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The core *phoD*-harboring bacteria promote wheat phosphorus uptake by enhancing alkaline phosphatase activity under long-term fertilization

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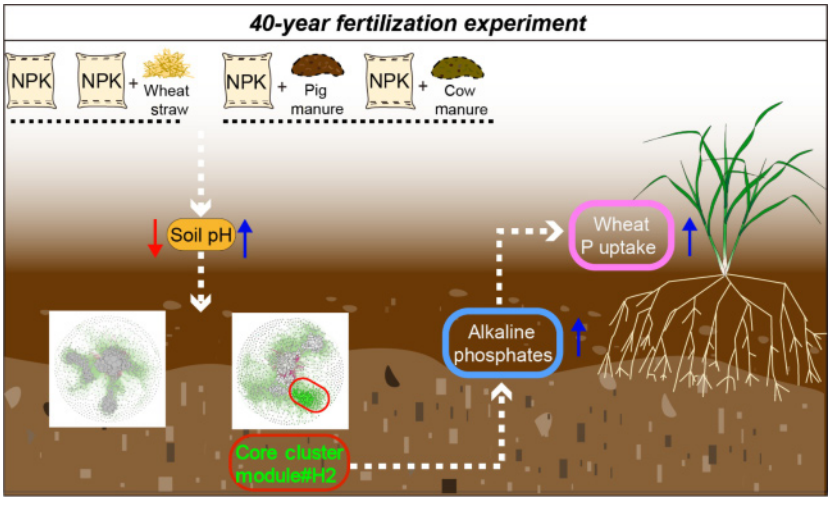
ABSTRACT

• Soil pH was the key factor influencing the *phoD*-harboring bacterial networks.

• Identification of a cluster positively linked to ALP activity and plant P uptake.

• Low soil pH resulted in a severe loss of *phoD*-harboring bacterial core cluster.



Fertilization treatments profoundly influence the bacterial communities associated with soil organic phosphorus (P) mineralization and alkaline phosphatase (ALP) activity. However, the relationships among the *phoD*-harboring bacterial communities associated with soil organic P mineralization, soil ALP activity, and plant P uptake under long-term fertilization remain unexplored. This study investigated these associations at the wheat rapid growth stage in a 40-year fertilization experiment. NPK fertilization led to a significant decrease in the diversity of *phoD*-harboring bacteria, which could be partially mitigated by the addition of organic materials. Soil pH emerged as the key factor influencing the structure and diversity of the *phoD*-harboring bacterial community. Furthermore, fertilizations involving manure additions resulted in more stable and cooperative *phoD*-harboring bacterial co-occurrence networks, compared to NPK fertilization. A functional *phoD*-harboring bacterial cluster, comprising genera *Nostoc*, *Bradyrhizobium*, and *Pseudomonas*, was identified, showing a positive association with soil ALP activity and plant P uptake. In summary, our study highlights the significant role of the identified core cluster of *phoD*-harboring bacteria in maintaining soil ALP activity and promoting plant P uptake, in decades of fertilization. Moreover, this study inferred a list of *phoD*-harboring bacterial genera from the core cluster, with established links to both plant P uptake and soil organic P mineralization. These findings offer valuable insights for sustainable agricultural practices.

Keywords *phoD*-harboring bacteria, co-occurrence network, long-term fertilization, wheat phosphorus uptake

1 Introduction

Wheat (*Triticum aestivum* L.) is one of the most important food crops in the world, covering 17% of the world's cultivated land (Laino et al., 2015). Phosphorus (P), a vital macronutrient for plant growth, is integral to various metabolic processes in plants, including carbohydrate metabolism, energy generation, photosynthesis, glycolysis nucleic acid synthesis, and nitrogen (N) fixation (Vance et al., 2003),

especially during the rapid growth phase, from jointing to flowering. This period demands substantial phosphorus accumulation for plant growth (Römer and Schilling, 1986). Therefore, sufficient P availability during this stage can greatly enhance growth, yeild, and grain quality.

Organic manure and crop straw are rich in organic P forms, such as phospholipids, phosphate monoesters, and phytic acid (Turner and Leytem, 2004), which could be mineralized to become phosphate in soil solution for uptake by nearby roots, and alkaline phosphatase (ALP) derived from bacteria can catalyze this mineralization process (Tabatabai, 1994). The *phoD* gene commonly serves as a biomarker, indicative of bacterial involvement in ALP production and organic P mineralization (Tan et al., 2013; Fraser et al., 2015; Ragot et al., 2016; Chen et al., 2019). Studies have highlighted the important role of *phoD*-harboring communities in driving soil ALP activity and increasing P availability in steppe ecosystems (Xu et al., 2022). Fertilization is a commonly employed practice to replenish nutrient elements to cultivated soil and enhance crop yields in agricultural production. However, the impact of various fertilization methods on *phoD*-harboring bacterial diversity, community structure, co-occurrence pattern, and ALP activity are inconsistent. For instance, long-term mineral fertilization decreased the total *phoD* gene abundance in maize cultivated soil, while manure fertilization had the opposite effect (Liu et al., 2021). Similarly, long-term inorganic nitrogen fertilization lowered soil pH and ALP activity compared with no fertilization in winter wheat–summer sweet potato rotation system soil (Wang et al., 2023). In contrast, both chemical and manure-straw-based compost organic fertilizer addition decreased the *phoD* gene abundance, the *phoD*-harboring bacterial diversity, and ALP activity in winter wheat–summer rice rotation system soil (Wang et al., 2022). A previous study found that the bacteria (including the *phoD*-harboring bacteria and the *phoC*-harboring bacteria) exhibited stronger connections in manure-amended treatments compared to those without manure-amended treatments (Luo et al., 2019). Additionally, the *phoD*-harboring bacterial network had higher modularity in pig manure or maize straw treatments than without organic amendments in the winter-spring cucumber–autumn winter tomato rotation greenhouse soil (Chen et al., 2023).

