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

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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. We compared microbial communities, assemblages of genes associated with microbial resistance to antibiotics, biocides and metals, and insertion sequences (ISs) in soils following 16 years of irrigation with groundwater (GW), RW or alternately with GW and RW in two greenhouses with different cropping systems, using shotgun metagenome sequencing. The results showed that cropping system exerted greater influence than irrigation on the profile of ISs and resistance genes. This influence was most strongly associated with concentrations of copper, mercury and perfloxacin in the soils. There was no significant difference in soil ARG profiles between continuous RW irrigation and alternating GW and RW irrigation. Proteobacteria, Actinobacteria and Firmicutes and a limited number of ISs were closely associated with the detected ARGs. Most ARGs were found to co-occur with metal and biocide resistance genes through the mechanism of efflux pumps. These findings highlight the significance of understanding and improving crop management in mitigating the dissemination of ARGs in soils irrigated with RW.

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 have made many countries in arid and semi-arid regions consider treated wastewater as a supplement for irrigation (Elgallal et al., 2016; Fatta-Kassinos et al., 2020; Pedrero et al., 2010; Pereira et al., 2002). However, most wastewater treatment plants discharge effluents containing contaminants such as 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 waters could release these contaminants to soil-plant systems increasing their potential 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, thereby compromising the efficacy of antibiotics in animal and human medicine (Pruden et al., 2006). This has become a public concern (Sorinolu et al., 2021). ARGs have several mechanisms to spread in soil, one of which is horizontal transfer through mobile genetic elements (MGEs) (Gatica and Cytryn, 2013). It was also found that ARGs often co-exist with metal resistance genes (MRGs) since they share the same MGEs (Baker-Austin et al., 2006).

Reclaimed wastewater contains antibiotics and ARGs, and continuous RW irrigation could cause their accumulation in soils (Kampouris et al., 2021b). RW-borne bacteria and associated ARGs can persist below detection levels in irrigated soils and have potential to increase in abundance under copiotroph conditions (Marano et al., 2021). Since RW irrigation changes bio-physicochemical conditions of soil and root-induced processes, which in turn alter antibiotic degradation and microbial community composition, the long-term effects of RW irrigation on dissemination of ARGs in soil are complicated and its principal determinants remain obscure. RW irrigation for 3–4 years has been shown to increase the abundance of ARGs in urban park soil,

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significantly increasing the diversity and abundance of ARGs and altering soil bacterial communities due to increased pH and decreased total N (Han et al., 2016). This was corroborated by a similar study that irrigating urban parks using RW for 1–10 years enriched ARGs in soil due to the increase in antibiotics and MGEs in soil (Wang et al., 2014b). The ARG burden of RW is an important driver influencing ARGs in soil following RW irrigation (Kampouris et al., 2021a). However, these studies overlooked the differences in pre-irrigation soil properties, as well as microclimates and plant coverage. It is thus difficult to determine 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). There are also reports that RW irrigation has no influence on ARG dissemination (Cui et al., 2018; Marano et al., 2019; McLain and Williams, 2014; Negreanu et al., 2012). For example, a study of ARG patterns in *Enterococcus* in pond sediments revealed that the levels of antibiotic resistance following long-term RW recharge were comparable to that with GW, and that bacterial multiple-antibiotic-resistance in the sediments from GW-filled ponds was significantly higher than that in RW-filled ponds (McLain and Williams, 2014). In a separate study, the abundance of four ARGs (*sul1*, *sul2*, *ermB*, and *ermF*) in soils irrigated with RW for 6–15 years was either unchanged or lower than that in soils irrigated with freshwater (Negreanu et al., 2012). Such inconsistent results regarding the influence of RW irrigation on ARG dissemination are a public concern: its mechanistic understanding is hampered due to a lack of experiments of sufficient duration to study the change in both ARGs and other biogeochemical properties of soil following RW irrigation, especially under field conditions with continual agricultural practices.

The effects of RW irrigation on ARG dissemination in soil depend on many factors. RW quality and irrigation methods control ARG input to soil (Fahrenfeld et al., 2013), and a change in soil biogeochemical properties due to RW irrigation can reshape microbial assemblages (Cui et al., 2018). Physiologically, roots could become electrically charged and they hence react with charged antibiotics via iron plaques or chemical functional groups on the root surface (Choi et al., 2016; Liu et al., 2018; Tai et al., 2018). Since morphological and electrical properties of roots and their rhizosphere vary with crop species and varieties (Granzow et al., 2017; Lu et al., 2018), it is envisaged that crops may also impose selective pressure on soil antibiotic-resistant microbes despite the lack of studies on their significance. Han et al. (2016) and Wang et al. (2014b) did not separate RW irrigation and plants, and it is hence impossible to distinguish the relative significance of RW irrigation and plants in their influence on ARGs in soil. Negreanu et al. (2012) investigated ARG assemblages in orchard soils with limited tillage and soils cultivated with cotton and wheat, but these plants have deep roots and do not require as much water as vegetables. It is unclear that the similarity in ARGs between the treatments was due to the crops or other factors.

