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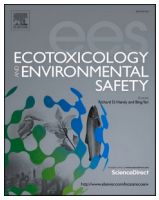

Reduction effect of individual N, P, K fertilization on antibiotic resistance genes in reclaimed water irrigated soil

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ABSTRACT

The transfer of antibiotic resistance genes (ARGs) in soil under reclaimed water irrigation poses a potential environmental risk. Regulation of NPK fertilizer could influence the behavior of bacterial communities, mobile genetic elements (MGEs), and soil properties, which determine the fate of ARGs. To identify the key element in NPK fertilizer and realize efficient regulation, we explored the effect of individual N, P, K fertilization on ARG variation in tomato rhizosphere and bulk soils. Compared with an unfertilized treatment, N fertilization resulted in greater decreases in the abundance of ARGs (decreases of 24.06%–73.09%) than did either P fertilization (increases of up to 35.84%, decreases of up to 58.80%) or K fertilization (decreases of 13.47%–72.47%). The influence of different forms of N ($CO(NH_2)_2$, $NaNO_3$, and NH_4HCO_3), P ($Ca(H_2PO_4)_2$ and $CaMgO_4P^+$), and K (KCl and $K_2(SO_4)$) fertilizers was also investigated in this study, and showed the influence of $NaNO_3$, $CaMgO_4P^+$, and $K_2(SO_4)$ on reducing ARGs abundance was greater in different types of N, P, K fertilizers. Bacterial communities showed the strongest response to N fertilization. The reduced bacterial diversity and abundance of ARG-host and non-host organisms explained the decline of total ARG abundance in soil. In soils fertilized with either P or K, the effect of soil properties, especially total nitrogen and pH, on ARG variation was greater than that of bacterial community and MGEs. These results suggest that N regulation of in NPK fertilizer may be an effective way to reduce the risks of ARGs in soil associated with reclaimed water irrigation.

1. Introduction

Antibiotic resistance genes (ARGs) are an emerging environmental contaminant attracting global public attention. One of their major reservoirs is reclaimed water because of the increasing use of antibiotic compounds and incomplete removal of antibiotics and ARGs in wastewater treatment plants (WWTP) (Marano et al., 2019). The use of reclaimed water to irrigate farmland has been advocated across China to reduce the reliance of agriculture upon groundwater resources. This has been accompanied by the introduction of specific limits for nutrients, metals, fecal coliforms, and Ascaris eggs (GB/T 18919-2002; GB 20922-2007). Recent studies have revealed the accumulation of unregulated contaminants, including polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and antibiotics, in soil following reclaimed water irrigation (Chen et al., 2005, 2011; Al Nasir and Batarseh, 2008). These may enhance the accumulation of ARGs in soil due to their selective pressures (Sataloff et al., 2018). Compared with groundwater irrigated soil, Fahrenfeld et al. (2013) and Cerqueira et al. (2019) observed an elevated abundance of ARGs. Other studies have revealed that reclaimed water irrigation of soil resulted in lower or similar abundance of ARGs (Negreanu et al., 2012; Marano et al., 2019; Shamsizadeh et al., 2021). Notably, these detected ARGs in soil can be transferred to the plant, air, and surface water in the whole ecosystem, potentially threatening human health (Wang et al., 2021). This threat necessitates the development of economically feasible approaches to reduce ARGs abundance in reclaimed water irrigated soil.

Nitrogen (N), phosphorus (P), and potassium (K) fertilizers are used as basic fertilizers for agricultural production. Application of these fertilizers also affects the bacterial community, soil properties, and mobile genetic elements (MGEs), which were important factors affecting ARG variation (Chen et al., 2016, 2018; Han et al., 2018; Xie et al., 2018; Sui et al., 2019; Wang et al., 2020). Since these variables have different responses to combined NPK fertilization, there is little consistent evidence regarding the influence of fertilization on the occurrence of ARGs,

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relative to unfertilized soil. In some cases, NPK application did not affect ARG levels (Lin et al., 2016; Sui et al., 2019), while in other instances, NPK application enriched ARG abundance (Chen et al., 2016; Xie et al., 2018; Sun et al., 2019). When controlling the fate of ARGs in soil under reclaimed water irrigation, determining the key element in chemical fertilizer has been addressed by comparing the effects of N, P and K individually. The regulation of N, P, K may shift the composition of bacterial communities, and the role of N fertilization is more significant than that of P and K fertilization (Pan et al., 2014; Yu et al., 2019). Since most studies demonstrate that the most important factor for ARG variation was the bacterial community (Chen et al., 2016, 2018; Han et al., 2018; Wang et al., 2020), we hypothesized the effect of P and K fertilization on the ARGs abundance in soil was weaker than N fertilization. Individual applications of different forms of N, P, and K fertilizers have also been shown to have diverse effects on the structure of bacterial communities (Zhong et al., 2010; Ramirez et al., 2012; Pan et al., 2014; Yu et al., 2019; Zhang et al., 2019; Wang et al., 2020). This may also affect the fate of ARGs.

In this work, the effect of individual applications of different forms of N, P, and K fertilizers on the structure and abundance of ARGs (285 primers) and MGEs (10 primers), bacterial community composition, and soil properties (pH, total nitrogen, and total phosphate) in reclaimed water irrigated rhizosphere and bulk soil was investigated. The relationship between ARGs and MGEs, and bacterial assemblages was determined, and potential ARGs hosts in soil following individual applications of N, P, and K fertilizer were explored. Finally, the indirect and direct effects of the bacterial community, soil properties, and MGEs revealed the dominant factor affecting ARG variation in the individual N, P, and K fertilization treatments. These findings help clarify which component of mixed fertilizer is most effective for eliminating ARGs in soil irrigated with reclaimed water, so that appropriate combined chemical fertilization strategies can be developed.

2. Materials and methods

2.1. Experimental design

The field trial was carried out from March 2015 to June 2016 in a commercial greenhouse at Xinxiang, Henan Province, China (35.19°N, 113.53°E). The soil type was fluvo-aquic according to the Genetic Soil Classification of China. Soil properties in the 0–20 cm layer were as follows: pH 7.6, organic matter 3.43%, total N 1.16 g kg^{-1}, total K 10.08 g kg^{-1}, total P 0.84 g kg^{-1}, available K 133.00 mg kg^{-1}, and available P 15.97 mg kg^{-1}. Groundwater was pumped from a well, and reclaimed water was the secondary effluent from a domestic sewage treatment plant in Xinxiang. The properties of these two irrigation waters are shown in Table S1. Salts of copper, zinc, lead, and cadmium ($CuSO_4{\cdot}5H_2O$, $Zn(CH_3COO)_2$, $(CH_3COO)_2Pb$, and $CdCl_2{\cdot}5/2H_2O$, respectively) were added to the reclaimed water to obtain the maximum concentrations specified in GB 20922-2007 (1.0 mg L^{-1}, 2.0 mg L^{-1}, 0.2 mg L^{-1}, and 0.01 mg L^{-1}, respectively).