The biodiversity or relative abundance of key ecological clusters within a co-occurrence network has been observed to positively influence maize yield and soil nitrogenase activity (Shi et al., 2021) and wheat yield (Qiu et al., 2022). Although previous studies have investigated the *phoD*-harboring bacterial diversity, community structure, network features, and soil ALP activity, foundational knowledge remains limited concerning how the *phoD*-harboring bacterial core cluster impacts ALP activity that ultimately determines the effect of fertilization on wheat P uptake.

This study evaluates the influence of different fertilization strategies on the *phoD*-harboring bacterial community and ALP activity in soils from a 40-year fertilization experiment during the wheat rapid growth stage. Specifically, the objectives of this study were to investigate 1) how long-term fertilization treatments affect the rhizosphere *phoD*-harboring bacterial community and 2) how the *phoD*-harboring bacteria, ALP activity, and plant P uptake are related under these long-term fertilization treatments.

2 Materials and methods

2.1 Experimental design and sampling

Soil samples were collected from long-term experimental sites established in 1982 in Mengcheng County, Anhui Province, China (33°13′ N, 116°35′ E; 40 m a.s.l.). This region experiences a warm temperate semi-humid monsoon climate with an average yearly temperature of 14.8°C, 214 days of frost-free weather, and 732 mm of precipitation. The soil is typical lime concretion black soil. The study compared five fertilizer treatments under wheat soybean rotation: 1) Control: no fertilizer; 2) NPK: mineral-only fertilizer input (180 kg N ha^{-1} $year^{-1}$ urea, 90 kg P_2O_5 ha^{-1} $year^{-1}$ superphosphate and 86 kg K_2O ha^{-1} $year^{-1}$ potassium chloride); 3) NPK+WS: the combination of mineral and wheat straw fertilizer (7 500 kg fresh wheat straw ha^{-1} $year^{-1}$); 4) NPK+PM: the combination of mineral and pig manure fertilizer (15 000 kg fresh pig manure ha^{-1} $year^{-1}$); 5) NPK+CM: the combination of mineral and cow manure fertilizer (30 000 kg fresh cow manure ha^{-1} $year^{-1}$).

Rhizosphere soil samples were collected in 2022 at seven time points during the wheat jointing stage, i.e., 26 February, 4 March, 11 March, 18 March, 30 March, 11 April, and 23 April 2022. After removing the loosely attached soil by shaking, we collected the soil tightly adhered to the roots. In total, 140 rhizosphere soil samples (four biological replicates per each of the five fertilization treatments and seven sampling times) were collected. All samples were sieved through a 2-mm mesh until homogenous and separated into two parts, one of which was stored at 4°C for physicochemical analysis. In contrast, the other was stored at −40°C for subsequent DNA extraction. For each sample, 10 healthy wheat plants were harvested from each plot randomly, and the above-ground plant tissue was dried at 60°C for 72 h to constant weight, weighed, and crushed before analysis.

2.2 Soil and plant physicochemical analysis

The air-dried soils were subject to physiochemical property measurements. Soil pH was measured at an air-dried soil sample to distilled water ratio of 1:5 (weight/volume) using a

pH meter (S210, Mettler Toledo, Germany). The ratio of evaporated water to dried soil was known as the soil moisture. A total organic carbon analyzer (Multi N/C 3100, Analytik Jena AG, Germany) was used to quantify dissolved organic carbon (DOC). Dissolved total nitrogen (DTN), nitrate nitrogen (NO_3^--N), and ammonium nitrogen (NH_4^+-N) were measured using a continuous flow analyzer (San++ system, Skalar, Holland), and dissolved organic nitrogen (DON) = DTN − NH_4^+-N − NO_3^--N. Total carbon contents (TC) and total nitrogen (TN) of soil and plant samples were determined using a Vario MAX CNS analyzer (Elementar Germany). Total phosphorus contents (TP) in soil and plant samples and available phosphorus contents (AP) in soil were measured using the Molybdenum blue method. Total potassium contents (TK) in soil and plant samples were determined according to the flame spectrophotometry method, and available potassium contents (AK) in soil were determined by the flame photometry method (FP640, INASA, China). Plant P uptake in wheat was calculated by multiplying the aboveground biomass by the P concentration. ALP activity was measured using a Full-wavelength microplate reader (MD SpectraMax190, USA), defined as the amount (mg) of p-nitrophenol released in 1 day per gram of soil samples (U d^{-1}). data obtained are detailed in Table S1 and Table S2.

2.3 High-throughput sequencing and bioinformatics analysis

Total DNA was extracted from approximately 0.5 g of fresh soil for DNA extraction using the Fast DNA SPIN Kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's standard operation protocol. Forward primer *phoD*-F733 (5'-TGGGAYGATCAYGARGT-3') and reverse primer *phoD*-R1083 (5'-CTGSGCSAKSACRTTCCA-3') were applied to amplify the *phoD* gene (Ragot et al., 2015). High-throughput sequencing was carried out in a reaction mixture consisting of, 20 μL of Pro Taq, 0.8 μL of 5 μM forward primer, 0.8 μL of 5 μM reverse primer, 10 ng μL^{-1} of template DNA, and ddH_2O to a final volume of 20 μL. The amplification protocol was as follows: 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with final extension at 72°C for 10 min. The PCR products were purified using an Agarose Gel DNA purification kit (TaKaRa Bio), and the PCR products were purified and sequenced on the Illumina MiSeq 300 PE platform at Majorbio Company, Shanghai. Raw sequencing reads with exact matches to the barcodes were assigned to the respective samples and identified as valid sequences. Pair-end raw reads were assembled and screened, and quality scores (⩽20) and less than 300 bp sequences were removed. The remaining sequence was clustered to OTUs (operational taxonomic units) using the UCluster method at 97% similarity in QIIME2 (Bolyen et al., 2019). Each representative sequence's taxonomy information was obtained from the NCBI nucleotide database using the Blast algorithm (available at the NCBI website) (Yang et al., 2023). The total sequences of each sample were rarified to 8326 sequences for the following analyses. Alpha-diversity metrics of *phoD*-harboring bacteria were computed on the Majorbio cloud platform (available at majorbio.com) with Mothur v1.30.1 at the OTU level (Schloss et al., 2009). All sequence information is available via the Microbiome Database (available at the platform Microbiome Database) under project number AMPLI-ba7178f647cc650797b9f853e582d5e0.