Large-scale wastewater treatment plants are usually associated with metropolitan areas and vegetable production in the suburbs can readily access RW for irrigation. Unlike staple crops, vegetables require intensive fertilization and irrigation. Their roots are shallow and root-induced biotic and abiotic processes are most active in the topsoil. We hence hypothesized that cropping exerts an important influence on microbial and biogeochemical properties of soil (Bengough, 2012), and consequently the proliferation or attenuation of ARGs. Since the changes in physical and biogeochemical properties of soil resulting from different irrigation waters and management are slow and take decades to stabilize (Wang et al., 2022), we selected two greenhouses grown with various vegetables and having received different RW irrigation treatments for 16 years, with groundwater (GW) irrigation as the control. We aimed to test: 1) how cropping and long-term RW irrigation affect ARG profiles in 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. Three irrigation treatments were compared: groundwater irrigation, alternate groundwater - reclaimed water irrigation, and reclaimed water irrigation. Each treatment has three replicates arranged across 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 plots 1–8 and plot 9 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 apart to avoid possible lateral water flow, and GW used for 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 the water properties are given in Tables S2 and S3.

Soils were sampled from the top layer (0–20 cm) at three randomly placed locations between the drip pipes in each plot, and they were then pooled. Sub-samples designated for nucleic acid extraction were immediately stored at −80 ºC and the remains were 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 because of their common usage in healthcare and livestock husbandry and their close association with ARG dissemination (Leng et al., 2020; Wang et al., 2014a, 2014b; Yan et al., 2018). Details of the antibiotics determination 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 soils (0.3 g) following the manufacturer's instructions. We did not extract DNA from the water for reasons detailed in the Supplementary information. The concentration of extracted DNA was determined using a Qubit Fluorometer and dsDNA BR Assay kit (Invitrogen, USA). Electrophoresis in a 1% agarose gel was used to check DNA quality. Genomic DNA (1 μg) was randomly fragmented using Covaris Focused-ultrasonicators (ME220, 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. Single strand circle DNA (ssCir DNA) 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).

2.4. Assembly, gene catalogue construction and annotation

Assembly of the clean reads was conducted for each sample respectively using megahit v1.1.3 (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, N50 for the samples ranging from 398,525 to 1021,693.

Open reading frames (ORFs) were predicted from contigs for each sample using MetaGeneMark (v2.10) software (Zhu et al., 2010), with 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) using the parameters -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, 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 $E < 1 \times 10^{-5}$.

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 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 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 the 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. Minkowski distance and Ward's agglomerative clustering algorithm were used for clustering. To identify the most diagnostic genes and insertion sequences characterizing assemblages 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 genes and insertion sequences. The RF classifier was built by growing 5000 classification trees. Only 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. 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 was 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 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* $= 11.5$, $p = 3 \times 10^{-5}$), 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. The overall pattern presented

