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# Elevated $CO_2$ alleviated the dissemination of antibiotic resistance genes in sulfadiazine-contaminated soil: A free-air $CO_2$ enrichment study

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## ABSTRACT

Climate change affects soil microbial communities and their genetic exchange, and subsequently modifies the transfer of antibiotic resistance genes (ARGs) among bacteria. However, how elevated $CO_2$ impacts soil antibiotic resistome remains poorly characterized. Here, a free-air $CO_2$ enrichment system was used in the field to investigate the responses of ARGs profiles and bacterial communities to elevated $CO_2$ (+200 ppm) in soils amended with sulfadiazine (SDZ) at 0, 0.5 and 5 mg $kg^{-1}$. Results showed that SDZ exposure induced the co-occurrence of beta-lactamase and tetracycline resistance genes, and SDZ at 5 mg $kg^{-1}$ enhanced the abundance of aminoglycoside, sulfonamide and multidrug resistance genes. However, elevated $CO_2$ weakened the effects of SDZ at 0.5 mg $kg^{-1}$ following an observed reduction in the total abundance of ARGs and mobile genetic elements. Additionally, elevated $CO_2$ significantly decreased the abundance of vancomycin resistance genes and alleviated the stimulation of SDZ on the dissemination of aminoglycoside resistance genes. Correlation analysis and structural equation models revealed that elevated $CO_2$ could directly influence the spread of ARGs or impose indirect effects on ARGs by affecting soil properties and bacterial communities. Overall, our results furthered the knowledge of the dissemination risks of ARGs under future climate scenarios.

**Keywords:** $CO_2$, Antibiotic resistome, Microbial communities, Sulfadiazine

## Environmental Implication

Rising antibiotic resistance genes (ARGs) is a huge challenge for global healthcare systems. The dissemination of ARGs may be modified by climate change. However, the impact of elevated $CO_2$ on ARGs remains poorly characterized, especially under realistic exposure scenarios. Here, we investigated responses of ARGs profiles to elevated $CO_2$ in soils amended with sulfadiazine using a free-air $CO_2$ enrichment system. Results revealed that elevated $CO_2$ alleviated the dissemination of ARGs by direct inhibition and indirectly affecting soil properties and bacterial composition. These results furthered our knowledge of the dissemination risks of ARGs under future climate scenarios.

## 1. Introduction

Rising antimicrobial resistance has become a global concern and a huge challenge for the medical and scientific community. It is estimated that drug-resistant diseases cause around 700,000 deaths annually, which will reach 10 million by 2050 (O'Neill, 2014). Meanwhile, climate change is considered as an important impetus of emerging infections worldwide and a potential "threat multiplier" of antibiotic resistance in human pathogens (Fouladkhah et al., 2020; Li et al., 2022). Thus, the dissemination and transfer of antibiotic resistance genes (ARGs) could potentially be modified by climate change, but this has seldom been studied (Zheng et al., 2022).

Under the background of global warming, the concentration of $CO_2$ in the atmosphere has been increasing since the pre-industrial era and is predicted to reach 430–1000 ppm by 2100 (IPCC, 2021). Elevated $CO_2$ can facilitate C flow, influence cell membrane permeability, and alter the interaction and function of microbiota (Yu and Chen, 2019), the effects of which may result in alterations of the transfer of ARGs between microbes (Liao et al., 2019a). Limited studies have reported that the dissemination of ARGs via plasmid-mediated conjugation is intensified under elevated $CO_2$ levels (Liao et al., 2019b). Increasing $CO_2$ was found to reduce intercellular repulsion, facilitate the mobilization and channel transfer of ARGs carried on plasmids, and increase proton motive force by providing more power for DNA uptake (Liao et al., 2019b). By contrast, we previously found that elevated $CO_2$ diminished the abundances

of sulfonamide resistance genes (*sul1* and *sul2*), tetracycline resistance genes (*tetG* and *tetM*) and the class 1 integron in paddy soil, and it also alleviated the effects of sulfamethazine on the sulfonamide resistance genes by inhibiting ARG hosts (Xu et al., 2020; Xu et al., 2021). Agricultural soil is a complex and dynamic ecosystem, and it also constitutes an important habitat for the exchange of ARGs among bacteria and a vital reservoir of antibiotics (Xiang et al., 2021). Yet, the complicated bioprocesses mediated by high $CO_2$ concentration and antibiotics remain unclear, which hindered our reliable predictions regarding the mechanism of ARG spread in changing climate conditions, and the development of management strategies for mitigating antibiotic resistance in soil ecosystems (Zhu et al., 2018c).