2.4 Co-occurrence network analysis

The OTUs that were selected to build the *phoD*-harboring bacterial co-occurrence network had a relative abundance higher than 0.1%. The correlations between OTUs with a Spearman's coefficient of more than 0.70 and a *P* value of less than 0.05 were saved (Zhang et al., 2018) with the R package "Hmsic". The main ecological clusters in the network were identified and visualized using Gephi (available at gephi.org). We computed the relative abundance of each ecological cluster by averagingthe standardized relative abundances (*z*-score) of all the species belonging to that cluster (Delgado-Baquerizo et al., 2018). The entire set of o-occurrence networks was divided into subnetworks using the subgraph function in the "igraph" package. The topological features (nodes, edges, average degree, transitivity, connectance, modularity) of the networks were also calculated with the "igraph" package. Two co-occurrence subnetworks were generated to identify the different co-occurrence patterns of the *phoD*-harboring bacterial communities in high pH (NPK+PM and NPK+CM) soil and low pH (NPK and NPK+WS) soil after long-term fertilization treatment.

2.5 Statistical analysis

To evaluate the homogeneity of variances and the normality of the distribution, Shapiro−Wilk and Bartlett's tests were used. The one-way ANOVA test was performed when normality assumptions were met, and the Kruskal–Wallis test was performed when normality assumptions were not met. The *phoD*-harboring bacterial community structure was analyzed using Bray−Curtis distance matrix similarity and ordinated using principal coordinated analysis (PCoA) with the R software package "vegan". Using PERMANOVA and the Adonis function, the relative contributions of several factors to community dissimilarity were investigated. The soil physiochemical variables were correlated against the alpha diversity indices and ALP activity of *phoD*-harboring bacterial community, the topological features of the subnetworks, and

the relative abundance of the main ecological clusters using the corr.test function of the "psych" package and Spearman's coefficients were calculated. DISTLM was used to examine the relative effects of the physiochemical variables on the *phoD*-harboring bacterial community composition (McArdle and Anderson, 2001). R packages "randomForest" and "rfPermute" were used to identify the key variables that affect the topological features of the subnetwork (Breiman, 2001). A 10-fold cross-validation method was used to determine the optimal set of OTUs correlated to the ALP activity.

2.6 Structural equation modeling

Structural equation model (SEM) analysis was performed to assess the direct and indirect effects of ALP activity and the relative abundance of the main ecological cluster (Module#H2) on plant phosphorus uptake using IBM SPSS Amos 26 (Chicago, IL: Amos Development Corporation). The physiochemical properties of the soil included DOC and NO_3^--N were included. In the model, the treatments (NPK + PM and NPK + CM) were set as categorical variables with 1 and 0, respectively. Based on prior knowledge that soil ALP activity could be affected by physiochemical properties (Wang et al., 2022) and the relative abundance of key ecological clusters played an important role in soil functions related to nutrient cycles and wheat production (Fan et al., 2020), we hypothesized that the changes of soil physiochemical properties induced by long-term application of NPK fertilizer and manure could increase ALP activity through influencing the core cluster of *phoD*-harboring bacteria, and promote crop P uptake. We constructed a priori model based on a literature review and our knowledge on how these predictors are related (Fig. S1), and the model was accepted when the model criteria were met, i.e., comparative fit index (*CFI*) > 0.95 and Root Mean Square Error (RMSEA) <0.05. The standardized total effects (STEs) of the fertilization treatments, the soil physicochemical properties, the relative abundance of Module#H2, and ALP activity on the plant phosphorus uptakes were calculated to interpret the SEM.

3 Results

3.1 Effects of fertilization on soil physiochemical properties

Long-term NPK and NPK+WS fertilization treatments significantly reduced soil pH compared with the control ($P < 0.05$). (Table S1). However, this decline was mitigated by manure additions (i.e., NPK+PM and NPK+CM), resulting in pH levels analogous to those observed in the control group. Relative to the NPK treatment alone, NPK+WS, NPK+PM and NPK+CM treatments significantly increased the TC, TN and DOC. Among all these treatments, the NPK+PM treatment had the highest TP and AP. In contrast, the NPK+CM treatment resulted in the highest ALP activity, followed by NPK+PM and the control treatments, whereas the NPK and NPK+WS treatments had the lowest ALP levels.

3.2 Effects of fertilization on *phoD*-harboring bacterial diversity and community composition

Our analysis of *phoD*-harboring bacterial communities across different fertilization treatments revealed substantial impacts. Adonis analyses revealed that fertilization treatment (R^2 = 0.66, $P < 0.001$) had stronger effects on the *phoD*-harboring bacterial community composition than plant growth time (R^2 = 0.02, P = 0.021) (Fig.1A). Pairwise Adonis comparison revealed that the *phoD*-harboring bacterial community structure varied significantly between fertilization treatments (Table S3), while such differences were not observed across different time points (Table S4).

Furthermore, distance-based linear modeling (DISTLM) showed that soil physicochemical properties could explain 63.1% of community variation, in which soil pH was the primary driver of the *phoD*-harboring bacterial community composition, which individually explained 35.9% of the variation (Table 1).

