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Elevated CO_2 alters antibiotic resistome in soil amended with sulfamethazine via chemical-organic fertilization

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ABSTRACT

Rising antimicrobial resistance (AMR) is an enormous challenge for global healthcare systems. The effects of elevated CO_2 (eCO_2) on AMR are poorly characterized. Using a free-air CO_2 enrichment system and high-throughput qPCR arrays, we investigated the response of soil antibiotic resistome and bacterial communities to eCO_2 (ambient + 200 ppm) in soils amended with sulfamethazine (SMZ) at 0.1 and 1 mg kg^{-1} via chemical-organic fertilizer (COL, COH). Results showed that under ambient condition, COH significantly enhanced the diversity of high-risk antibiotic resistance genes (ARGs), relative abundance of low risk ARGs, unassessed ARGs and total ARGs compared to COL. Nevertheless, eCO_2 mitigated the effects of COH, with no significant difference found between COL and COH on the above high risk, low risk, unassessed and total ARGs. Meanwhile, eCO_2 decreased the relative abundance of *spcN*, *ermA*, *olec, oprD, sulA-olP, tetB, tetT* and *vanXD* in COL, and alleviated the enrichment of *pikR2*, *ampC, lunC*, *oprD and pncA* caused by the application of SMZ at 1 mg kg^{-1}. Correlation and network analysis illustrated that changes of certain bacteria biomarkers and horizontal gene transfer of integrase gene were associated with the altered response of ARGs abundance to eCO_2. This study adds knowledge of the potential risk of antibiotic resistance in agricultural exposure scenarios under increasing CO_2 concentration.

Keywords: Antibiotics resistance genes; Free-air CO_2 enrichment; Sulfamethazine; Soil bacterial community; Chemical-organic fertilizer.

1. Introduction

Antimicrobial resistance has been highlighted as a major challenge threatening human health in the 21st century (Carr et al., 2020; Wang et al., 2021). It has been estimated that 4.95 million peoples' deaths associated with bacterial antimicrobial resistance (AMR) in 2019, including 1.27 million deaths attributable to bacterial AMR (Antimicrobial Resistance, 2022). International action plans are needed to better understand and prevent the risks caused by antimicrobial resistance in the environment (UNEP, 2017). Agricultural soil is a major sink for antibiotic resistance genes (ARGs) due to the intensive anthropogenic activities, such as fertilization (Cerqueira et al., 2019; Li et al., 2022; Sanz et al., 2022). Chemical-organic fertilization can maintain and even enhance the crop yields (Bi et al., 2009), but it's a main pathway for the release of antibiotic resistant microorganisms and ARGs into agricultural soil (Karkman et al., 2019; Zhu et al., 2013). Meanwhile, this practice could also introduce a suite of chemicals known to be drivers of AMR such as antibiotics and metals (Holzel et al., 2012; Zhao et al., 2010). Although significant effects on the soil resistome following fertilization have been found, the risk of soil ARGs under elevated CO_2 levels remains largely unclear (Li et al., 2022; Rzymski et al., 2024).

By 2100, the rising carbon dioxide (CO_2) level will reach between 430 ppm and 1000 ppm (IPCC, 2021). Elevated CO_2 (eCO_2) can promote carbon flow, alter soil biophysical properties and microbial communities, the combined effect of which has the potential to alter the environmental risks of soil resistome (Liao et al., 2019a; Liao et al., 2019b; Qiu et al., 2023a; Qiu et al., 2023b). It has been found that eCO_2 may impact the spread of ARGs through influencing cell contact and plasmid transfer (Liao et al., 2019b). However, several researches into the effects of eCO_2 on soil ARGs have reported disparate observations. For example, eCO_2 has no obvious effect on the total

