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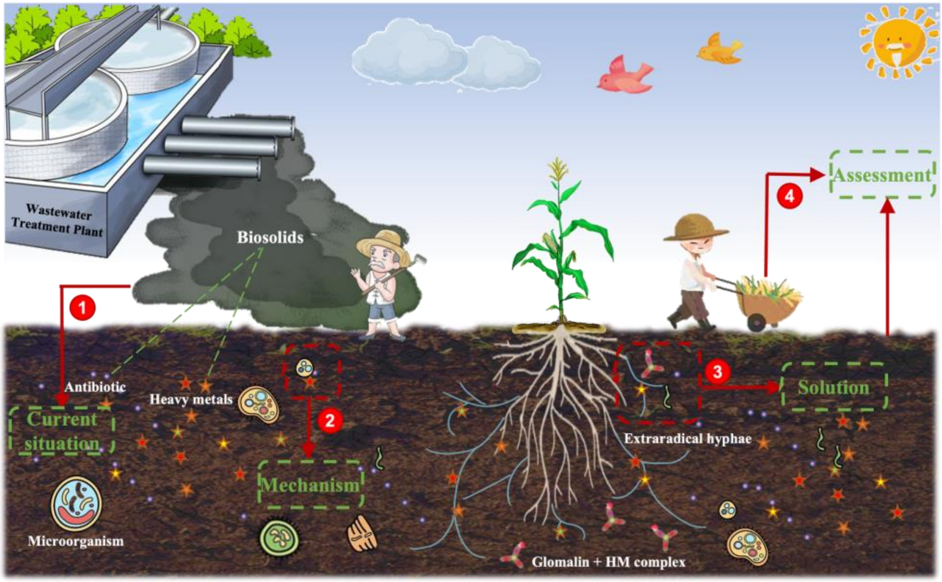
Mechanisms on the fate of heavy metal and antibiotic resistance genes in co-contaminated farmland after long-term application of biosolids

Tao Sun



Supervisor: Prof. Pierre Delaplace
Co-Supervisor: Prof. Jianfeng Zhang
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COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Mechanisms on the fate of heavy metal and antibiotic resistance genes in co-contaminated farmland after long-term application of biosolids

Tao Sun

Étude des mécanismes régissant le devenir des métaux lourds et des gènes de résistance aux antibiotiques dans des sols agricoles co-contaminés après une application prolongée de biosolides

Promoteur: Prof. Pierre Delaplace
Co-Promoteur: Prof. Jianfeng Zhang
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Abstract

Tao Sun (2025). "Mechanisms on the fate of heavy metal and antibiotic resistance genes in co-contaminated farmland after long-term application of biosolids" (PhD Dissertation in English).

Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liege.
186 pages, 54 figures, 14 tables.

Summary:

The application of biosolids is considered a sustainable strategy to improve soil fertility and crop productivity, but it also poses risks of heavy metals and antibiotics accumulation, which may impact soil microbial communities and ecological functions. In order to explore the impacts of biosolids application on soil microbial communities and reduce the negative effects of heavy metals and antibiotics on soil and plants, we sampled soil from a 16-year long-term field study, conducted a microcosm experiment with the addition of heavy metals and antibiotics, and pot experiment with arbuscular mycorrhizal fungi inoculation. The field study revealed that biosolids application significantly altered soil bacterial, fungal, and AM fungal communities. While bacterial communities were predominantly governed by stochastic processes, fungal and AM fungal assemblies were mainly shaped by deterministic processes. Heavy metals were identified as major drivers (Hg, Cu, Cd, and Zn for bacteria, Pb and Cr for fungi, and As and Ni for AM fungi) of the community assembly process. The results of microcosm showed that heavy metals exerted a dominant selective pressure on microbial communities and resistance gene profiles during the co-selection of heavy metals and antibiotics. Furthermore, the core microbiome explained the change of antibiotic genes and supported microbial community stability. Next, the pot experiment was conducted to assess the role of AM fungi inoculation in mitigating environmental risks. We found AM fungal inoculation significantly enhanced plant biomass, reduced soil and plant heavy metal contents, increased microbial network complexity, and enriched beneficial heavy metal-resistant microorganisms. Furthermore, AM fungal inoculation reduced the abundance of mobile genetic elements, potentially limiting the spread of antibiotic resistance genes. Finally, we evaluated soil health and multifunctionality by integrating field and pot experiments. Biosolids application could improve the soil health index and multifunctionality. Soil bacterial and AM fungal diversity were positively related to multifunctionality. AM fungal inoculation significantly increased soil multifunctionality by increasing soil carbon, nitrogen, phosphorus, and sulfur gene abundance. Overall, this study provides novel insights into the ecological risks and management strategies associated with biosolids use, offering guidance for sustainable agriculture development.

Key words: Biosolids, soil microbial community assembly process, arbuscular mycorrhizal fungi, antibiotic resistance genes, metal resistance gene, core microbiome, soil health.

Résumé

Tao Sun (2025). " Étude des mécanismes régissant le devenir des métaux lourds et des gènes de résistance aux antibiotiques dans des sols agricoles co-contaminés après une application prolongée de biosolides" (thèse de doctorat en anglais).

Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège.
186 pages, 54 figures, 14 tableaux.

Résumé:

L'épandage de biosolides est considéré comme une stratégie durable pour améliorer la fertilité des sols et la productivité des cultures, mais il présente également des risques d'accumulation de métaux lourds et d'antibiotiques, susceptibles d'affecter les communautés microbiennes et les fonctions écologiques du sol. Afin d'explorer les impacts de l'épandage de biosolides sur les communautés microbiennes du sol et de réduire les effets négatifs des métaux lourds et des antibiotiques sur le sol et les plantes, nous avons échantillonné le sol d'une étude de terrain à long terme de 16 ans, mené une expérience en microcosme avec ajout de métaux lourds et d'antibiotiques et une expérience en pots avec inoculation de champignons mycorhiziens à arbuscules. L'étude déposée a révélé que l'épandage de biosolides modifiait significativement les communautés bactériennes, fongiques et mycorhiziennes du sol. Alors que les communautés bactériennes étaient principalement régies par des processus stochastiques, les assemblages fongiques et mycorhiziens étaient principalement façonnés par des processus déterministes. Les métaux lourds ont été identifiés comme les principaux moteurs (Hg, Cu, Cd et Zn pour les bactéries, Pb et Cr pour les champignons, et As et Ni pour les champignons mycorhiziens) du processus d'assemblage des communautés. Les résultats des expérimentations en microcosmes ont montré que les métaux lourds exerçaient une pression sélective dominante sur les communautés microbiennes et les profils de gènes de résistance lors de la co-sélection des métaux lourds et des antibiotiques. De plus, le microbiome central expliquait la modification des gènes des antibiotiques et soutenait la stabilité de la communauté microbienne. Ensuite, l'expérience en pots a été menée pour évaluer le rôle de l'inoculation de champignons AM dans l'atténuation des risques environnementaux. Nous avons constaté que l'inoculation de champignons AM augmentait significativement la biomasse végétale, réduisait les teneurs en métaux lourds du sol et des plantes, augmentait la complexité du réseau microbien et enrichissait les microorganismes bénéfiques résistants aux métaux lourds. De plus, l'inoculation de champignons AM réduisait l'abondance des éléments génétiques mobiles, limitant potentiellement la propagation des gènes de résistance aux antibiotiques. Enfin, nous avons évalué la santé et la multifonctionnalité du sol en intégrant des expériences sur le terrain et en pots. L'application de biosolides pourrait améliorer l'indice de santé du sol et sa multifonctionnalité. La diversité bactérienne et fongique AM du sol était positivement corrélée à la multifonctionnalité. L'inoculation de champignons AM augmentait significativement la multifonctionnalité du sol en augmentant l'abondance des gènes associés au carbone, à l'azote, au phosphore et au soufre. Dans l’ensemble, cette étude fournit de nouvelles perspectives sur les risques écologiques et les stratégies de gestion associés à l’utilisation des biosolides, offrant des conseils pour le développement d’une agriculture durable.

Mots clés: Biosolides, processus d'assemblage de la communauté microbienne du sol, champignons mycorhiziens arbusculaires, gènes de résistance aux antibiotiques, gène de résistance aux métaux, microbiome de base, santé du sol.

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Tao Sun
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孙涛
2025/04/29 于比利时让步鲁

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List of Abbreviations

AMF	Arbuscular mycorrhizal fungi
ARGs	Antibiotic resistance genes
MRGs	Metal resistance genes
MGE	Mobile genetic element
SHI	Soil health index
SMF	Soil multifunctionality
HGT	Horizontal gene transfer
MAT	Average annual temperature
MAP	Average yearly precipitation
NMDS	Non-metric multidimensional scaling
BC	Buffer chamber
RC	Root chamber
HC	Hyphal chamber
ROS	Reactive oxygen species
AG	α-1,4 Glucosidase
BC	β-Cellubiosidase
BG	β-1,4 Glucosidase
BX	β-1,4 Xylosidase
NAG	β-1,4- Nacetylglucosaminidase
LAP	Leucine-aminopeptidas
ACP	Acid phosphatase
SUL	Sulfatase
TEG	Total glomalin-related soil protein
EEG	Easily extractable glomalin
PCA	Principal component analysis
PCs	Principal components
Cu	Copper
Zn	Zinc
SOM	Soil organic matter
TN	Total nitrogen
Nmin	Inorganic nitrogen
AP	Available phosphorous
TP	Total phosphorus
AK	Available potassium
TK	Total potassium
MBC	Microbial biomass carbon
MBN	Microbial biomass carbon
DOC	Dissolved organic nitrogen
DON	Dissolved organic nitrogen
Ca	Calcium
Mg	Magnesium
Na	Sodium
POXC	Permanganate oxidizable carbon

Chapter 1

General introduction

1.Research background

The global population is projected to approach 10 billion by 2050, placing unprecedented pressure on food production systems to meet increasing demand. To sustain this growth while minimizing environmental degradation, it is essential to maintain and improve soil health. Healthy soils not only support crop productivity but also regulate water, store carbon, and sustain biodiversity (Jia et al., 2025). Central to this is the concept of soil multifunctionality, defined as the simultaneous delivery of multiple ecosystem services such as nutrient cycling, climate regulation, and pathogen suppression (Creamer et al., 2022). However, modern agricultural practices, especially intensive monocultures and excessive agrochemical inputs, have led to declines in soil biodiversity and functionality, challenging the long-term resilience of agroecosystems (Gupta et al., 2022).

Biosolids are nutrient-rich organic materials derived from the treatment of municipal sewage sludge. After undergoing stabilization processes such as anaerobic digestion, composting, or heat drying, these materials become suitable—under regulatory control—for land application **(Fig. 1-1)**. Their agricultural use is increasingly promoted as a sustainable strategy to recycle waste resources, reduce reliance on synthetic fertilizers, and restore degraded soils (Nascimento et al., 2020). Their application to agricultural land has become increasingly common, offering an effective method to recycle waste resources and enhance ecosystem functions. Fermented biosolids contain substantial amounts of organic and inorganic nutrients, making them viable substitutes for conventional fertilizers. However, the presence of potentially toxic heavy metals and residual antibiotics often limits their widespread use (Singh and Agrawal, 2010). Of particular concern is the presence of antibiotic resistance genes (ARGs), which represent a significant risk to soil and crop health. Once ARGs are transferred to bacteria, they can persist and proliferate in environmental microbial communities (Collignon et al., 2018; Zhuang et al., 2021). Additional environmental stressors may further select for these resistance determinants, increasing the probability of ARG transmission between environmental bacteria and human pathogens. For example, Qin et al. (2022) found that long-term biosolid application increased not only crop biomass and soil nutrient availability but also the relative abundance of ARGs and mobile genetic elements (MGEs) in the rhizosphere. Similarly, Wolters et al. (2022) demonstrated that repeated biosolid amendment can alter rhizosphere microbial community composition and enhance the horizontal gene transfer potential in plant-associated bacteria. Furthermore, Sun et al. (2025) reported that biosolid-derived heavy metals, particularly Cu and Zn, accumulated in edible plant tissues when high application rates were used, raising food safety concerns. These findings suggest that while biosolids improve soil fertility and plant productivity, they also introduce complex microbiological and ecological risks that must be carefully managed. In response to these concerns, many countries have established legislative thresholds for heavy metals in biosolids to ensure environmental and food safety. For example, the United States Environmental Protection Agency (EPA) under the 40 CFR Part 503 rule sets ceiling limits for cadmium (85 mg/kg), copper (4300 mg/kg), lead (840 mg/kg), and zinc (7500 mg/kg).