A total of 16 398 OTUs were obtained from all samples, predominantly assigned to *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Cyanobacteria*, *Planctomycetes*, *Firmicutes*, *Chloroflexi*, *Verrucomicrobia*, and others (Figs. 1B and S2). *Proteobacteria* emerged as the most abundant taxa, constituting 47.5% of the total reads. Long-term NPK fertilization significantly decreased the relative abundance of the *Cyanobacteria* and *Actinobacteria*, notably increasing *Proteobacteria* and *Planctomycetes* abundance in contrast to the control (Table S5). Additionally, compared with NPK treatment, NPK+WS treatment did not affect the abundance of *Cyanobacteria* and *Actinobacteria* but decreased the relative abundance of the *Proteobacteria*, NPK+PM and NPK+CM treatments increased those of the *Cyanobacteria* and *Actinobacteria* and reduced the relative abundance of the *Proteobacteria* significantly, and NPK+CM treatment had the highest relative abundance of *Cyanobacteria*. Notably, positive correlations were observed between *Cyanobacteria* and soil pH and nutrients, while a negative correlation was evident between *Proteobacteria* and soil pH (Table S6).

Long-term NPK fertilization also significantly reduced OTU richness and Shannon index compared with the control (Figs. 1C, S3, and S4). In contrast, treatments with straw or manure additions, such as NPK+WS, NPK+PM, and NPK+CM, significantly increased the *phoD*-harboring bacterial OTU richness compared to NPK fertilization. Moreover, the richness index was positively correlated with soil pH, TP, and AP (Table S7).

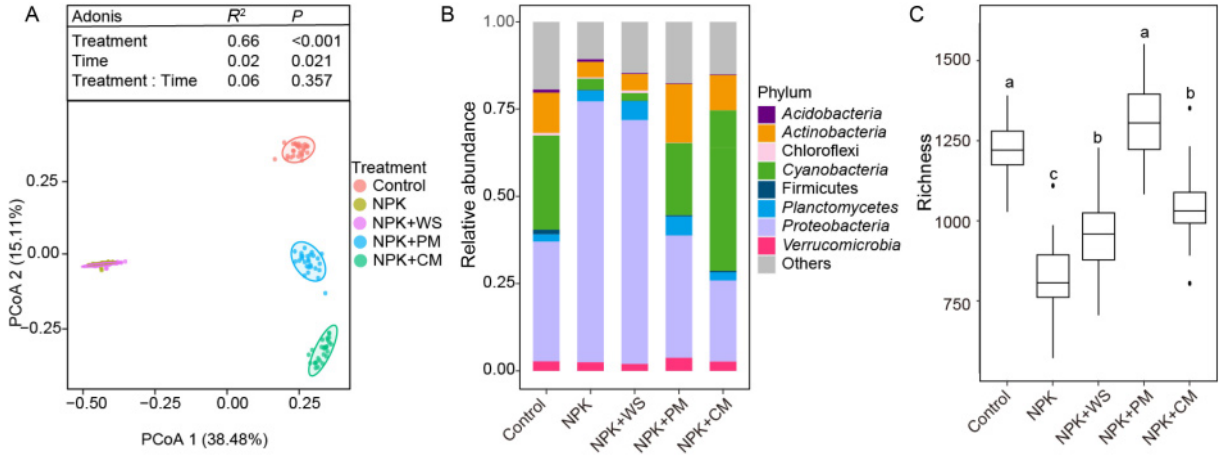


Fig. 1 (A) Principal coordinate analysis (PCoA) of *phoD*-harboring bacterial communities based on the Bray-Curtis distance. (B) Relative abundance of the main *phoD*-harboring taxa in the phylum level across different fertilization treatments. (C) The *phoD*-harboring bacterial alpha diversity under each fertilization treatment. Different lowercase letters indicate statistically significant ($P < 0.05$) differences as detected using Kruskal-Wallis tests. Control: non-fertilization; NPK: nitrogen-phosphorus-potassium mineral fertilizer only; NPK+WS: mineral fertilizer plus wheat straw; NPK+PM: mineral fertilizer plus pig manure. NPK+CM: mineral fertilizer plus cow manure.

Table 1 Influencing factors of the *phoD*-harboring bacterial community compositions based on the DISTLM model.

Variable	Pseudo-F	*P*	R^2 (conditional)	R^2 (cumulative)
pH	77.22	0.001	0.359	0.359
TN	39.78	0.001	0.144	0.503
AP	28.24	0.001	0.085	0.589
DOC	4.18	0.001	0.012	0.601
NH_4^+-N	2.70	0.001	0.008	0.609
SM	2.53	0.001	0.007	0.616
AK	2.21	0.001	0.006	0.622
NO_3^--N	1.69	0.014	0.005	0.627
TP	1.43	0.045	0.004	0.631

AP, available phosphorous; TN, total nitrogen; DOC, dissolved organic carbon; NH_4^+-N, ammonium nitrogen; SM, soil moisture; AK, available potassium; NO_3^--N, nitrate nitrogen; TP, total phosphorus.