77 relative abundance of efflux pumps genes (Qiu et al., 2023a), whereas
78 decreased the relative abundance of aminoglycoside resistance genes,
79 tetracycline resistance genes, sulfonamide resistance genes in agricultural soil
80 (Xu et al., 2021; Xu et al., 2023), while increased that of multidrug ARGs
81 (Wang et al., 2023). These may associate with the concentration and types of
82 antibiotics, the fertilizer used, heavy metal concentration and properties of
83 agricultural soil. Therefore, the effect of increasing CO_2 levels on the soil
84 antibiotic resistome is yet to be comprehensively investigated. Notably, not all
85 ARGs pose a serious threat to public health (Zhang et al., 2021), more
86 attention is needed to focus on the “high-risk” ARGs that with high mobility and
87 enriched in human-associated environments under rising CO_2 levels (Zhu et
88 al., 2018).
89 Sulfonamides are broad-band bacteriostatic antibiotics that act against the
90 reproduction of bacteria by inhibiting bacterial folate synthesis, and are utilized
91 to protect animal health worldwide (Li et al., 2021; Schauss et al., 2009).
92 Previous studies have reported that sulfonamides are widely detected in the
93 animal residues, and pose a great selection pressure on soil microbes,
94 enriched the diversity and abundance of soil ARGs (Chen et al., 2023; Wohde
95 et al., 2016; Wu et al., 2023). Therefore，the soil ARGs would be exposed to
96 the combined pressure of increasing CO_2 levels and sulfonamide residues
97 under future. Nevertheless, the effects of eCO_2 on the soil antibiotic resistance
98 in soil amended with sulfamethazine via chemical-organic fertilization,
99 especially using realistic agricultural exposure scenarios are poorly explored,
100 which hampers our understanding on the evolution and development of
101 antibiotic resistance under climate change conditions. Here, a free-air CO_2
102 enrichment (FACE) platform was carried out in the paddy field. Using
103 high-throughput qPCR (HT-qPCR) with 296 primer sets and illumina
104 sequencing (Zhu et al., 2013), we aimed to determine whether eCO_2 could

change the effect of sulfamethazine on the antibiotic resistome of soil amended with chemical-organic fertilizer.

2. Materials and methods

2.1. Materials

Organic fertilizer was pig manure compost collected from an organic fertilizer company. It contained an average N of 1.20%, P of 2.61% (as a weight % P_2O_5) and K of 1.15% (as a weight % K_2O), Cu of 310.5 mg kg^{-1}, Pb of 35.6 mg kg^{-1}, Zn of 2053.5 mg kg^{-1}, Ni of 21.9 mg kg^{-1} and pH of 8.1. The soil used for the experiment was classified as Shajiang Aquic Cambosols (Zhu et al., 2016) and obtained from adjacent farmland (top 20 cm). The characteristics of soil were as follows: sand, 57.8%; silt, 28.5%; clay, 13.7%; pH, 6.4; total nitrogen content, 1.11 g kg^{-1}; organic matter content, 18.6 g kg^{-1}. The concentrations of antibiotics including SMZ, sulfadiazine, sulfamerazine, sulfamethoxazole, sulfamethoxypyridazine, ciprofloxacin, enrofloxacin and ofloxacin in organic fertilizer and soil were below the routine limit of quantification by 0.67 μg $kg^{-1}$and 0.08 μg kg^{-1}, respectively. Organic fertilizer was amended with sulfamethazine ($C_{12}H_{14}N_4O_2S$, CAS 57-68-1, purity ≥ 98%) in this study.

2.2. FACE platform

The FACE platform was established in the Village of Zongcun, Jiangsu Province, China (119°42′0″ E, 32°35′5″ N), a typical Chinese rice production area with an average temperature of 24.9 °C during the rice season. Previous study has provided a detailed description of the FACE platform (Zhu et al., 2015). It comprised three ambient CO_2 plots (aCO_2, ~370 ppm) and three FACE plots (eCO_2, aCO_2 + 200 ppm). An octagonal ring tube (with a diameter of 12.5 m) was placed around each FACE plot and released pure CO_2 gas above the rice canopy during its growth. The CO_2 level of FACE plot was managed by a computer program using an algorithm based on wind direction

and speed. Each ambient plot was distanced 90 m away from FACE plot and received no additional CO_2 (Zhu et al., 2015).