The experimental field was 44 m × 8 m, and protection lines were established around it to reduce marginal effects. Each 1 m × 6 m plot was designed using a randomized block arrangement following shallow tillage. Ridges created in each plot were 30 cm high and 20 cm wide. A plastic film was buried at a depth of 60 cm to separate each plot from neighboring ones, preventing mixing due to surface irrigation. Taking water quality (groundwater and reclaimed water) and separate fertilization (N, P, or K) as variables, nine experimental treatments were conducted, with three replicates for each treatment. Groundwater irrigation with no fertilizer addition (GCK) was considered as the control treatment, and the eight treatments with reclaimed water irrigation were as follows: RCK (no fertilizer additions), RN1 (urea: $CO(NH_2)_2$), RN2 (sodium nitrate: $NaNO_3$), RN3 (ammonium bicarbonate: NH_4HCO_3), RP1 (superphosphate: $Ca(H_2PO_4)_2$), RP2 (calcium magnesium phosphate: $CaMgO_4P^+$), RK1 (potassium chloride: KCl), RK2 (potassium sulfate: $K_2(SO_4)$). The application rates of N, P, K fertilizer were converted from plant uptake amount considering the fertilizer utilization ratio, and their values were 1443 kg N ha^{-1}, 2936 kg P_2O_5 ha^{-1}, 572 kg K_2O ha^{-1}, respectively. P and K fertilizers were applied once, while N fertilizers were applied at three stages: 60% as basal fertilizer, then 20% at the first and third stages of fruit expansion, respectively. When there were 4–5 true leaves in the tomato seedling bed, we selected individual plants of the same height, and transplanted them into the field plots with 30 cm spacing between plants and between rows. Each field plot contained 40 tomato plants. Soil moisture probes were arranged longitudinally at a 10 cm interval in each plot, and the soil moisture content was maintained at 75% of the field capacity during the whole growing period by irrigation with 1800 L reclaimed water or groundwater. All other field managements of tomato were consistent with local farmers' practice.

2.2. Sample collection and DNA extraction

Whole tomato plants were harvested at the end of the growing period (last harvest), when fruits were fully ripe. Five plants per replicate plot were selected from positions along the diagonal and dug from the soil using a spade. Roots were shaken gently to collect bulk soil, and then brushed to remove the rhizosphere soil adhering to them. Soil samples were mixed evenly to obtain a composite sample. This was separated into two portions: one was air-dried in the shade and used for measurement of pH, total nitrogen (TN), and total phosphate (TP) as described by Guo et al. (2018); the second portion was lyophilized and ground to pass through a 2.0 mm mesh, and DNA was then extracted using the FastDNA SPIN Kit for Soil (MP Biomedical, Solon, OH, USA). The concentration of DNA (ng μL^{-1}) was determined using an ultra-micro spectrophotometer (NanoDrop ND-2000c; Thermo Scientific, Waltham, MA, USA). Extracted DNA was stored at −80 °C until ARGs and bacterial communities were analyzed.

2.3. High-throughput quantitative PCR

We employed 296 PCR primers including those targeting the 16S rRNA gene, 10 MGEs, and 285 ARGs to detect ARGs and MGEs in soil samples using the Wafergen SmartChip Real-time PCR system (Wafergen, Fremont, CA, USA) (Chen et al., 2017). Detailed descriptions of the reaction system and thermal cycling parameters for analyses of ARGs/MGEs by HT-qPCR have been provided elsewhere (Cui et al., 2018). A positive sample should have more than two technical replicates and a threshold cycle (C_t) less than 31. The formula used to calculate the relative abundance of ARGs and MGEs on the same chip was as follows: relative abundance $= 10^{(31-CtARG/MGE)/(10/3)}/10^{(31-Ct16S\ rRNA)/(10/3)}$. To minimize errors arising from differences in the amount of extracted DNA among samples, the normalized abundance of ARGs/MGEs was obtained by multiplying the relative abundance by 4.1 to give the number of copies per bacterial cell. The absolute abundance of ARGs/MGEs was calculated by multiplying the relative abundance by the abundance of 16 S rRNA determined by qPCR analysis. The fold-change (FC) of ARGs and MGEs abundance was calculated using the $2^{-\Delta\Delta Ct}$ method. These values indicated the increase or decrease of ARGs/MGEs in fertilization treatments compared with the non-fertilizer treatment (Chen et al., 2017).

2.4. 16S rRNA gene high-throughput sequencing

Using extracted DNA as the template, forward (515F) and reverse primers (907R) were used to amplify the V4–V5 hypervariable region of the 16S rRNA gene (Chen et al., 2018). To distinguish each sample, forward and reverse primers were tagged with a unique 10-nucleotide barcode (Rastogi et al., 2012). After quality filtering of raw single-end reads, operational taxonomic units (OTUs) were identified at the 97% similarity level (Edgar, 2010). Each sequence's taxonomic identity

(from phylum to species level) was classified with a 70% confidence threshold (Li et al., 2018).

2.5. Statistical analysis

Statistical analyses, including ANOVA and Spearman's rank correlation coefficient, were performed using SPSS 24. When determining the differences among samples, $P < 0.05$ indicated a significant difference. Using R version 3.4.4, the diversity of ARGs and bacterial communities was analyzed with vegan 2.5–3, and heatmap analysis was conducted with pheatmap 1.0.10. Shifts in ARG assemblages and bacterial community composition resulting from different fertilizer treatments were analyzed with Permutational Multivariate Analysis of Variance (Permanova) using the adonis function in vegan based upon Bray-Curtis dissimilarity. Mantel tests were conducted to determine associations between bacterial community and ARGs assemblages. Significant bacterial taxa associated with different fertilizers were identified by LEfSe (linear discriminant analysis effect size) and STAMP (statistical analysis of taxonomic and functional profiles). Partial least-squares path modeling (PLS-PM), network analysis, and variation partition analysis (VPA) were used to reveal the mechanisms underlying variations in ARGs.