In contrast, the European Union (Directive 86/278/EEC) provides stricter ranges, allowing member states to define limits between 20–40 mg/kg for cadmium and 2500–4000 mg/kg for zinc. China's national standard (GB 4284–2018) adopts even more conservative limits, such as 300 mg/kg for lead and 20 mg/kg for cadmium, reflecting local soil background levels and food safety priorities. These regulatory differences underscore the need for region-specific risk assessments when applying biosolids to agricultural land. Increasingly, emerging contaminants such as antibiotic resistance genes (ARGs), microplastics, and per- and polyfluoroalkyl substances (PFAS) are also being considered in future legislative revisions. Therefore, it is essential to not only assess the nutrient benefits of biosolids but also monitor the long-term accumulation of ARGs, HMs, and microbial community shifts, particularly in cropping systems with repeated or high-rate applications (Qin et al., 2022). A holistic understanding of these trade-offs is crucial for balancing soil health restoration with environmental safety in biosolid-amended agriculture **(Fig. 1-1)**.

Biosolids also contain multiple heavy metals (HMs) that can select metal-tolerant microorganisms (Sun et al., 2024). Land application of biosolids elevates HM concentrations in soils, and HMs have been widely identified as co-selective agents for ARGs in wastewater treatment plants (Sun et al., 2025), biosolid composting systems (Zhang et al., 2016a) and agri-food production chains. Evaluating the relationship between resistant microorganisms, ARGs and soil HM concentrations is necessary to identify the metals that drive co-selection process (Engin et al., 2023). Continuous biosolid amendment may lead to the accumulation of Cr, Cu, Pb, Zn and Cd, yet few studies have addressed the shift of ARGs under soil with long-term biosolids application. Importantly, HMs derived from biosolids are highly persistent and exert sustained selective pressure, potentially facilitating the transfer of ARGs into plant-associated microbial communities (Wang et al., 2022a). The combined presence of these contaminants poses considerable epidemiological risks (Wei et al., 2020). Bacterial co-selection mechanisms involve either co-resistance—where separate resistance genes for metals and antibiotics are located on the same mobile genetic element (MGE)—or cross-resistance, where a single resistance mechanism confers protection against both stressors (Buta et al., 2021). Understanding the distribution of ARGs in soil is therefore critical for mitigating antibiotic resistance in agricultural ecosystems.

Soil microorganisms play a central role in global biogeochemical cycling and constitute the most taxonomically and functionally diverse communities on Earth (Crowther et al., 2019). A single gram of soil may contain billions of microorganisms and tens of thousands of species, including bacteria, fungi, archaea, protists, and viruses. Bacteria and fungi dominate in both abundance and biomass, with bacteria driving key processes such as nitrogen cycling and organic matter decomposition, while fungi—including both saprotrophs and mutualists—contribute to carbon turnover, soil aggregation, and plant nutrition. Among them, arbuscular mycorrhizal (AM) fungi are obligate root symbionts that associate with most terrestrial plants (Smith and Read, 2008). Their extraradical mycelia extend into the surrounding soil beyond the rhizosphere, enhancing the uptake of phosphorus, nitrogen, and water, thereby promoting photosynthesis, carbon assimilation, and plant tolerance to stress (Sharif and Claassen, 2011; Shi et al., 2023). Through the secretion of carbohydrate-rich exudates, AM hyphae also shape the surrounding hyphosphere microbiota, selectively recruiting beneficial microbial consortia that can reciprocally influence fungal growth and function. Some of these hyphosphere microbes have been shown to tolerate heavy metals and antibiotics (Wu et al., 2019), suggesting potential cooperative mechanisms with AM fungi to alleviate environmental stress. In parallel, recent studies have identified the existence of core microbiota—microbial taxa that are consistently present across diverse soils and management regimes, and are functionally important for plant growth and soil functioning (Jiao et al., 2019). These core taxa are often more resilient to disturbances and contribute to microbial community stability through functional redundancy, resistance to invasion, and sustained metabolic activity. Their persistence and dominance make them crucial for maintaining ecological functions under environmental pressures, including those imposed by biosolid amendments. Understanding how AM fungi and core microbiota interact to shape stable, functional communities is essential for developing microbiome-based strategies in sustainable agriculture.

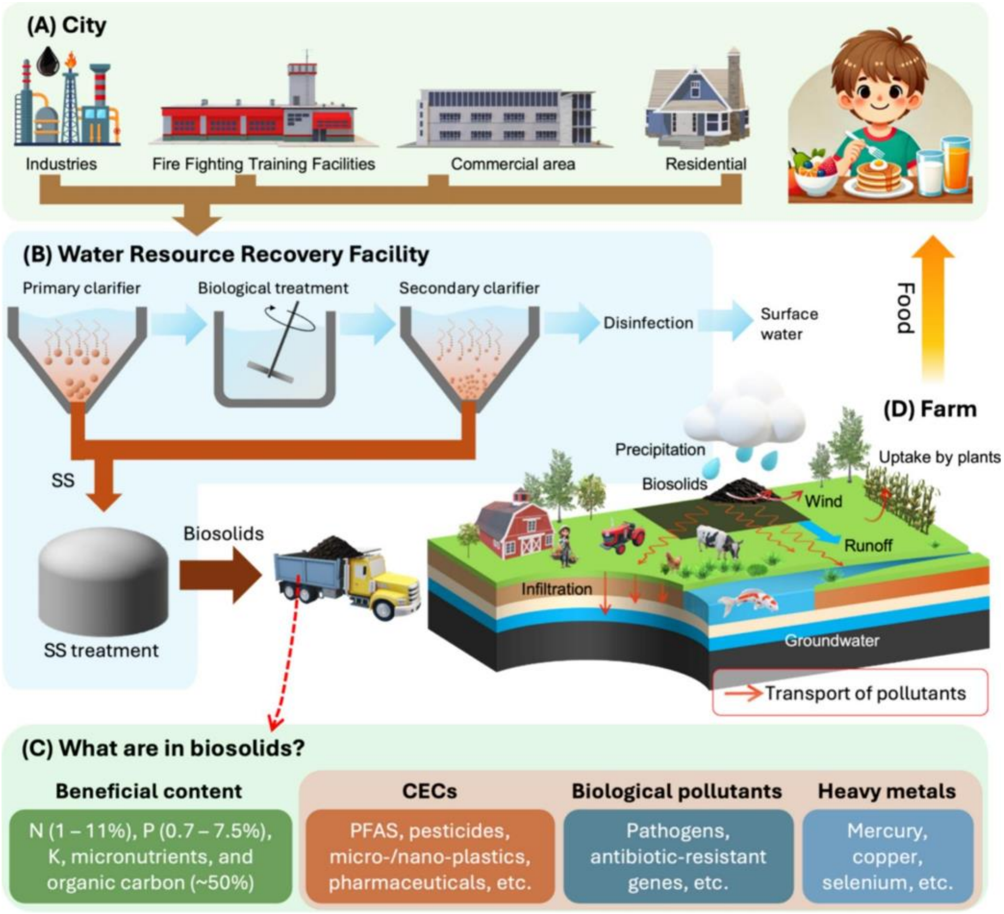

Figure 1-1: The benefit and risk of biosolid application on soil (Xue et al., 2025).

2.Research current advances

2.1. Environmental persistence and transfer of heavy metals and antibiotics in biosolids

Industrial activities have increased pollutant loads in wastewater, including HMs that partition into biosolids (Cheng et al., 2014; Di Cesare et al., 2016a). Metals occur in soluble, precipitated or adsorbed forms, most commonly Zn, Cu, Cr, Ni, Pb and Cd, with As and Se also present. Their abundance typically follows Zn > Cu > Cr > Ni > Pb > Cd (Fijalkowski et al., 2017). Although HM concentrations rarely exceed legal thresholds, long-term accumulation can pose serious environmental risks **(Fig. 1-2)** (Iglesias et al., 2018). Field studies confirm that biosolid application elevated soil heavy metal contents (Chagas et al., 2021). Heavy metals can accumulate in the soil solution and migrate to groundwater or crops (Yakovets, 2021). Their persistence depends on soil structure, cation exchange capacity, root exudates and, especially, pH and metal speciation (Bali et al., 2020). Even at low concentrations, metals are readily taken up by roots and may accumulate in edible tissues (Jolly et al., 2013). Shi et al. (2016) observed that wheat roots accumulated metals fastest, followed by leaves, stems and grains; Cu and Ni were most abundant, Pb and Cd least. Surveys of supermarket vegetables revealed contamination by Pb, Cd and Cr (Hu et al., 2013), while Balkhair and Ashraf (2016) documented eight metals in edible parts, with Ni, Pb, Cd and Cr frequently exceeding WHO limits. Continuous biosolid application intensifies metal accumulation in plough layers. For example, Mossa et al. (2020) recorded significant Cu and Zn enrichment after four years of application of biosolids. And Campbell et al. (1988) reported elevated soluble Cu, Zn and Cd in subsoils (40–60 cm) after seven years of application of biosolids, indicating possible downward migration of heavy metals.

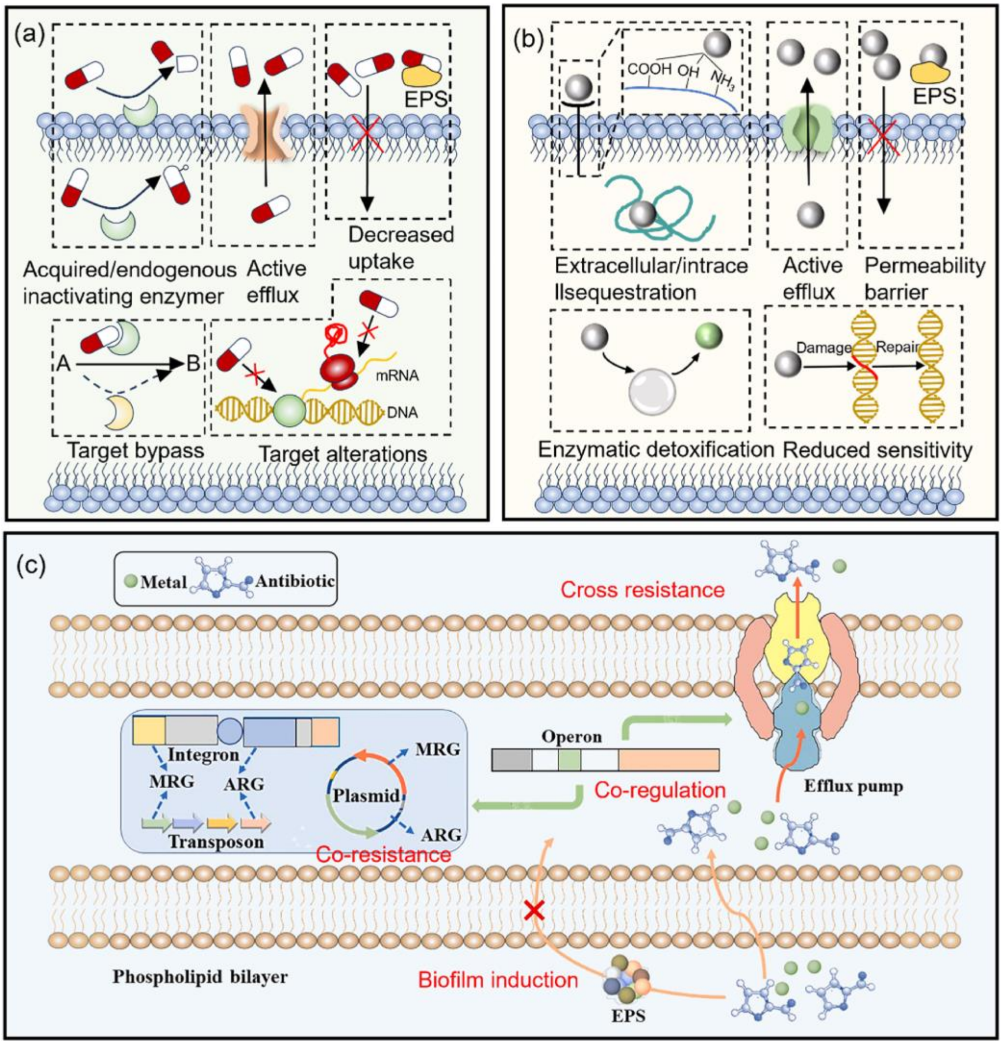

Figure 1-2: Main resistance mechanisms of microorganisms to antibiotics (a); Main resistance mechanisms of microorganisms to HMs (b); Co-selective resistance mechanisms of microorganisms to antibiotics and HMs (c). (Shu et al., 2024).