The presence of antibiotics may encourage the transfer of ARGs and impose potent selection pressures on microbial communities (Berendonk et al., 2015). Sulfonamides are currently the most popular class of broad-band bacteriostatic antibiotics and are widely used to protect the health of animals and humans worldwide (Haack et al., 2012; Schauss et al., 2009). They are poorly absorbed in the bodies of animals, and about 90% of sulfonamides are excreted into the environment as parent compounds or metabolites (Cheng et al., 2020). Driven by manure application and wastewater irrigation, the detected concentration of sulfonamides at the range of 1.29-2.45 mg $kg^{-1}$ in agricultural soils adjacent to feedlots, and the contamination level has been still increasing (Ji et al., 2012). Sulfonamides were previously reported to affect microbial

diversity, composition and functions (Haack et al., 2012; Ma et al., 2014), while simultaneously promoting the occurrence and spread of ARGs and mobile genetic elements (MGEs) including plasmids, transposons and integrons (Cleary et al., 2016; Makowska et al., 2016; Zhao et al., 2020). These MGEs are responsible for the genetic exchange between different microbes via assisting the horizontal gene transfer (HGT) of ARGs among bacteria (Wang et al., 2022). This further induces the emergence and dissemination of pathogenic antibiotic-resistant bacteria (ARB) and multi-resistant bacteria, posing a threat to clinical therapeutics and human health (Xie et al., 2022). In the long term, the soil antibiotic resistome will inevitably be exposed to the joint stress of sulfonamide residues and climate change as the environmental burden of sulfonamides increases at its present rate. However, there is a paucity of data on the emergence and dissemination of ARGs in sulfonamide-contaminated soil under future $CO_2$ levels, limiting our ability to predict the risks of ARGs under future scenarios.

In the present study, a free-air $CO_2$ enrichment (FACE) experiment was conducted in the field to explore the responses of soil antibiotic resistome and microbial communities to sulfadiazine (SDZ) under different $CO_2$ levels. The key purposes were: (1) to research the impacts of elevated $CO_2$ on the antibiotic resistome in SDZ-contaminated soil with high-throughput quantitative polymerase chain reaction (HT-qPCR), (2) to identify the responses of soil microbial communities to elevated $CO_2$ by sequencing the bacterial 16S rRNA

gene, and (3) to explore the potential mechanism of the effect of elevated $CO_2$ on the dissemination of ARGs in soil. The hypotheses are: (1) elevated $CO_2$ may alter the effects of SDZ on ARGs; and (2) changes in soil properties, MGE abundance or bacterial communities under elevated $CO_2$ might drive shifts in the soil antibiotic resistome.

## 2. Materials and methods

### *2.1. FACE system*

This study was conducted using the FACE platform located in Xiaoji Town, Yangzhou City, Jiangsu Province, China (119°42′0″ E, 32°35′5″ N). The FACE system has been described in detail in previous studies (Guo et al., 2011; Zhu et al., 2012). The system consisted of three comparative octagonal rings of ambient $CO_2$ (ambient plots, $CO_2$ concentration at about 390 ppm) and three rings with the target $CO_2$ concentration at about 200 ppm higher than the ambient conditions (FACE plots, the concentration was based on the predicted $CO_2$ concentration in 2100) (IPCC, 2021). FACE plot were encircled with an octagonal ring (an area of 80 $m^2$) that injected pure $CO_2$ gas above the plant canopy throughout the growth of rice. The target $CO_2$ concentration within FACE plots was controlled by a computer program with an algorithm based on wind speed and direction. Ambient plots did not receive any supplemental $CO_2$ and were 90 m away from FACE plots (Zhu et al., 2016). The site is situated in a typical Chinese rice-growing region with a mean annual precipitation of 980 mm and a temperature of 15 °C (Zhu et al., 2014).

153 *2.2. Materials and experiment setup*

154 SDZ (purity ≥98%) was acquired from Dr. Ehrenstorfer GmbH (Augsburg,
155 Germany). Surface soil (top 20 cm), classified as Shajiang-Aquic-Cambosols,
156 was collected from adjacent farmland for the pot experiment. The soil properties
157 are listed in Table S1. Fresh soil was air-dried and ground to less than 5 mm.
158 SDZ was dissolved in methanol and spiked in a sub-portion of soil (500 g), with
159 the control soils also treated with the same volume of methanol. After removal
160 of the methanol by evaporation in a fume hood for 24 h, the spiked soils were
161 progressively diluted and mixed thoroughly with the untreated soils, leading to
162 final SDZ concentrations of 0, 0.5 and 5 mg $kg^{-1}$ soil (dry weight). The used
163 SDZ concentrations in this study are within the same order of magnitude of
164 sulfonamide concentrations reported in soil from agricultural fields and animal
165 feedlots (Ji et al., 2012). A 4 kg aliquot of treated soil was packed into a plastic
166 pot (17 cm in diameter, 20 cm in height). Each treatment was carried out in
167 triplicate and evenly distributed in both the FACE and ambient rings. Rice
168 (*Oryza sativa* L. cv. Wuyunjing 23) plantlets were transplanted on 22 June with
169 two hills per pot and three plantlets per hill. Destructive sampling was conducted
170 at the harvest stage on 30 October 2017. Field management of the pots
171 followed the agricultural practice of local farmers. The soil in pots was
172 submerged in water until 10 days before harvest. All pots were fertilized three
173 times with compound fertilizer ($N–P_2O_5–K_2O$=15–15–15), with 50%, 25%, and
174 25% of the total nitrogen (22.5 g $m^{-2}$) applied before transplanting, tillering, and

heading, respectively (Xu et al., 2023).