2.3. Experiment design

For each CO_2 concentration, two treatments were set, which included COL and COH: soils amended with SMZ via chemical-organic fertilizer. SMZ in methyl alcohol was added to organic fertilizer. After methyl alcohol evaporated, the spiked organic fertilizers were gradually mixed thoroughly with soils, leading to a final soil concentration of 0.1 mg kg^{-1} (COL) and 1 mg kg^{-1} (COH) dry weight. The concentrations of SMZ used in this experiment are within the same order of magnitude of sulfonamides concentration reported in soil from agricultural fields and animal feedlots (Ji et al., 2012). Each treatment had four replicates and each pot was filled with 4 kg soil. Rice plants (*Oryza sativa* L. cv. Wuyunjing 23) were manually transplanted into two hills (two plants per hill) in each pot on 22 June and harvested on 30 October. The water management was as follows: soils in pots were submerged in water from June 19th to July 21st; manually wet-dry cycles from July 22nd to August 10th; then submerged again and no irrigation after October 20th. Organic fertilizer was applied before transplanting, chemical fertilizer was applied at the tillering and heading stages. The application of chemical fertilizer with organic fertilizer was based on studies that demonstrated this practice could maintain or even improve the crop yield (Bi et al., 2009; Singh et al., 2016). Further information about nitrogen treatments is detailed in Table S1.

2.4. Soil sampling, physiochemical parameters analysis and DNA extraction

After harvest, rhizosphere soil samples were collected by mixing subsamples from five different points, sealed in sterile plastic bags and transported to the lab on ice. Soil SMZ concentrations were analysed by liquid chromatography-tandem mass spectrometry (Xu et al., 2021). The average

SMZ concentrations of COL and COH soils were 10.6-12.7 μg kg^{-1} and 106.5-114.6 μg kg^{-1}, respectively (Table S2). Soil heavy metal contents were determined by atomic absorption spectrometry. Total phosphorus and nitrogen contents were analysed according to previously reported methods (Bremner, 2009; Han et al., 2012).

Soil DNA was extracted with a Fast DNA Spin Kit (MP Biomedicals, CA) following the manufacturer instructions. A Nano Drop ND-1000 spectrophotometric (Wilmington, DE) was used to measure the DNA concentration and quality. Soil DNA was stored at −80°C for Illumina sequencing analysis and HT PCR.

2.5. Soil bacterial communities analysis

To assess the soil bacterial communities in different treatments, the 16S V4-V5 region was amplified using the primer pair F515/R907 (Chen et al., 2017). All samples were run on a Miseq 300 instrument with Illumina MiSeq Kit v2 by Majorbio (Shanghai, China). The high-quality sequencing data was analyzed by Qiime version 1.9.1 (Caporaso et al., 2010). The UPARSE was used to cluster Operational taxonomic units (OTUs) at 97% similarity level (Edgar, 2010). The alpha-diversity was performed based on the OTU table with a random sampling depth of minimal sequencing number among samples (Yuan et al., 2016). Difference in abundant bacterial taxa among treatments was determined using the linear discriminant analysis effect size (LEfSe) method (Segata et al., 2011). All raw sequences sets have been deposited into the NCBI's Sequence Read Archive under project accession number PRJNA758632 (SRR15686232-235, 237-246, 248-257, 259-262).