2.2. Persistence and transfer mechanisms of antibiotics and ARGs

Approximately 70% of unmetabolized antibiotics present in wastewater adsorb onto biosolids during wastewater treatment processes (Xu et al., 2015). Among these antibiotics, tetracyclines, quinolones, macrolides, and sulfonamides are frequently detected and have raised significant environmental concerns due to their persistence

(Yang et al., 2021). Excessive and widespread antibiotic use in medical treatment, animal husbandry, and agriculture has accelerated the proliferation and dissemination of antibiotic resistance genes (ARGs) globally, now recognized as a critical environmental and public health challenge (Serwecińska et al., 2020). Even at sub-inhibitory concentrations, antibiotics impose selective pressure, enhancing the propagation and persistence of ARGs in microbial communities. ARGs can be disseminated vertically, via inheritance from parent to offspring, and more prominently through horizontal gene transfer (HGT). HGT typically occurs via mobile genetic elements (MGEs), such as plasmids, integrons, and transposons, facilitating rapid ARG dissemination among diverse microbial taxa and ecosystems (Lerminiaux and Cameron, 2019; Hu et al., 2020). In addition, heavy metals, commonly co-existing with antibiotics in biosolids and other environmental matrices, can co-select for ARGs through mechanisms of co-resistance or cross-resistance. This co-selection further exacerbates the potential for antibiotic resistance proliferation, increasing environmental reservoirs of resistant microorganisms and significantly amplifying associated health risks. Understanding the complex interactions and synergistic effects of antibiotics, heavy metals, and ARGs is thus essential for assessing and mitigating antibiotic resistance in soil environments receiving biosolids amendments (**Fig. 1-2**).

2.3. Bacterial co-resistance to heavy metals and antibiotics

Heavy metals impose selection pressures on environmental microorganisms, driving the evolution and dissemination of both metal and antibiotic resistance genes (Menz et al., 2018). Mobile genetic elements, particularly conjugative plasmids and integrons, play pivotal roles as carriers of co-resistance genes, enabling their rapid horizontal gene transfer (Di Cesare et al., 2016b). Heavy metal resistance genes (MRGs) have been widely detected not only in wastewater treatment plants and biosolids but also in agricultural soils and crops, indicating their extensive environmental distribution. For example, Pitondo-Silva et al. (2016) identified arsenic, cobalt, copper, and mercury resistance genes in Brazilian plantation soils, highlighting their prevalence beyond industrial or municipal settings. Despite growing evidence, however, the specific impacts of biosolids-borne metals on the abundance and spread of HMRGs in agricultural soils remain insufficiently explored.

Co-resistance can arise through two main pathways: (i) co-location, where distinct ARGs and MRGs reside together on the same MGE, thereby facilitating simultaneous transfer; and (ii) cross-resistance, wherein shared resistance mechanisms, such as multidrug efflux pumps, regulatory proteins, or enzymatic detoxification pathways, provide microorganisms tolerance to both heavy metals and antibiotics (Baker-Austin et al., 2006). Consequently, microbial populations exposed to heavy metals can indirectly become reservoirs for antibiotic resistance. Indeed, field surveys in Dutch agricultural soils have shown significantly elevated ARG abundances when antibiotics co-occur with metals such as copper, nickel, and zinc, compared to contamination by antibiotics or metals alone (Davies et al., 2010). Such synergistic interactions underscore the critical role of heavy metals in amplifying ARG proliferation, emphasizing the importance of understanding metal-antibiotic resistance co-selection mechanisms to effectively manage antibiotic resistance risks in soils amended with biosolids (**Fig. 1-2**).

2.4. Symbiosis between AM fungi, plants, and associated microbes

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with the roots of most terrestrial plants, potentially exchanging mineral nutrients uptake, particularly phosphorus, for plant-derived photosynthates (**Fig. 1-3**). Carbon transfer from plants to fungi typically ranges from 4–20% of daily photosynthate production (Peng, 1993). The symbiotic relationship's nature—whether mutualistic or parasitic—depends on the balance of exchanged benefits and costs, influenced by environmental conditions and nutrient availability (Johnson et al., 1997). The AM fungi's extraradical mycelia extend significantly beyond the root zone, facilitating nutrient uptake and improving plant resilience to environmental stresses. Importantly, AM fungi positively influence soil biogeochemical cycling, promoting the mineralization and mobilization of soil carbon, nitrogen, and phosphorus. These fungi enhance soil aggregation and stability, contributing to improved soil structure, increased organic carbon storage, and nutrient availability, thus boosting soil fertility and plant productivity (**Fig. 1-3**). Additionally, AM fungal hyphae release exudates rich in sugars, organic acids, and amino acids, selectively stimulating or suppressing the growth of specific soil microbes in the hyphosphere (Wahab et al., 2023). Such microbial communities, termed hyphosphere microbiota, reciprocally influence fungal fitness and ecosystem functioning by modulating fungal growth, sporulation, nutrient acquisition, and suppression of plant pathogens (Wang et al., 2024). The interactions between AM fungi and hyphosphere microbes significantly influence soil nutrient dynamics and microbial community structure, although the precise mechanistic links remain poorly understood (Duan et al., 2024). Furthermore, the synergistic interactions between AM fungi and functional microbes are increasingly recognized for their potential to mitigate heavy metal (HM) toxicity and antibiotic resistance gene (ARG) proliferation in soils. AM fungi immobilize and compartmentalize heavy metals within root systems, reducing their translocation to aboveground plant tissues (Chen et al., 2003). Concurrently, associated microbes may directly detoxify metals through mechanisms such as sorption, precipitation, complexation, and redox transformations, or indirectly support phytoremediation by enhancing plant growth (Yang et al., 2018). Given that fungal communities strongly modulate ARG dynamics, targeted management of AM fungi–microbe associations present a promising strategy to reduce biosolids-derived pollutant toxicity and enhance sustainable soil management practices.

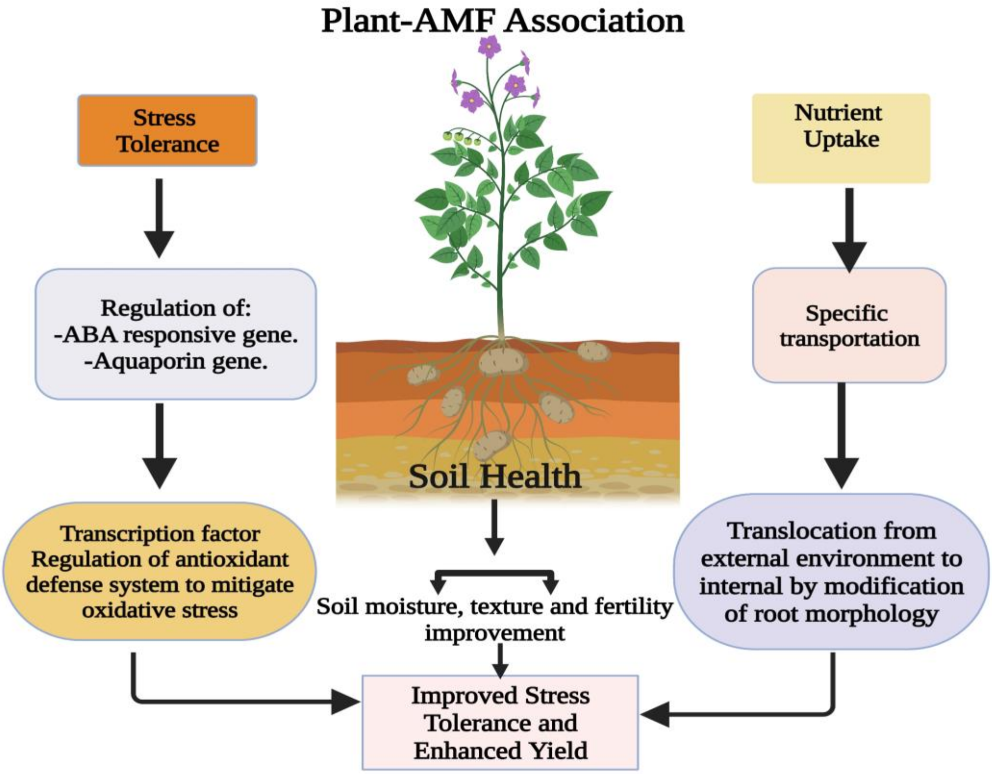

Figure 1-3: Arbuscular mycorrhizal fungi enhance soil fertility and the ability of plants to tolerate stress and uptake more nutrients. (Wahab et al., 2023).

3.Research objectives

Although many studies have examined the impacts of biosolids-derived heavy metals (HMs) on soils, crops, and water resources, comprehensive risk assessments that integrate microbial community responses remain limited. The environmental carrying capacity and safe utilization guidelines for biosolids fermentation products in agriculture are still unclear, posing significant challenges for their sustainable application. Furthermore, the mechanisms underlying interactions between hyphosphere microbial communities and arbuscular mycorrhizal (AM) fungi under biosolids amendments are not fully understood. Consequently, the combined fate and ecological risks of HMs, antibiotics, and antibiotic resistance genes (ARGs) associated with long-term biosolids applications need further elucidation. Our study aims to address these knowledge gaps by combining field study, incubation experiments, and pot experiments. Specifically, the objectives are to: (i) quantify the responses of soil microbial community to biosolids applications; (ii) elucidate the mechanism of heavy metals and antibiotics driving soil microbial communities; and

(iii) explore the mechanism of AMF in reducing the risk of heavy metals and antibiotics, and (iv) assess the effects of biosolids on soil health and how to improve soil functions by improve soil microbial diversity.

4.Thesis structure

4.1. Research roadmap

The research roadmap is displayed in **Figure 1-4**.

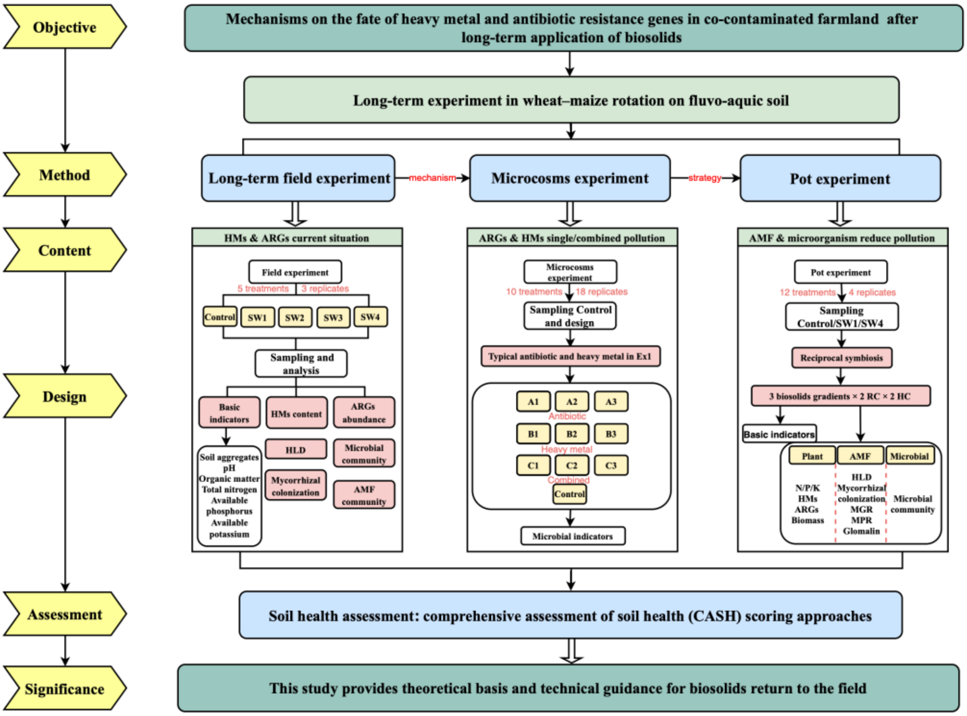

Figure 1-4: Research roadmap.

4.2. Outline

In Chapter 2, we investigated the impacts of long-term biosolids application on soil microbial community structure and assembly processes in an open field system. This chapter presents the design and outcomes of a 16-year field experiment, focusing on how biosolids influence microbial diversity and community composition under realistic agricultural conditions.

In Chapter 3, building upon the findings of the long-term field experiment, we designed a controlled microcosm experiment to further explore the effects of antibiotic and heavy metal co-contamination on soil microbial community structure and assembly. This chapter details the experimental setup and evaluates the interactive impacts of these stressors on microbial dynamics and resistance gene profiles under controlled conditions.

In Chapter 4, a greenhouse pot experiment was conducted to evaluate the effectiveness of arbuscular mycorrhizal fungi (AMF) inoculation in reducing the environmental risks associated with long-term biosolids application. This chapter explores how AMF influence heavy metal accumulation, reshape soil microbial communities, and affect the abundance of metal resistance genes (MRGs), antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs), providing insights into their role in enhancing soil resilience and remediation potential under co-contaminated conditions.