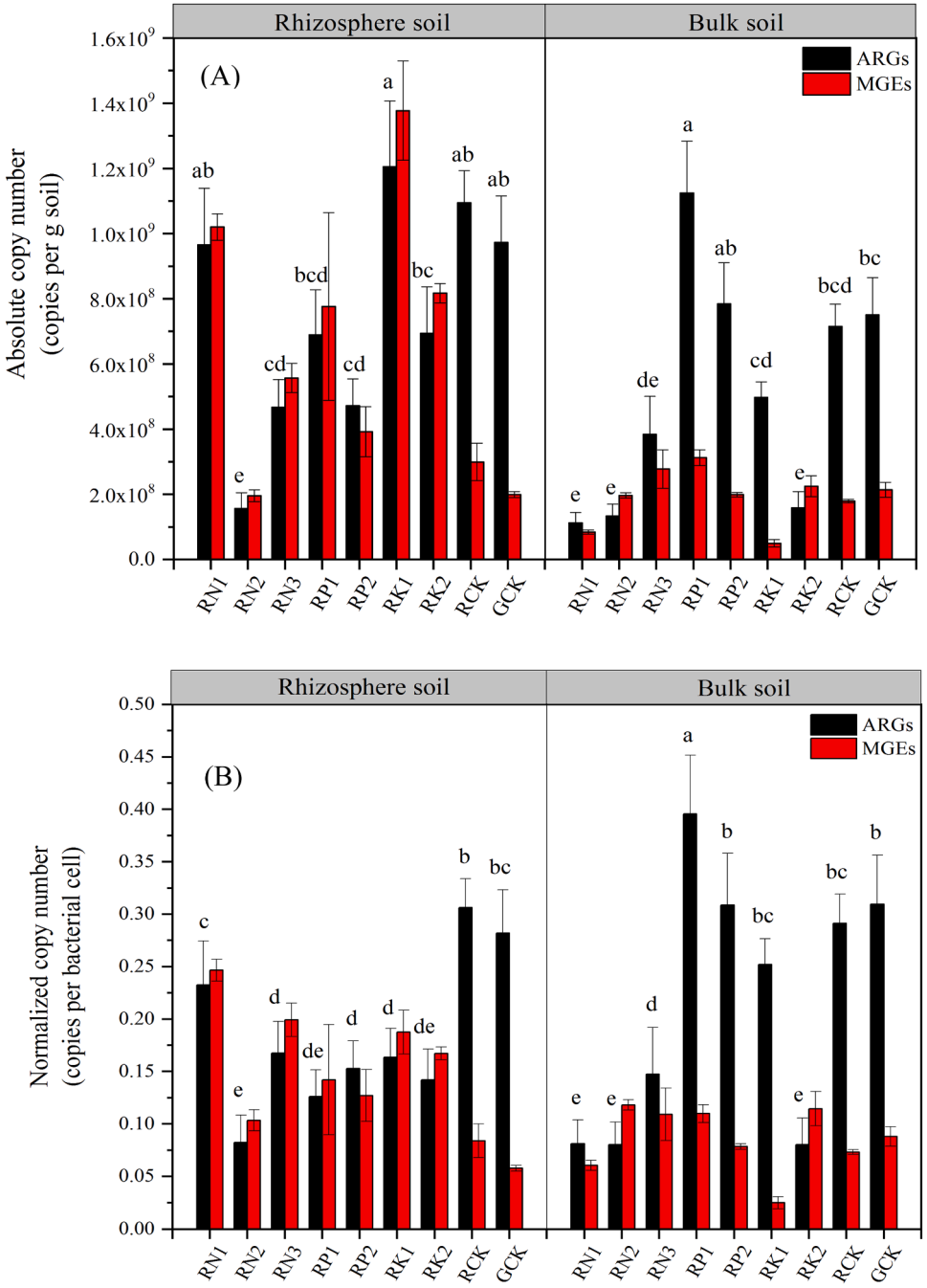

Fig. 1. Influence of individual N, P, or K fertilization on abundance of antibiotic resistance genes (ARGs) in tomato rhizosphere and bulk soil. (A) Absolute copy number of ARGs (number per g soil sample). (B) Normalized copy number of ARGs (number per bacterial cell). Different letters indicates difference of ARGs among different treatments.

3. Results

3.1. Effects of fertilizers on tomato yield and soil chemical properties

Tomato fruit yield was slightly higher ($P > 0.05$) and soil pH was significantly higher ($P < 0.05$) in RCK than in GCK fertilized soils. Compared with RCK, treatments with separate applications of N, P, and K fertilizers had no significant effect on fruit yields, but decreased soil pH and increased soil fertility. All fertilizer additions except NH_4HCO_3 resulted in higher TN in rhizosphere soil than that in bulk soil. All fertilizer treatments except $CO(NH_2)_2$ treatment resulted in higher TP in bulk soil than in rhizosphere soil (Table S2).

3.2. Effects of chemical fertilizers on ARGs patterns

A total of 159 ARGs were detected across all samples (range of 62–93 per sample). The total absolute ARGs abundance in the rhizosphere and bulk soil in RCK (1.1×10^9 and 7.2×10^8 copies g^{-1}, respectively) was similar to that in GCK (9.7×10^8 and 7.5×10^8 copies g^{-1}, respectively) (Fig. 1a). Similarly, the normalized ARG abundance in rhizosphere and bulk soil was 0.30 and 0.29 copies per bacterial cell, respectively, in RCK, and 0.28 and 0.31 copies per bacterial cell, respectively, in GCK (Fig. 1b). Under reclaimed water irrigation, individual fertilization with N, P, or K decreased the total absolute ARG abundance in rhizosphere soil, but only N and K fertilization decreased absolute ARG abundance in bulk soil. Moreover, except in P fertilization treatments, the total absolute ARG abundance was greater in rhizosphere soil than that in bulk soil (Fig. 1a). Changes in normalized ARG abundance in the different fertilizer treatments exhibited a similar trend to those of absolute ARG abundance (Fig. 1b). The different fertilization treatments were ranked as follows from largest to the smallest reduction of ARGs in rhizosphere soil: N (24.06–73.09% decrease) > P (50.13–58.80% decrease) > K (46.52–53.64% decrease). The rank order for bulk soil was as follows: N (49.43–72.44% decrease) > K (13.47–72.47% decrease) > P (increase of up to 35.84%). The lowest ARG abundance was observed under N fertilization, especially with $NaNO_3$. Additionally, the effect of $CaMgO_4P^+$ and $K_2(SO_4)$ on reducing ARG abundance was greater than other forms of P and K fertilizers.

The ARGs diversity in rhizosphere soil responded more to chemical fertilization than in bulk soil. ARG profiles in rhizosphere soil also differed significantly from that in bulk soil (Permanova, $R^2 = 0.15$, $P = 0.001$). Compared with non-fertilization treatments, individual N, P, K fertilization treatments increased the ARGs diversity in both rhizosphere and bulk soils ($P < 0.05$). Similarly, significant variations of ARG assemblages in individually N, P, K fertilized and unfertilized soils were found, except for the difference between P fertilized and unfertilized bulk soils (Table 1). Among the nine ARG classes, the most frequently detected were aminoglycoside, beta_lactamase, multidrug, MLSB, and tetracycline resistance genes (Fig. S1). In rhizosphere soil, individual applications of different forms of N, P, or K led to a significant reduction in the abundance of multidrug resistance genes, but significant increases in the abundance of other types of ARGs. The exception to this was a remarkable decrease of sulfonamide resistance genes following K fertilization. The effect of fertilization of different forms of N on ARG subtypes in bulk soil was similar to that in rhizosphere soil. However, fertilization with different forms of P only increased the abundance of sulfonamide resistance genes in bulk soil. For K fertilization, application of KCl increased only the abundance of vancomycin resistance genes ($P < 0.05$) in bulk soil, $K_2(SO_4)$ fertilizer had a similar effect on the change of ARGs in bulk soil as that in rhizosphere soil (Fig. S2).