### *2.3. Soil analysis and bacterial DNA extraction*

At harvest, the rice was uprooted and shaken to remove the loose soil, and the remaining soil near the roots (<1 cm) was collected as rhizosphere soil (Dong et al., 2021). The rhizosphere soil was sampled in five portions randomly to form one composite sample and immediately transported to the laboratory using sterile plastic bags and an ice box. The Kjeldahl method was adopted to determine soil total nitrogen (Bremner, 2009). The molybdenum-blue colorimetry method was used to determine total phosphate (Han et al., 2012). The concentrations of Cu, Zn, Pb and Ni were measured using a flame atomic absorption spectrometer (FAAS, Hitachi Z-2000, Tokyo, Japan) after digestion with $HNO_3$-HF-$HClO_4$ (5:3:3, v/v/v) (Wang et al., 2020). The concentration of antibiotics in soil was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Xu et al., 2020).

A FastDNA Spin Kit (MP Biomedical, France) was used to extract soil DNA following the manufacturer's recommendations (Zhou et al., 2019). The DNA samples were kept at –80 °C for further analyses. The DNA was tested for concentration and quality using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

### *2.4. High-throughput quantitative PCR (HT-qPCR)*

HT-qPCR was conducted with a SmartChip Real-time PCR platform (Wafergen Inc., USA) to determine the composition and abundance of ARGs

and MGEs (Zhu et al., 2013). The HT-qPCR included 296 primer sets for one 16S rRNA gene, 10 MGEs, and 285 ARGs conferring resistance to eight classes of antibiotics: aminoglycoside, beta-lactamase, MLSB (macrolide-lincosamide-streptogramin B), multidrug, FCA (fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol), sulfonamide, tetracycline and vancomycin (Zhu et al., 2013). The reaction system and conditions of HT-qPCR were kept the same as the previous study (Zhu et al., 2018b). The HT-qPCR data were analyzed using the SmartChip qPCR software (v2.7.0.1, WaferGen Biosystems, Inc.; Takara Bio). The qPCR results were manipulated based on the following criteria, i.e. wells with multiple melting peaks or amplification efficiency beyond the range (1.8–2.2) were removed from the analysis (Zhu et al., 2018a). To minimize the error produced by DNA extraction and bacterial abundance, the abundances of ARGs and MGEs were normalized based on 16S rRNA genes and expressed as ARG/MGE copies per bacterial cell.

### *2.5. 16S rRNA gene sequencing*

The V4–V5 region of the 16S rRNA gene was amplified using the primers 515F (GTGCCAGCMGCCGCGG) and 907R (CCGTCAATTCMTTTRAGTTT) for the assessment of bacterial communities. Sequencing was performed with a MiSeq 300 instrument with Illumina MiSeq Kit v2 (Majorbio BioPharm Technology Co. Ltd., Shanghai, China) (Liu et al., 2014). Low-quality reads, and ambiguous nucleotides and barcodes were filtered before the assembly of raw pair-end reads. Qiime version 1.9.1 was used to process and analyze high-

quality sequences (Caporaso et al., 2010). Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE and assigned to taxonomy using the Ribosomal Database Project Classifier (Edgar, 2013). The raw sequencing data (project accession number PRJNA758632) were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra/).

### *2.6. Statistical analysis*

Data were presented as mean and standard deviations that were generated using IBM SPSS Statistics v26 (IBM Corporation, Armonk, NY, USA). This software was also used to detect the significant differences among treatments using one-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) tests. Alpha-diversity indexes of bacterial communities were calculated using Mothur v1.30.2. The structures of ARGs and bacterial communities were revealed by principal coordinates analysis (PCoA) based on the Bray–Curtis distance using Past software and visualized in OriginPro 2018 (OriginLab, USA) (Xu et al., 2023). Heatmaps were used to display the composition patterns of ARG subtypes and dominating bacterial genera, which were produced by the "pheatmap" package in R. Ordinary least squares regression analysis was performed using the R package "reshape" (Xiang et al., 2019). To explore the co-association between bacterial taxa and ARGs, network analysis was carried out using the R software "psych" package and visualized in Gephi 0.9.2 (Chen et al., 2016). OTUs with relative

abundance >0.1% were excluded for network analysis to explore the response of bacterial co-occurrence patterns to elevated $CO_2$ (Halary et al., 2010). Structural equation models (SEMs) were generated based on the procedures described by Chen et al., (2018). Before SEM construction, we performed pairwise correlation analysis to assess the importance of environmental factors, including soil properties (e.g., soil pH, TN, TP, Zn, Ni, Cu and Pb concentration) and bacterial diversity (including Chao1, Ace, Simpson and Shannon indexes). Soil variables that were screened out as significant predictors were introduced to the SEM construction (Du et al., 2020; Han et al., 2018). The first PCoA axis was incorporated into the SEM as the bacterial structure (Chen et al., 2019). The SEMs were constructed using AMOS 22 (SPSS Inc., Chicago, USA) with maximum-likelihood estimation.