In Chapter 5, a comprehensive soil health evaluation was conducted by applying established indicators to assess the effects of long-term biosolids application and AMF bioaugmentation. Based on results from field and pot experiments, this chapter examines how biosolids and microbial interventions influence soil microbial diversity and multifunctionality, using soil health indices to quantify overall ecological impact in a practical, management-oriented context.

Chapter 6: General discussion, conclusion, and perspectives.

Chapter 2

Microbial community assembly dynamics in farmland with long-term biosolids application

Sun Tao, Li, G., Mazarji, M., Delaplace, P., Yang, X., Zhang, J., & Pan, J. Heavy metals drive microbial community assembly process in farmland with long-term biosolids application. *Journal of Hazardous Materials*, 2024, 468, 133845. https://doi.org/10.1016/j.jhazmat.2024.133845

Abstract

Biosolids are considered an alternative to chemical fertilizers due to their rich nutrients. However, long-term biosolids application can lead to heavy metals accumulation, which severely affect soil microbial community compositions. The factors influencing soil microbial community assembly were explored under a 16-year long-term experiment with biosolids applications. Our results indicated that biosolids application significantly increased fungal richness while not for bacterial and arbuscular mycorrhizal (AM) fungal richness. Besides, biosolids application significantly affected soil bacterial, fungal and AM fungal compositions. Soil microorganisms were clustered into different modules with bacterial and AM fungal communities were affected by both organic matter and heavy metals, while fungal communities were affected by heavy metals (Cr, Ni, and As). The soil bacterial community assembly was dominated by stochastic processes while the fungal and AM fungal community assemblies were mainly driven by deterministic processes. Random forest analysis showed that heavy metals were identified as major drivers (Hg, Cu, Cd, and Zn for bacteria, Pb and Cr for fungi, and As and Ni for AM fungi) of the community assembly process. Overall, our study highlights the significant role of heavy metals in shaping microbial community dynamics and give a guide for controlling biosolids application.

Keywords: Biosolids, heavy metals, soil microbial community, community assembly process.

The graphical abstract is displayed in **Figure 2-1**.

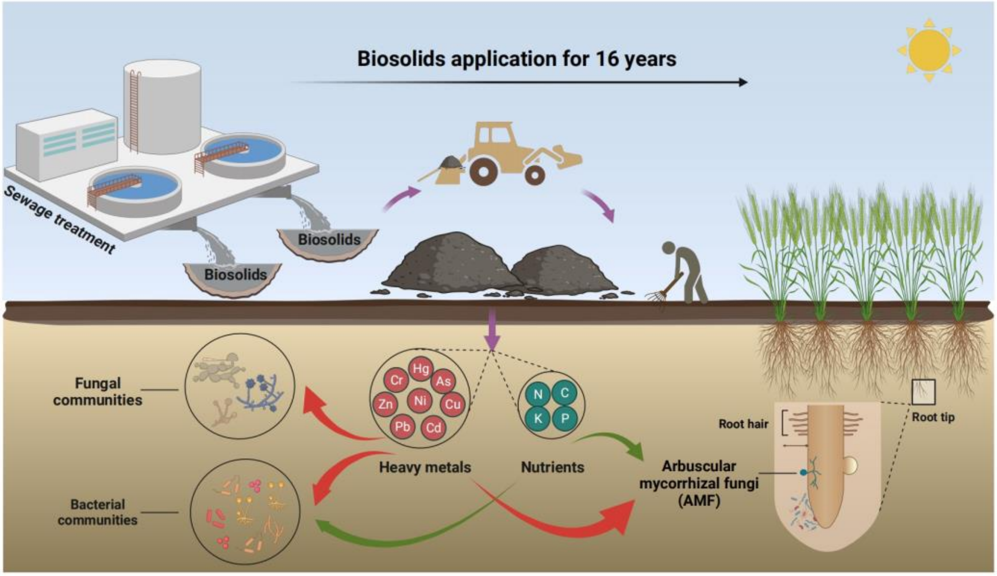

Figure 2-1: Chapter 2 graphical abstract.

1.Introduction

Biosolids are mainly composed of organic matter, which can serve as a valuable nutrient source for plants, especially in terms of nitrogen (N), phosphorus (P), and potassium (K) (Marchuk et al., 2023; Kirchmann et al., 2017). Incorporating biosolids into soils could improve soil properties such as soil aeration, organic matter, and nutrient contents (Alvarenga et al., 2015, 2017). In addition, biosolids application could increase soil respiration, improve soil microbial activity, elevate microbial biomass, and alter microbial community compositions (Wang et al., 2017; Hu et al., 2019). However, repeated or long-term application of biosolids might potentially lead to heavy metals accumulation in soils and plants, adversely affecting soil biology (Mossa et al., 2020; Qin et al., 2022). In this instance, the presence of heavy metals has been linked to a decrease in soil microbial activity, abundance, and diversity, affecting plant growth, and eventually crop yield (Chen et al., 2014a; Shahid et al., 2015). However, the co-existence of the potentially positive effects of nutrients and the adverse effects of heavy metals from the application of biosolids make it complex to understand their actual impacts on soil ecosystem (Mossa et al., 2017).

Soil microbial communities are susceptible to alternation following the application of biosolids, mainly as the consequence of soil properties changes (Sullivan et al., 2006). However, soil microorganisms may respond differently to biosolids due to their various physiological and ecological traits (Wood et al., 2017). Soil nutrients and organic matter contents changes may influence the equilibrium between oligotrophic microorganisms, which grow slowly but exhibit a relatively high carbon use efficiency, and saprotrophic microorganisms, which grow quickly but demonstrate a lower carbon use efficiency (Adingo et al., 2021; Drigo et al., 2008). It has been reported that adding biosolids has the potential to change microbial life strategy, leading to a transition of microbial community from chemolithotrophic to heterotrophic, primarily due to the enhanced soil carbon content (Vardanyan et al., 2019). Furthermore, different microorganisms respond differently to heavy metal stress due to their different tolerance levels to those heavy metals (Zhang et al., 2022a). For example, prolonged exposure to heavy metals could cause an increase in the population of stress-tolerant microbes, such as Firmicutes, and Actinobacteria (Kasemodel et al., 2019). Conversely, the presence of heavy metals often leads to a decrease in the population of sensitive or low-tolerance microbes (Kürsten et al., 2015; Piotrowska-Seget et al., 2005). Therefore, the changes in soil nutrients, carbon content, and heavy metals resulting from the application of biosolids can impact diverse microbes occupying specific niches, engaging in various biogeochemical cycles, and contributing to ecosystem functions (Sanchez-Monedero et al., 2018). Understanding the interplay among heavy metals, soil nutrients, and carbon content, as well as their comprehensive effects on microbial communities is crucial for improving soil health and maintaining ecosystem functions.

Soil microbial assembly processes are crucial in establishing community composition, directly impacting microbial functions within the ecosystem (Nemergut et al., 2013). Deterministic processes govern the assembly of communities, relying on species-specific traits, interactions among organisms, and environmental factors like soil moisture, pH, and salinity (Stegen et al., 2015). They could result in community structures that exhibit distinct patterns of phylogenetic relatedness and turnover (Stegen et al., 2012). In contrast, stochastic processes are expected to produce phylogenetic community structures that are not significantly different from randomly assembled communities (Stegen et al., 2012). However, studies of microbial assembly processes in soils with long-term application of biosolid fertilizers are limited, and the effects of soil environmental parameters on these processes remain unclear. In this context, a previous study has revealed that the determinant roles of stochastic processes to bacterial and archaeal communities decreased with the increase of heavy metals (Zhang et al., 2022a). Nevertheless, the driving process of heavy metals was reported to shift from stochastic to deterministic and finally changed to stochastic again (Zhong et al., 2023). The variability in microbial community responses adds to the puzzle of understanding the ecological processes that govern microbial assembly in soils subjected to the long-term application of biosolids.

The objectives of our study were to determine the effects of the long-term application of biosolids on soil microbial community structure and assembly dynamics while identifying the key factors influencing soil microbial community. This study hypothesizes that (i) the biosolids applications could improve soil microbial diversity due to increased nutrient and organic matter contents, and (ii) the input of heavy metals resulting from the application of biosolids would induce a shift of soil microbial community and act as the primary drivers of microbial community assembly process.

2.Materials and methods

2.1 Site description and experiment design

The long-term experiment took place at Changping Soil Quality National Observation and Research Station in Beijing, China, situated at coordinates 40◦10′N, 116◦14′E. This site experiences an annual rotation of winter wheat and summer maize and is classified as flavor-aquic soil. This area experiences a sub-humid continental monsoon climate characterized by an average annual temperature (MAT) of 12.4°C and an average yearly precipitation (MAP) of 628.9 mm.

The field experiment has been running since 2006 and comprises five triplicate treatments: (1) only chemical fertilization (Control), (2) 4.5 t ha^{-1} biosolids with chemical fertilization (SW1), (3) 9 t ha^{-1} biosolids with chemical fertilization (SW2), (4) 18 t ha^{-1} biosolids with chemical fertilization (SW3), and (5) 36 t ha^{-1} biosolids with chemical fertilization (SW4). The detailed information regarding fertilization can be found in **Table 2-1**. All treatments except the control received the same amounts of chemical fertilizers: 150 kg ha^{-1} yr^{-1} of nitrogen (as urea), 79 kg ha^{-1} yr^{-1} of phosphorus (as superphosphate), and 199 kg ha^{-1} yr^{-1} of potassium (as potassium sulfate). The control treatment received only phosphorus and potassium fertilizers, without nitrogen or biosolids. Biosolids were applied at increasing rates of 4.5, 9.0, 18.0, and 36.0 t ha^{-1} yr^{-1} in treatments SW1 to SW4, respectively. This design allows for assessing the effects of increasing biosolid inputs under constant levels of chemical fertilizer supply. The biosolids utilized in this study underwent an air-drying process before their application in October 2021. The application procedure was akin to that of chemical fertilizers. The biosolids for this study were sourced from municipal domestic sewage, specifically acquired from Beijing Drainage Group Co., Ltd. in Beijing, China. The basic properties of the biosolids are given in the Supplementary Materials **(Table 2-2)**. The application of biosolids occurred once every year between the planting cycles of maize and wheat. Spreading fertilizers evenly over the entire farm surface and then ploughing the soil.

Table 2-1: The rate of biosolids and mineral fertilizers used in the long-term field experiment (2006–2022).

Treatment	Chemical fertilizers (kg ha− 1 yr− 1)			Biosolids
	Nitrogen	Phosphorus	Potassium	t ha− 1 yr− 1
Control	0	79	199	0
SW1	150	79	199	4.5
SW2	150	79	199	9.0
SW3	150	79	199	18.0
SW4	150	79	199	36.0

Table 2-2: Basic characteristics of biosolids applied in this study.

Item	Biosolids
pH	7.61±0.41
Organic matter (%)	36.0±7.7
N (%)	2.68±0.58
P_2O_5 (%)	4.06±1.0
K_2O (%)	0.57±0.26
Zn/mg·kg^{-1}	1 033±369
Cu/mg·kg^{-1}	190±52
Cd/mg·kg^{-1}	1.79±1.2
Cr/mg·kg^{-1}	84.2±45
As/mg·kg^{-1}	13.0±6.9
Hg/mg·kg^{-1}	9.50±4.1
Pb/mg·kg^{-1}	38.6±20
Ni/mg·kg^{-1}	30.9±17

2.2 Soil sampling and analysis

In June 2022, soils (0-20 cm) were sampled from each treatment. After removing any residues, five soil cores were sampled from each plot and mixed into a single sample. Following collection, samples were promptly transported while chilled to the laboratory, where they underwent sieving through a 2.0-mm mesh sieve. Afterward, these samples were divided into three separate subsamples for further analysis or experimentation. One subsample was kept at 4°C for measuring biological indicators, another was kept at –20°C for DNA sequencing, and the remaining subsample was air-dried for soil physicochemical properties analyses.

Soil moisture, pH, soil organic matter (SOM), total nitrogen (TN), inorganic nitrogen (Nmin), available phosphorous (AP), total phosphorus (TP), available potassium (AK), total potassium (TK), microbial biomass carbon (MBC) and nitrogen (MBN), dissolved organic carbon (DOC) and nitrogen (DON), exchangeable calcium (Ca), magnesium (Mg), sodium (Na) and permanganate oxidizable carbon (POXC), were measured as described as Jia et al. (2024). The soil's heavy metal contents underwent analysis using inductively coupled plasma optical emission spectrometry.