The fold-change (FC) values indicate an increase or decrease in ARGs abundance in each fertilizer treatment compared with unfertilized soils under reclaimed water irrigation. The abundance of *fox5*, *mexF*, *oprJ*, and *tetL-02* genes in rhizosphere soil and *sul1*, *aacC4*, *emrD*, and *oprJ* genes in bulk soil decreased under most fertilizer treatments. In rhizosphere soils receiving different individual N fertilizer forms, $CO(NH_2)_2$ fertilization increased the abundance of *tetG-02* and *vanC-03* genes by 139- and 210-fold, respectively; $NaNO_3$ fertilization increased the abundance of *oleC* and *vanC-03* genes by 99-fold and 77-fold, respectively; NH_4HCO_3 fertilization increased the abundance of *bacA-01* and *oleC* genes by 96- and 134-fold, respectively. In bulk soil following $CO(NH_2)_2$, $NaNO_3$, and NH_4HCO_3 fertilization, the most enriched genes were *bacA-01* (72-fold), *aacC* (100-fold), and *oprD* (143-fold), respectively. Other genes also showed greatest enrichment following N fertilization, such as *floR* (14- to 59-fold), *erm(36)* (9- to 25-fold), and *mphA-02* (23- to 58-fold) in rhizosphere soil, and *oleC* (77- to 89-fold) and *sul2* (6- to 31-fold) in bulk soil. Following P fertilization as $Ca(H_2PO_4)_2$ or $CaMgO_4P^+$, *vanC-03/oprD* and *putative multidrug/oprD* showed the most significant increases in rhizosphere and bulk soil, respectively. In addition, *vanc-03* and *aacC* were enriched by 153-fold and 47-fold in rhizosphere and bulk soil, respectively, following KCl fertilizer application. The abundance of *oleC* was increased by 152-fold and 77-fold in rhizosphere and bulk soil, respectively, following $K_2(SO_4)$ fertilizer application. The maximum increase in MGEs was in bulk soil following KCl fertilizer application (e.g., 229-fold for *tnpA-05*) (Fig. S3).

3.3. Variations in bacterial community after chemical fertilization

Similar to ARG patterns in rhizosphere and bulk soils, the 16S rRNA-based diversity of bacterial communities was significantly greater in rhizosphere soil than that in bulk soil (Fig. S4). The structure of bacterial communities also differed between rhizosphere and bulk soil (Permanova, $R^2 = 0.07$, $P = 0.001$). In all soil samples, *Actinobacteria* was the dominant phylum (29.48–43.49%), followed by *Proteobacteria* (13.31–25.51%), and *Chloroflexi* (9.17–13.38%) (Fig. S4). But in general, individual N, P, or K fertilizer applications resulted in bacterial community structures that were different from those in unfertilized soils (Table 1). Differences in composition of soil bacterial assemblages between unfertilized and fertilized soils were identified by LEfSe analysis using an LDA score > 3.3. Twenty-two taxa (2 phyla, 7 classes, and 13 orders) in rhizosphere soil and 37 taxa (5 phyla, 13 classes, and 19 orders) in bulk soil differed between the fertilization treatments (Fig. 2). The strongest responses to fertilization, in terms of changes in the bacterial community, were following N fertilization. Applications of P and K individually had stronger effects on bacterial communities in bulk soil than rhizosphere soil. For example, individual N applications significantly increased the abundance of *Gitt_GS_136* and *Thermomicrobia* (phylum *Chloroflexi*) and *Alphaproteobacteria* (phylum *Proteobacteria*) in rhizosphere soil; and *Flavobacteria* and *Cytophagia* (phylum *Bacteroidetes*), *Caldilineae* and *Thermomicrobia* (phylum *Chloroflexi*), *Planctomycetacia* (phylum *Planctomycetes*), and *Betaproteobacteria* (phylum *Proteobacteria*) in bulk soil. Individual P applications significantly increased the abundance of the phylum *Cyanobacteria* in rhizosphere soil

Table 1
Permanova results of the similarity of ARGs and bacterial communities.

	Rhizosphere soil of ARGs		Bulk soil of ARGs		Rhizosphere soil of OTUs		Bulk soil of OTUs	
	R^2	P	R^2	P	R^2	P	R^2	P
N fertilized vs unfertilized	0.59	0.008	0.53	0.005	0.24	0.003	0.16	0.016
P fertilized vs unfertilized	0.78	0.011	0.27	0.075	0.22	0.017	0.16	0.160
K fertilized vs unfertilized	0.93	0.014	0.31	0.019	0.21	0.024	0.20	0.051