## 3. Results

### *3.1. Diversity of ARGs and MGEs*

In total, 9 MGEs (4 transposase genes and 5 integrons) and 123 ARGs were detected among all soil samples. The numbers of ARGs and MGEs detected in different treatments are listed in Table 1. Among these samples, the top 4 dominant types were aminoglycoside, beta-lactamase, multidrug and tetracycline resistance genes. Under ambient conditions, SDZ at 0.5 and 5 mg $kg^{-1}$ increased the number of detected ARGs from 71 to 75 and 81, respectively, with a notable increase in the number of ARGs resistant to beta-lactamase and tetracycline. Comparably, 68 and 78 ARGs were detected in 0.5SDZ and 5SDZ treatments under FACE conditions, respectively, indicating that elevated $CO_2$

potentially alleviated the stimulation of SDZ on ARG diversity. Comparing control soils from ambient and FACE conditions, elevated $CO_2$ itself did not impact the diversity of ARGs and MGEs.

The bipartite network highlights shared and unique ARG subtypes between different treatments (Figure 1). Compared with the controls, 14 unique ARGs (*aadA9, bla-ACC-1*, *blaGES*, *blaOXA1_blaOXA30*, *blaPER*, *blaSFO*, *erm(34)*, *ermK-01*, *mdtA*, *oleC*, *emrD*, *yceL_mdtH*, *tetB* and *tetL*) were observed in SDZ-amended soils under both ambient and FACE conditions. These shared ARGs consisted of beta-lactamase (5), MLSB (4), multidrug (2), tetracycline (2) and aminoglycoside (1). In addition, 2 shared ARGs (*blaSHV* and *spcN*) were identified between control and SDZ-amended soils under ambient conditions (Figure 1a), whereas 3 shared ARGs (*catB3*, *bacA* and *tetA*) were found between control and FACE conditions (Figure 1c); this indicated that elevated $CO_2$ levels exert selective pressure on the expression of these genes.

### *3.2. Abundance of ARGs and MGEs*

The abundances of ARGs varied from 0.005 to 0.026 copies per bacterial cell, and the abundances of MGEs ranged from 0.001 to 0.007. As shown in Figure 2a, SDZ at 5 mg $kg^{-1}$ significantly increased the abundance of total ARGs by 1.21- and 0.82-fold under ambient and FACE conditions, respectively, compared with the control soils. Comparing 0.5SDZ under ambient conditions with 0.5SDZ under FACE conditions, elevated $CO_2$ significantly lowered the total ARG abundance by 47.3% and that of MGEs by 65.0%. At the ARG class level, all classes remained unchanged under the low-concentration treatment. Under ambient conditions, SDZ at 5 mg $kg^{-1}$ significantly increased the

abundance of aminoglycoside, multidrug and sulfonamide resistance genes. Elevated $CO_2$ significantly decreased the abundance of vancomycin resistance genes in both control and SDZ-amended soils. A comparison of 5SDZ from FACE conditions and ambient conditions showed that elevated $CO_2$ reduced the abundance of aminoglycoside resistance genes. For MGEs, SDZ at 0.5 mg $kg^{-1}$ increased the abundance of transposase genes, which was not observed under FACE conditions (Figure 2b). PCoA revealed that SDZ treatments, especially SDZ at 5 mg $kg^{-1}$, were significantly separated from the control soils through the first axis, which explained 38.4% of the ARG variance. Samples from ambient conditions were separated from FACE conditions mainly through the second axis, which explained 15.7% of ARG variance (Figure 2c).

The relative abundance of ARG subtypes is displayed in a heatmap (Figure 3). Elevated $CO_2$ by itself diminished the abundance of *aacC* and *int-1*(clinic), whereas SDZ at different concentrations exerted pressure on particular subtypes of ARGs. SDZ at 0.5 mg $kg^{-1}$ significantly boosted the abundance of *ttgA*, *vanXD* and *vanYD*, but abated the abundance of *aacC* and *int-1*(clinic), whereas SDZ at 5 mg $kg^{-1}$ increased the abundance of *aadA2*, *blaOXA10* and *vanYD*. Comparatively, under the FACE conditions, SDZ application changed the abundance of more resistance genes. For instance, selected genes, like *aacC*, *aadA2*, *amPC*, *blaCTX-M*, *blaOXA10*, *erm (36)* and *vanC* were detected at lower abundance, whereas the abundance of some ARGs (*strB*, *blaVEB*, *blaSFO*, *qacEdelta1*, *ttgB*, *sul-olP*, *vanA*, *vanSB* and *vanXD*) were elevated

under combined treatments.

