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The publisher's version can be accessed at:

- https://doi.org/10.1016/j.jhazmat.2021.128046

The output can be accessed at: https://repository.rothamsted.ac.uk/item/98751/cropping-system-exerts-stronger-influence-on-antibiotic-resistance-gene-assemblages-in-greenhouse-soils-than-reclaimed-wastewater-irrigation.

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Cropping system exerts stronger ~~impact~~ influence on antibiotic resistance gene assemblages in greenhouse soils than reclaimed wastewater irrigation

Yuan Liu[a], Andrew L. Neal[b], Xiaoxian Zhang[c], Haiyan Fan[d], Honglu Liu[d], Zhongyang Li[a,*]

[a] *Farmland Irrigation Research Institute, Chinese Academy of Agricultural Sciences, Xinxiang 453002, China.*

[b] *Department of Sustainable Agriculture Sciences, Rothamsted Research, North Wyke, Devon EX22 2SB, UK.*

[c] *Department of Sustainable Agriculture Sciences, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK.*

[d] *Beijing Engineering Research Center for Non-conventional Water Resources Utilization and Water Saving, Beijing Water Science and Technology Institute, Beijing 100048, China*

*Corresponding author. E-mail address: lizhongyang1980@163.com.

Abstract

The effects of reclaimed wastewater (RW) irrigation on the spread of antibiotic resistance genes (ARGs) in soil is modulated by a myriad of biotic and abiotic factors and their relative significance remains vague. ~~Herein, w~~We compared ~~the~~ microbial communities, assemblages of genes associated with microbial ~~resistant~~ resistance to antibiotics, biocides and metals, and insertion sequences (ISs) in soils following 16~~-~~ years of crop irrigation with groundwater (GW), RW or ~~alternate~~ alternately with GW and RW in two greenhouses with different cropping systems, using shotgun metagenome sequencing. The results showed that ~~it was the~~ cropping system exerted greater influence ~~rather~~ than ~~the RW~~ irrigation on ~~that impacted~~ the profile of ISs and resistance genes ~~more significantly, . and the impact~~This influence was most strongly associated with concentrations of copper, mercury and perfloxacin in the soils. There was no significant difference in ~~the~~ soil ARG~~s~~ profiles between continuous RW irrigation and ~~alternate~~ alternating GW and RW irrigation~~, . and the bacteria of~~ Proteobacteria, Actinobacteria and Firmicutes and ~~some~~ a limited number of ISs were closely associated with the detected ARGs. Most ARGs were found to co-occur with metal~~s~~ and biocide~~s~~ resistance genes through the mechanism of efflux pump~~s~~. These findings highlight the significance of understanding and improving crop management in mitigating the dissemination of ARGs in soils irrigated with RW.

Key words: Reclaimed wastewater; cropping system; metagenomic analyses; ARGs; irrigation.

1. Introduction

Agricultural production consumes approximately 50-80% of freshwater globally (Boretti and Rosa 2019, Palese et al. 2009). Over the past few decades, dwindling water resources ~~combined with increased demand for water due to population growth~~ has~~ve~~ made many countries in arid and semi-arid regions ~~look for~~consider treated wastewater as a supplementary water resource for irrigation (Elgallal et al. 2016, Fatta-Kassinos et al. 2020, Pedrero et al. 2010, Pereira et al. 2002). ~~Although available wastewater treatment technologies are able to meet stringent standards (Levine and Asano 2004), they are not currently practical because of the high costs. As a result~~However, most wastewater treatment plants ~~using conventional treatment measures will continue to~~ discharge ~~the~~ effluent~~s~~ ~~with~~ contaminant~~s~~ with ~~including~~ heavy metals, antibiotics, antibiotic resistance genes (ARGs) and microbes harboring ARGs, into water bodies (Cacace et al. 2019, Ding et al. 2020, Teijon et al. 2010). Irrigation with ~~such~~ these waters could release ~~these~~ contaminants to soil-plant system~~s~~ ~~with~~ increasing the potential for them to end up in the food chain (Al-Jassim et al. 2015). The selective pressure of antibiotics on soil microorganisms following reclaimed wastewater (RW) irrigation could disseminate ARGs and compromise the efficacy of antibiotics in animal and human medicine (Pruden et al. 2006), ~~which~~ and this has become a public ~~health~~ concern (Sorinolu et al. 2021). ARGs have several ~~pathways~~ mechanisms by which they ~~to~~ spread in soil, one of which is horizontal transfer through mobile genetic elements (MGEs) (Gatica and Cytryn 2013). Co-selection of ARGs ~~together~~ with metal resistance genes (MRGs) is promoted since the genes often ~~occur on~~share the same MGEs (Baker-Austin et al. 2006).

Reclaimed wastewater contains antibiotics and ARGs, and continuous RW irrigation ~~with RW~~

could cause their accumulation in soils (Kampouris et al. 2021b). RW-borne bacteria and associated ARGs can persist ~~at~~ below detection level~~s~~ in irrigated soils and have potential to ~~re-grow~~increase in abundance under copiotroph conditions (Marano et al. 2021). Since RW irrigation ~~could~~ may change bio-physicochemical conditions of soil and root-induced ~~biotic and abiotic~~ processes~~,~~ ~~which could alter~~altering antibiotic degradation and microbial community composition, the long-term effects of RW irrigation on dissemination of ARGs in soil is complicated and its ~~dominant~~ principal determinant remains obscure. ~~For example, one study found that~~ RW irrigation for 3-4 years has been shown to increase~~d~~ the abundance of ARGs in urban park soil, ~~and~~ significantly ~~enriched~~ increasing the diversity and abundance of ARGs and ~~altered~~ altering soil bacterial communities ~~carrying these ARGs after irrigating the urban parks using RW for 3-4 years~~ due to ~~the~~ an increase in pH and ~~the~~ decrease in total N (Han et al. 2016). This was corroborated by a similar study in China that irrigating urban parks using RW for 1-10 years led to an ARGs enrichment in soil due to the increase in antibiotics and MGEs in soil (Wang et al. 2014b). ~~In addition to these, it is reported that~~The ~~ARGs~~ ARG burden ~~load~~ of RW ~~were wa~~is ~~the~~ an important driver~~s to impacting~~ influencing ARGs in ~~the~~ soil following RW irrigation (Kampouris et al. 2021a). However, these studies overlooked ~~the~~ difference~~s~~ in soil properties before irrigation, microclimate~~s,~~ and plant cover~~s~~, among ~~others~~factors, therefore it is difficult to ~~identify~~ determine ~~that~~ whether the effect of RW irrigation on ARGs was caused by the irrigation itself or other factors (Christou et al. 2017, McLain and Williams 2014). ~~In contrast, t~~There ~~were~~ are also reports that RW irrigation ~~did not~~has no influence ~~have promoting impacts~~on ~~on~~ the dissemination of ARGs (Cui et al. 2018, Marano et al. 2019, McLain and Williams 2014, Negreanu et al. 2012). For example, a comparative study ~~on~~ of ARG~~s~~ patterns in *Enterococcus* found in pond~~s~~ sediment~~ss~~ ~~associated *Enterococcus* isolated from~~