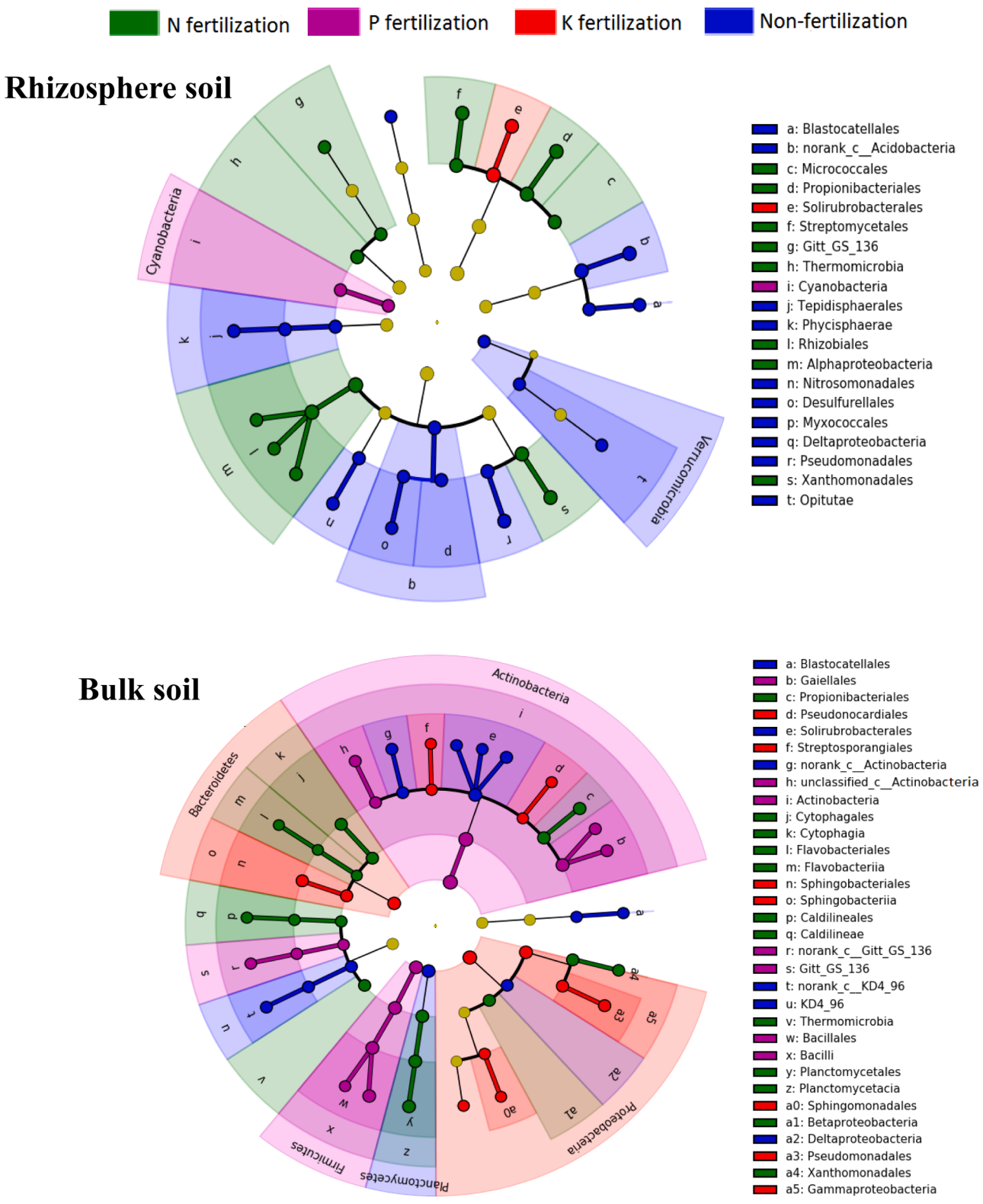

Fig. 2. Cladograms showing results of linear discriminant analysis effect size analysis of rhizosphere and bulk soil following individual N, P, or K fertilization.

and the phyla *Actinobacteria* and *Firmicutes* in bulk soil. Individual K applications markedly increased *Solirubrobacterales* (phylum *Actinobacteria*) abundance in rhizosphere soil and the abundance of the phyla *Bacteroidetes* and *Proteobacteria* in bulk soil.

3.4. Relationship between ARGs and bacterial community

Mantel tests identified associations between assemblages of bacteria and ARGs following applications of N (rhizosphere soil: $R = 0.91$, $P < 0.001$; bulk soil: $R = 0.21$, $P > 0.05$), P (rhizosphere soil: $R = 0.60$, $P < 0.01$; bulk soil: $R = 0.42$, $P < 0.05$), and K (rhizosphere soil:

$R = 0.61$, $P < 0.001$; bulk soil: $R = 0.30$, $P < 0.05$) fertilizers. Microbial taxa (family level, > 1% in any sample) potentially carrying ARGs were identified by network analysis based on a strong and significant Spearman's rank correlation ($R > 0.8$, $P < 0.05$). We identified 21, 5, and 8 bacterial families as potential ARG hosts following individual applications of N, P, K fertilizers, respectively. These bacterial families had the closest relationship with multidrug resistance genes, followed by beta-lactamase, aminoglycoside, and MLSB resistance genes (Table S4). Most of these potential ARG hosts (> 70%) belonged to the *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria*. Relatively simple correlations between ARGs and bacterial families were detected following individual P or K fertilizer applications when compared with N fertilizer applications. For P fertilization, associations between ARGs and bacterial families were more common in bulk soil than in rhizosphere soil: only the correlation between *mtrD-03* and *Sphingobacteriaceae* was found in rhizosphere soil. Following K fertilization, *Nocardiaceae*, *Pseudomonadaceae*, and *Rhizobiaceae* were identified as potential ARG-harboring taxa in rhizosphere and bulk soil, but they were associated with different ARGs (Fig. 3).

Following N fertilization, *Streptomycetaceae*, *Alicyclobacillaceae*, *Hyphomicrobiaceae*, *Microbacteriaceae*, *Phyllobacteriaceae*, and *Sphingomonadaceae* were most closely associated with ARGs in rhizosphere soil, while *Nocardiaceae* and *Nocardiopsaceae* were identified as most closely associated in bulk soil (Fig. 3). Individual N fertilization treatments slightly increased the proportion of all ARG hosts by 1.53%–4.49% and 0.24%–1.62% in rhizosphere and bulk soil, respectively. Still, there was an apparent decline of total ARGs due to a smaller percentage of putative ARG hosts (27.91%–32.40% in rhizosphere soil and 9.36%–10.99% in bulk soil) and a positive and negative correlation between putative host taxa and ARGs. For example, a decline in *Alicyclobacillaceae* abundance resulted in an increase of its apparently associated ARGs, while the decline of *Pseudomonadaceae* abundance led to a decrease of its related ARGs. To reveal how the total ARG assemblage changed following N fertilization, we found that the bacteria with a good relationship with total ARG abundance was inconsistent with the host bacteria of ARGs. *Gemmatimonadaceae*, *Nocardiaceae*, *Nocardiopsaceae*, *Nitrosomonadaceae*, *Sphingobacteriaceae* were positively correlated with total ARG abundance in rhizosphere soil, and *Caldilineaceae* and *Longimicrobiaceae* were negatively correlated. In bulk soil, *Nocardiaceae*, *Solirubrobacteraceae*, and *Elev-16S-1332* were positively correlated with total ARG abundance, and *Caldilineaceae and Bacillaceae* were negatively correlated (Fig. S5). In addition, the change in putative bacteria affected the abundance of some unique ARGs. Enrichment of *floR* and *mphA-02* genes was associated with an increased abundance of *Hyphomicrobiaceae*, *Phyllobacteriaceae*, *Microbacteriaceae*, and *Streptomycetaceae*. In contrast, enrichment of the *erm(36)* gene was associated with an increased abundance of *Nocardioidaceae* and *Streptomycetaceae* (Figs. 3 and S5).

3.5. Direct and indirect roles of various factors on ARG assemblages

The data from the fertilization treatments were subjected to PLS-PM analyses to determine how sample type, fertilizer application, soil properties (pH, TN, and TP), bacterial community (family level), and MGEs (normalized abundance) affected ARG patterns (normalized abundance) (Fig. 4). Results showed that the pattern of ARGs was negatively influenced by fertilizer application and sample type in all treatments. In individual N applications, the bacterial community had the most significant effect on variations in ARGs abundance and was a more critical controlling factor than soil properties and MGEs. The direct role of the bacterial community on ARG abundance was more important than its indirect role. In contrast, following individual P or K applications, soil properties explained the largest proportion of variations in ARG abundance, followed by bacterial communities and MGEs. Soil properties had a negative effect under P fertilization, but a positive effect under K fertilization. The direct effects of soil properties on ARGs abundance were 3.60- and 6.74-times their indirect effects following individual P or K applications, respectively. However, in the three kinds of fertilizers treatments, although the role of MGEs was less pronounced, remarkable positive correlations between MGEs and most ARG types were observed: the correlation coefficients were much higher in rhizosphere soil (R=0.73–0.99) than that in bulk soil ($R = 0.69$–0.97) (Table 2).