2.3 Microbial DNA extraction, PCR, and Illumina sequencing

Total DNA was extracted from 0.5 grams of soil using the MagaBio Kit (Bioer Technology, China). PCR amplifications targeted different regions: the V4 region for bacteria using primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') / 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Walters et al., 2016),, the ITS region for fungi using primers ITS3F (5'-GCATCGATGAAGAACGCAGC-3') / ITS4R (5'-GCATCGATGAAGAACGCAGC-3') (Op De Beeck et al., 2014), and a specific region for AM Fungi with primers AML1F (5'-ATCAACTTTCGATGGTAGGATAGA-3'), AML2R (5'-GAACCCAAACACTTTGGTTTCC-3'), AMV4.5NF (5'-AAGCTCGTAGTTGAATTTCG-3'), and AMDGR (5'-CCCAACTATCCCTATTAATCAT-3') (Van Geel et al., 2014) synthesized by Invitrogen. PCR reactions were performed using 25 µL 2x Premix Taq (Takara Biotechnology, Dalian Co. Ltd., China), 1 µL each primer (10 µM), and 3 µL DNA (20 ng/µL) template in a 50 µL volume, subjected to thermocycling: 5 min at 94°C for initialization; 30 cycles of 30s denaturation at 94°C, 30s annealing at 52°C, and 30s extension at 72°C; followed by a final 10 min elongation at 72°C using the BioRad S1000 instrument. PCR products' length and concentration were assessed via 1% agarose gel electrophoresis. Samples showing normal, bright main strips were selected for subsequent experiments. The PCR products were equidensity-ratio mixed using GeneTools Analysis Software (SynGene). This mixture was purified with the E.Z.N.A. Gel Extraction Kit (Omega, USA). Sequencing libraries were generated using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA) with added index codes. Library quality was evaluated using the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA). Sequencing was conducted on an Illumina Nova6000 platform, producing 250 bp paired-end reads (Guangdong Magigene Biotechnology Co., Ltd. Guangzhou, China)/ Quality control was performed on initial pair-end reads using Trimmomatic v0.39 (Bolger et al., 2014) to remove sequences with a quality score below 20. Subsequently, FLASH v1.2.7 combined the pair-end reads, followed by denoising and chimera filtering using the 'USEARCH' algorithm (Edgar, 2010). Reads shorter than 200 bp were excluded. The sequencing reads, acquired from paired-end sequencing, were initially sorted into individual samples using unique barcode. Post sorting, the reads underwent merging and were subsequently subjected to denoising and chimera filtering through DADA2. Taxonomic assignment for bacteria utilized the SILVA v138 database (Green et al., 2022), while the UNITE v8.0 database (Eshaghi et al., 2021) was employed for fungi and the Maarj*AM* database (Öpik et al., 2010) was employed for AM fungi.

2.4 Statistical analyses

R (version 4.1.0) was utilized for statistical analyses. One-way ANOVAs assessed variations in soil properties, microbial diversity, and community composition. Non-metric multidimensional scaling (NMDS) was performed to evaluate the influence of biosolids application rates on soil bacterial, fungal, and AM fungal communities. Soil microbial co-occurrence networks were constructed using the 'microeco' package (Liu et al., 2021). Robust correlations (Spearman's $\rho > 0.60$) with FDR-corrected p-values < 0.01 were used to construct networks. And subsequent network property analysis was used by the 'igraph' package. Random networks with identical edges and nodes were generated, and the topological properties of these networks were calculated using the Erdős–Rényi model (Cheng et al., 2023). The iCAMP method was used to evaluate the stochastic and deterministic processes in contributing soil microbial community. The detailed process of iCAMP analyses of the data was in the supporting Materials section, which contains comprehensive information about these procedures. Bacterial, fungal, and AMF βNTI differences were examined using one-way ANOVA. Random forest regressions identified significant drivers of soil microbial community assembly.

3.Results

3.1 Effect of biosolids application on soil parameters

Soil physical, chemical, and biological parameters were significantly affected by the application of biosolids **(Table 2-3)**. Notably, soil AP, TN, SOM, and DOC contents increased with the application of biosolids. In this context, SW4 had the highest contents and control had the lowest contents. However, AK content was highest in the SW4 treatment. Furthermore, MBC and MBN were highest in the SW3 treatment. MBC and MBC increased by 11.47%, 29.70%, 64.59%, 64.40%, and 17.74%, 31.55%, 56.14%, and 42.66% in the SW1, SW2, SW3, and SW4 treatments compared to the control, respectively.

The heavy metals contents of Cu, Zn, Cd, and Hg in the soil increased with the increase in the application doses of biosolids **(Fig. 2-2)**. However, although Cr and Pb contents increased with the increased application of biosolids from SW1 to SW4, Cr and Pb content was decreased in SW1 treatment **(Fig. 2-2A and 2-2G)**. There was no significant difference in Ni and As content among various treatments **(Fig. 2-2B and 2-2E)**.

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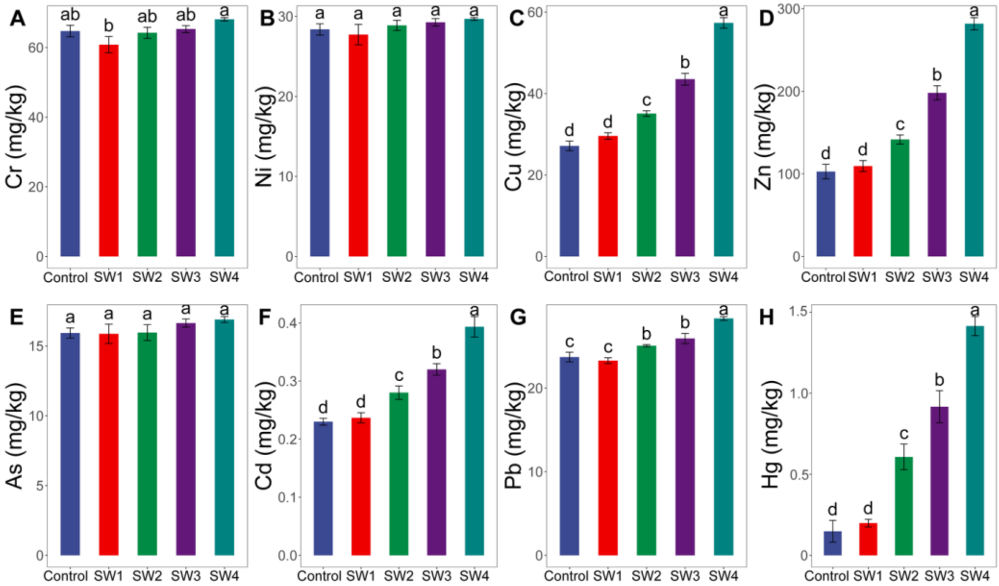

Figure 2-2: The effect of biosolids application on the contents of different types of heavy metals.

Table 2-3: The effect of biosolids applications on soil parameters.

Parameter	Control	SW1	SW2	SW3	SW4
pH	7.13 ± 0.07a	7.14 ± 0.03a	7.15 ± 0.02a	7.08 ± 0.088a	7.12 ± 0.07a
Moisture (%)	15.48 ± 4.33a	14.51 ± 0.72a	11.18 ± 2.26a	20.43 ± 8.35a	16.85 ± 5.18a
AP (mg/kg)	52.71 ± 23.45c	71.09 ± 8.45bc	97.43 ± 11.72b	191.48 ± 8.67a	217.12 ± 21.58a
TP (g/kg)	1.41 ± 0.19a	1.46 ± 0.18a	1.67 ± 0.23a	1.48 ± 0.14a	1.28 ± 0.57a
AK (mg/kg)	289.61 ± 76.49b	199.82 ± 7.63b	306.91 ± 66.28b	296.41 ± 78.96b	606.45 ± 186.40a
TK (g/kg)	19.91 ± 0.73a	19.05 ± 0.91a	21.24 ± 0.99a	21.89 ± 2.25a	17.77 ± 6.36a
TN (g/kg)	1.06 ± 0.10d	1.30 ± 0.09d	1.67 ± 0.13c	2.44 ± 0.14b	3.42 ± 0.30a
SOM (g/kg)	18.83 ± 1.94e	25.30 ± 2.85d	32.67 ± 2.55c	40.91 ± 4.27b	58.95 ± 1.53a
EC (ds/cm)	138.40 ± 20.21a	140.27 ± 5.06a	134.03 ± 6.89a	193.23 ± 64.29a	183.37 ± 39.19a
NH_4^+(mg/kg)	21.96 ± 2.64a	22.24 ± 1.19a	22.09 ± 0.399a	19.96 ± 3.58ab	17.68 ± 0.51b
NO_3^- (mg/kg)	11.06 ± 5.85c	14.36 ± 1.89bc	15.40 ± 1.38bc	32.66 ± 16.18ab	37.81 ± 15.61a
Nmin (mg/kg)	33.02 ± 5.52c	36.61 ± 1.31bc	37.48 ± 1.34bc	52.62 ± 13.23ab	55.49 ± 15.19a
Mg (mg/kg)	436.11 ± 19.84b	418.67 ± 7.80b	422.34 ± 10.51b	440.44 ± 56.17b	500.52 ± 28.66a
Na (mg/kg)	131.87 ± 31.95a	132.30 ± 9.72a	91.23 ± 24.05ab	96.60 ± 28.87ab	75.53 ± 6.24b
Ca (mg/kg)	3819.01 ± 284.58ab	3844.08 ± 195.99ab	3676.27 ± 64.07b	3720.44 ± 320.02b	4222.01 ± 34.14a
MBC (mg/kg)	309.48 ± 52.18b	344.97 ± 15.70b	401.38 ± 48.66ab	509.33 ± 100.71a	508.58 ± 55.24a
MBN (mg/kg)	57.71 ± 11.36b	67.95 ± 4.63ab	75.92 ± 9.85ab	90.11 ± 27.82a	82.33 ± 17.42ab
DOC (mg/kg)	62.68 ± 9.85c	73.64 ± 3.14c	80.04 ± 5.21c	123.48 ± 18.60b	150.14 ± 15.82a
DON (mg/kg)	32.44 ± 9.33b	37.75 ± 1.29b	38.09 ± 0.95b	76.30 ± 21.01a	85.65 ± 17.07a
POXC (mg/kg)	0.20 ± 0.05b	0.19 ± 0.04b	0.18 ± 0.05b	0.25 ± 0.01ab	0.33 ± 0.08a

3.2 Effect of biosolids application on soil microbial richness and community composition

Soil bacterial richness was not significantly different among various treatments **(Fig. 2-3A)**. Fungal richness increased with the increase in biosolids application rates, which was highest in SW4 treatment compared to the control, the fungal richness increased by 4.43%, 2.88%, 6.68%, and 7.48% in the SW1, SW2, SW3, and SW4 treatments **(Fig. 2-3B)**. Compared with control, AM fungal richness was significantly increased in SW4 treatment. However, there was no significant difference among other treatments. Compared to the control, the AM fungal richness increased by 0.77%, 17.39%, and 41.46% in SW1, SW3, and SW4 treatments while exhibiting a decrease of 7.92% in the SW2 treatment **(Fig. 2-3C)**.

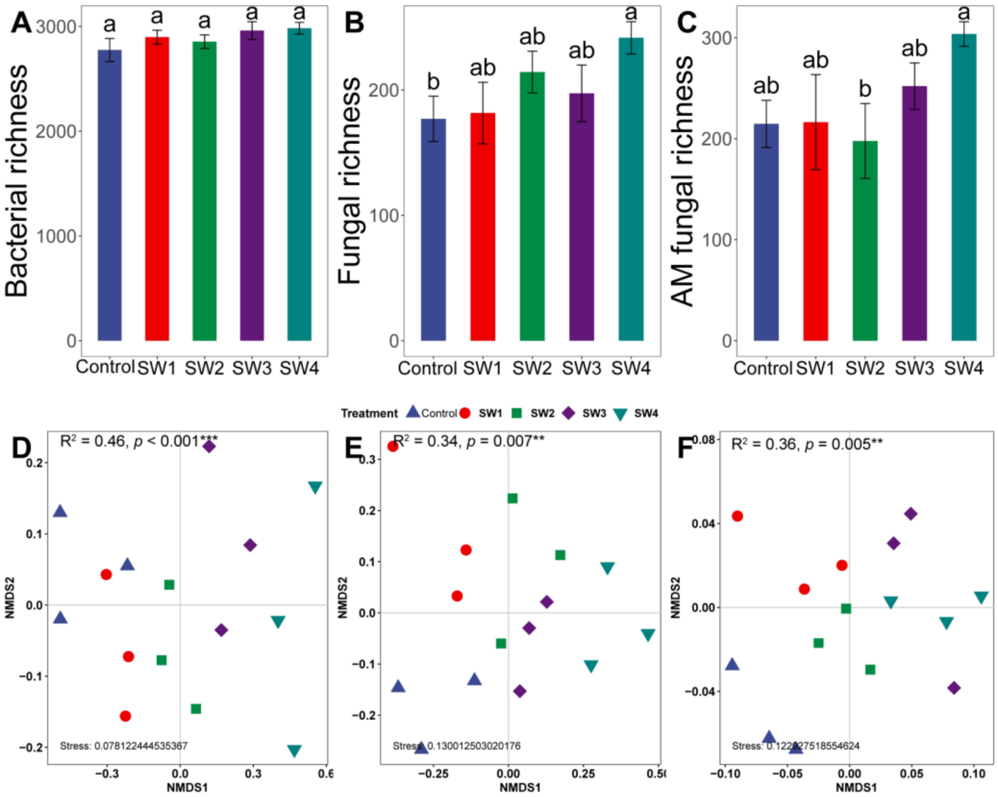

Figure 2-3: The bacterial (A, D), fungal (B, E), AM fungal (C, F) richness and community compositions after biosolids application.