3.3 Variation in *phoD*-harboring bacterial co-occurrence subnetwork across fertilization treatments

Using rhizosphere soil samples from different fertilization treatments, the corresponding overall co-occurrence networks of the *phoD*-harboring bacterial communities were constructed, resulting in a network with 2061 nodes and 71180 edges. Subnetworks for each treatment were generated to explore the impact of fertilization treatments on the network patterns of the *phoD*-harboring bacterial communities (Fig. 2A). The *phoD*-harboring bacterial co-occurrence subnetwork of the non-fertilization treatment had more nodes (1077) yet fewer edges (31268) than the NPK fertilization treatment (with 960 nodes and 35831 edges) (Table S8). Compared with NPK treatment, NPK+WS, NPK+PM, and NPK+CM treatments decreased the number of edges (32232 edges, 25563 edges, and 21824 edges), NPK+WS and NPK+CM treatments reduced the number of nodes (948 nodes and 946 nodes), but NPK+PM increased the number of nodes (1095 nodes). Our results showed that mineral fertilization augmented the complexity (average degree, transitivity, and connectance) of the *phoD*-harboring bacterial community whilst diminishing its modularity relative to the control (Fig. 2B). The topological properties of the NPK+WS subnetwork were highly consistent with that of NPK, while the manure application (NPK+PM and NPK+CM) subnetworks had a lower average degree, transitivity, and connectance, but higher modularity. Further investigation revealed that the *phoD*-harboring bacterial co-occurrence subnetworks in the NPK and NPK+WS treatments exhibited more negative correlations compared to the Control, NPK+PM, and NPK+CM treatments.

Random forest analysis identified pH, TC, TN, TP, and AP as key predictors of topological features of these subnetworks (Table S9). A subsequent correlation analysis further highlighted substantial and significant associations between pH levels and these topological subnetwork characteristics ($R = 0.74$ with nodes; $R = 0.58$ with modularity; $R = 0.66$ with positive correlations; $R = -0.58$ with transitivity; $R = -0.66$ with negative correlations) (Fig. S5).

3.4 Ecological clusters in *phoD*-harboring bacterial co-occurrence subnetworks

The community structure and the topological characteristics of the *phoD*-harboring bacterial co-occurrence subnetwork were significantly different between soils of high and low-pH

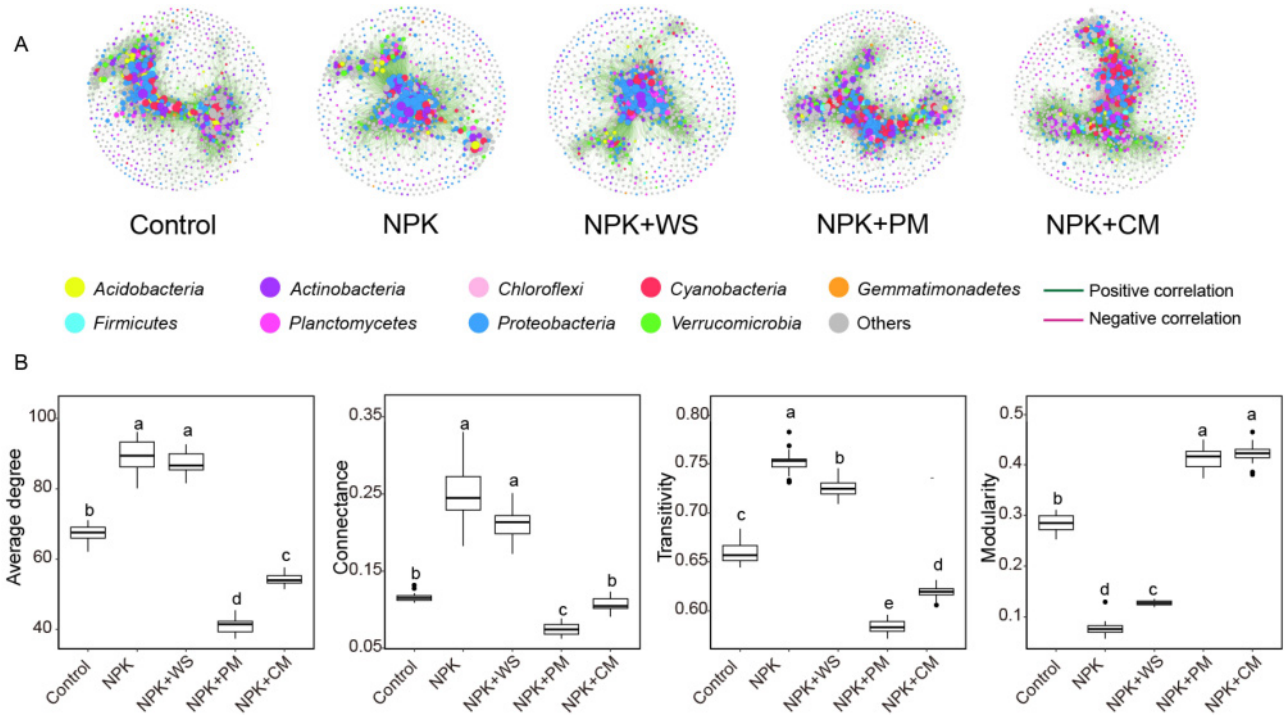


Fig. 2 (A) Co-occurrence subnetworks of different fertilization treatments. The subnetworks of five fertilization treatments were visualized to show the significant associations (R > 0.70, P < 0.05) between *phoD*-harboring bacterial operational taxonomic units (OTUs) filtered from the overall co-occurrence networks of each fertilization treatment. Each dot symbolizes an OTU; phyla are represented by different colors; node size indicates node degree; and important correlations between OTUs are indicated by edges. (B)Topological features of subnetwork across different fertilization treatments. Different lowercase letters indicate the values that differ significantly among treatments at P < 0.05 (Kruskal-Wallis tests). Control: non-fertilization; NPK: nitrogen-phosphorus-potassium mineral fertilizer only; NPK+WS: mineral fertilizer plus wheat straw; NPK+PM: mineral fertilizer plus pig manure. NPK+CM: mineral fertilizer plus cow manure.

levels resulting from different fertilization treatments. This led to the generation of two distinct co-occurrence subnetworks to identify the different co-occurrence patterns of the *phoD*-harboring bacterial communities in high pH (NPK+PM and NPK+CM) soil and low pH (NPK and NPK+WS) soil after long-term fertilization treatments (Fig. 3A). This investigation aimed to identify the key ecological clusters in microbial co-occurrence networks under different pH levels and their relationships with ALP activity. Regression analysis was performed to assess the relationship between the relative abundance of main ecological clusters and ALP activity. There was a significant and positive linear relationship between ALP activity and the relative abundance of Module#H2 (R^2 = 0.15, P = 0.003) in the high pH condition (Fig. 3B). However, no significant association was detected between the relative abundance of main ecological clusters of the low pH level subnetwork and ALP activity (Fig. S6). *Proteobacteria*, *Cyanobacteria*, and *Actinobacteria* were the main phyla in Module#H2 (Fig. 3C), and within this module, relatively abundant genera included *Nostoc*, *Bradyrhizobium*, and *Prosthecobacter* (Table S10).