To understand the specific soil properties influencing ARGs abundance following individual P or K fertilization better, the data were subjected to variance partitioning analysis (VPA). Three variables (pH,

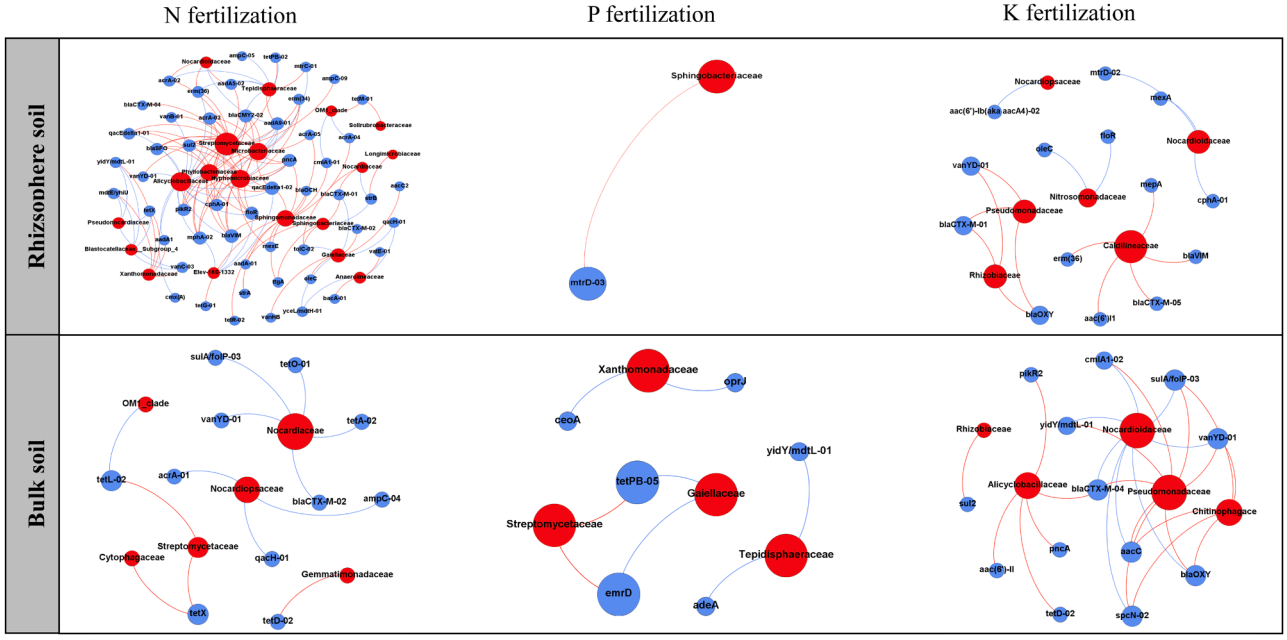

Fig. 3. Relationship between antibiotic resistance genes (ARGs) and microbes (family level) under individual N, P, or K fertilization. The Spearman's correlation coefficient adjusts to $R > 0.8$ and $P < 0.05$. The size of each node is proportional to the number of the connections (also call degree). The red lines mean positive correlation, and blue lines mean negative correlation.

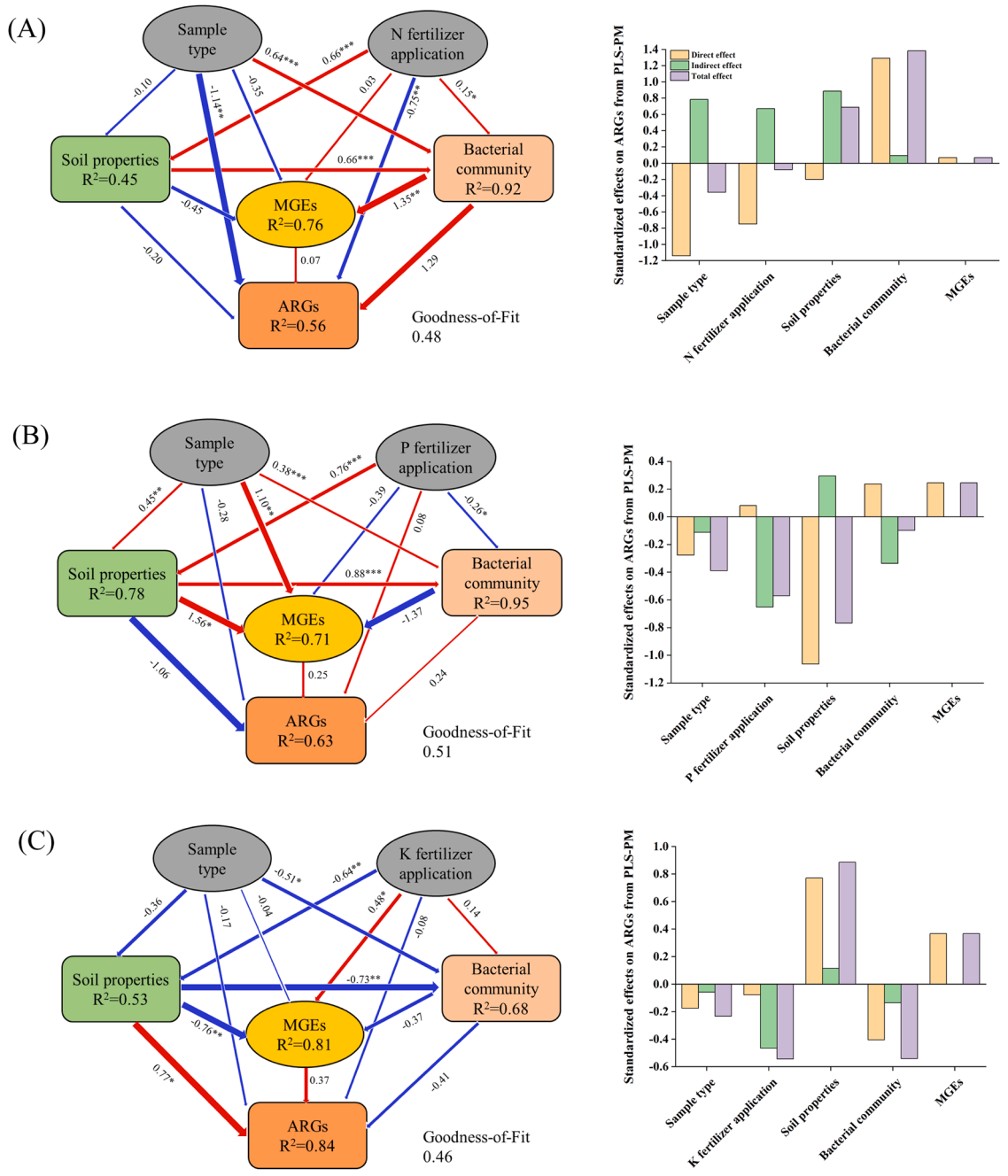

Fig. 4. Partial least-squares path model (PLS-PM) of the effects of soil properties, bacterial community, and mobile genetic elements (MGEs) on abundance of antibiotic resistance genes (ARGs) following individual applications of N fertilizer (A), P fertilizer (B) and K fertilizer (C). Larger path coefficients are shown as wider arrows, and red and blue colors indicate positive and negative effects, respectively. Path coefficients and coefficients of determination (R^2) were calculated after 999 bootstraps, and significance levels are indicated by * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

TN, and TP) explained a total of 63.23% and 50.02% of the variance in ARG abundance following P or K fertilization, respectively. Interestingly, under P fertilization, the interactive effect of pH and TN explained the largest proportion of variation (25.70%), followed by TN (21.55%). For K fertilization, the factors explaining the most variation in ARG abundance were pH (27.91%), TN (3.64%), TP (5.77%), and the interaction among them (3.46%) (Fig. 5). These results suggested that the abundance of ARGs was influenced by different mechanisms under separate N, P and K fertilization.