### *3.3. Microbial community assembly*

The effects of either SDZ or elevated $CO_2$ alone were not statistically significant on bacterial alpha-diversity concerning the Shannon index. A comparison of 5SDZ from ambient and FACE conditions showed that elevated $CO_2$ significantly decreased bacterial diversity ($p<0.05$) (Figure 4a). PCoA showed that the overall patterns of the bacterial community were altered by elevated $CO_2$. Soil from ambient and FACE conditions were significantly separated (Adonis test, $p = 0.019$) along the PCoA 1 axis (explaining 23.8% of variance), whereas the SDZ treatments did not alter bacterial structure under either elevated and ambient $CO_2$ conditions (Figure 4b). Bacterial compositions were also affected by elevated $CO_2$ and SDZ treatments. At the phylum level, Proteobacteria, Chloroflexi, Acidobacteria and Actinobacteria dominated across all samples. SDZ and elevated $CO_2$ alone had no significant effects on bacterial phylum. However, combined elevated $CO_2$ and SDZ at 5 mg $kg^{-1}$ significantly boosted the abundance of Actinobacteria and Firmicutes, but reduced the abundance of Bacteroidetes and Nitrospirae (Figure 4c, Table S3). The heatmap revealed that the composition patterns of dominant genera showed that soils from the same $CO_2$ level shared a similar microbiome composition and that microbiomes of the same treatment were significantly dissimilar under different $CO_2$ levels (Figure S1). Exposure to SDZ exhibited significant effects on the abundance of *o_Chloroplast*, *Geothrix* while elevated $CO_2$ levels

significantly increased the abundance of *Gaiella*, *Nocardioides*, *Haliangium*, *Defluviicoccus* and *Bacillus*.

Changes in responses of bacterial co-occurrence patterns were also observed under elevated $CO_2$ levels. The FACE network contained a comparable number of nodes but more edges than did the ambient network (Table S4), which indicated that more microbial interactions existed under high $CO_2$ concentrations. Additionally, elevated $CO_2$ decreased the modularity module and average path length, but increased the average clustering coefficient, graph density and average degree (Figure 4d–e, Table S4).

### *3.4. Multiple factors shaping ARG profiles*

HGT plays a vital role in ARG dissemination mediated by MGEs. Integrons and transposons are major MGEs associated with the capture, mobilization and spread of ARGs among bacteria. Integron-encoded integrase can recombine gene cassettes, and some classes of integron are responsible for the emergence and wide dissemination of ARGs, especially among Gram-negative clinical isolates (Makowska et al., 2016). Transposase is an important catalyst involved in DNA cleavage and jointing reactions, and transposase-encoded genes are important transport in shaping and organizing ARGs (Nicolas et al., 2017). Thus, Ordinary least squares (OLS) regression analysis was used to determine the relationships between the pattern of ARGs and MGEs estimated with the relative abundance of ARGs at the class level, integrase and transposases (Fig. 5). Results showed that the abundance of

aminoglycoside resistance genes was linearly and positively correlated with integrase abundance ($R^2$=0.4286, $p$=0.0032), and the abundance of sulfonamide resistance genes was positively correlated with the abundance of transposase ($R^2$=0.2391, $p$=0.0395) (Figure 5). Additionally, soil properties can also affect the patterns of ARGs (Han et al., 2018). Spearman's correlation analysis showed that the abundance of total vancomycin resistance genes was negatively correlated with soil total nitrogen (TN, r=−0.773, $p$<0.001) (Table S5). Concerning the ARG subtypes, soil TN expressed a significant negative correlation with the abundances of *aacC*, *erm(36)* and *vanC* (Figure S2).

Network analysis showed that a total of 34 bacterial genera were identified as having a strong and significant correlation with ARGs (Figure 6, detailed data provided in Table S6). Proteobacteria, Firmicutes and Actinobacteria were highly connected with multiple ARG subtypes and were thought to be important potential hosts harboring ARGs. For example, five genera affiliated with Firmicutes (*Clostridium*_sensu_stricto_1, *Clostridium*_sensu_stricto_10 *Clostridium*_sensu_stricto_8, *Fonticella* and *Tumebacillus*) whose abundances were increased under combined SDZ and elevated $CO_2$ treatments were correlated with three ARG subtypes (*vatB*, *erm(36)* and *tetL*/*aacC*) (Table S6). Additionally, we found that a single ARG was correlated with different bacterial taxa. The vancomycin resistance genes subtype (*vanC*) was negatively correlated with *Defluviicoccus* and *Solirubrobacter*, whose abundances also

increased under combined exposure to SDZ and elevated $CO_2$ (Table S6).

The SEM results showed that SDZ was the most powerful positive feature shaping the ARG profiles, followed by bacterial diversity and MGEs, whereas $CO_2$ and soil variables had negative effects on the abundance of ARGs (Figure 7). Specifically, SDZ directly impacted the abundance of ARGs (λ=0.812***, $p$<0.001). $CO_2$ directly impacted the abundance of ARGs (λ=−0.645***, $p$<0.001) and imposed indirect effects on ARGs by influencing soil variables (λ=0.831***, $p$<0.001) and bacterial diversity (λ=0.829*, $p$=0.028). Soil variables indirectly influenced ARGs by affecting bacterial diversity (λ=−0.844*, $p$=0.025). Moreover, bacterial structure indirectly affected the abundance of ARGs by influencing the abundance of MGEs which had a direct positive effect on the abundance of ARGs (λ=0.180*, $p$=0.019).

