d	29.92 $\pm$ 0.29a	29.13 $\pm$ 0.19b
NH_4^+ (mg kg ⁻¹)	63.66 $\pm$ 5.55c	88.83 $\pm$ 8.53ab	71.28 $\pm$ 7.22bc	82.22 $\pm$ 8.97ab	81.60 $\pm$ 8.29ab	91.50 $\pm$ 6.94a
NO_3^- (mg kg ⁻¹)	64.55 $\pm$ 6.02c	67.67 $\pm$ 8.82c	145.73 $\pm$ 12.33a	62.92 $\pm$ 3.88c	107.66 $\pm$ 5.62b	110.12 $\pm$ 12.03b
P_{os} (mg kg ⁻¹)	11.92 $\pm$ 3.61bc	6.45 $\pm$ 2c	22.68 $\pm$ 5.79b	8.75 $\pm$ 0.35c	44.69 $\pm$ 5.9a	41.82 $\pm$ 10.48a
ACP (mg kg ⁻¹)	482.36 $\pm$ 10.91c	479.99 $\pm$ 8.25c	520.28 $\pm$ 10.01b	475.63 $\pm$ 11.6c	567.07 $\pm$ 13.74a	560.51 $\pm$ 9.96a
ALP (mg kg ⁻¹)	43.97 $\pm$ 4.34b	44.79 $\pm$ 4.68b	82.34 $\pm$ 7.59a	54.07 $\pm$ 7.53b	75.43 $\pm$ 2.52a	74.00 $\pm$ 2.24a
PDE (mg kg ⁻¹)	78.07 $\pm$ 8.48b	82.07 $\pm$ 8.88b	107.99 $\pm$ 2.79a	85.52 $\pm$ 3.27b	116.55 $\pm$ 11.13a	124.47 $\pm$ 5.94a
Yield (Mg ha ⁻¹)	4.94 $\pm$ 0.16c	6.23 $\pm$ 0.23b	7.00 $\pm$ 0.31a	4.59 $\pm$ 0.28c	7.05 $\pm$ 0.38a	6.98 $\pm$ 0.27a
16S rRNA gene (copies $\times$ 10 ¹² g ⁻¹ soil)	3.74 $\pm$ 0.17 ^c	4.64 $\pm$ 0.32 ^c	6.01 $\pm$ 2.20 ^{bc}	6.06 $\pm$ 0.48 ^{bc}	11.91 $\pm$ 3.63 ^a	9.19 $\pm$ 0.58 ^{ab}

Table 2. Significance test using the nonparametric multivariate statistical approach (Adonis) to assess the effects of fertilization on the *phoD*- and *ppqC*-harboring bacterial communities.

Adonis comparisons	<i>phoD</i> gene composition			<i>ppqC</i> gene composition		
	F	R ²	P	F	R ²	P
CK vs CM(P)	2.276	0.275	0.109	1.172	0.163	0.434
CK vs CM(-P)	10.992	0.647	0.031*	4.843	0.447	0.028*
FP vs CM(P)	2.907	0.326	0.042*	1.468	0.197	0.176
FP vs CM(-P)	11.335	0.654	0.027*	8.444	0.585	0.038*
CM(P) vs CM(-P)	8.606	0.589	0.032*	7.023	0.539	0.034*

Note: CK: control without fertilizer; NK: chemical NK fertilizers with normal NK doses; FP: chemical fertilizers with normal NPK doses; M(20%P): organic manure (OM) alone with 20% of normal P doses; CMP: OM (20%P) + chemical fertilizers (80%P) with the same total nutrient doses as FP treatment; CM(-P): OM (20%P) + chemical fertilizers (64% P) with the same total NK doses and 84% P dose as NPK treatment. Significant differences ($P < 0.05$) are indicated in bold.

Figures

Fig. 1 The abundance and diversity of *phoD*- and *ppqC*-harboring bacterial communities. The gene copy number of (a) *phoD* and (b) *ppqC* (g^{-1} soil), and the Shannon diversity index for (c) *phoD* and (d) *ppqC*. Error bars represent the standard deviation of four independent replicates. Different lower-case letters refer to significant differences between treatments based on Tukey's test ($P < 0.05$ and $P < 0.001$).

Fig. 2 Heatmap of *phoD* (a) and *pqqC* (c) gene communities at the genus level based on Bray-Curtis distances. Only the relative abundance of the genus in one sample > 1% was shown. The relative abundance of the four most abundant and distinct genera under the six treatments in *phoD* (b) and *pqqC* (d) gene communities.

Fig. 3 Ternary plots of all enriched OTUs in the (a) *phoD*- and (b) *pqqC*-harboring communities ($\log_2$ fold change ≥ 2 , Q value > 0.05) for CK (the control), FP (conventional chemical fertilization) and CM(-P) (organic-inorganic fertilization with reduced P fertilizer) treatments, and its size represents the average across all three treatments. Colored circles represent OTUs enriched in one treatment compared with the others. Taxonomic classification using the enriched OTUs of each compartment is also shown at the phylum level (The *Proteobacteria* and *Firmicutes* classes are included). The size of each segment in the chart is proportional to the cumulative relative abundance of OTUs assigned to the indicated taxa

Fig. 4 Networks resulting from Non-P fertilizer treatments (CK and NK) (a and d for *phoD*- and *pqqC*-harboring communities, respectively) and organic-inorganic fertilization treatments (CM(P) and CM(-P)) (b and e for *phoD*- and *pqqC*-harboring communities, respectively) based on RMT analysis from OTU profiles. The right panel shows the correlations indicating the module eigengenes of respective networks and the coefficient values (r). Modules larger than five nodes are labeled with different colors in the respective network. The edges with red color indicate positive interactions, and those with blue indicate negative interactions. The OTUs acting as generalists were labeled in the networks. Zi-Pi plot showing the distribution of OTUs based on their topological roles (c and f for *phoD*- and *pqqC*-harboring communities, respectively). Each symbol represents an OTU in each network. The threshold values of Zi and Pi for categorizing OTUs were 2.5 and 0.62, respectively.

Fig. 5 Structural equation model (SEM) showing the influences of *phoD* and *pqqC* gene diversity, abundance, and composition on phosphatase (ALP, ACP, and PDE) activity, as mediated by pH, total bacterial abundance, Pt, and available P (*Posl*) in the soil. Black and gray arrows illustrate positive significant and negative significant relationships, respectively. The width of the arrows indicates the strength of the standardized path coefficient, and R^2 values represent the proportion of the variance explained for each endogenous variable. The total standardized effects of these factors on different phosphatase activities are shown.

Highlights:

- *phoD* gene abundance and available P content were the highest in organic-inorganic fertilization with or without reduced P input.
- Long-term organic-inorganic fertilization with reduced P input promoted the growth of *Bradyrhizobium* and *Methylobacterium*.
- Soil pH was the strongest determinant in P mobilization and utilization by shaping *phoD*- and *pqqC*-harboring bacterial communities.