4. Discussion

4.1. Effects and mechanism of individual N fertilization on ARG variation

When assessing the sustainability of crop irrigation with reclaimed water, farmers are concerned more about crop yield than soil quality (Khanpae et al., 2020). Although this study showed that reclaimed water irrigation did not reduce crop yield, the behavior of emerging pollutants (e.g., ARGs) in soil must also be considered. Irrigation of farmland with reclaimed water harboring ARGs and antibiotics can add vast numbers of ARGs to soil and exert selection pressure on soil native bacteria, so that both indigenous and exogenous ARGs may be maintained in soil. Surprisingly, antibiotic-resistant elements entering soil via reclaimed

Table 2
Spearman rank correlations between normalized antibiotic resistance gene (ARG) abundance and normalized mobile genetic elements (MGEs) abundance. Bold value indicates the significant correlation.

	Rhizosphere soil			Bulk soil		
	N fertilization	P fertilization	K fertilization	N fertilization	P fertilization	K fertilization
Aminoglycoside	**0.90*** *	**0.92*** *	**0.80*** *	**0.69***	-0.27	**0.75***
Beta_Lactamase	0.50	0.00	**0.88*** *	0.18	0.55	-0.22
Chloramphenicol	**0.97*** *	**0.87*** *	**0.93*** *	0.29	**0.73***	0.55
MLSB	**0.91*** *	**0.88*** *	**0.95*** *	**0.82*** *	**0.73***	**0.90*** *
Multidrug	-0.36	-0.40	-0.35	0.19	**0.72***	-0.35
Others	**0.94*** *	**0.75***	**0.95*** *	-0.34	**0.90*** *	0.63
Sulfonamide	**0.99*** *	**0.98*** *	0.40	0.09	0.48	-0.48
Tetracycline	**0.84*** *	**0.73***	0.58	-0.08	**0.90*** *	0.57
Vancomycin	**0.93*** *	**0.93*** *	**0.93*** *	**0.97*** *	**0.72***	0.33
Total ARGs	-0.36	-0.30	-0.37	0.05	**0.85*** *	-0.37

Note: *$P < 0.05$, **$P < 0.01$.

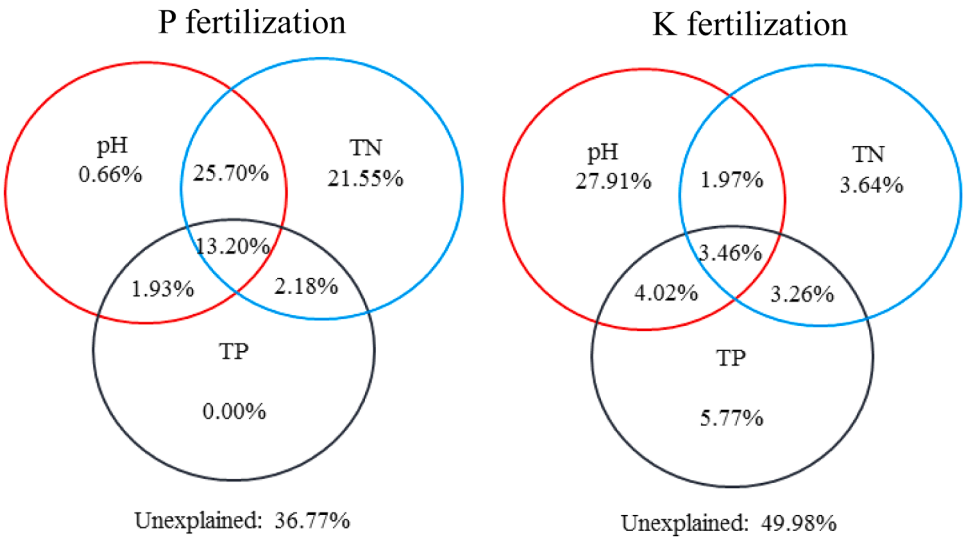

Fig. 5. Variation partitioning analysis (VPA) comparing the effects of soil pH, total nitrogen (TN), and total phosphorus (TP) on antibiotic resistance gene (ARG) profiles under individual P or K fertilization.

water rarely survive for long in soil (Negreanu et al., 2012). Therefore, results showed that irrigation with reclaimed water did not enrich 285 ARGs abundance compared with irrigation with groundwater, which was consistent with previous studies which revealed the no significant change of five or six resistant genes in reclaimed water irrigated soil (Negreanu et al., 2012; Marano et al., 2019; Shamsizadeh et al., 2021). However, the higher the abundance of ARGs in reclaimed water irrigated soil, the more potential risk of ARG migration to groundwater and humans. It was previously reported that chemical fertilization influenced ARG occurrence in soil (Chen et al., 2016; Xie et al., 2018; Sun et al., 2019). However, considering the three elements in NPK fertilizer, it is important to understand which component of chemical fertilizer exerts the greatest influence upon the persistence and spread of ARGs in soil. Results showed that individual N fertilization more strongly affected ARG patterns than individual P or K fertilization, consistent with the hypothesis. Fertilization with N alone significantly decreased total ARG abundance in fluvo-aquic soil irrigated with reclaimed water, and the reduction following $NaNO_3$ application was greater than that following $CO(NH_2)_2$ or NH_4HCO_3 application, indicating that more attention should be paid to N fertilization management in the application of combined fertilizers. In contrast, a previous study found individual $CO(NH_2)_2$ application did not affect ARGs abundance in fluvo-aquic soil with groundwater irrigation (Wang et al., 2020). Compared with the decreasing total ARG abundance in both rhizosphere and bulk soil with the addition of NH_4^+-N or NO_3^--N fertilizer into reclaimed water irrigated soil, the addition of them to biosolid amended soil led to an increase of *blaTEM-1*, *cmlA*, *str*, *sul1*, and *tetO* gene abundance in soil (Sun et al., 2020). These results indicated that chemical fertilizer management should be considered in combination with irrigation water resources or biosolid application. In addition, multidrug resistance genes were the main ARGs class in chemical fertilizer amended fluvo-aquic soil, which was also observed in red soil (Wang et al., 2018; Xie et al., 2018). This may be because chemical fertilization markedly increased the abundance of efflux pump genes (Xie et al., 2018), and the over-expression of efflux pump genes led to the emergence of multidrug-resistant bacteria (Nikaido and Pages, 2012). Moreover, total ARG abundance in soil receiving only N fertilization was higher in rhizosphere soil than in bulk soil. On one hand, the higher bacterial diversity in rhizosphere soil was most likely due to the presence of root exudates, which makes the rhizosphere a beneficial habitat for microorganisms and increases microbial growth rates compared with those in bulk soil (Wolters et al., 2018). On the other hand, the apparent relationships between MGEs or microbes and ARGs were more complex in rhizosphere soil than bulk soil, indicating that the rhizosphere is a "hot spot" for horizontal gene transfer (HGT) (Chen et al., 2018).