## 4. Discussion

### *4.1. Co-selection of SDZ on the occurrence and dissemination of ARGs*

Following SDZ exposure, we observed significantly increased diversity of ARGs and enhanced occurrence of non-corresponding ARG types including beta-lactamase and tetracycline resistance genes, indicating important co-selection effects of SDZ on the development of resistance. Similarly, previous investigation found that the application of a single antibiotic could enrich multiple ARGs associated with other antibiotic classes, which are widely reported in the resistomes of animals and the human gut, and systems for wastewater treatment (Zhao et al., 2020). The development of co-resistance may be attributed to the evolution of multi-resistant microorganisms or acquired

399 through HGT or gene mutations (Zhao et al., 2021). It was previously reported
400 that beta-lactams exhibited effects on bacterial mutations and recombination
401 rates, whereas sulfonamide and tetracycline resistance genes were frequently
402 carried by plasmids and co-occurred in environment samples (Gutierrez et al.,
403 2013; Zhao et al., 2021).

404 In addition to the enrichment of multiple ARG types caused by SDZ
405 exposure, the dissemination and abundance of different ARGs were also
406 affected in this study. SDZ at 5 mg $kg^{-1}$ significantly increased the abundance
407 of ARGs resistant to aminoglycoside, sulfonamide and multidrug without
408 affecting other types of ARGs. Generally, the multidrug, sulfonamide and
409 aminoglycoside resistance genes were more easily inducible than other ARG
410 classes (Zhao et al., 2021). Additionally, some aminoglycoside resistance
411 genes were found to occur in the same genetic elements with sulfonamide
412 resistance genes (Chung et al., 2015). This combined with our correlation and
413 network analysis confirmed that SDZ exposure may induce bacterial intrinsic
414 resistance to aminoglycoside or facilitate the transfer of exogenous genes
415 mediated by MGEs (Zhu et al., 2017). It is worth noting that the abundance of
416 multidrug resistance genes was dramatically elevated following to SDZ
417 exposure under both ambient and FACE conditions. The multidrug resistance
418 genes, especially the clinically relevant multidrug efflux pump clusters, are
419 usually chromosomally encoded and more likely to co-occur with other ARGs
420 (Zhao et al., 2020). Genes encoding multidrug efflux pumps on the
421 chromosome may favor bacteria with an intrinsic mechanism to develop

resistance to antibiotics, and potentially induce the evolution of multi-resistant bacteria (Zhao et al., 2020). Thus, the over-expression of these genes poses a great threat to resistance levels in the environment. Further understanding of co-resistance mechanisms, including mutations and ARG transmission, especially in multi-resistant bacteria, is warranted as a step toward mitigating the dissemination of ARGs.

### *4.2. Elevated $CO_2$ alleviated the dissemination of ARGs*

Our research was the first to show that elevated $CO_2$, directly and indirectly, impacted the soil antibiotic resistome by altering soil variables, bacterial diversity and composition. In this research, elevated $CO_2$ significantly decreased the abundance of total ARGs and MGEs under low-concentration treatments of SDZ (0.5 mg $kg^{-1}$), which indicated that elevated $CO_2$ potentially alleviated the transmission of ARGs through HGT mediated by MGEs. The 0.5 mg $kg^{-1}$ treatment is considered a conservative treatment concentration under future high $CO_2$ concentrations. At the ARG class level, elevated $CO_2$ significantly decreased the abundance of ARGs resistant to vancomycin. This may be attributed to increased TN, which showed a significant negative correlation with the abundance of vancomycin resistance genes. Previous publications have reported that adding nutrients, including N and P, induced greater resistance to tetracycline and ciprofloxacin antibiotics without affecting vancomycin resistance (Ali et al., 2016; Li et al., 2020). A factor responsible for the divergent responses could be the difference in the mechanism of resistance

444 to the antibiotics. For example, the main mechanism for vancomycin resistance
445 is cellular protection, and the main mechanism for tetracycline resistance is
446 efflux pump (Wang et al., 2019b). Nevertheless, the abundances of two
447 bacterial genera (*Defluviicoccus* and *Solirubrobacter*), which were negatively
448 correlated with the *vanC* gene, were increased under elevated $CO_2$ levels, with
449 these bacterial genera potentially being capable of inhibiting the survival or
450 dissemination of vancomycin resistance genes (Peng et al., 2016). Furthermore,
451 elevated $CO_2$ significantly reduced the dissemination of aminoglycoside
452 resistance genes in the SDZ treatment at 5 mg $kg^{-1}$. The abundances of
453 aminoglycoside resistance genes and integrase genes were positively
454 correlated. Thus, we infer that elevated $CO_2$ may inhibit the HGT of
455 aminoglycoside resistance genes via diminishing the abundance of integrase
456 genes. Moreover, the abundance of *Defluviicoccus,* which was negatively
457 correlated with the *aacC* gene, was increased under the combined treatment,
458 potentially inhibiting the survival of the ARGs (Peng et al., 2016).