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***.

The result of heatmaps showed that there were significantly positive correlations between AP, DOC content and bacterial richness and AM fungal richness **(Fig. 2-4)**. Soil moisture and DON content exclusively showed a positive correlation with AM fungal richness **(Fig. 2-4)**. Positive correlations were found among TN and SOM content with fungal and AM fungal richness ($p < 0.05$) **(Fig. 2-4)**. As for heavy metals, no statistically significant correlations were observed between bacterial richness and any of the heavy metals **(Fig. 2-4)**. All heavy metals showed significantly positive correlations with fungal and AM fungal richness. Concurrently, Cr, Ni, As, Cd, Pb, and Hg displayed a positive association with fungal and AM fungal Shannon diversity **(Fig. 2-4)**.

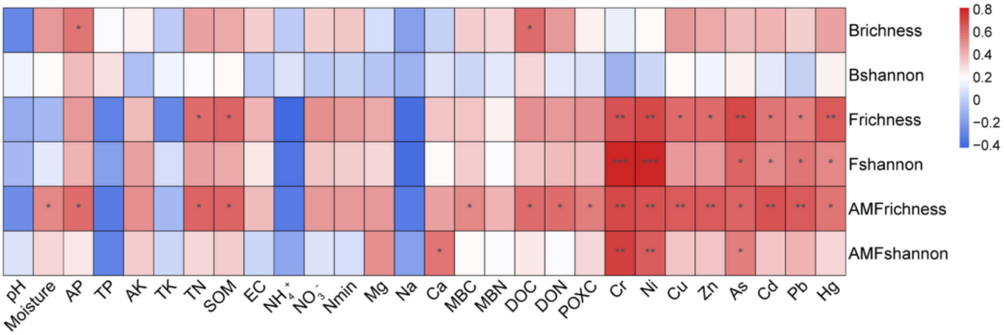

Figure 2-4: The relationships between soil nutrients and heavy metal contents and bacterial, fungal, as well as AM fungal richness and Shannon diversity.

Both soil bacterial, fungal, and AM fungal communities were significantly influenced by the application of biosolids, which were cleared and separated along NMDS1 **(Figs. 2-3D and 2-3E)**. AM fungal community of SW1 treatment was observed to have a higher resemblance with SW2 treatment than other treatments. A higher resemblance could also be observed between SW3 and SW4 treatments **(Fig. 2-3F)**.

Proteobacteria, *Acidobacteria*, and *Ascomycota*, *Basidiomycota* were the two most abundant bacterial and fungal phyla, respectively, in all treatments **(Fig. 2-5A and 2-5B)**. *Glomeraceae* and *Pycnomycaceae* were the two most abundant families in all treatments **(Fig. 2-5)**. The mantel tests indicated that the soil bacterial and AM fungal community structures were markedly influenced by SOM, AP, TN, Nmin, DOC, Cu, Pb, Cd, Zn, and Hg contents. Furthermore, the fungal community structure was significantly affected by Cr, Ni, and As contents **(Fig. 2-6)**. As for bacteria, Cu, Zn, Pb, and Hg contents exhibited positive correlations with the abundances of *Actinobacteriota* and *Chloroflexi* while showing negative associations with the abundances of *Acidobacteriota*, *Bacteroidota*, *Verrucomicrobiota*, *Armatimonadota* and *Elusimicrobiota* **(Fig. 2-7A)**. Furthermore, Cr content was positively related to *Actinobacteriota* while negatively related to *Actinobacteriota* and

Verrucomicrobiotav **(Fig. 2-7A)**. For fungi and AM fungi, Cu, Zn, Cd, Pb, and Hg contents were positively related to the abundances of *Rozellomycota* and *Ambisporaceace*, respectively **(Fig. 2-7B and 2-7C)**. Moreover, the abundance of *Blastocladiomycota* increased with the increased Cd content **(Fig. 2-7B)**. The abundance of *Archaeosporaceae* reduced with the increase of Cr, Ni, and As contents **(Fig. 2-7C)**.

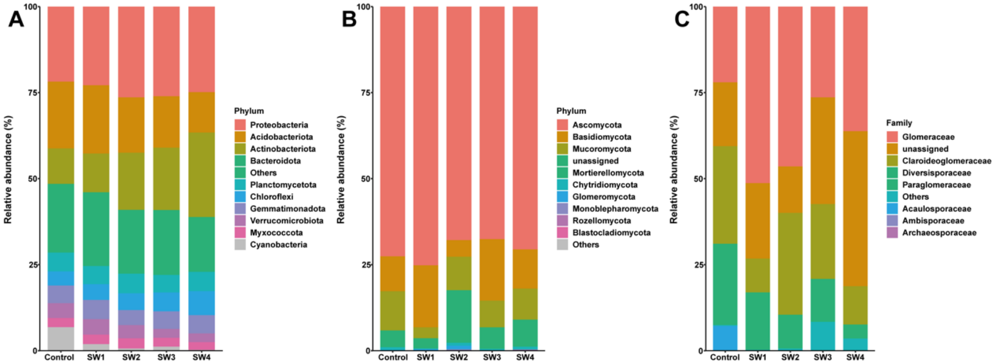

Figure 2-5: The relative abundance (%) of bacterial (A), fungal (B) phyla, and AM fungal family (C) in different treatments.

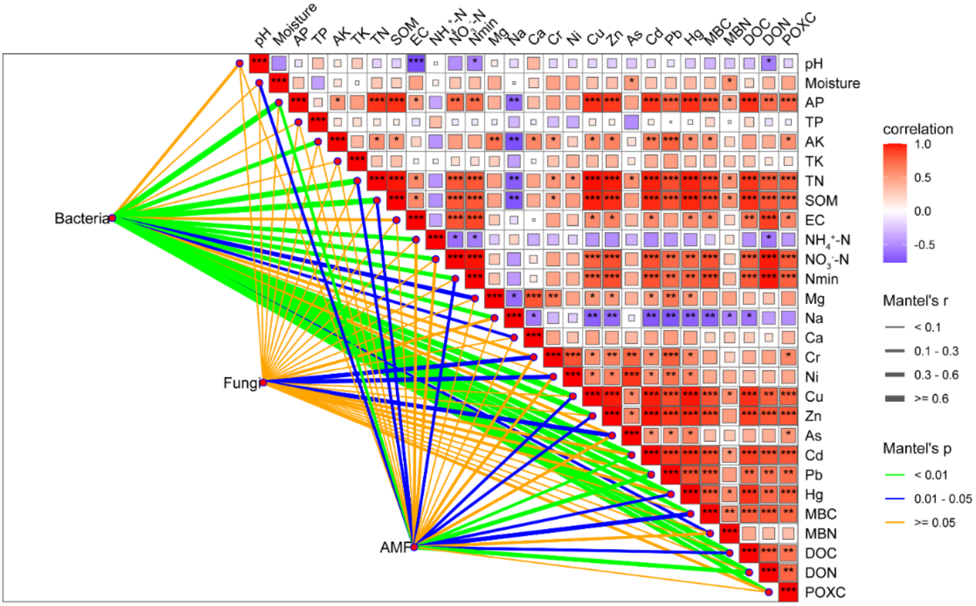

Figure 2-6: Mantel test analysis showed the influence of soil parameters on bacterial, fungal, and AM fungal community composition.

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***.

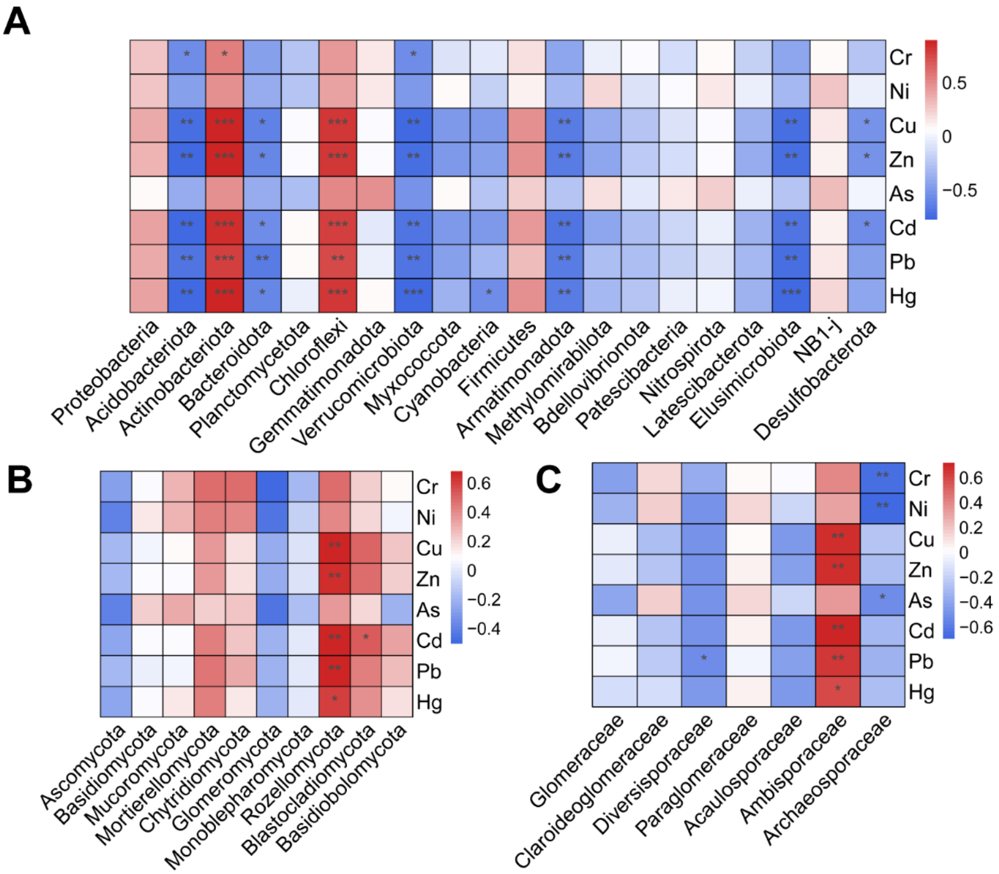

Figure 2-7: The correlations between soil nutrients, heavy metal contents, and bacterial (A) and fungal (B) phyla, as well as AM fungal family (C) abundance.

Microbial co-occurrence patterns were examined for a more in-depth characterization of the impact of biosolids application on microbial interactions **(Fig. 2-8)**. The bacterial network comprises 639 nodes and 1483 edges, the fungal network consists of 494 nodes and 1113 edges, and the AM fungal network encompasses 250 nodes and 972 edges. Other microbial network properties such as average degree and modularity were also the highest in the soil bacterial network **(Table 2-4)**. Furthermore, there were 5, 8, and 8 modules in bacterial, fungal, and AM fungal networks, respectively **(Fig. 2-8A, B, C)**. For the bacterial network, the relative abundance in module 1 and module 5 showed a significantly negative relationship with Cu, Zn, Cd, Pb and Hg contents **(Fig. 2-8D)**. Microbial abundance in module 2 showed positive relationships with Cr, Cu, Zn, As, Cd, Pb, and Hg contents **(Fig. 2-8D)**. Moreover, microbial abundance in module 4 was positively related to Cr and As **(Fig. 2-8D)**. For the fungal network, positive relationships were found between the relative abundance of module 1 and Cr, Cu, Zn, Cd, Pb, and Hg contents. And microbial abundance in module 2 was negatively related to Cu and Hg contents **(Fig. 2-8D)**. In the AM fungal network, negative relationships were observed between the relative abundance of module 1 and Cr, Ni, and As contents **(Fig. 2-8D)**. Furthermore, notable negative relationships were observed between microbial abundance of module 2 and Ni content **(Fig. 2-8D)**. And there were incredibly positive correlations between microbial abundance in module 3 and Cr, Ni, Cu, Zn, As, Cd, Pb, and Hg content. Cu and Zn content were found to be positively related to module 7 abundance ($p < 0.05$*) **(Fig. 2-8D)**.