water storage basins in central Arizona revealed that the levels of bacterial antibiotic resistance following long-term RW recharge were equal to that with GW, and that bacterial multiple-antibiotic-resistance determined by culture-based isolate methods in the sediments from GW-filled ponds was significantly higher than RW-filled ponds (McLain and Williams 2014). TIn a separate study, his was consistent with the experimental study of Negreanu et al. (2012) that the levelsabundance of selected four ARGs (*sul1*, *sul2*, *ermB*, and *ermF*) in soils irrigated with RW for 6-15 years were either the sameunchanged as or even lower than that in soils irrigated with freshwater (Negreanu et al. 2012). Such conflicting results about the impactregarding the influence of RW irrigation on ARGs dissemination is a of public concern, —: while our mechanistic understanding of the underlying mechanisms is hampered due to the a lack of experiments which are long enoughof sufficient duration to see off the transition instudy the change in both ARGs and other biogeochemical properties of soil following RW irrigation, especially under field conditions with continual agricultural practices not modified.

The effects of RW irrigation on ARGs dissemination in soil depends on many abiotic and abiotic factors. Physicochemically, both Physically, tthe quality of RW and irrigation methods controls the input of ARGs to the soil (Fahrenfeld et al. 2013), and the bio-physicochemical soil properties of the soil (Ma et al. 2018), including pH, nitrogen cycle (Han et al. 2016), organic matter (Chen et al. 2015), electrical conductivity (Tan et al. 2019), heavy metals and soil aggregation. control the introduction of ARGs to soil. MicrobiallyBiologically, changes in soil biogeochemical properties could may reshape microbial composition assemblages (Cui et al. 2018).

Physiologically, the charged roots could may adsorb interact with the chargedpolar and

~~ionizable~~ antibiotics via ~~the~~ iron plaques or ~~the~~ chemical functional groups ~~interaction with carboxyl, amido and hydroxyl on~~at the root surface (Choi et al. 2016, Liu et al. 2018, Tai et al. 2018). ~~As~~ Since morphological and electrical properties of roots as well as the rhizosphere vary with crop species and varieties (Lu et al. 2018)~~, and each crop has its unique rhizosphere and associated microbial communities~~ (Babin et al. 2019), it is envisaged that crops may also impose ~~different~~ selective pressure~~s~~ on soil antibiotic-resistant ~~microbiome~~microbes. However, little is known about how significant the influence of cropping systems could be. For example, the ~~experiment~~ studies of Han et al. (2016) and Wang et al. (2014b) did not separate RW irrigation and plants, ~~and it is hence difficult to~~preventing their ability to distinguish between RW irrigation or plant heterogeneity as having the greater influence on ~~that the~~ ARG assemblages in soil~~s~~ ~~difference in ARGs between treatments was due to RW irrigation or plant heterogeneity~~. Other studies, for example Negreanu et al. (2012), have studied ARG assemblages in soils used to cultivate cotton and wheat, crops whose deep roots typically do not require as much water as vegetables. ~~In contrast, the experimental study on the managed orchard had limited tillage, and while cotton and wheat with deep roots normally have deep rooting and do not require as much water as water vegetables need (Negreanu et al. 2012). Therefore, i~~It remains ~~is~~ unclear ~~that~~ whether ~~the insignificant differences~~similar level~~s~~ ~~in~~ of ARGs between treatments was ~~due to the~~a result of cropping or other factors ~~which suppressed ARGs proliferation~~. ~~Cropping alters nutrient cycle and reshapes microbial community composition due to fertilization, nitrogen fixation (Sainju et al. 2003), irrigation, tillage and crop rotations. How these combine to affect ARGs dissemination in soil is largely unknown.~~

Large-scale wastewater treatment plants are usually ~~in~~ associated with metropolitan ~~regions~~

areas and ~~the~~ vegetable ~~bases~~ production in the suburbs can ~~easily~~ readily access ~~use~~ RW for irrigation. Unlike staple crops, vegetables ~~are water-demanding and need~~ require intensive fertilization and irrigation; their roots are ~~much~~ shallow and ~~the~~ root-induced biotic and abiotic processes are most active in the very top soil. We ~~hence~~ hypothesized that cropping ~~might~~ exerts an important ~~impact~~ influence on microbial and biogeochemical properties of soil (Bengough 2012), and ~~thereby~~consequently the proliferation~~/~~ or attenuation of ARGs. Since the changes in physical and biogeochemical properties of soil resulting from different irrigation water sources and management are likely to be manifest over an extended period of time(Wang et al. 2022), ~~which mediate microbial activity and ARGs profile, after management practice change is a slow process and takes decades to reach new equilibria (Wang et al. 2022), and testing this hypothesis needs long-term experiment. We~~ we ~~hence~~ selected two greenhouses grown with various vegetables and having received different RW irrigation treatments for 16 years, ~~with~~ using ~~the~~ groundwater (GW) irrigation as a comparator (control) ~~with groundwater (GW) taken as the control~~. ~~We used the shotgun metagenomic analyses to simultaneously analyse the massive amount of genes in each soil sample.~~ We aimed to test: 1) how cropping and long-term RW irrigation affect ARG~~s~~ profiles in ~~the~~ soil, and 2) ~~the~~ associations between soil ARGs and the potential propagators.