459 The changes in the topological properties of bacterial networks may also
460 modify the soil antibiotic resistome. The nodes with higher betweenness,
461 degree and closeness centrality in the bacterial network under elevated $CO_2$
462 than that at ambient conditions may alter ARGs co-occurrence and regulate
463 ARGs transfer (Xiang et al., 2023). Besides, significant direct inhibition of
464 elevated $CO_2$ on the abundance of ARGs was revealed by SEM analysis.
465 However, only very few studies described that elevated $CO_2$ could impact cell

membrane permeability, carbon transfer efficiency, biofilm formation and the leakage of intracellular substances, which further alters microbial activities and influences the joint transfer of ARGs within genera (Liao et al., 2019b). Since soil is a complex ecosystem, further mechanistic studies are warranted to identify the mechanisms underlying ARG dissemination in future climate scenarios (Zhu et al., 2019).

### *4.3. Elevated $CO_2$ and SDZ differentially influenced soil bacterial communities*

Antibiotics may restrain bacterial growth or cause bacterial death, leading to changed soil microbial community structure and functions (Xie et al., 2018; Xu et al., 2016). However, our results indicated that SDZ application showed little effect on soil microbial communities after the full life cycle of rice (about 130 days exposure), perhaps because of two main factors: (1) SDZ is rapidly degraded with a half-life in the range of 3–17 days in silty-clay soil and the metabolites may not be toxic to bacteria (Kreuzg and Holtge, 2005; Wang et al., 2019a), and (2) SDZ has a high affinity to the soil matrix and may become non-extractable (non-bioavailable) with previously published research demonstrating non-extractable residues in the range of 35%–58% of the applied amount within the first day (Hammesfahr et al., 2008; Kreuzg and Holtge, 2005; Xu et al., 2016).

Compared with SDZ, elevated $CO_2$ showed significant effects on soil microbial composition and structure. Numerous studies have demonstrated that elevated $CO_2$ alters nutrient cycling, and changes quantities and qualities of

rhizodeposition and root exudates (Drigo et al., 2013; Wang et al., 2019b; Williams et al., 2000). This may further affect microbial enzyme synthesis and change microbial community composition (Garcia-Palacios et al., 2015; Guenet et al., 2012). Additionally, we found that elevated $CO_2$ significantly decreased bacterial alpha-diversity estimated by the Shannon evenness index in SDZ-amended soil at 5 mg $kg^{-1}$. One possible reason for this is disequilibrium between sensitive bacteria and resistant bacteria such as ARG hosts (Wang et al., 2018). As shown above, combined exposure to elevated $CO_2$ and SDZ significantly increased the abundance of potential ARG hosts including many genera affiliated with the phyla Proteobacteria, Firmicutes and Actinobacteria, and this was positively correlated with ARG resistance to MLSB and tetracycline. Another possible reason is that, as revealed by SEM analysis, soil properties negatively impacted bacterial diversity. Other studies have reported similar results (Drigo et al., 2013; Wang et al., 2019b). Elevated $CO_2$ can increase the C/N ratio in the rhizosphere, which favors organisms with lower nutrient requirements and constrains the responses of other genera under high $CO_2$ concentrations, resulting in changed species distribution (Garcia-Palacios et al., 2015; Wang et al., 2019b). Our further network analysis also confirmed that the higher degrees of centralization, transitivity and complexity indicated greater susceptibility of bacteria to external interferences under future higher $CO_2$ conditions.

## 5. Conclusions

In summary, our study investigated the response of the antibiotic resistome to future elevated $CO_2$ levels in sulfonamide-contaminated soils under field conditions. The presence of SDZ exhibited co-selection on the occurrence and dissemination of non-corresponding ARG types. Elevated $CO_2$ alleviated the effects of SDZ at 0.5 mg $kg^{-1}$ on total ARG abundance, and it also inhibited the spread of vancomycin and aminoglycoside resistance genes. Modified soil properties and bacterial communities under elevated $CO_2$ levels contributed to the altered dissemination of ARGs in SDZ-amended soils. Our results highlight that the future elevated $CO_2$ levels could alleviate the risks of soil ARGs to some extent, but sustainable management strategies are warranted to mitigate antibiotic contamination. The mechanisms underpinning this effect still need to be uncovered in future studies.

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**Figure captions:**

**Figure 1.** Network analysis revealing shared and unique ARGs among treatments. (a) Two ARGs shared between the control and SDZ-amended soil under ambient conditions; (b) 14 ARGs unique to SDZ-amended soil; (c) 3 ARGs shared between the control and SDZ-amended soil under FACE conditions; (d) 2 ARGs detected in two treatments; (e) 9 ARGs detected in three treatments; (f) 17 ARGs detected in four treatments; and (g) 54 ARGs were found in at least five treatments.