Random forest regression identified 100 OTUs that showed strong positive correlations with ALP activity (Fig. S7). Notably, these OTUs were mainly present in module #H2 (29/100) compared to other ecological clusters (Table S11; Fig. S8); Specifically, the relative abundances of three genera, i.e., *Micromonospora* (OTU3879, R = 0.52, P < 0.001), *Bradyrhizobium* (OTU3945, R = 0.43, P < 0.001) and *Pseudomonas* (OTU 3800 and OTU4298, R = 0.46, P < 0.001; R = 0.39, P < 0.01) were positively correlated with ALP activity and thus they were potentially the key bacteria affecting ALP activity (Table S12).

3.5 Linking the ecological cluster with ALP activity and plant P uptake under long-term fertilization

SEM analysis revealed direct and indirect associations between the main ecological cluster of the *phoD*-harboring bacteria and plant P uptake under high-pH soils. This exploration provides deeper insights into the effects of fertilization on ALP activity and plant P uptake, considering multiple environmental factors simultaneously (Fig. 4A). We found a strong positive and direct association between the relative abundance of module#H2 and ALP activity (SPC = 0.35, P < 0.001). Moreover, ALP had a positive effect on plant P uptake (SPC =0.32, P < 0.001).

We also identified the bacteria within Module#H2, which showed a strong association with ALP activity through

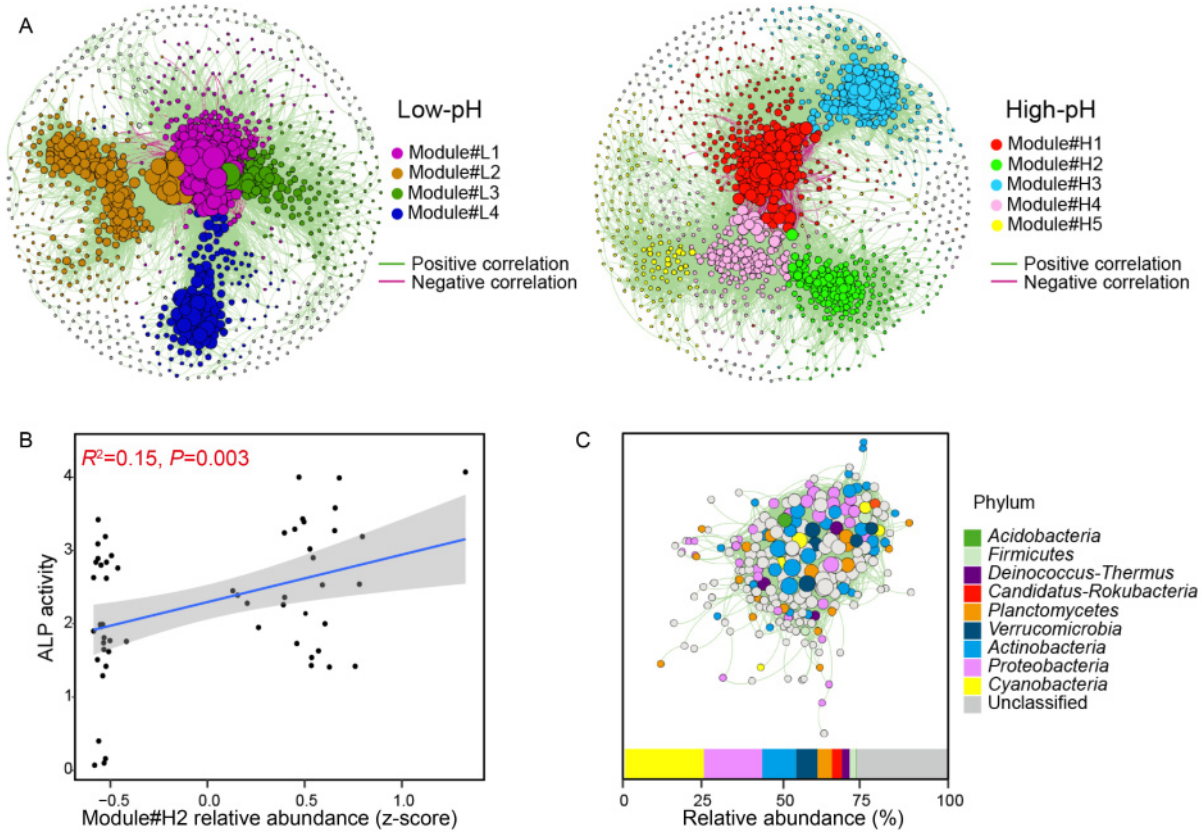


Fig. 3 (A) *phoD*-harboring bacterial co-occurrence subnetworks under different pH levels. Each dot represents a microbial OTU, color represents different ecological clusters, and node size represents the degree of the node. (B) Linear regression between the relative abundance of Module#H2 (z-score) and alkaline phosphatase (ALP) activity. (C) Network diagram of Module#H2. The green line indicates a positive correlation; and the relative proportions of the dominant *phoD*-harboring bacterial taxa are summarized at the phylum level in Module#H2.

random forest regression. Further their abundance varied considerably among different fertilization treatments (Fig. 4B). The long-term combined organic and inorganic fertilization notably enriched their abundance. Contrastingly, no fertilization practice led to a reduction of their abundance while both NPK+WS and NPK treatments resulted in a more severe loss.