2. Materials and methods

2.1 Field experiment and soil sampling

The experiment was conducted in two greenhouses at the Yongledian Experimental Station for Water-Saving Irrigation Research, managed by Beijing Water Science and Technology Institute (39° 20′ N, 114° 20′ E; 12 m above sea level). The greenhouses intercept rainwater and

use hot water pipes to maintain a minimum temperature approximately at 20 ºC between November and February. The mean annual temperature and precipitation were 11.0 -12.0 ºC and 565 mm respectively, with > 70% of the precipitation falling between June and August. The topsoil (0-20 cm) is silty loam (<0.002 mm, 7.0%; 0.002-0.05 mm, 54.7%; 0.05-2 mm, 38.3%), and its properties were: bulk density 1.4 g cm^{-3}, pH 8.4, electrical conductivity (EC) 36.0 mS cm^{-1}, organic matter (OM) 24 g kg^{-1}, total-N 1.13 g kg^{-1}, total-P 1.24 g kg^{-1}, total-K 20.7 g kg^{-1}, available-N 162.9 mg kg^{-1}, available-K 319.2 mg kg^{-1}, available-P 134.7 mg kg^{-1}.

The experiment was established in December 2002, and all crops were drip-irrigated. T~~he~~ ~~t~~hree irrigation treatments we<u>re</u> compared ~~are~~: groundwater irrigation, alternate groundwater - reclaimed water irrigation, and reclaimed water irrigation. Each treatment has three replicates arranged ~~in~~ <u>across</u> two greenhouses (referred to as Greenhouse A and Greenhouse B respectively). Consistent agronomic management (application of chemical fertilizer and chicken manure, weed control, irrigation time and volume per hectare) was adopted for all treatments except irrigation water quality in each greenhouse. The plot arrangement (Fig. S1) and cultivation histories (Table S1) in the two greenhouses are described in the supplementary information. At the time of soil sampling (December 5, 2018), the crop in Greenhouse A was long beans (*Vigna unguiculata* L.) arranged in nine plots, with the area of ~~the 1th - 8th~~ plots <u>1 to 8</u> and ~~the ninth~~ plot <u>9</u> being 30 m^2 and 20.4 m^2 respectively; the crop in Greenhouse B was purple cabbages (*Brassica oleracea* var. *capitata rubra*) arranged in nine plots, each having an area of 34 m^2. Crop systems in the two greenhouses have been kept different for 16 years, and the experiments were not designed to compare individual plants but the legacy of cropping history. Adjacent plots in each greenhouse were spaced 30 cm <u>apart</u> to avoid possible lateral water flow, and GW used ~~in the~~<u>for</u> irrigation

was pumped from a borehole 8.0 m below the ground surface. RW was the secondary effluent water taken from the Gaobeidian Wastewater Treatment Plant, Beijing, and water properties are listed in Tables S2 and S3.

Soils were sampled ~~randomly~~ from the top layer (0 - 20 cm) at three randomly placed locations between the drip pipes in each plot; ~~they~~ these were ~~then~~ pooled, ~~with~~ and ~~soil~~ sub-~~sampled~~ samples ~~from which~~designated for nucleic acid extraction ~~being~~ were immediately stored at -80 ºC. ~~and t~~The remaining sample was ~~being~~ air-dried for chemical analysis. Soil pH, EC, OM, total N, NO_3^--N, NH_4^+-N, available-P, available-K, total heavy metals were analyzed using the methods detailed in our previous studies (Liu et al. 2019b). Soil available Hg, Cr, Cu, Zn, Pb and Cd were extracted by DTPA-TEA solution (5 mmol L^{-1} DTPA with 10 mmol L^{-1} $CaCl_2$ and 100 mmol L^{-1} triethanolamine); soil available As was extracted by 0.5 mol L^{-1} NaH_2PO4 (Guo et al. 2018), and measured by ICP-OES iCAP7400 (ThermoFisher, USA).

2.2 Antibiotic compounds analysis

Thirty-three antibiotic compounds including 14 quinolones, 15 sulfonamides and 4 tetracyclines were selected for content determination (Table S4). We selected the test antibiotic classes ~~due to~~because of their common usage in healthcare and livestock husbandry and their close association with ARG~~s~~ ~~spread~~ dissemination (Leng et al. 2020, Wang et al. 2014a, Wang et al. 2014b, Yan et al. 2018). Details of the specific antibiotics ~~determination~~ of interest are provided in the supplementary information.

2.3 DNA extraction and library construction

The NucleoSpin Soil Kit (Macherey-Nagel, Germany) was used to extract total DNA from ~~the~~ soil~~s~~ (0.3 g) following the manufacturer's instructions. We did not extract DNA from the water, and

the explanations ~~were~~ are detailed in the Supplementary Information. The concentration of extracted DNA was determined ~~with~~ using a Qubit Fluorometer ~~using~~ and dsDNA BR Assay kit (Invitrogen, USA)~~.~~, ~~and~~ Electrophoresis in a 1% agarose gel ~~electrophoresis~~ was used to check ~~the~~ DNA quality. Genomic DNA (1 μg) was randomly fragmented ~~by~~ using Covaris Focused-ultrasonicators (ME220, ~~America~~Covaris, Woburn, MA). The fragmented DNA was selected by Magnetic beads to an average size of 200-400 bp. The selected fragments were through end-repair, 3' adenylated, adapters-ligation, PCR amplifying and the products were purified by the Magnetic beads. The double stranded PCR products were heat-denatured and circularized by the splint oligo sequence. ~~The s~~Single strand circle DNA (ssCir DNA) ~~were~~ was formatted as the final library and qualified by Quality control (QC). The qualified libraries were sequenced on BGISEQ-500 platform (BGI, China). QC of the raw reads was conducted using the SOAPnuke (v1.5.6) software (Kravchenko and Guber 2017) with the following parameters: -l 20 -q 0.2 -n 0.05 -Q 2 -d -c 0 -5 0 -7 1. Over 300 million reads were generated for each sample after QC (Table S5). ~~The qualified libraries were sequenced on BGISEQ-500 platform (BGI, China).~~

2.4 Assembly, gene catalogue construction and annotation

Assembly of the clean reads was conducted for each sample respectively using ~~the~~ megahit ~~(~~v1.1.3~~)~~ ~~software~~ (Li et al. 2015) with the following parameters: --min-count 2 --k-min 33 --k-max 83 --k-step 10. A total of 13,911,093 contigs were assembled, ~~with the~~ N50 for ~~each of~~ the samples ranging from 398,525 to 1,021,693.