2.6. Soil ARGs analysis

To determine the ARGs profile in soil, HT-qPCR was performed using the SmartChip Real-time PCR system (Warfergen Inc. USA) as described previously (Su et al., 2015; Zhu et al., 2013). The 296 (285 ARGs, 10 MGEs

and one 16S rRNA gene) primers and specfiec operation steps used for this study were refer to Xiang et al.(2019). ARG copies/16S rRNA gene copies were used as the normalized abundance of ARGs. The risk rank of ARGs were evaluated according to human-associated-enrichment, gene mobility, and host pathogenicity (Zhang et al., 2021). Based on the study, the high and low risk ARGs were defined as Rank Ⅰ (21) and Ⅱ (3), Rank Ⅲ (10) and Ⅳ (26), respectively. The detail information of ARGs risk levels was shown in Table S3.

2.7. Statistical analysis

Data averages and analysis were obtained from IBM SPSS Statistics 26. One-way ANOVA and T-test were employed to analyze treatment differences. Spearman's correlation analysis was calculated using SPSS software and displayed by using the pheatmap package within R 3.5.2. Network analysis was performed using the psych package within R to investigate co-occurrence patterns between bacterial taxa and ARGs. Gephi 0.9.2 was then employed to visualise the results (Bastian et al., 2009).

3. Results and discussion

3.1 Diversity of soil antibiotic resistance genes

An average total of 65-77 genes were detected in all samples (Table 1). The main resistance mechanisms of these ARGs were efflux pump (42%) and antibiotic deactivation (39%) (Fig. S1). Under aCO_2, compared with that in COL, the number of detected high risk ARGs increased in COH, with an increase in the number of Rank Ⅰ ARGs. Comparably, the number of detected high risk ARGs and Rank Ⅰ ARGs have little change between COL and COH groups under eCO_2. Our results indicated that eCO_2 potentially alleviated the stimulation effect of SMZ concentration on the diversity of high risk ARGs.

3.2 Abundance of soil antibiotic resistance genes

The normalized abundance of ARGs ranged from 0.012 to 0.015 copies

per 16S rRNA (Fig. 1). Under aCO_2, the total ARGs abundance in COH was higher than that in COL. Comparably, the total ARGs abundance has little change between COL and COH groups under eCO_2. For ARGs risk grades, high risk ARGs abundances were 0.0016-0.0020 copies per 16S rRNA, low risk ARGs abundances ranged from 0.004 to 0.006 copies per 16S rRNA and unassessed ARGs abundances ranged from 0.005 to 0.008 copies per 16S rRNA (Fig. 1C and Fig. 1D). Under aCO_2, COH showed higher abundance of low risk ARGs and unassessed ARGs than those in COL. Comparatively, no significant difference was found between COH and COL under eCO_2. These results indicated that the SMZ promoted the abundance of ARGs, while eCO_2 potentially mitigated the stimulation effect of SMZ concentration on the ARGs abundance in soil amended with chemical-organic fertilizer.

The ARG subtypes with statistically significant difference between treatments were listed in Table S4. Under aCO_2, the normalized abundances of ten ARG subtypes and two MGE subtype (*intI-1LC*, *tnpA-05*) in COH were significantly higher than those in COL (Table S4 and Fig. 2). Comparatively, under eCO_2, COH changed the abundance of four ARG subtypes compared to COL. Comparing treatments under aCO_2 with that under eCO_2, the eCO_2 decreased the normalized abundance of *spcN*, *ermA*, *olec, oprD, sulA-olP, tetB, tetT* and *vanXD* in COL. Besides, eCO_2 partially alleviated the enrichment of five ARG subtypes (*ampC, pikR2*, *oprD*, *ttgB*, *pncA*) caused by the application of SMZ at 1 mg kg^{-1}, but it increased the normalized abundance of *cphA and tetPB*.

A total of 3-6 MGEs were detected for each sample, with the normalized abundance of MGEs varied from 0.004 to 0.006 copies per 16S rRNA. Under aCO_2, the abundances of integrase gene in COH were significantly higher than those in COL (Fig. 1B). Comparatively, under eCO_2, COH showed little effect on integrase genes compared to COL.