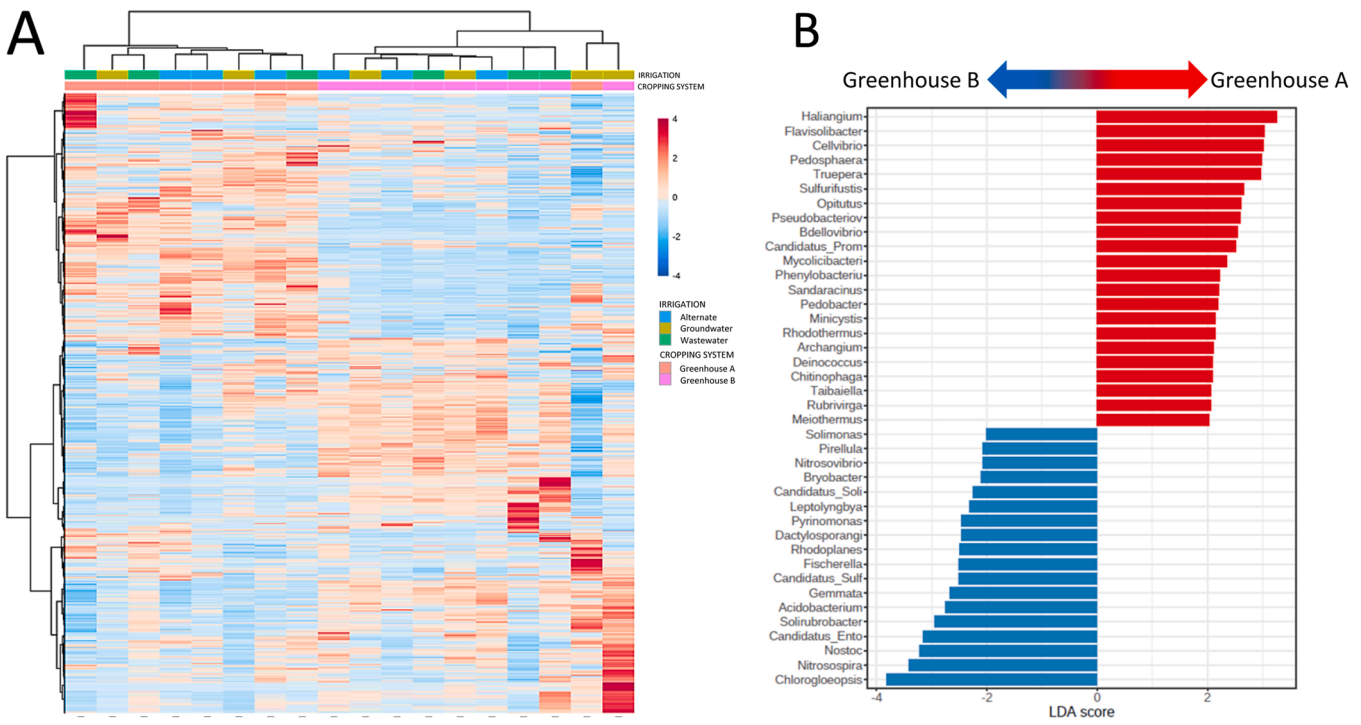

Fig. 1. Influence of irrigation water source and cropping system upon soil microbial communities. A – Heat map of two-dimensional hierarchical clustering analysis of microbial assemblages in soil irrigated using groundwater, reclaimed wastewater, or alternately with the two water sources, and under contrasting cropping systems. Relative organism abundance was determined from shotgun metagenomics and centered log-ratio transformed. Euclidean distance and Ward's agglomerative clustering algorithm cluster determination. Heatmap colors represent the relative organismal abundance; blue indicates the lowest abundance and red the highest abundance. The color scale bar is shown at the top right corner of the figure. B – Linear Discriminant Analysis Effect Size (LEfSe) analysis showing organisms that were significantly differentially abundant ($p_{adj} < 0.05$) between the two cropping systems, ranked by effect size (all LDA scores >2).(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Basic properties of soil with groundwater (GW) irrigation, alternate irrigation with GW and reclaimed water (RW), and RW irrigation. The data are expressed as the mean and standard deviation in every bracket. Different lower case letters represent significant difference between treatments at $p < 0.05$.

Treatment		pH	EC (mS m^{-1})	OM (g kg^{-1})	Total N (%)	NO_3^--N (mg kg^{-1})	NH_4^+-N (mg kg^{-1})	Available P (mg kg^{-1})	Available K (mg kg^{-1})
Cropping	Irrigation								
Greenhouse A	GW	8.10a (0.32)	33.13a (22.51)	32.53a (4.07)	0.17ab (0.00)	188.38c (123.40)	4.12a (0.52)	221.22ab (46.40)	606.09a (262.89)
	Alternate irrigation	7.73ab (0.17)	53.80a (19.22)	29.71a (2.23)	0.16abc (0.02)	351.27abc (22.43)	3.36a (0.16)	228.70a (12.94)	672.42a (72.15)
	RW	7.87ab (0.20)	43.13a (14.80)	27.53a (2.48)	0.14c (0.03)	367.16ab (94.81)	3.68a (0.93)	184.60ab (7.46)	594.33a (86.60)
Greenhouse B	GW	7.99ab (0.15)	28.07a (5.74)	30.02a (0.44)	0.15bc (0.01)	214.40bc (97.23)	3.47a (0.89)	201.21ab (10.65)	492.06a (14.22)
	Alternate irrigation	7.77ab (0.23)	48.10a (32.03)	31.17a (1.49)	0.17ab (0.02)	349.69abc (69.43)	3.29a (1.24)	199.89ab (18.57)	577.77a (70.73)
	RW	7.63b (0.18)	57.43a (24.53)	32.51a (2.97)	0.18a (0.01)	406.29a (70.00)	5.97a (5.46)	182.50b (19.11)	598.45a (128.56)

by PCA (Fig. 2) could not separate the soils based on water quality or cropping system, suggesting that neither factor influenced soil pH, EC, OM, NH_4^+-N, available-P and available-K 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 sources, 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, a similar level to 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

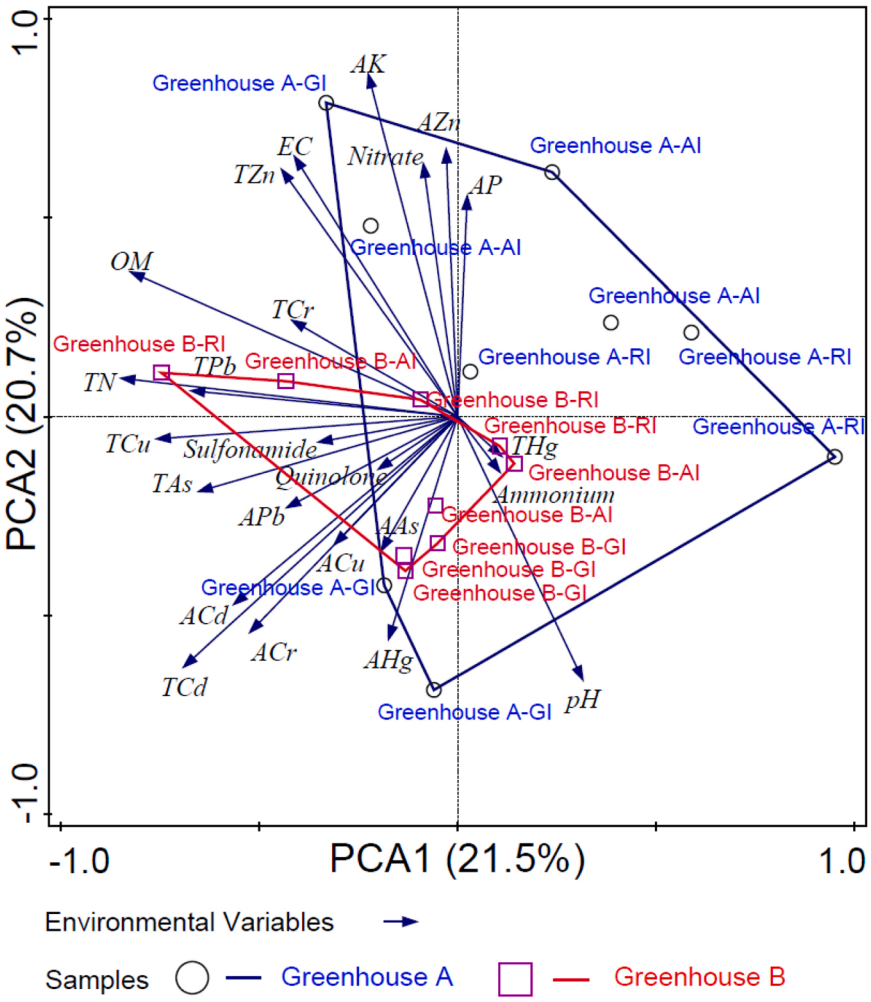

Fig. 2. Principal Component Analysis depicting the influence of irrigation water source and cropping system upon soil chemistry. Cropping systems (Greenhouse A and Greenhouse B) are identified by symbols and irrigation water source by letter: GI – groundwater irrigation, RI – reclaimed wastewater irrigation, AI – alternating irrigation using the two water sources. Cropping system space is described by convex envelopes. Associations between cropping system, irrigation water source and edaphic factors presented in Tables 1 and 2 are represented as vectors. The length of each vector indicates the strength of correlation of the respective parameter with the samples.