**Figure 2.** (a) The relative abundance of total ARGs and MGEs. (b) The relative abundance of each class of ARGs and MGEs. (c) Principal coordinate analysis based on the Bray−Curtis distance showing the distribution pattern of ARGs. Control. A, ambient (390 μmol $mol^{-1}$) control; Control. F, FACE (590 μmol $mol^{-1}$) control; 0.5SDZ. A, ambient with 0.5 mg $kg^{-1}$ SDZ; 0.5SDZ. F, FACE with 0.5 mg $kg^{-1}$ SDZ; 5SDZ.A, ambient with 5 mg $kg^{-1}$ SDZ; 5SDZ.F, FACE with 5 mg $kg^{-1}$ SDZ. Data are means ± standard deviation for triplicate samples. Different letters among bars indicate statistically significant differences at $p < 0.05$.

**Figure 3.** Heatmap of the normalized abundance of ARG subtypes in different treatments. Control. A, ambient (390 μmol $mol^{-1}$) control; Control. F, FACE (590 μmol $mol^{-1}$) control; 0.5SDZ. A, ambient with 0.5 mg $kg^{-1}$ SDZ; 0.5SDZ.F, FACE with 0.5 mg $kg^{-1}$ SDZ; 5SDZ.A, ambient with 5 mg $kg^{-1}$ SDZ; 5SDZ.F, FACE with 5 mg $kg^{-1}$ SDZ. The "*" indicates a significant difference between the control soil (Control. A) and other treatments (Significance level: 0.05).

**Figure 4.** (a) Alpha-diversity indexes for the bacterial communities estimated by Shannon index. (b) Principal coordinate analysis of bacteria based on Bray–Curtis metric distance. (c) The composition of bacteria at the phylum level in different treatments. Control, 0.5SDZ and 5SDZ indicate the soil amended with SDZ at 0, 0.5 and 5 mg $kg^{-1}$, respectively. A and F indicate the ambient and FACE conditions, respectively. The network co-occurrence analysis of bacterial OTUs with abundance > 0.1% in soils under ambient (d) and FACE (e) conditions.

**Figure 5.** Ordinary least squares (OLS) regression showing the relationship between the relative abundance of ARGs at different classes and those of integrase and transposases.

**Figure 6.** Network analysis of co-occurring ARGs and the dominant bacterial genera with abundance > 0.1%. Each connection represented a strong (Spearman's coefficient r > 0.6) and significant ($p$< 0.05) correlation. The nodes are colored according to ARG types and bacterial taxa. The size of the nodes is proportional to the number of connections. Red and blue lines represent positive and negative correlations, respectively. The detailed abundances of bacterial genera correlated with ARGs and the correlation coefficients can be found in Table S6.

**Figure 7.** The impacts of $CO_2$, SDZ, soil properties, bacterial communities and MGEs on ARG abundances as estimated using structural equation models. The red and blue lines indicate significant positive and negative relationships,

850 respectively. Indexes with non-significant positive and negative relationships
851 are presented as gray solid and dashed lines, respectively. The bacterial
852 diversity is estimated by the Shannon index, while the bacterial structure is
853 represented by the first PCoA axis. Significance levels are indicated: *$p < 0.05$,
854 **$p < 0.01$, and ***$p < 0.001$. The hypothetical models fit our data well: $\chi^2 = 0.10$,
855 $p = 0.995$, Df = 2, GFI = 0.999 and RMSEA = 0.000. Standardized effects (total,
856 direct and indirect effects) were derived from the structural equation models.
857

858 **Table 1.** The number of ARGs and MGEs detected in different treatments

| | Ambient | | | FACE | | |
|---|---|---|---|---|---|---|
| | Control | 0.5SDZ | 5SDZ | Control | 0.5SDZ | 5SDZ |
| FCA | 3 | 2 | 5 | 4 | 4 | 4 |
| Aminoglycoside | 11 | 10 | 11 | 11 | 9 | 11 |
| Beta-Lactamase | 13 | 18 | 18 | 12 | 12 | 11 |
| MLSB | 8 | 8 | 8 | 7 | 9 | 12 |
| Multidrug | 14 | 13 | 15 | 14 | 13 | 14 |
| Sulfonamides | 2 | 2 | 2 | 1 | 2 | 4 |
| Tetracycline | 10 | 13 | 13 | 12 | 11 | 12 |
| Vancomycin | 9 | 8 | 9 | 7 | 6 | 8 |
| Others | 1 | 1 | 0 | 2 | 2 | 2 |
| Total ARGs No. | 71 | 75 | 81 | 70 | 68 | 78 |
| Integrase | 2 | 1 | 3 | 2 | 2 | 2 |
| Transposase | 4 | 4 | 4 | 4 | 3 | 4 |
| Total MGEs No. | 6 | 5 | 7 | 6 | 5 | 6 |

859 Control, 0.5SDZ and 5SDZ indicate soil amended with SDZ at 0, 0.5 and 5 mg
860 $kg^{-1}$, respectively. The genes observed in at least two samples in one treatment
861 were defined as detected.