Overall, the application of SMZ at 1 mg kg^{-1} via chemical-organic fertilizer significantly increased not only the diversity of soil high risk ARGs but also the relative abundance of low risk ARGs and unassessed ARGs, compared to SMZ at 0.1 mg kg^{-1} via same fertilizer. The increased ARGs possibly caused by the susceptible microorganisms developing resistance via the horizontal transfer of genetic material that encodes resistance (Alt et al., 2021; Chen et al., 2017; Ghosh and LaPara, 2007). However, eCO_2 alleviated the stimulation effect of SMZ concentration on diversity of Rank Ⅰ ARGs, abundance of low risk ARGs and unassessed ARGs. Rank Ⅰ ARGs represent genes contributing to new or multidrug resistance in pathogens at present (Zhao et al., 2023). These indicated that eCO_2 might partly alleviate the stimulation effect of SMZ concentration on antibiotic resistance crisis in soil amended with chemical-organic fertilizer. The impact of eCO_2 on ARGs abundance under natural conditions should be explored in the future works.

3.3 Compositions and co-occurrence networks of soil bacteria

The effect of SMZ concentration was not statistically significant with respect to the bacterial alpha-diversity index under both aCO_2 and eCO_2 (Fig. 3A). Meanwhile, eCO_2 had an insignificant effect on the dominant bacteria (> 10%) of soil amended with SMZ via chemical-organic fertilizer (Fig. 3B). This may be related to the fact that soil respiration constantly kept soil [CO_2] higher than aCO_2, and the direct effect of eCO_2 on soil dominant bacteria may be negligible (Qiu et al., 2023b). LEfSe was performed to find robustly differential species (biomarkers) between treatments (Fig. 3C). Results showed that COH.A resulted in higher abundance of o_Micrococcales, o_Fusobacteriales, f_Sandaracinaceae, c_Fusobacteriia and *g_Lacunisphaera.* This may be related to the fact that some potential ARGs hosts were affiliated with order Fusobacteriales (Jia et al., 2020). Meanwhile, COH.F led to the higher abundance of f_Kineosporiaceae, f_Intrasporangiaceae, f_Paludibacteraceae,

f_Veillonellaceae, f_Xanthobacteraceae, f_Magnetospirillaceae, f_Geodermatophilaceae and *g_Roseimarinus*. Similarly, Luo et al. (2021) also observed that eCO_2 significantly increased the abundance of *Xanthobacteraceae* in soil. The effect of eCO_2 on soil bacteria may be influenced by the exposure time of eCO_2, eCO_2 concentration, cultivated plants and soil properties (Qiu et al., 2023b). Our results indicated that eCO_2 does not have a major impact on the main phylum composition of bacterial communities, although eCO_2 can increase carbon source and the abundance of specific bacteria (Qiu et al., 2023b).

Furthermore, responses of bacterial co-occurrence patterns to eCO_2 was shown in Fig. 3D and Fig. 3E. The eCO_2 network contained fewer total/negative edges than did the aCO_2 network (Table S5). The edges represent the strong and significant correlations between two functional species (nodes) (Newman, 2006; Yu et al., 2018). Less edges led by eCO_2 suggested eCO_2 might weak the interactions among species (Newman, 2006; Xu et al., 2019). Additionally, eCO_2 decreased graph density and average degree (Table S5). The differences in the topological properties of bacterial networks between eCO_2 and aCO_2 might affect soil antibiotic resistance (Xiang et al., 2023).