4 Discussion

4.1 *phoD*-harboring bacterial diversity, community, and co-occurrence pattern responses to different long-term fertilizations

A previous study found that the bacterial community structure in *Arabidopsis thaliana* rhizosphere soil did not change significantly from the vegetative to the flowering stage (Chaparro et al., 2014). Similarly, we observed little difference in the *phoD*-harboring bacterial community in wheat rhizosphere soil among seven sampling times during the wheat rapid growth period. However chemical fertilization led to a significant reduction in *phoD*-harboring bacterial diversity, whereas the addition of manure demonstrated a capacity to mitigate such reduction. Therefore, this observation aligns with previous findings indicating that the addition of chemical fertilizers significantly decreased the *phoD*-harboring bacterial diversity in soil from the winter wheat-summer maize rotation system (Chen et al., 2017). The reduction in the diversity of these bacteria could be due to soil acidification caused by long-term mineral fertilizer addition (Wang et al., 2023). Further, their community structure was distinct across five treatments, with pH, TN, and AP as the main influencing factors. Such observation is consistent with previous studies (Chen et al., 2017; Hu et al., 2018; Chen et al., 2019; Wang et al., 2023), and further confirmed the vital role of soil pH in determining the structure of soil bacterial communities (Ragot et al., 2016).

Co-occurrence network analysis serves as a valuable tool for exploring potential microbial interactions (including competition, facilitation, and inhibition) in agricultural ecosystems and their responses to different fertilization managements (Fan et al., 2019). Our study found that chemical fertilization treatment increased the complexity and lowered the modularity of the *phoD*-harboring bacterial