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 soils, there was a significant effect of cropping system on ARG assemblages (PERMANOVA, *pseudo-F* = 10.6, $p = 9 \times 10^{-5}$) 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 involved in molybdate/tungstate import, as 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 involved in ferric ion import and *acn* encodes iron-regulated aconitate hydratase; *znuC* is involved in zinc import. The gene *arsM* contributes to the 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 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, $p = 2 \times 10^{-5}$) (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 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*

Table 2
Concentration of total and available metals in soil with groundwater (GW) irrigation, alternate irrigation with groundwater and reclaimed water, and reclaimed water (RW) irrigation. The data are expressed as the mean and standard deviation in every bracket. Different lower case letters represent significant difference between treatments at $p < 0.05$.

Treatment		Total Cd (mg kg^{-1})	Total Cr (mg kg^{-1})	Total Cu (mg kg^{-1})	Total Pb (mg kg^{-1})	Total Zn (mg kg^{-1})	Total As (mg kg^{-1})	Total Hg (mg kg^{-1})
Cropping	Irrigation							
Greenhouse A	GW	0.20a (0.02)	59.51a (2.06)	29.17 cd (2.00)	21.63a (0.73)	111.97a (8.12)	5.96a (0.40)	0.11a (0.02)
	Alternate irrigation	0.15b (0.01)	63.78a (7.83)	29.30bcd (1.36)	21.51a (0.29)	112.29a (2.31)	5.55a (0.45)	0.13a (0.02)
	RW	0.16b (0.02)	58.43a (2.93)	27.56 d (1.79)	20.59a (0.86)	104.92a (3.16)	5.43a (0.85)	0.12a (0.01)
Greenhouse B	GW	0.21a (0.02)	62.28a (3.74)	32.91a (0.62)	21.80a (0.66)	108.18a (1.43)	5.79a (0.24)	0.12a (0.09)
	Alternate irrigation	0.21a (0.01)	60.44a (2.88)	31.83abc (0.57)	21.35a (0.85)	105.83a (2.48)	5.77a (0.40)	0.10a (0.02)
	RW	0.20a (0.02)	58.30a (3.44)	32.36ab (2.69)	22.05a (0.90)	109.60a (6.13)	5.50a (0.88)	0.15a (0.08)
Treatment		Avialable Cd (mg kg^{-1})	Avialable Cr (mg kg^{-1})	Avialable Cu (mg kg^{-1})	Avialable Pb (mg kg^{-1})	Avialable Zn (mg kg^{-1})	Avialable As (mg kg^{-1})	Avialable Hg (μg kg^{-1})
Cropping	Irrigation							
Greenhouse A	GW	0.04a (0.00)	0.04a (0.00)	1.60bc (0.09)	0.83a (0.05)	9.58ab (1.81)	0.29a (0.01)	0.04abc (0.01)
	Alternate irrigation	0.04ab (0.00)	0.04ab (0.00)	1.55c (0.05)	0.73ab (0.06)	9.76a (0.52)	0.30a (0.00)	0.03c (0.00)
	RW	0.04b (0.00)	0.04b (0.00)	1.46c (0.04)	0.68b (0.05)	7.96b (0.34)	0.31a (0.02)	0.03bc (0.01)
Greenhouse B	GW	0.04ab (0.00)	0.04a (0.00)	1.88a (0.04)	0.74ab (0.06)	8.04ab (0.37)	0.31a (0.01)	0.04ab (0.00)
	Alternate irrigation	0.04ab (0.00)	0.04ab (0.00)	1.73ab (0.12)	0.70ab (0.09)	7.87b (0.68)	0.31a (0.00)	0.05a (0.01)
	RW	0.04ab (0.00)	0.04ab (0.00)	1.79a (0.14)	0.76ab (0.10)	8.75ab (0.88)	0.30a (0.01)	0.05a (0.01)