3.4 Multiple factors shaping antibiotic resistome

The spread of ARGs was mainly through the propagation of their host cells (vertical transfer) or horizontal transfer mediated by MGEs (Yan et al., 2023). Network analysis was used to explore the interaction profile between microbial taxa and ARGs, and track potential ARGs hosts (Fig. 4) (Chen et al., 2017; Li et al., 2015). A total of 47 microbial biomarkers were significantly correlated with 22 changed ARG subtypes ($|r| > 0.6$, $p < 0.05$). For example, o_Fusobacteriales was positively correlated with *lnuC*, *erm(34)* and *pncA* (Fig. 4), which were significantly enriched in COH.A (Fig. 2 and 3C). The

g_Lacunisphaera was positively correlated with *marR-01*，*aacC* and *erm(34)*. Besides, f_Peptostreptococcaceae and f_Saprospiraceae, whose abundances were higher in COL under eCO_2 than that under aCO_2 (Fig. 3C), were positively correlated with *aadD* (Fig. 4). Previous researches have reported that o_Fusobacteriales and f_Saprospiraceae were the potential ARGs hosts (Deng et al., 2023; Jia et al., 2020). These results suggested eCO_2 may change the spread of ARGs through affecting the potential source bacteria for ARGs, such as f_Peptostreptococcaceae and f_Saprospiraceae (Peng et al., 2016). The Saprospiraceae had the potential metabolic capacity for complex molecular degradation, nitrogen removal, and storage of intracellular polymers, which could facilitate pollutant removal and bacterial growth (Jin et al., 2024; Kondrotaite et al., 2022).

Spearman correlation analysis was used to explore relationships between ARGs and environment variables. Results showed that the SMZ had strong positive correlation with ARGs and integrase (Fig. S2). Yan et al. (2023) observed that the SMZ can promote the horizontal transfer of ARGs through improving cell membrane permeability and regulating the expression of oxidative stress, the SOS reaction, and conjugation transfer related genes. Moreover, SMZ was significantly positively correlated with *blaSHV*, *oprD*, *erm(34)*, *Inuc*, *marR-01*, *pikR2*, *aacC* and negatively correlated with *ermA* and *blaTEM* (Fig. S3). Ohore et al. (2020) also found *blaTEM* was negatively related with sulfonamide. Besides, SMZ could favor the selection of non-corresponding ARGs. These may associate with the roles of MGEs (Wang et al., 2024). One-variable linear regression was used to determine the relationship between the abundance of MGEs and ARGs (including total ARGs, high risk ARGs, low risk ARGs and unassessed ARGs) (Fig. 5). There were linear positive relationships among the abundance of integrase gene and total ARGs ($R^2 = 0.72$, $p = 0.0016$), low risk ARGs ($R^2 = 0.81$, $p < 0.001$) and

unassessed (R^2 = 0.56, p = 0.023). Horizontal gene transfer constitutes an important component in the ARGs transmission (Sun et al., 2022), which occurs through conjugation, transformation, transduction and extracellular vesicles (Martínez, 2008; Qin et al., 2022). The eCO_2 could alter conjugative and transformation transfer frequency of ARGs within genera through influencing the cell-to-cell contact, mobilization, channel transfer of plasmid and power for DNA uptake (Liao et al., 2019a; Liao et al., 2019b). Our results highlighted the importance of horizontal gene transfer mediated by integrase gene in the spread of ARGs under elevated CO_2 concentration. Further efforts using various metagenomics surveys will provide a deep understanding of the mechanisms underlying ARGs transmission under the complex agricultural system.

4 Conclusions

In summary, this study revealed that the eCO_2 changed the response of the soil antibiotic resistome to the different concentration of SMZ in soils amended with chemical-organic fertilizer. SMZ at 1 mg kg^{-1} enhanced the abundance of soil ARGs compared to that with SMZ at 0.1 mg kg^{-1}. However, eCO_2 alleviated the impact of SMZ at 1 mg kg^{-1} on the diversity of high risk ARGs, relative abundance of total ARGs, low risk ARGs and unassessed ARGs. Changes of certain bacterial biomarkers and horizontal gene transfer mediated by the integrase gene were linked to the altered response of ARGs abundance to eCO_2 in soils amended with SMZ via chemical-organic fertilizer. The above results highlight that the risk of ARGs caused by sulfamethazine in chemical-organic fertilizer soil could be mitigated to some extent under elevated CO_2 level, but sustainable strategies are needed to mitigate the spread of ARGs, particularly high risk ARGs.