Open reading frames (ORFs) were predicted from contigs for each sample using MetaGeneMark (v2.10) software (Zhu et al. 2010), with ~~the~~ a minimum ORF length of 101 bases via the parameter -l 100. To construct the unique gene catalogue for the samples, all predicted genes

from each of the 18 samples were grouped. Redundant genes were identified and removed using CD-Hit version 4.6.6 (Li and Godzik 2006) ~~with~~ using the parameters ~~set as follows:~~ -c 0.95 -aS 0.9 -M 0 -d 0 -g 1. A total of 10,683,999 unique genes were included in the gene catalogue.

The protein sequences of the unique genes in the gene catalogue were annotated against NCBI_nr (only bacterial, fungal and virus sequences were selected and included in this alignment) [release 2018-08-14] (Pruitt et al. 2006), BacMet databases (Pal et al. 2013) using DIAMOND (v0.8.23.85) software (Buchfink et al. 2015) with the cutoff value of *E*-value of 1×10^{-5} to infer the function of predicted genes. Simultaneously, insertion sequences (ISs), one important component of MGEs, were annotated against ISfinder (Siguier et al. 2006) using BLAST (Altschul et al. 1990, Altschul et al. 1997), and ARGs were annotated against CARD (Jia et al. 2016) using the Resistance Gene Identifier (RGI). The numbers of the annotated genes against each database were listed in Table S6.

Taxonomic association of the genes was based on the annotation of the protein sequences against the NCBI_nr database (as described above) [release 2018-08-14] with the cutoff values of identity greater than 30%, coverage greater than 50% and $E < 1\times10^{-5}$.

2.5 Statistical Analysis

Abundances of individual genes were determined by aligning high-quality reads to the total clean reads in each sample. Bioinformatic analysis ~~described~~ generated organism and gene (associated with antibiotic, heavy metal and xenobiotic resistance mechanisms and insertion sequence) abundance tables. In each case, we tested our hypothesis that the source of irrigation water influenced organism and gene distribution using a two-factor permutational multivariate analysis of variance (PERMANOVA) after having confirmed an absence of significant

heterogeneity of multivariate dispersion using the PERMDISP test. Probabilities associated with permutational test were based upon 99,999 permutations. Where PERMANOVA identified a significant effect of an experimental factor, we used linear discriminant analysis effect size (LEfSe) (Segata et al. 2011) to identify biomarkers (organisms or genes) associated with significant differences in abundance between treatments. We employed LEfSe cut-offs of p_{adj} = 0.05 and $\log_{10}$ linear discriminant scores ranging between 1.0 and 1.5, depending upon gene group. We ~~routinely~~ generated organism or gene profiles to identify taxa or genes that remain unchanged in their composition independent of treatment based on sample prevalence and relative abundance, as well as bi-hierarchical clustering and heatmap representation of the abundance of features according to treatment. In this latter case, organism or gene abundance data were centered log-ratio (CLR) transformed, generating the log of the ratio between each observed abundance and the geometric mean abundance across all treatments. ~~Euclidean~~ Minkowski distance and Ward's agglomerative clustering algorithm were used for clustering. To identify the most diagnostic ~~features~~ genes and insertion sequences characterizing communities of each soil, we used supervised Random Forests (RF), a classification algorithm approach based upon a collection of unpruned decision trees (Cutler et al. 2007), each built using a bootstrap sample of training data using a randomly selected subset of ~~OTUs~~genes and insertion sequences. The RF classifier was built by growing 5,000 classification trees. Only ~~significant~~ biomarker genes and insertion sequences associated with significantly different abundance between treatments as determined by LEfSe were used as potential determinants in RF. The prediction performance and confusion matrices were determined using out-of-bag cross-validation. ~~Percent~~ The mean decrease in accuracy of the importance matrix was used to select taxa that were most predictive of each

microbiome assemblage. RF was employed as implemented in MicrobiomeAnalyst (Dhariwal et al. 2017).

To model the contribution of edaphic factors to the observed distributions of those resistance genes and insertion sequences for which PERMANOVA and LEfSe identified significant treatment effects, we employed distance-based redundancy analysis (dbRDA, (Anderson and Legendre 1999) using Hellinger distance metrics. In this approach, multivariate multiple regression of principal coordinate axes on predictor variables is used to identify linear combinations of predictor variables which explain the greatest variation in the multivariate dataset. Edaphic factors, listed in sections 2.1 and 2.2, were employed as potential predictor variables and were selected according to which were best in explaining the variation in treatments. The small sample corrected Akaike Information Criterion (AICc) was used to identify the best combination of variables to describe the observed distribution of treatments. These steps were performed in PRIMER PERMANOVA+ version 7.0.20 and were based upon 99,999 permutations.

3. Results

3.1 Microbial Community Assemblages

The dominant phyla in ~~the~~ all soils were Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Gemmatimonadetes, Thaumarchaeota, Bacteroidetes, Cyanobacteria, *Candidatus* Rokubacteria, Planctomycetes and Unclassified phyla (Fig. S2A). PERMANOVA indicated a significant influence of cropping system upon soil bacterial assemblages (*pseudo-F* = 11.5, $p = 3\times10^{-5}$), but no significant influence of the different irrigation water types (*pseudo-F* = 1.1, $p = 0.333$). Heatmap-based hierarchical clustering supported this observation (Fig. 1A). The

prokaryotic populations in all soils were dominated by *Nitrososphaera*, *Sphingomonas*, *Nitrospira*, and closely related to Gemmatimonadetes *Gemmatirosa* and *Gemmatimonas* (Fig. S2B). In total, twenty-two organisms were found to be significantly more associated with Greenhouse A soil within the LEfSe parameters used (Fig. 1B). Eighteen organisms were identified as significantly more associated with Greenhouse B soil.

3.2 Environmental variables

Soil properties were shown in Table 1~~,~~. ~~and t~~The overall pattern presented by PCA (Fig. 2) could not separate the soils based on water quality or cropping system, suggesting that ~~none of them~~neither factor influenced soil pH, EC, OM, NH_4^+-N, available-P and available-K ~~significantly~~ appreciably (Table 1). RW irrigation did increase soil NO_3^--N significantly compared to GW irrigation, regardless of cropping system. Total-N in soil showed the same trend as NO_3^--N in Greenhouse B soil, while the opposite was true for soil in Greenhouse A.

There was no significant difference in total heavy metal concentrations between irrigation water source, except for total cadmium in Greenhouse A soil which was significantly reduced following the RW irrigation and the alternate irrigation (Table 2). Soil available heavy metals were reduced following RW irrigation with a few exceptions in Greenhouse A but not in Greenhouse B.