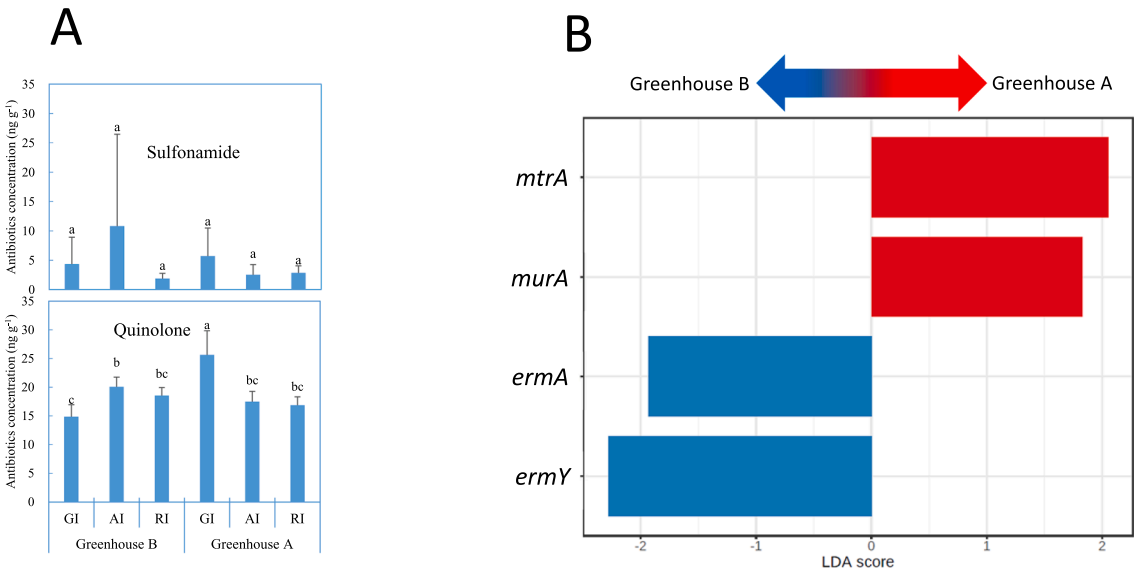

Fig. 3. Influence of irrigation water source and cropping system upon soil antibiotic concentration and the distribution of antibiotic resistance genes in microbiomes associated with irrigated soils. A – Concentrations of Sulfonamide and Quinolone classes of antibiotic compounds detected in irrigated soils under contrasting cropping systems (Greenhouse A or Greenhouse B). GI – groundwater irrigation, RI – reclaimed wastewater irrigation, AI – alternating irrigation using the two water sources. The mean concentration is shown, error bars represent standard deviation. Different letters associated with each bar indicate groups between which a significant difference in concentration was detected by ANOVA and *post hoc* pairwise comparison. B – Linear Discriminant Analysis Effect Size (LEfSe) analysis showing antibiotic resistance genes that were significantly differentially abundant ($p_{adj} < 0.05$) between the two cropping systems, ranked by effect size (all LDA scores >1.5). No significant effect of irrigation water source upon gene assemblages was detected.

and *aioB,* which code for an arsenite oxidase are involved in arsenic detoxification; *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 silver 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

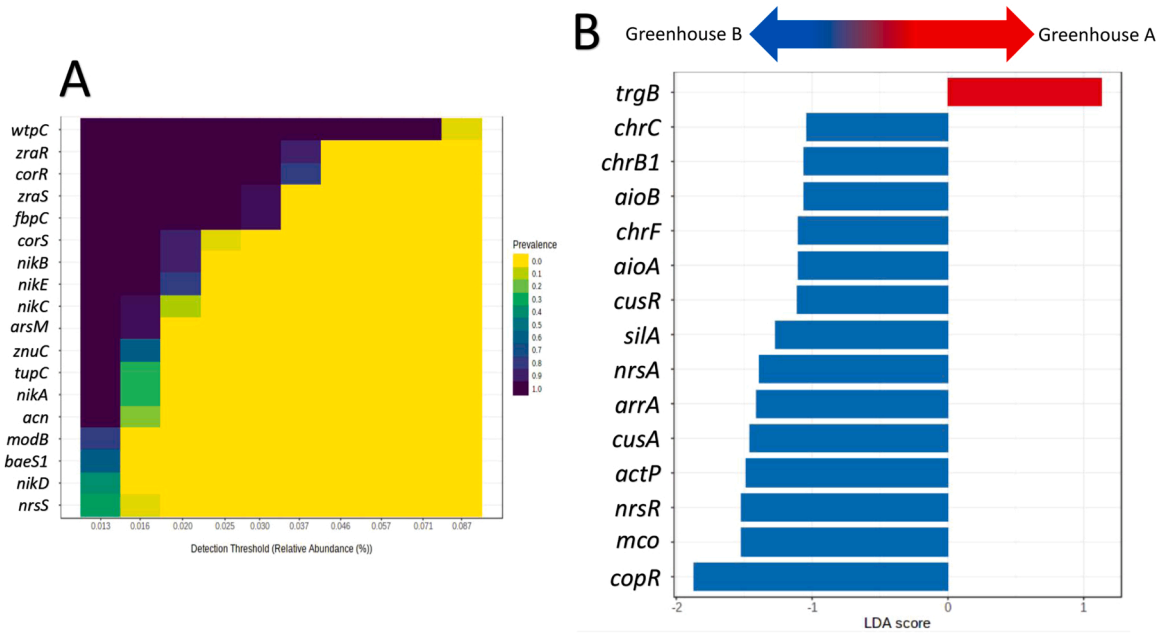

Fig. 4. Influence of cropping system upon the distribution of metal resistance genes in microbiomes associated with irrigated soils. A – Prevalence of the most widely distributed metal resistance genes in the experimental soils. The distribution of each gene is represented as a heatmap ranging from yellow (present in no sample) to blue (present in all samples) and is associated with a detection threshold based upon gene relative abundance in the metagenomes in which it is present. These widely distributed genes are generally associated with metal acquisition processes. B – Linear Discriminant Analysis Effect Size (LEfSe) analysis showing genes that were significantly differentially abundant ($p_{adj} < 0.05$) between the two cropping systems, ranked by effect size (all LDA scores >1). These differentially abundant genes were associated with metal resistance mechanisms.

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 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 soil metagenomes.

A significant influence of cropping system was observed on biocide resistance gene assemblages in the soils (PERMANOVA, *pseudo-F* = 8.7, $p = 3 \times 10^{-5}$), but as with the other gene families studied here, there was no significant influence of water source (PERMANOVA, *pseudo-F* = 1.0, $p = 0.389$): 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.