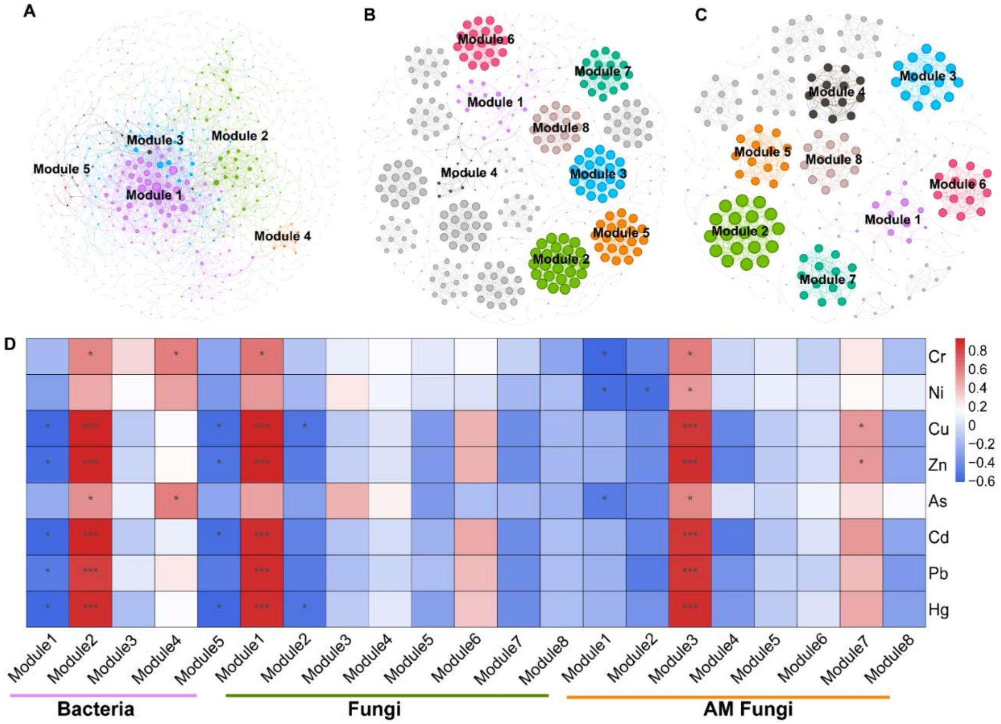

Figure 2-8: Network visualization of microbial co-occurrence patterns of (A) bacterial, (B) fungal, and (C) AM fungal community. And the relationships between heavy metal contents and the microbial network abundances (D).

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***.

Table 2-4: Properties of soil bacterial, fungal, and AM fungal co-occurrence networks.

	Node	Edge	Average degree	Average path length	Network diameter	Clustering coefficient	Density	Heterogeneity	Centralization
Bacteria	639	1483	4.64	4.26	12	0.33	0.01	1.29	0.06
Fungi	94	113	2.4	1.01	2	0.98	0.03	0.78	0.05
AM Fungi	250	972	7.78	1.15	4	0.98	0.03	0.7	0.04

3.3 The effect of biosolids application on soil microbial community assembly processes

The weighted βNTI combined with RCbray was used to calculate and assess the deterministic and stochastic aspects of microbial community assembly **(Fig. 2-9)**. Stochastic processes of drift predominantly governed bacterial community assembly, accounting for 80% of the observed dynamics. In comparison, deterministic methods of homogeneous selection were prevalent in shaping fungi (80%). AM fungal (80%) community assembly **(Fig. 2-9)**. Bacterial βNTI decreased with the increase in biosolids application doses, except for the SW4 treatment **(Fig. 2-9A)**. However, βNTI of fungal and AM fungal showed an increasing trend with the increasing application rates of biosolids **(Fig. 2-9B and 2-9C)**. Deterministic factors' role in shaping the assembly of fungal communities decreased by 33.33% in the SW1, SW2, and SW3 treatments compared to the control. And the role of deterministic processes in shaping AM fungal communities exhibited an increase of 33.33% and 66.67% in both SW3 and SW4 treatments, compared to the control **(Fig. 2-9D)**.

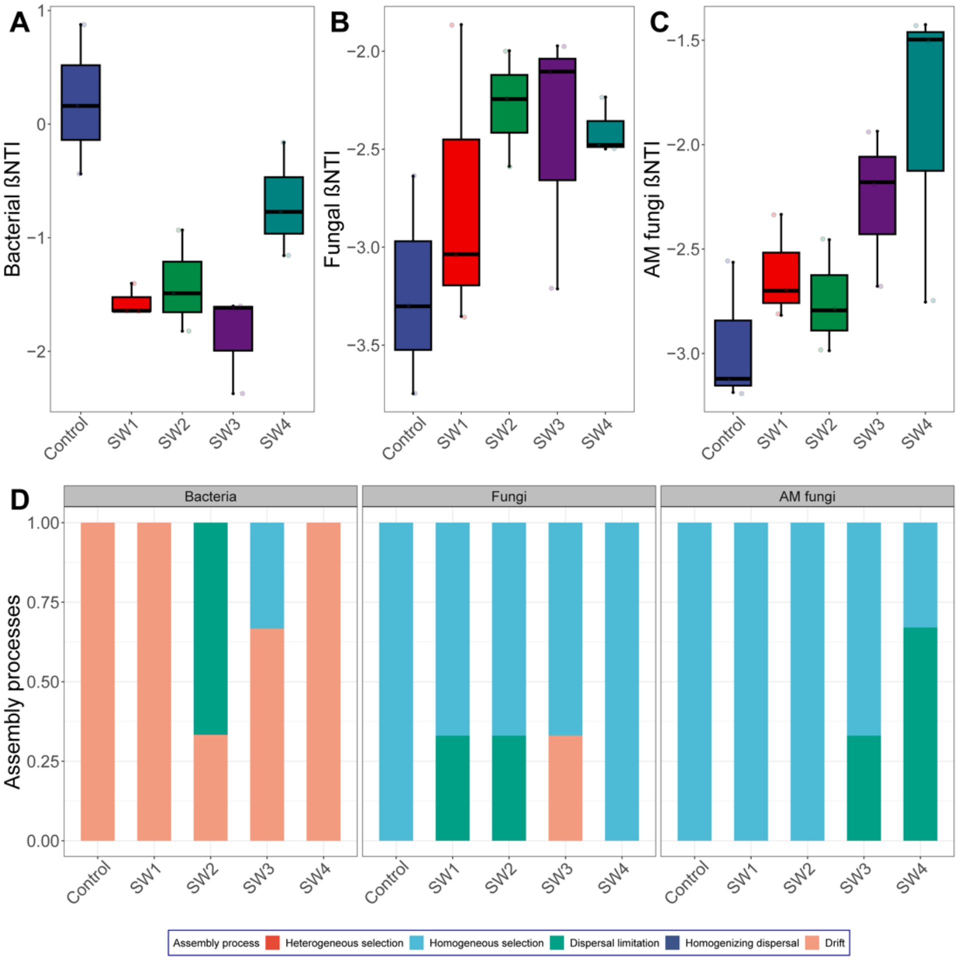

Figure 2-9: Effects of biosolids application on bacterial (A), fungal (B), and AM fungal (C) βNTI and community assembly processes (D).

Results of mantel test demonstrated that the bacterial community assembly processes could be correlated with AP, AK, TK, TN, SOM, NH_4^+, NO_3^-, Mg, Na, Cu, Zn, Cd, Pb, Hg, MBC, DON, and POXC **(Table 2-5)**. Furthermore, fungal community assembly processes exhibited significant associations with AP, TP, Ni, Cu, and Zn contents **(Table 2-5)**. AM fungal community assembly processes could be related to soil parameters such as pH, EC, NO_3^-, Nmin, Mg, Cu, Zn, MBC, MBN, DON, and POXC contents **(Table 2-5)**.

Table 2-5: The correlation between soil parameters and bacteria, fungi, and AM fungi community assembly processes by mantel test analysis.

Parameter	Bacteria		Fungi		AM fungi	
	R	*p*	R	*p*	R	*p*
pH	0.03	0.62	0.02	0.43	0.25	0.03*
Moisture	0.08	0.21	-0.01	0.55	0.21	0.06
AP	0.37	0.01**	0.21	0.05*	0.02	0.42
TP	0.18	0.1	0.19	0.05*	0.13	0.2
AK	0.31	0.01*	-0.04	0.63	0.18	0.1
TK	0.23	0.03*	-0.03	0.59	0.17	0.13
TN	0.55	<0.001***	0.14	0.1	0.19	0.06
SOM	0.53	<0.001***	0.17	0.06	0.09	0.21
EC	0.18	0.1	0.03	0.42	0.26	0.03*
NH_4^+	0.4	<0.01**	-0.03	0.6	0.12	0.17
NO_3^-	0.34	<0.01**	-0.07	0.73	0.26	0.03*
Nmin	0.26	0.03*	-0.03	0.6	0.33	0.02*
Mg	0.39	<0.01**	-0.06	0.71	0.31	0.01**
Na	0.34	<0.01**	-0.17	0.95	-0.27	0.99
Ca	0.19	0.07	0.02	0.56	0.19	0.03*
Cr	0.15	0.1	0.17	0.06	-0.04	0.64
Ni	0.05	0.26	0.24	0.03*	-0.11	0.79
Cu	0.53	<0.001***	0.23	0.04*	0.35	<0.01**
Zn	0.55	<0.001***	0.23	0.04*	0.36	<0.01**
As	0.05	0.36	0.12	0.15	-0.08	0.75
Cd	0.55	<0.001***	0.11	0.18	0.15	0.1
Pb	0.51	<0.001***	0.11	0.18	0.11	0.18
Hg	0.5	<0.001***	0.16	0.07	0.08	0.24
MBC	0.33	<0.01**	0.12	0.15	0.21	0.04*
MBN	0.1	0.2	0.12	0.15	0.3	0.01*
DOC	0.46	<0.001**	0.13	0.13	0.16	0.1
DON	0.41	<0.01**	0.1	0.2	0.32	0.01*
POXC	0.28	0.02	0.24	0.03	0.28	0.02*

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***

The results of random forest analysis regressions further illuminated the most crucial drivers of the community assembly processes. Spontaneous forestry regressions explained 66.67%, 36.71%, and 41.18% of variations of bacterial, fungal, and AM fungal community assembly process, respectively **(Fig. 2-10)**. AP, TN, Hg, SOM, Cu, NH_4^+, Cd, DOC, MBC, and Zn emerged as primary predictors of bacterial community assembly processes **(Fig. 2-10A)**. In the case of the fungal community assembly processes, POXC, Pb, SOM, Mg, and Cr were recognized as significant predictors **(Fig. 2-10B)**. Additionally, TN, As, and Ni were identified as significant predictors of AM fungal community assembly process **(Fig. 2-10C)**.

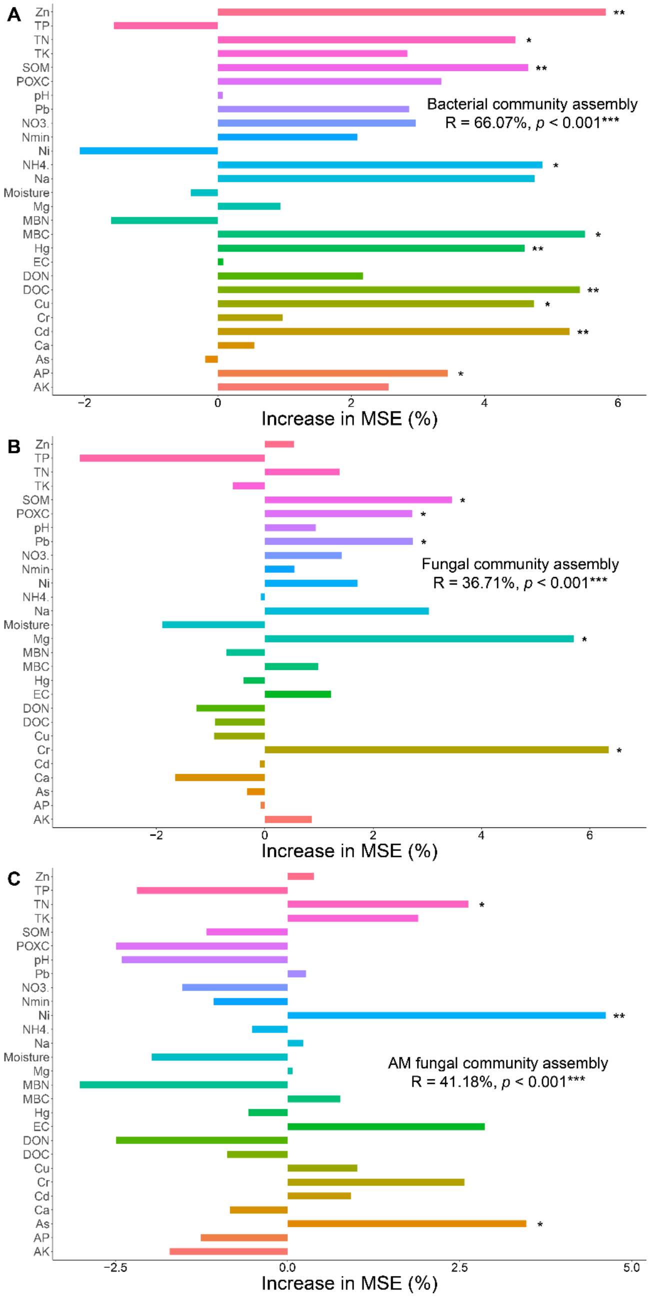

Figure 2-10: Important soil parameters as predictors of microbial community processes characterized by random forest regression.