3.3 ARGs

Antibiotic concentrations in soil are shown in Fig. 3A and Table S7. Sulfamethoxypyridazine, sulfametoxydiazine, sulfamonomethoxine, sulfathiazole, sulfacetamide sodium, difloxacin, sarafloxacin, lomefloxacin, flumequine, and the four tetracycline antibiotics were almost all below detectable levels. The concentration of each antibiotic in GW-irrigated soils

was not more than 10 ng g^{-1} in this study, ~~which was at~~a similar level ~~compared~~ to ~~with~~ other studies (Chen et al. 2011, Cui et al. 2018, Liu et al. 2019b, Ma et al. 2018). Neither continuous nor alternate irrigation with RW influenced the total concentration of antibiotics in either greenhouse. The total concentration of quinolones was higher than that of sulfonamides. For sulfonamides, the two RW irrigation treatments did not alter their concentrations significantly compared to GW irrespective of the cropping system. For quinolones, their concentration in GW-irrigated soils was significantly higher than that in soils associated with RW in Greenhouse A, but lower in Greenhouse B.

Thirteen ARGs were detected in all soils (Fig. S3A), of which the *oqxB* gene was particularly widespread (Fig. S3B). A comparison of the combined relative abundance of all ARGs (Box-Cox transformed to stabilize the variance: lambda = -0.795, log likelihood = 222.9) indicated that there was no significant influence of irrigation water sources upon the relative abundance of ARGs in the metagenomes (ANOVA, $F = 0.6$, $p = 0.582$): however, there was a significant influence of cropping system (ANOVA, $F = 17.4$, $p = 0.0013$) with greater relative abundance associated with Greenhouse A (1.73×10^{-5}) than Greenhouse B (1.04×10^{-5}).

As with the distribution of organisms between ~~the~~ soils, there was a significant effect of cropping system on ARG assemblages (PERMANOVA, *pseudo-F* = 10.6, $p = 9\times10^{-5}$) but no effect of the irrigation water sources (PERMANOVA, *pseudo-F* = 1.7, $p = 0.145$). ARG biomarkers for each soil were identified with LEfSe (Fig. 3B). The genes *mtrA* and *murA* were identified as more associated with Greenhouse A soil, while the Greenhouse B soil was more associated with the genes *ermA* and *ermY*.

3.4 Metal Resistance Genes

Alignment against the BacMet database showed that a total of 445 types of MRGs were detected in the soils. Several genes were present in all soils (Fig. 4A), the most abundant of which was the *wtpC* gene ~~which was~~ involved in molybdate/tungstate import, as ~~does~~ is a second gene *tupC*. The genes *nikA*, *nikB*, *nikC* and *nikE* are associated with a nickel importing ATP-binding cassette (ABC). The genes *zraR* and *zraS* are associated with a membrane-associated protein kinase that phosphorylates ZraR in response to high concentrations of zinc or lead. The genes *corR* and *corS* code for a copper-responsive two-component system that induces carotenoid production and regulates copper metabolism. The gene *fbpC* is ~~involves~~ involved in ferric ion import and~~. The gene~~ *acn* encodes iron-regulated aconitate hydratase; ~~and~~ *znuC* is ~~involves~~ involved in zinc import. The gene *arsM* contributes to the ~~methylating~~ methylation of arsenite to volatile trimethylarsine. A comparison of the combined relative abundance of all MRGs indicated no significant influence of either irrigation water source (ANOVA, $F = 1.7$, $p = 0.225$) or cropping system (ANOVA, $F = 0.2$, $p = 0.654$) on their relative abundance in ~~the~~ soil metagenomes.

Although gene relative abundance was not altered, a significant effect of cropping system was observed on MRG assemblages within the soils (PERMANOVA, *pseudo-F* = 8.2, $p = 2\times10^{-5}$) (Fig. S4). However, there was no significant effect of irrigation water source (PERMANOVA, *pseudo-F* = 1.1, $p = 0.313$). In contrast to the widely distributed genes, predominantly associated with metal acquisition from the environment, genes identified as biomarkers of the different cropping systems were largely associated with metal resistance mechanisms (Fig. 4B). The only gene identified by LEfSe to be significantly more abundant in the Greenhouse A was *trgB*, which together with *trgA* (not identified by LEfSe) forms an operon coding for a membrane~~-~~-associated complex which confers tellurite resistance. A greater number of MRGs were associated with ~~the~~

Greenhouse B. These included *chrB1*, *chrF* and *chrC*, which code for regulatory proteins and an iron-dependent superoxide dismutase respectively and associate with chromium resistance; *aioA* and *aioB*, which code for an arsenite oxidase ~~and~~ are involved in arsenic detoxification; ~~and~~ *arrA*, which codes for an arsenate respiratory reductase; *cusR* and *cusA*, which encode a response regulator and part of a cation efflux system; *actP* coding a P-type ATPase; *copR* coding a transcriptional activator protein; and *mco* coding a multicopper oxidase all of which are associated with various aspects of copper (and silver) resistance; *silA* coding a component of the sil cation-efflux system (*silABC*) that also confers resistance to silver; and *nrsA* and *nrsR* coding part of a cation or drug efflux system protein and its response regulator respectively associate with nickel resistance.

3.5 Biocide resistance genes

Several biocide resistance genes (BRGs) were distributed widely in the soils (Fig. 5A). The most abundant and widely distributed of these was *fabL*, which confers resistance to the antibacterial and antifungal compound triclosan. Also widely distributed were the genes *evgS* and *evgA* of a two-component system conferring multidrug tolerance. In addition, several widespread genes were associated with ~~the~~ resistance to quaternary ammonium compounds (QACs), including *mdeA*, *cpxR*, *smrA*, and *vcaM*. A comparison of the combined relative abundance of all biocide resistance genes indicated no significant influence of either irrigation water source (ANOVA, $F = 1.4$, $p = 0.283$) or cropping system (ANOVA, $F = 3.0$, $p = 0.106$) on gene relative abundance in ~~the~~ soil metagenomes.