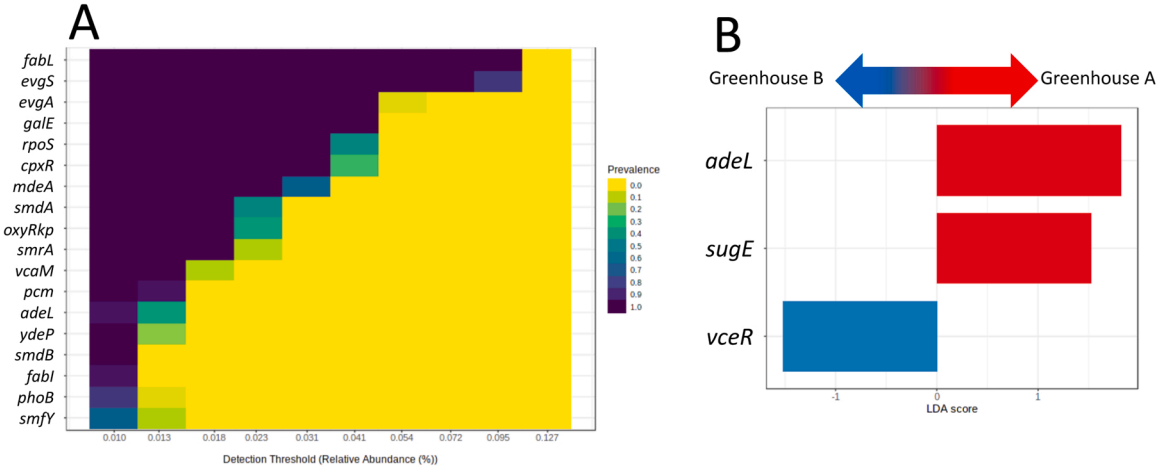

Fig. 5. Influence of cropping system upon the distribution of biocide resistance genes in microbiomes associated withirrigated soils. A – Prevalence of the most widely distributed biocide resistance genes in the experimental soils. Thedistribution of each gene is represented as a heatmap ranging from yellow (present in no sample) to blue (present in allsamples) and is associated with a detection threshold based upon gene relative abundance in the metagenomes in which it ispresent. B – Linear Discriminant Analysis Effect Size (LEfSe) analysis showing genes that were significantly differentially abundant ($p_{adj} < 0.05$) between the two cropping systems, ranked by effect size (all LDA scores >1.5). No significant effect of irrigation water source upon gene assemblages was detected.

3.6. Insertion sequences

Alignment of metagenome-derived sequences against the ISfinder database showed that a total of 2628 ISs were detected in the soils, 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 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 modeling 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, $p_{perm} = 0.0365$) from all the edaphic factors as exerting significant influence upon the assemblages of 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 cropping system 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 fifteen features identified by RF classification to be the most characteristic of each cropping system in Fig. 7B. Six of these fifteen features were characteristic of the Greenhouse A soil: the BRGs *sugE* and *adeL*; the tellurium resistance gene *trgB*; the ARGs *murA* and *mtrA*; and the IS*1595* insertion sequence. The majority of these fifteen features were identified as characteristic of the Greenhouse B soil: seven MRGs (*aioB*, *copR*, *chrC*, *aioA*, *chrB1*, *nrsR* and *cusR*) and two ISs (IS*NCY* and IS*701*).

3.8. Contributions of microbes, MRGs, BRGs and ISs to ARG propagation

The microbial phyla and MRGs/BRGs information corresponding to the gene sets containing ARGs 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 genes *oqxB*, *qacH* and *soxR* were associated with BRGs resistant to phenolic compounds, alkane, aromatic hydrocarbons, QACs, halogens, biguanides, organo-sulfates, acridine, phenanthridine, azin, and paraquat. Among 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 efflux pumps, which may explain their interdependence.

As for the associations between ISs and ARGs, only *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 investigated the effect of irrigating vegetable crops using RW from municipal treatment plants as an alternative to GW and cropping system upon ARG dissemination. We were interested specifically in irrigation and cropping effects upon the incidence of various prokaryotic resistance mechanisms to heavy metals, biocides, and antimicrobial compounds in the irrigated soils. Soil samples were collected, and metagenomes generated after sixteen years of continuous