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Author contributions

Fen Xu: Investigation, Formal analysis, Writing-original draft. **Qian Xiang**: Investigation, Writing-review & editing. **Mei-Ling Xu**: Formal analysis, Writing-review & editing. **Laura J. Carter**, **Wen-Chao Du**, **Ying Yin**, and **Rong Ji**: Writing − review & editing. **Chun-Wu Zhu** and **Fu-Xun Ai**: Resources, **Hong-Yan Guo**: Conceptualization, Supervision, Writing − review & editing.

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537

538 **Figure captions**

539 **Fig. 1.** The relative abundance of total ARG (A), integrase and transposases
540 (B), different risk grades of ARGs in soils under ambient CO_2 (C) and elevated
541 CO_2 concentration (ambient + 200 ppm) (D). COL and COH, soils amended
542 with sulfamethazine (0.1 mg kg^{-1} or 1 mg kg^{-1}) via chemical-organic fertilization.
543 Different letters above the bars indicate a significant difference at $p < 0.05$
544 among treatments.
545 **Fig. 2.** Heatmap of the normalized abundance of ARGs in soils under ambient
546 CO_2 (A) and elevated CO_2 concentration (F, ambient + 200 ppm); COL and
547 COH, soils amended with sulfamethazine (0.1 mg kg^{-1} or 1 mg kg^{-1}) via
548 chemical-organic fertilization.
549 **Fig. 3.** Changes in bacterial communities of soils under ambient CO_2 and
550 elevated CO_2 concentration (F, ambient + 200 ppm). (A) Alpha diversity
551 estimated by Shannon index; (B) phylum distribution of bacterial communities
552 (C) LEfSe analysis showing differentially abundant bacterial taxa among

553 treatments, based on $p < 0.05$ and linear discriminant analysis score > 2.5.
554 The networks co-occurrence analysis of OTUs with abundance > 0.1% under
555 (D) ambient or (E) elevated CO_2 concentration. Red and blue lines indicate
556 positive and negative correlations, respectively. Connections represented
557 strongly significantly ($p < 0.01$) and ($|r| > 0.8$) correlations. The size of each
558 node is proportional to number of connections. COL and COH, soils amended
559 with sulfamethazine (0.1 mg kg^{-1} or 1 mg kg^{-1}) via chemical-organic
560 fertilization.
561 **Fig. 4.** The networks co-occurrence analysis of significantly changed ARGs
562 with soil bacteria biomarkers. Red and blue lines indicate positive and negative
563 correlations, respectively. Connections represented strongly significantly ($p <$
564 0.05) and ($|r| > 0.6$) correlations. The size of each node is proportional to
565 number of connections.
566 **Fig. 5.** One-variable linear regression showing the relationship between the
567 relative abundance of MGEs and ARGs (including total ARGs, high risk ARGs,
568 low risk ARGs and unassessed ARGs).

569 **Table 1** The number of different risk grades of ARGs and MGEs detected in
570 soils amended with sulfamethazine (COL, 0.1 mg kg^{-1}; COH, 1 mg kg^{-1}) via
571 chemical-organic fertilization under different CO_2 levels.

	Ambient CO_2 level		Elevated CO_2 level	
	COL	COH	COL	COH
Rank Ⅰ ARGs	14	17	14	13
Rank Ⅱ ARGs	2	2	2	3
Rank Ⅲ ARGs	8	9	7	9
Rank Ⅳ ARGs	20	17	17	19
Unassessed ARGs	27	32	25	30
High risk ARGs	16	19	16	16
Low risk ARGs	28	26	24	28
Total ARGs NO.	71	77	65	74
Integrase	1	1	1	1
Transposase	3	3	4	2
Total MGEs NO.	4	4	5	3

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