A significant influence of cropping system was observed on ~~the distribution of~~ biocide resistance gene assemblages in the soils (PERMANOVA, *pseudo-F* = 8.7, $p = 3\times10^{-5}$), but as ~~were~~

with the other gene families studied here, there was no significant influence of water source (PERMANOVA, *pseudo-F* = 1.0, p = 0.389): ~~and~~ this is evident from hierarchical clustering (Fig. S5). Very few BRGs were identified by LEfSe to characterize the different cropping systems (Fig. 5B): *adeL*, a regulator of the *adeFGH* efflux system which confers resistance to organosulfates, phenanthridines, azins and acridines, was significantly more abundant in Greenhouse A soil, as was *sugE* coding a QACs efflux pump; *vceR* which regulates the *vceCAB* operon associated with bile acid resistance was more abundant in Greenhouse B soil.

3.6 Insertion Sequences

Alignment of metagenome-derived sequences against the ISfinder database showed that a total of 2,628 ISs were detected in the soils, ~~which~~ these could be classified into twenty-nine IS families. The distribution of ISs showed a very similar response to cropping system and irrigation as the other genes studied here. There was a significant effect of cropping system on insertion sequence assemblages (PERMANOVA, *pseudo-F* = 10.6, p = 0.0002), but no significant influence of the irrigation water sources (PERMANOVA, *pseudo-F* = 1.5, p = 0.185) (Fig. S6). Several ISs were distributed widely within the soils (Fig. 6A) including IS*3*, IS*5*, IS*21*, IS*66*, IS*110*, IS*256* and IS*630*. Nine ISs were determined by LEfSe ~~were~~ to be significantly more abundant in Greenhouse B soil (Fig. 6B).

3.7 Characteristic resistance genes and insertion sequences associated with cropping systems

We identified thirty-four genes or ISs which displayed sensitivity to the different cropping systems based on LEfSe criteria. Using these features, distance-based linear modelling identified total cadmium (marginal test: *pseudo-F* = 5.8, p_{perm} = 0.0032), total (marginal test: *pseudo-F* = 6.4, p_{perm} = 0.0019) and available (marginal test: *pseudo-F* = 5.5, p_{perm} = 0.0043) copper, available

mercury (marginal test: *pseudo-F* = 5.5, p_{perm} = 0.0044), and the quinolone perfloxacin (marginal test: *pseudo-F* = 3.3, p_{perm} = 0.0365) ~~out of~~from all the edaphic factors as exerting significant influence upon the assemblages of ~~sensitive~~ responsive genes. Distance-based redundancy analysis (Fig. S7A) suggested that total and available copper, available mercury and perfloxacin were statistically largely associated with separation of the two cropping system gene assemblages with metal concentrations being greater in Greenhouse B and perfloxacin concentrations being greater in Greenhouse A. However, this does not mean that other antibiotics did not play a role. Total cadmium showed little influence upon the assemblages characterizing the cropping systems. Hierarchical clustering of the thirty-four genes with the experimental factors is shown in Fig. 7A and there is clear evidence for separation according to ~~the~~ cropping system~~s~~ in each greenhouse. To generate a general view of the association of groups of resistance genes and ISs we used these thirty-four genes and IS as features in a supervised Random Forest classification (Fig. S7B). Using the mean decrease in accuracy of the model as a guide, we show the ~~RF classification identified ten~~ fifteen ~~of these~~ features identified by RF classification ~~as~~ to be the most ~~discriminatory~~ characteristic of ~~one or the other~~each cropping system ~~(in~~ Fig. 7B~~)~~. ~~The majority~~Six of these ~~ten~~ fifteen most discriminatory features were characteristic of the Greenhouse A soil: the BRGs ~~*adeL,*~~ *sugE* and ~~*actP*~~*adeL*; the tellurium resistance gene *trgB*; the ARGs *murA* and *mtrA*; and the IS*1595* ~~and IS*L3*~~ insertion sequence~~s~~. ~~Only two~~The majority of these fifteen features were identified as characteristic of the Greenhouse B soil: ~~the~~ these were all associated with metal resistance mechanisms, including *aioB, copR, chrC, aioA, chrB1, nrsR* and *cusR*. ~~chromium resistance gene *chrB1* and the IS*NCY*~~Two ISs were identified as characteristic of Greenhouse B, IS*NCY* and IS*701*. This distribution of characteristic features is consistent with the observation that metal concentrations were greater in

Greenhouse B, discussed above and shown in Fig. S7A.~~.~~

3.8 Contributions of microbes, MRGs, BRGs and ISs to ARGs propagation

The microbial phyla and MRGs/BRGs information corresponding to the gene sets containing ARGs ~~was~~ is listed in Table S8. The most abundant ARG *oqxB* was largely associated with Proteobacteria which also promoted the spread of *sul1* and *soxR*. The genes *sul2*, *ANT(6)-Ia*, *ErmC*, *qacH* are mainly related to Unclassified phylum, and the propagation of *rspL*, *gyrA*, *mtrA* and *murA* was mainly ascribed to Actinobacteria. The genes *ErmY* and *ErmC* were correlated with Firmicutes. The BRGs ~~genes~~ *oqxB*, *qacH* and *soxR* ~~were~~ are associated with ~~BRGs~~ resistan~~t~~ce to ~~Phenolic~~ phenolic compounds, ~~Alkane~~alkane, ~~Aromatic~~ aromatic hydrocarbons, QACs, ~~Halogens~~halogens, ~~Biguanides~~biguanides, ~~Organo~~organo-sulfate~~s~~, ~~Acridine~~acridine, ~~Phenanthridine~~phenanthridine, ~~Azin~~azin, and ~~Paraquat~~paraquat. Among ~~all~~ these genes, only *mtrA* was relevant to the MRG *czcR* conferring resistance to cadmium, zinc and cobalt. MRGs/BRGs *oqxB*, *qacF* and *czcR* are located at plasmid, and others at chromosome. It is worth mentioning that the MRGs/BRGs-linked ARGs all confer resistance through ~~the~~ efflux pump~~s~~, which ~~can well~~may explain their interdependence.

As for the associations between ISs and ARGs, only ~~do~~ *ANT(6)-Ia* and IS (IS*Cco2*) belonging to the IS*1595* family coexist in a gene set. Therefore, we conducted a correlation analysis of between the relative abundance of ARGs and the biomarker ISs and found that IS*1182*, IS*1595*, IS*256*, IS*30*, IS*66* and IS*L3* was related with most ARGs. The genes *oqxB* and *sul2* were only positively associated with IS*21* and IS*66* respectively at a significant level, while *qacH* was not linked to any ISs.