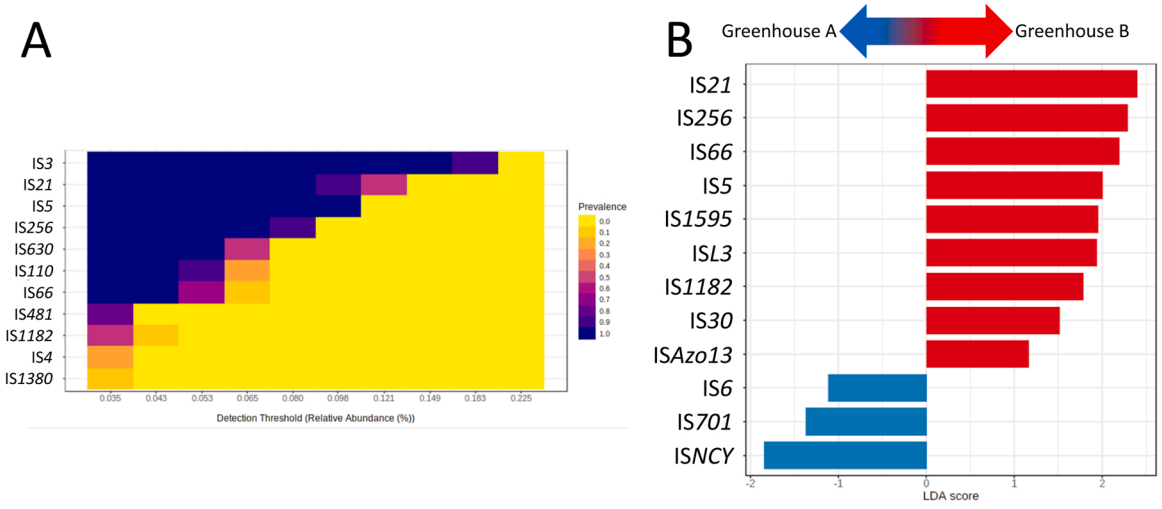

Fig. 6. Influence of cropping system upon the distribution of insertion sequences in microbiomes associated withirrigated soils. A – Prevalence of the most widely distributed insertion sequences in the experimental soils. The distributionof each gene is represented as a heatmap ranging from yellow (present in no sample) to blue (present in all samples) and isassociated with a detection threshold based upon gene relative abundance in the metagenomes in which it is present. B –Linear Discriminant Analysis Effect Size (LEfSe) analysis showing genes that were significantly differentially abundant ($p_{adj} < 0.05$) between the two cropping systems, ranked by effect size (all LDA scores >1). No significant effect of irrigation watersource upon insertion sequence assemblages was detected.

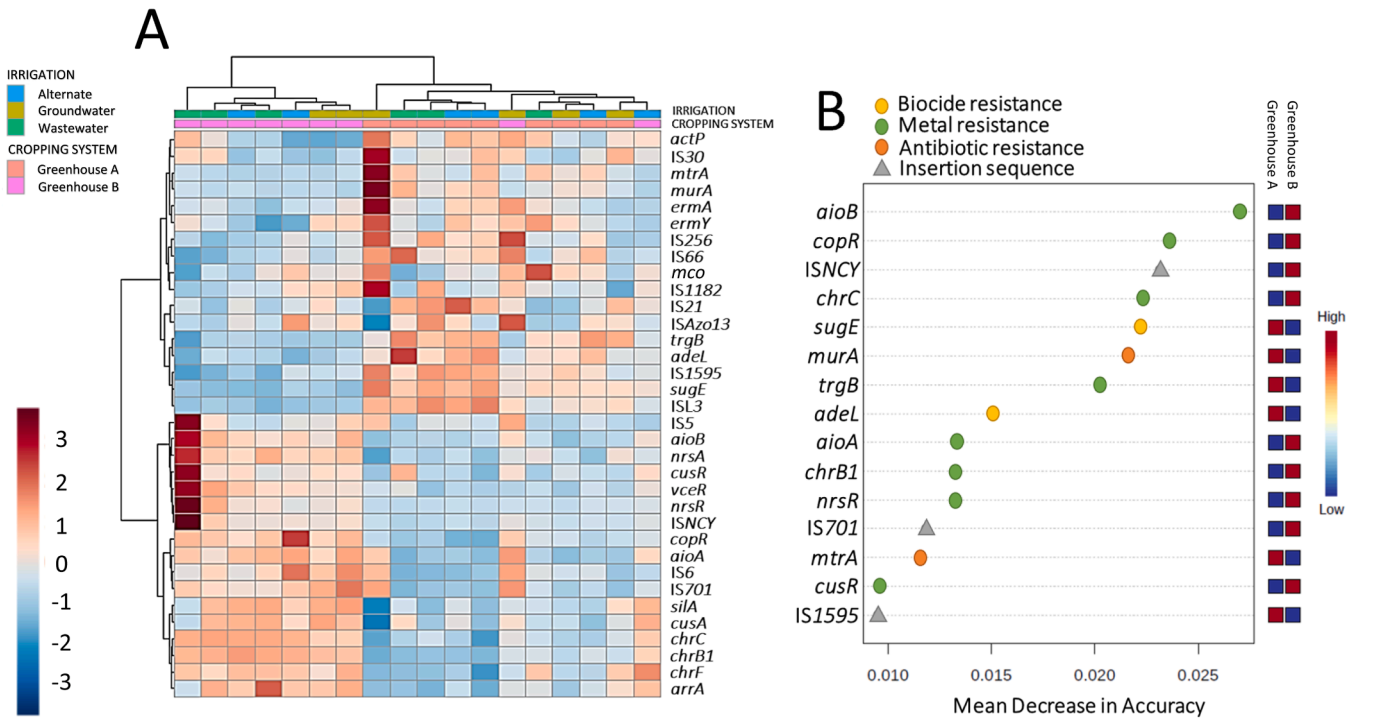

Fig. 7. Summary of the influence of irrigation water source and cropping system upon resistance gene and insertion sequence assemblages. A – Heat map of two-dimensional hierarchical clustering analysis of assemblages of significantly differentially abundant genes and insertion sequences in soil irrigated using groundwater, reclaimed wastewater, or alternately with the two water sources, and under contrasting cropping systems (identified in Figs. 3–6). Relative abundance was determined from shotgun metagenomics and centred log-ratio transformed. Minkowski distance and Ward's agglomerative clustering algorithm was employed for cluster determination. Heatmap colours represent the relative organismal abundance; blue indicates the lowest abundance and red the highest abundance. The colour scale bar is shown at the bottom left corner of the figure. B – Random forest classification identifying biomarkers of each cropping system based on the predicative accuracy of each gene or insertion sequence (indicated by the mean decrease in model accuracy using out-of-bag cross validation). The fifteen genes or insertion sequences associated with the highest predictive accuracy are shown. Heat map on the right indicates the relative abundance of each gene or insertion sequence in the different cropping system. The complete set of importance values are shown in Fig. S7B.