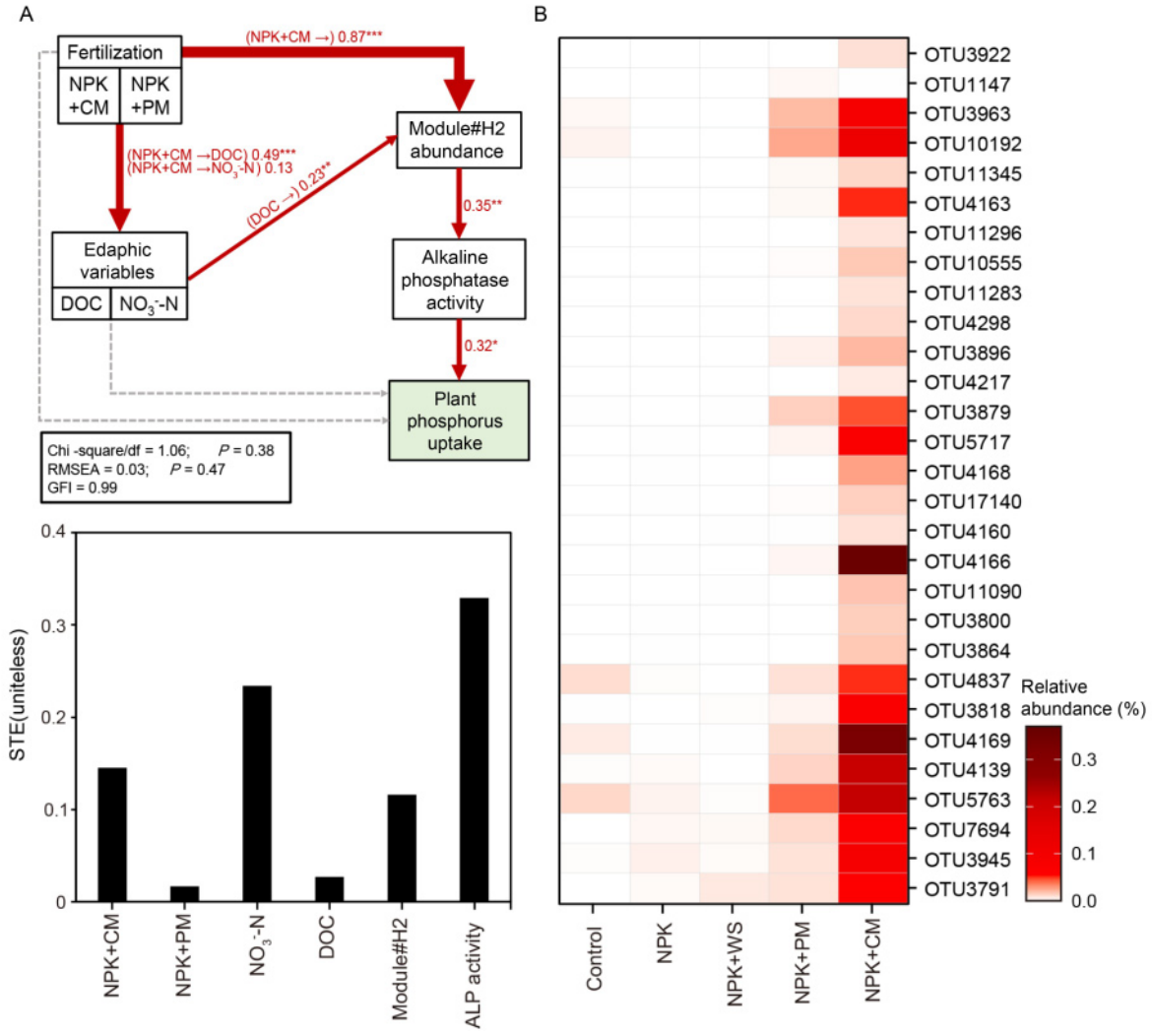


Fig. 4 (A) Structural equation model (SEM) showing the relationships among fertilization treatments (NPK+PM, NPK+CM), edaphic variables (DOC, NO_3^--N), alkaline phosphatase (ALP) activity, the main ecological cluster (Module#H2) in high-pH co-occurrence subnetwork and the plant phosphorus uptake (n = 28, χ^2 = 5.23, P = 0.38, degrees of freedom (df) = 5; RMSEA = 0.03, P = 0.47, GFI = 0.99). A number above each arrow represents the value of the standardized path coefficients (SPCs). Red lines indicate significant positive effects, gray lines indicate no significant effects. Significance levels of each predictor are *P < 0.05, **P < 0.01, ***P < 0.001. Standardized total effects (STE) of every variable are shown in the left lower corner. This is the total of each variable's direct and indirect impacts on the uptake of phosphorus by plants. (B) The shared phoD-harboring bacterial OTUs of Module#H2 and ALP indicators were detected using random forest regression and their relative abundances under different fertilization treatments.

community networks, and this observation is consistent with a previous study (Li et al., 2023). According to Stephen Vacation, system modularity decreases as stress levels intensify (Bertness and Callaway, 1994; Hernandez et al., 2021). The decreased modularity could resulted from the increased environmental stress due to soil acidification, observed in long-term chemical fertilization and straw addition treatments. Conversely, manure addition treatments showed an ability to mitigate soil acidification thereby increasing the modularity. Higher modularity in manure addition treatments might reflect that there were potentially more operational communities and suggest that these soils might have more ecologically similar functional and ecological characteristics and bacterial groups speculated to positively impact soil biological fertility (Williams et al., 2014; Ling et al., 2016). The positive effect of manure addition fertilization on the *phoD*-harboring bacterial co-occurrence pattern is also presented in the fact that manure addition treatments had more positive correlations than chemical fertilization treatment and straw addition treatment. Positive correlations within co-occurrence networks often stem from facilitation and mutualism among taxa or reflect ecological or functional similarity, while negative correlations can indicate competition among taxa or reflect divergent niche requirements (Chaffron et al., 2010; Zelezniak et al., 2015). This prevalence of cooperative relationships or similar niche requirements

among *phoD*-harboring bacterial members in soil treated with organic manure additions is suggested by these findings. Moreover, the higher ALP activity observed in the two organic manure additions treatments supports the idea that higher microbial network modularity and more positive correlations were beneficial to soil ecosystem functions such as nutrient cycling (Ling et al., 2016). These observations indicated that the manure amendment mitigated the soil acidification caused by long-term chemical fertilizer input, and alleviated the environmental stress on the *phoD*-harboring bacterial community, thereby sustaining high organic P mineralization potential in the rhizosphere soil during the wheat rapid growth stage.

4.2 Core cluster is positively associated with ALP activity and plant P uptake

Our comprehensive analyses identified a core ecological cluster (Module#H2) with a robust positive correlation with ALP activity after decades of continued fertilization. This cluster encompassed diverse bacterial genera known for advantageous roles in soil nutrient cycling and plant growth. Among these, *Nostoc* emerged as the most abundant genus in Module#H2 renowned for its versatile P utilization capacity and ubiquity in various environments (Dong et al., 2019). A study indicated that applying *Nostoc* in nitrogen-poor soils increased the maize dry matter yeild and enhanced soil C and N contents (Maqubela et al., 2009). Furthermore, the exopolysaccharides and indole 3-acetic acid (IAA) produced by *Nostoc* fortified soil aggregate stability while fostering wheat germination and growth (Gheda and Ahmed, 2015). Despite their lower abundances in Module #H2, the relative abundances of *Pseudomonas* and *Bradyrhizobium*, were also positively correlated with ALP activity. According to a previous study, *Bradyrhizobium* exhibited ALP activity (Zhu et al., 2021). In addition, *Bradyrhizobium* was an important nitrogen-fixed member integral to soil N cycling (Tao et al., 2021), and contributed to plant growth enhancement by producing IAA (Masciarelli et al., 2014). Similarly, *Pseudomonas* isolated from wheat rhizosphere demonstrated an array of plant growth-promoting effects such as phosphate solubilization, IAA production, and siderophore production (Gontia-Mishra et al., 2017), alongside inducing plant systemic resistance against foliar pathogen (Wen et al., 2021).

Soil DOC positively affects the relative abundance of Module#H2, ultimately increasing the ALP activity and plant P uptake. The positive correlation between DOC and the relative abundance of Module#H2 implied an association between soil *phoD*-harboring bacterial proliferation and labile C turnover (Fei et al., 2021). Previous studies only found a positive association between soil DOC and ALP activity (Deng et al., 2019), while our study identified the core cluster involved in this process.

Our study revealed that all correlations within Module#H2 were positive, indicating that mutual facilitations dominated this ecological cluster. It is important to note that the effect of appying individual plant growth-promoting bacteria might not be consistently stable and long-lasting. The addition of multiple bacteria synergistic functional is more conducive to the maintenance of agricultural yield increase (Gholami et al., 2012; Bechtaoui et al., 2019; Shirmohammadi et al., 2020). These results hold potential for future biotechnological applications, especially in developing synthetic microbiota inoculants aimed to enhance the ALP activity and plant P uptake.

5 Conclusion

The identification of the core cluster of *phoD*-harboring bacteria, which was positively linked to soil ALP activity and plant P uptake at the wheat rapid growth stage, underscores the impact of long-term chemical fertilization in depleting the *phoD*-harboring bacteria, whereas manure addition mitigated such shifts. However, this study focused solely on the rapid growth period of wheat under long-time fertilization, leaving a gap in understanding the contributions of the core cluster to wheat P uptake and final yield throughout the entire growth cycle. Altogether, this study provides insights into regulating the soil organic P mineralization process by manipulating the core cluster, supporting sustainable agricultural development.

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Electronic supplementary material

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