4. Discussion

This study ~~was to~~ investigate~~d~~ the effect of irrigating vegetable crops using RW from municipal

treatment plants as an alternative to GW and cropping system upon ARG~~s~~ dissemination. We were ~~specifically~~ interested specifically in irrigation and cropping effects upon the incidence of various prokaryotic resistance mechanisms to heavy metals, ~~biocides~~biocides, and antimicrobial compounds in the irrigated soils. Soil samples were collected, and metagenomes generated after sixteen years of continuous irrigation of greenhouses grown with different cropping systems. We found that specific genes from each broad family of interest were widely distributed in the irrigated soils, irrespective of the water sources. The most broadly distributed genes are shown in Figs. 4A, 5A and S3. Collectively, they are associated with resistance to ~~the~~ biocidal compounds (Triclosan and QACs)~~,~~ and antimicrobial compounds (olaquindox, quinolones and chloramphenicol), as well as several metal acquisition mechanisms.

4.1 Irrigation effects

Within this background of endemic genes, we could identify no significant influence of water sources (GW *versus* RW) or irrigation management (continuous *versus* alternating) upon the distribution of prokaryotic organisms, ISs or genes conferring resistance to metals, biocides or antibiotics in the soils in ~~each~~ either greenhouse. This suggests that the use of RW for crop irrigation as an alternative to GW ~~does~~ did not result in significantly increased resistance gene burdens in irrigated soils, possibly because the abundance of such genes (e.g. *sul1*) in Chinese agricultural soils is already high (Peng et al. 2017, Tan et al. 2019, Wang et al. 2014a, Wang et al. 2018). However, the assemblages of ARGs in soils ~~received~~ receiving RW irrigation was markedly different between the~~se~~ two greenhouses, and this effect was observed even when GW was used to irrigate the crops. This suggests that the risk of increased or altered ARGs and other resistance genes profiles in the GW-irrigated soils should be of concern in the future.

4.1.1 The ARGs dissemination in soils irrigated with GW

ARGs are not novel soil pollutants and exist in pristine habitats with no direct anthropogenic exposure (D'Costa et al. 2006). It is possible that poor irrigation management (Yi et al. 2011), particularly the use of poor-quality wastewater irrigation at Yongledian ~~town~~ where the experimental station is located before the development of the comprehensive wastewater collection and treatment systems, has resulted in heavy metal, antibiotic, biocide or other possible selective pressures for ARGs propagation to GW - especially those associated with low degradation and adsorption such as ofloxacin and sulfamethoxazole (Avisar et al. 2009, Lyu et al. 2019, Ma et al. 2018). In addition, air pollution may be another cause of the detected antibiotics and ARGs in GW-irrigated soils (Hsiao et al. 2020, Ling et al. 2013). The application of chicken manure, a well-known reservoir of ARGs, may be another reason for the detection of ARGs in GW-irrigated soils.

4.1.2 ~~The~~ ARG~~s~~ dissemination in soils irrigated with RW

As for the inconsistent effects of RW irrigation on the dissemination of ARGs in soil, a recent study at Braunschweig, ~~in~~ Germany showed that only ARGs ~~(e.g. *sul1*)~~ which were initially more abundant in the RW (e.g. *sul1*) increased in soil following RW irrigation, while ARGs which were ~~(e.g. bla_{TEM})~~ initially sparse in the RW (e.g. bla_{TEM}) did not increase and even decreased under certain circumstances (Kampouris et al. 2021a). ~~These, however, do not apply to~~This phenomenon is not evident in our study in which the *sul1* and *sul2* were more abundant in GW and RW respectively (obtained from Liu ~~(2019a)),~~ (2019a)). ~~but t~~There was no significant difference in their abundance between ~~all~~ soils in each greenhouse, probably because soil properties, climate and crops in their study differed from ours. For example, the soil pH in our soils was 7.6~~3~~-8.1~~0~~, compared to 3.~~78~~-~~5.97~~6.0 in Braunschweig~~their study~~. Our results ~~was~~ are consistent with ~~that~~ those of

Shamsizadeh et al. (2021) obtained from an experiment conducted under a semi-arid climate, and showing that irrigation water sources had no significant ~~impact~~ influence on the abundance of ARGs including *sul1* in soils, and that RW can be used in agriculture in semi-arid regions; however, since the soil samples taken from fields cultivated with different crops were pooled in their study, it is difficult to ~~glean~~ determine whether ~~that~~ the ~~no~~ lack of effect of RW irrigation on ARGs was caused by ~~the~~ cropping or other factors. Most previous studies on ARGs under RW irrigation have focused on irrigation ~~only~~ alone and ~~overlooked~~ have not considered the possible impact of other factors~~, .~~ ~~while i~~In our study, all variables but the irrigation water source were kept the same in each greenhouse. ~~In the meantime, w~~We also measured ~~the detailed~~several soil properties including pH, nutrients, heavy metals, antibiotics as well as the profile of ARGs, MRGs, ISs and microbial community. Comparatively speaking, our study excluded other factors and demonstrated the influence of irrigation water sources.

Some studies suggest~~ed~~ that ~~the~~ resistant bacteria ~~from~~ in RW that enter~~ed~~ soils ~~were~~ are not able to compete or survive in the ~~new~~ soil environment ~~(Negreanu et al. 2012),~~ (Negreanu et al. 2012). This partly ~~explaining~~ explains the similar level~~s~~ of ARGs between RW- and GW-irrigated soils in each ~~of the two~~ greenhouses in our study. The ~~impact~~ influence of RW-associated bacteria on the soil microbiome is ~~inappreciable~~ not quantifiable ~~on average~~ and in the long-term, they are unlikely to ~~significantly~~ increase antibiotic resistance significantly. Another possibility is that the primary ecological role of naturally-produced antibiotics is to inhibit the growth of other soil organisms ~~in soils~~ (Kelsic et al. 2015), ~~thus~~ alleviating ~~their~~ competition for scarce resources. The microbes in RW-irrigated soils receive more carbon and nitrogen while facing less competition for resource, and they thus reduce the energy-costing expression of ARGs for antibiotic production

(Martínez and Rojo 2011), and offset the increase in ARGs induced by RW which is rich in antibiotics, ARGs and antibiotic resistant microbes.

4.2 Cropping effects

~~On the c~~Contrary to the limited influence of irrigation water sources, cropping system as exemplified by the two greenhouses exerted a strong, statistically significant and consistent influence upon assemblages of metal, biocide and antibiotic resistance genes and ISs, and in the case of ARGs, a significant difference in the relative abundance of ~~the~~ genes as well.