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 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) and 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 either greenhouse. This suggests that the use of RW for crop irrigation as an alternative to GW 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, 2018). However, the assemblages of ARGs in soils receiving RW irrigation was markedly different between the 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 due to different managements should be of concern in the future.

4.1.1. ARG 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 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 ARG 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. ARG 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, Germany showed that only ARGs (e.g. *sul1*) which were initially more abundant in the RW increased in soil following RW irrigation, while ARGs (e.g. bla_{TEM}) which were initially sparse in the RW did not increase and even decreased under certain circumstances (Kampouris et al., 2021a). These, however, do not apply to our study in which the *sul1* and *sul2* were more abundant in GW and RW respectively (obtained from Liu et al., 2019a), but there was no significant difference in their abundance between 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 7.63–8.10, compared to 3.77–5.97 in Braunschweig. Our results are consistent with those of Shamsizadeh et al. (2021) obtained from an experiment conducted under a semi-arid climate, showing that irrigation water sources had no significant 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 determine that whether the lack of effect of RW irrigation on ARGs was caused by cropping or other factors. Most previous studies on ARGs under RW irrigation have focused on irrigation without considering the possible impact of other factors, while in our study, all variables, except the irrigation water source, were kept the same in each greenhouse. We also measured soil properties including pH, nutrients, heavy metals, antibiotics as well as the profile of ARGs, MRGs, BRGs, ISs and microbial community. Therefore, our study comparatively excluded other factors and demonstrated the influence of irrigation water sources.

Some studies postulated that resistant bacteria in RW that enter soils are not able to compete or survive in the soil environment (Negreanu et al., 2012). This partly explains the similar levels of ARGs between RW- and GW-irrigated soils in each greenhouses in our study. The influence of RW-associated bacteria on the soil microbiome is not quantifiable and in the long term, they are unlikely to increase antibiotic resistance significantly as shown in our study. Another possibility is that the primary ecological role of naturally-produced antibiotics is to inhibit the growth of other soil organisms (Kelsic et al., 2015), alleviating competition for scarce resources. The microbes in RW-irrigated soils receive more carbon and nitrogen while facing less competition for resource; 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

Contrary to the inappreciable 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 genes as well.

4.2.1. 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, 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 than in the latter (10.35). This may facilitate microbial activity to mineralize organic N in Greenhouse A soil. The difference in total N and OM between the two greenhouses could result from 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-associated microbes belonged to Proteobacteria, Actinobacteria and Firmicutes, consistent with previous studies (Wu et al., 2021).

4.2.2. 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-borne MRGs-associated ARGs possessed a high horizontal transfer probability. The high correlation between ARGs and ISs (Table S9) also indicated that MGEs are crucial to the ARG spread. The IS*Cco2* and other MGEs were also found to be dominant in other environments and play a key role in ARG 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 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 interact to shift the ARGs making them differ between the two cropping systems.

4.3. Implications for future research

Our results suggest 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 were associated with the biocide resistance genes *sugE* and *adeL*, the metal resistance gene *trgB* and the antibiotic resistance genes *murA* and *mtrA* together with IS*1595* in Greenhouse A. In contrast, Greenhouse B were characterized largely by metal resistance genes and associated with the insertion sequences IS*NCY* and IS*701*. We are unable to determine whether the characteristic resistance genes in each soil were structurally associated with the characteristic ISs, but the data is suggestive of associations between specific resistance genes and ISs in the two greenhouse soils. Our unpublished data from a separate experiment suggests that this is a consistent response of soil, where irrigation with livestock wastewater with different cropping systems showed cropping had a significant influence on ARG dissemination in soil, despite the underlying mechanisms remaining elusive. This is corroborated by our experiments that legume roots absorb more antibiotics than grass roots due to the differences in their root properties (http://kd.nsfc.gov.cn/advancedQuery/personInfo/b86bca4a5e8002797998c5bc02c04feb). The experiment studied in this paper did not allow us to determine that the differences in gene distributions between the cropping systems are the legacy of different cropping regimes over the 16 years or just the short-term effects of legume versus brassicaceae 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 (pH, EC, OM, NH_4^+-N, available-P and available-K) and the total concentration of antibiotics of soil at significant levels after RW irrigations for 16 years. The concentration of soil available heavy metals was reduced following RW irrigation with a few exceptions, while the assemblages of ARGs, MRGs, BRGs, ISs and microbial taxa in soils irrigated with RW were similar to those irrigated with GW. Although alternate GW and RW irrigation reduced the total input of ARGs and the associated ARG propagators to the soils, it did not influence ARG dispersal significantly. We showed that differences in cropping regimes, which have been unaccounted for in previous studies, can exert a greater influence upon the distribution of resistance genes in soils than irrigation waters. Our results implicate that the influence of factors other than irrigation water, such as planting, on ARG diffusion in soil warrants more research effort.

CRediT authorship contribution statement

Yuan Liu: Conceptualization, Funding acquisition, Data curation, Visualization, Writing – original draft. **Andrew Neal:** Conceptualization, Funding acquisition, Supervision, Bioinformatics analysis and visualization, Writing – review & editing. **Xiaoxian Zhang:** Conceptualization, Investigation, Writing – review & editing. **Haiyan Fan:** Conceptualization, Resources, Investigation, Data curation. **Honglu Liu:** Supervision, Project administration, Investigation. **Zhongyang Li:** Conceptualization, Supervision, Resources, Funding acquisition.

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

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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.jhazmat.2021.128046.

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