In PLS-PM analysis having a goodness-of-fit value greater than 0.35, sample type, fertilizer application, soil properties, bacterial community, and MGEs could all directly or indirectly affect ARGs abundance (Liao et al., 2019). The total effects of sample type and fertilizer application were smaller than the effects of soil properties, the bacterial community, and MGEs. The bacterial community explained the most significant proportion of variation in the soil resistome because bacteria harbor

ARGs. Similar findings have been reported when soil was amended with different manures (Han et al., 2018) and biochar (Chen et al., 2018). Despite distinct correlations between ARG subtypes and MGEs, the abundance of MGEs increased as the total ARG abundance decreased. Therefore, compared with the bacterial community and soil properties, MGEs had a smaller role in controlling ARG variations.

4.2. Bacterial hosts of ARGs following individual N fertilization

The strong association between bacterial assemblages and ARGs suggested that microbial community change was an important factor driving the behavior of ARGs in N-fertilized soil. When using LDA effect size to identify the taxa that differed among the individual N fertilization and non-fertilization treatments, many taxa responded to N fertilization treatments. This is likely to be because microorganisms do not need to mineralize and compete for nitrogen following N fertilizer applications (Pan et al., 2014). Chemical fertilizer application may either accelerate or limit the proliferation of soil indigenous microbes carrying ARGs (Xie et al., 2018; Sui et al., 2019; Sun et al., 2019). Phyla of *Acidobacteria*, *Actinobacteria, Bacteroidetes, Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Planctomycetes*, and *Proteobacteria* were reported as the bacterial hosts carrying ARGs (Duan et al., 2017; Han et al., 2018; Xie et al., 2018; Liao et al., 2019; Wang et al., 2020). Previous studies have suggested that the changes of these phyla following N fertilizer applications are explained not only by copiotroph-oligotroph trade-offs, but also soil texture and nutrient conditions. For example, fertilization with 160 kg N^{-1} ha^{-1} resulted in an increased representation of *Actinobacteria* and a reduced representation of *Firmicutes* in heavy-clay soil (Pan et al., 2014). However, another study found that applying 1250 kg N^{-1} ha^{-1} to different soil types led to increased abundance of *Actinobacteria* and *Firmicutes*, and decreased abundance of *Acidobacteria* (Ramirez et al., 2012). Consequently, due to the combined action of several factors, the change of these phyla was not apparent in this study, but the change of some families was significant.

Combining Spearman's rank correlation, STAMP analysis, and network analysis, a total of 21 bacterial taxa (family level) was detected with a close association with ARGs based on a non-random relationship (Li et al., 2015). Variation in the abundance of putative ARG host and non-host taxa was responsible for the decreased total abundance of ARGs in rhizosphere and bulk soil. The significant decrease in total ARGs in rhizosphere soil was associated with a significant decrease in the abundance of putative ARG host (e.g., *Sphingobacteriaceae*) and a remarkable decline of non-host bacteria (e.g., *Nitrosomonadaceae*). In bulk soil, the abundance of the putative ARGs host, *Nocardiaceae*, did not change. Therefore, the obvious lower ARG abundance was mainly due to a decrease in the abundance of non-host bacteria, including *Elev-16S-1332* and *Solirubrobacteraceae* ($P < 0.05$) (Fig. S5). Apart from the change in the composition of the bacterial community, altered bacterial diversity can also influence the occurrence of ARGs (Chen et al., 2018; Han et al., 2018; Liao et al., 2019). We observed that the application of N alone decreased soil microbial diversity, partly explaining the decrease of total ARG abundance.

4.3. Individual P or K fertilization had different underlying mechanisms in shaping ARGs profiles

The main mechanisms underlying the changes in ARG profiles under fertilization with either P or K alone were different from those following fertilization with N alone. Rather than the bacterial community, soil properties most strongly influenced ARG abundance under P or K fertilization. The role of the bacterial community was more important than MGEs in the control of ARGs, consistent with previous results (Chen et al., 2016, 2018). Additionally, the form of chemical fertilizer affected the associations between ARGs and microbes. A relatively simpler associations were observed following P or K fertilization than following N fertilization, which indirectly reflected a reduced role for the bacterial community in ARG variation under P or K fertilization.

Direct effects of soil properties were more prominent than indirect effects, thus soil properties play a considerable role in the variation of bacteria harboring ARGs. Moreover, the results indicated that the interaction of TN and pH greatly influenced the soil resistome under P fertilization. In contrast, pH was found to exert a greatest influence under K fertilization. The effect of pH on bacterial community composition was greater than that of other environmental factors (e.g., TN) (Xie et al., 2018). The decrease in pH may inhibit the survival of some bacteria. In addition, the change of pH affects antibiotic compound sorption and desorption process in soil (Tang et al., 2015), and the accumulation of antibiotics in bacterial cells (Zarfl et al., 2008), which ultimately alter the fate of ARGs. Although the apparent increase of TN plays a considerable role in the growth and reproduction of ARGs host bacteria (Guo et al., 2018), the non-host bacteria that account for a large proportion consume much more TN than the host bacteria with low proportion, resulting in the reduction of ARGs.

5. Conclusion

In this study, we evaluated the effects of reclaimed water irrigation combined with individual N, P, or K fertilization on the ARG profiles in rhizosphere and bulk soil. We observed a greater role of N fertilization than P or K fertilization. Compared with individual P or K fertilization, the composition of the bacterial community and its association with ARGs responded most obviously to individual N fertilization. Bacterial community change was the dominant factor controlling the decrease in total ARG abundance under N fertilization. In contrast, edaphic factors exerted the greatest influence following fertilization with either P or K. These findings shed light on the importance of critical element regulation in NPK fertilizer when controlling the transfer of ARGs in soil under reclaimed water irrigation. Future investigation should be designed to reveal how the management of N in combined NPK fertilizers influences ARG variation in soil and plants.

CRediT authorship contribution statement

Erping Cui: Conceptualization, Investigation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing. **Xiangyang Fan:** Funding acquisition, Resources, Project administration, Investigation. **Chao Hu:** Data curation, Investigation. **Andrew L. Neal:** Writing – review & editing. **Bingjian Cui:** Funding acquisition, Writing – review & editing. **Chuncheng Liu:** Formal analysis, Data curation. **Feng Gao:** Resources, Supervision, Project administration.

Declaration of Competing Interest

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113185.

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