4.2.1 The differences in basic properties and microbial composition of soil between the two cropping systems

There were no significant differences in the properties of RW-irrigated soils between the two greenhouses except for total Cd, available and total Cu, ~~total Cu,~~ available Hg and total N. Given that the difference in nitrate and ammonium between the two RW-irrigated soils was small, the difference in total N might be due to the difference in organic N (Kelley and Stevenson 1995). Though total N and OM in Greenhouse A soil were lower than that in Greenhouse B (Table 1), the C/N ratio was higher (11.82) in the former ~~(11.82)~~ than ~~in~~ the latter (10.35~~),~~). ~~which~~ This may ~~might~~ facilitate microbial activity to mineralize N and propagate ARGs. The difference in total N and OM between the two greenhouses could ~~be caused by~~arise as a result of planting, and chemical and chicken manure fertilization. In the long term, all these could shift microbial community and alter their associated genes. For example, the relative abundance of Proteobacteria, Bacteroidetes, Verrucomicrobia and *Ca*. Tectomicrobia were lower in Greenhouse B than in Greenhouse A, while Acidobacteria, Cyanobacteria, *Ca*. Rokubacteria, Planctomycetes, and Deinococcus-Thermus trended in the opposite direction (Fig. S2A). We found that most ARG~~s~~-associated microbes

Commented [AN1]: how?

belonged to Proteobacteria, Actinobacteria and Firmicutes, consistent with previous studies (Wu et al. 2021).

4.2.2 The associations between soil ARGs and the potential propagators

Cross-resistance of ARGs and MRGs/BRGs (e.g. *oqxB*) in our study mainly functioned through efflux of structurally dissimilar antibiotic compounds and biocides/metals using the same mechanisms. The plasmid-~~located~~ borne MRGs-associated ARGs possessed a high horizontal transfer probability. The high correlation between ARGs and ISs (Table S9) also indicated that MGEs ~~was~~ are crucial to the ARGs spread. The Is*Cco2* and other MGEs were also found to be dominant in other environments and play a key role in ARGs transfer (Zhang et al. 2021). It was postulated that the critical system in *Acinetobacter* for increasing their resistance level could be due to the existence of ISs in the genome, such as the IS*Abc1* (IS*1595* family) that can insert ~~itself into~~at the 5´-end of existing resistance genes, equipping them with strong promoters and up-regulating gene expression (Gootz and Marra 2008). All these bio-physicochemical differences ~~worked together~~interact to shift the ARGs making them differ between the two cropping systems.

4.3 Implications for future research

Our results suggests that the concentrations of a limited number of metals including copper and mercury and the antibiotic compound perfloxacin accounted for the differences in the resistance gene assemblages between the two cropping systems (Fig. 7A). The most characteristic genetic markers of each cropping system ~~grouped~~ associated the biocide resistance genes *adeL* and~~,~~ *sugE* ~~and *actP*~~, the metal resistance gene *trgB* and the antibiotic resistance genes *murA* and *mtrA* together with ~~, insertion sequences~~ IS*1595* ~~, and IS*L3*~~ in Greenhouse A. In contrast, Greenhouse B were characterized largely by ~~a high abundance of the~~ metal resistance genes ~~chrB1 and~~associated with

the insertion sequences ~~*ISL3*~~*ISNCY* and *IS701*. ~~Although w~~We are unable to ~~ascertain~~ determine whether the characteristic resistance genes ~~characteristics of~~in each soil were ~~physically~~ structurally associated with the ~~identified~~ characteristic ISs~~,~~. However, the data is ~~suggested~~ suggestive of associations between specific resistance genes and ISs the ~~differences between~~in the two greenhouse soils. ~~This appears to be~~Our unpublished data suggests that this is a ~~–~~consistent ~~rather~~ response of the soil resistome~~than exceptional~~ as in a separate experiment investigating the impact of irrigation with livestock wastewater, we found cropping systems have a significant ~~impact~~ influence on ARG~~s~~ dissemination in soil. However, ~~though~~ the underlying mechanisms remain undetermined~~obscure (unpublished)~~. This is ~~further~~ corroborated by our experiments showing that legume roots absorb~~ed~~ more antibiotics than the grass roots due to ~~the~~ difference~~s~~ in their root properties (http://kd.nsfc.gov.cn/advancedQuery/personInfo/b86bca4a5e8002797998c5bc02c04feb). ~~Our~~ This experiment was not designed to allow us to determine the differences in gene distributions due to the ~~difference~~ specific differences in the cropping regimes in the 16-year ~~cropping systems~~experiment, or because of the short-term effects of legume *versus* brassica crops. Our results strongly suggested that the influence of cropping systems upon resistance gene distributions warrants further research.

5. Conclusions

We found that neither RW irrigation nor cropping system influenced edaphic factors ~~soil~~ (pH, EC, OM, NH_4^+-N, available-P and available-K) ~~and~~ or the total concentration of antibiotics to any significant degree~~ly in the soils with~~employing different RW irrigations for 16 years~~ in two greenhouses grown with different cropping systems, . while t~~The concentration of soil available

heavy metals ~~were~~ was reduced following RW irrigation with a few exceptions. ~~The a~~Assemblages of ARGs, MRGs, BRGs, ISs and microbial ~~community~~ taxa in soils irrigated with RW was not altered relative to that irrigated with GW. Although alternate irrigation with groundwater and reclaimed water reduced the total input of ARGs and the associated ARG propagators in the soil, ~~the~~ ARG~~s~~ dispersal in the soils was not influences significantly ~~affected~~. We showed that ~~cropping~~ differences in cropping regimes, which ~~had~~ have been ~~overlooked~~ unaccounted for in previous studies, ~~could~~ can exert ~~the~~ a greater influence upon the distribution of resistance genes in soils than ~~the~~ the source of irrigation water. Our results ~~unveiled~~ revealed that the influence of factors other than irrigation water, such as planting, on ARG diffusion in soil warrants more research effort.

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.

Acknowledgments

This work was financed by the Scientific and Technological Project of Henan Province (202102110215), the UK-China Centre for the Sustainable Intensification of Agriculture (CSIA 18-11), the National Natural Science Foundation of China (41701265), the National Key Research and Development Program of China (2017YFD0801103-2), the Central Public-interest Scientific Institution Basal Research Fund (Farmland Irrigation Research Institute, CAAS) (FIRI2019-04-02).

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