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***.

4.Discussion

4.1 Distinct responses of bacterial, fungal, and AM fungal communities to biosolids application

The application of biosolids could change soil physicochemical properties, such as soil pH, some essential nutrients, and organic matter contents (Urra et al., 2019), which could shift the composition of soil microbial communities (Hu et al., 2019). However, the application of biosolids had the potential to elevate the contents of heavy metals in soils. The presence of these heavy metals could potentially pose risks to both public health and the overall ecosystem functions (Koupaie et al., 2015).

In this study, soil microbial diversity was found to be positively correlated with SOM and nutrient contents **(Fig. 2-4)**, supporting our first hypothesis that the application of biosolids increased soil microbial diversity by increasing nutrient and organic matter contents. Biosolids application significantly increased soil organic matter and nutrient contents, such as AP, and TN **(Table 2-3)**. SOM functions as a vital energy and nutrient source for microorganisms, playing a pivotal role in shaping microbial communities due to its availability (Jia et al., 2023). Soil organic matter improves microbial diversity by supplying soil substrates and stimulating indigenous organisms' growth (Bastida et al., 2021). Furthermore, soil AP and DOC content were observed to play a crucial role in describing the bacterial community patterns compared to fungi and AM fungi. This finding was similar to a previous study, which found bacteria were generally considered to be fast-growing, whose population fluctuates with soil nutrient concentrations (Wang et al., 2018). Fungal and AM fungal richness are more sensitive to heavy metal contents, in particular to Cr and Ni than those SOM and nutrient contents. In contrast, bacterial diversity was only correlated with soil nutrient contents **(Fig. 2-4)**. The different responses of bacterial and fungal richness may be due to their phylogenetic and evolution characteristics (Amend et al., 2016). In addition, fungi, especially certain species, are known to respond more sensitively to heavy metals than bacteria. Heavy metals like Cr and Ni can have toxic effects on fungal metabolism and growth, influencing fungal richness (Zeng et al., 2020a). AM fungi create symbiotic relationships with plant roots and play a crucial role in nutrient uptake, including metals. Their sensitivity to heavy metal concentrations might be linked to their role in plant-metal interactions and the potential for metal accumulation in their structures (Dhalaria et al., 2020). Their sensitivity to metal concentrations suggests a dual role: facilitating metal uptake by plants, potentially influencing metal bioavailability, and acting as reservoirs for heavy metals within their structures, such as arbuscules and hyphae (Priyadarshini et al., 2021). This sensitivity is likely associated with their known ability to modulate plant responses, playing a crucial role in coping with various stresses, including heavy metal stress. (Ghori et al., 2019).

The mantel test result showed that soil bacterial and AMF community compositions were significantly influenced by SOM and heavy metals **(Fig. 2-6)**. These results were consistent with previous study that organic matter could shift soil bacterial community composition by affecting heavy metals mobility and bioavailability (Lin et al., 2019).

Organic matter, such as humic and fulvic acids, has a high affinity to bind to heavy metals, thereby reducing the concentration of soil heavy metals (Lian et al., 2022).

Hence, the intricate influence of biosolids application on soil bacterial communities stems from the simultaneous introduction of nutrients and heavy metals, resulting in complex alterations in community dynamics. AM fungi act as a symbiont fungus that could help plants capture nutrients such as phosphorus and nitrogen from the soil (Van Der Heijden et al., 2008; Artursson and Jansson., 2006). Meanwhile, plants could supply carbon to AM fungi (Bennett and Groten, 2002). The interactions between plants and AM fungi improved plant growth by facilitating nutrient uptake from the soil. As plants thrive and gain access to more soil nutrients, they allocate more carbon compounds to AM fungi. This increased carbon supply to AM fungi is crucial in shaping the AM fungal community (Artursson and Jansson., 2006). In addition, previous studies found that symbiotic relationships with host plants can be facilitated by the rapid growth of hyphae, particularly those that thrive under metal toxicity and challenging environmental conditions (Cabral et al., 2015; Cui et al., 2019; Riaz et al., 2021). Therefore, variations of soil nutrients and heavy metals could alter the AM fungal community.

However, the fungal community was significantly affected by heavy metals, including Cr, Ni, and As. Fungi were generally more sensitive to heavy metals than bacteria, exhibiting diverse responses to such contamination (Pasqualetti et al., 2012; Zarei et al., 2010). The shift in the fungal community may be due to the selection of heavy metals-tolerance fungi phyla (Shen et al., 2023). This study also identified positive relationships between heavy metals and fungal phyla, notably *Rozellomycota* and *Blastocladiomycota*, known for their robust tolerance to heavy metals (Lin et al., 2019). In addition, the fungal network further confirmed the selection of fungi. Our results revealed a positive relationship between fungal abundance in module 1 and heavy metals. Notably, *Ascomycota*, the dominant fungi in module 1, was also found to have a solid tolerance to heavy metals (Shen et al., 2023). Basidiomycota is another important fungus in module 1, which is widely found in agricultural soils and effectively transfers heavy metals (Lin et al., 2019). Our results showed soil bacteria, fungi, and AM fungi respond distinctly to long-term application due to their phylogenetic characteristics, and indicate the impact of heavy metals on the soil microbial community appears to be more pronounced compared to the influence of soil nutrients.

4.2 Different ecological processes governing bacterial, fungal, and fungal assemblies

In this study, the stochastic process dominated the bacterial community, while deterministic methods shaped the fungal and AM fungal communities. The bacterial community exhibited substantial variability driven by stochastic drift processes in most soil samples. Drift represents stochastic changes in species abundance, leading to more significant than expected turnover (Silva et al., 2015). The bacterial community may enhance its capacity to adapt to environmental changes by employing self-resistance mechanisms and participating in synergistic interactions with other microorganisms (Zhang et al., 2022a). Furthermore, compared with fungi, bacteria often demonstrate higher rates of phylogenetic and taxonomic turnover, along with increased adaptability in resource utilization. This versatility enables them to swiftly adapt to evolving conditions, establish their presence, and effectively compete in various environments (Jia et al., 2023). However, the contribution of the stochastic process to bacterial community dynamics decreased with biosolids application, except for SW4 treatment. This shift may be due to selective pressure imposed by environmental factors (Evans et al., 2017). The development of random forest showed that Zn, MBC, DOC, and Cd played significant roles in shaping the bacterial community assembly. Heavy metals like Zn and Cd likely exert selective pressure leading to the reduction or elimination of bacterial species with limited heavy metal tolerance (Giller et al., 1998). Cd and Zn have the potential to diminish bacterial biomass and diversity, as well as hinder bacterial enzyme activities within soils contaminated by heavy metals (Tang et al., 2022). Meanwhile, DOC could indeed have a positive impact on the diversity of soil bacterial communities, as it serves as a significant energy source that promotes bacterial growth and metabolic activities (Xu et al., 2021). The findings that showed dispersal limitation increased in SW4 treatment suggest that the beneficial impacts of DOC on bacterial communities surpass the selective pressures imposed by heavy metals.

Homogeneous selection, and deterministic processes, appeared to significantly drive fungal communities' turnover (Wang et al., 2020). Homogeneous selection commonly becomes a predominant factor in shaping communities during stable states following disturbances and is often associated with physicochemical variables (Wang et al., 2020). In the context of long-term biosolids application, it is possible that the fungal community reached a relatively stable state as fungi gradually adapted to elevated heavy metal levels. Homogeneous selection implies that particular fungi strains or groups within a population possess genetic traits that enhance their tolerance to heavy metal exposure. As a result, these fungi thrive and become more prevalent in environments with high heavy metal concentrations (Schlatter et al., 2017).

Furthermore, the dominance of homogenous selection in shifting fungal community may stem from fungi's heightened adaptability and tolerance to heavy metals and metalloids, surpassing that of bacteria (Shukla et al., 2019). Additionally, dispersal limitation contributes to shaping fungal and AM fungal community assembly. Heavy metals such as Cr and Ni recognized as the primary drivers of fungal and AM fungal community assembly, could impose more substantial dispersal limitations on the microbial community (Zhong et al., 2023). The change in dispersal limitation and homogeneous selection may stem from variations in microorganisms' dispersal/diffusion abilities across different domains (Zhang et al., 2022a). Our result indicated that heavy metals drive the microbial community assembly process in long-term biosolids application farmland.

5.Conclusion

In conclusion, our study showed a significant influence of the long-term application of biosolids on various aspects including soil physicochemical parameters, heavy metal concentrations, and microbial community composition and assembly process. Notably, soil microbial communities exhibited distinctive responses to biosolids applications. While both soil organic matter (SOM) and heavy metals (Cu, Pb, Cd, Zn, and Hg) influenced bacterial and arbuscular mycorrhizal fungal communities, only the fungal community responded to heavy metals (Cr, Ni, and As). Microbial co-occurrence network analysis identified unique modules within soil microorganisms, each exhibiting differential responses to heavy metal contents. Remarkably, stochastic processes primarily governed bacterial community assembly, whereas deterministic processes had more influence on fungal and AM fungal communities. Furthermore, heavy metals played a significant role in steering microbial community assembly, with specific heavy metals acting as crucial drivers for distinct microbial groups. Zinc, Chromium, and Nickel emerged as the most influential drivers for bacterial, fungal, and AM fungal community assembly, respectively. These findings provide insights into the distinct responses of bacterial, fungal, and AMF communities to biosolids applications, offering valuable guidance for biosolids application practices.

Chapter 3

Microbial and antibiotics resistance genes responses to simulated co-contamination of antibiotics and heavy metals

Sun Tao, Delaplace, P., Li, G., James, A., Pan, J., & Zhang, J. Biosolids application to agricultural soil: heavy metal as key driver of antibiotic resistance under co-occurrence of multiple selective pressures. Under Review.

The long-term field experiment demonstrated that biosolids significantly influence soil microbial communities. However, field conditions often involve multiple interacting factors, making it difficult to isolate their individual effects. Therefore, this chapter presents a controlled microcosm experiment designed to clarify the respective roles of heavy metals and antibiotics introduced through biosolids.

Abstract

The application of biosolids can improve soil fertility and nutrient cycling but also can pose risks of heavy metals and antibiotics introduction. The individual effects of heavy metals and antibiotics accumulation on soil microbial communities and functions have been reported, however, their combined effects during biosolids application to agricultural soil remain unclear. In order to explore the effects of long-term biosolids application on soil microbial communities, we sampled soil from a field experiment spanning 16 years of biosolids application and determined the abundance of resistance genes in the soil. The results showed that long-term biosolids application significantly increased the abundance of antibiotic resistance genes (ARGs) and metal resistance gene (MRGs), especially of aminoglycoside and Zn resistance genes. Based on these results we conducted a microcosm experiment that involved the addition of both a heavy metal and an antibiotic to the soil. This experiment showed that both the heavy metal and the antibiotic significantly affected soil microbial communities, while heavy metal acted as the dominant selective force under their co-selection. Further, core microbiome abundance was positively related to ARGs, MRGs and mobile genetic elements (MGEs) abundance, and explained the variations in antibiotic genes. In addition, core microbiome abundance showed positive relationship with microbial community stability. These findings emphasize the need for careful management of biosolids application to mitigate the risks associated with resistance genes accumulation, which could pose long-term ecological and agricultural challenges. Additionally, the positive relationship between core microbiome abundance and microbial community stability underscores the potential role of microbial communities in enhancing soil resilience against environmental stressors.

Keywords: Metal resistance genes; mobile genetic elements; core microbiome; microbial community shift.

The graphical abstract is displayed in **Figure 3-1**.

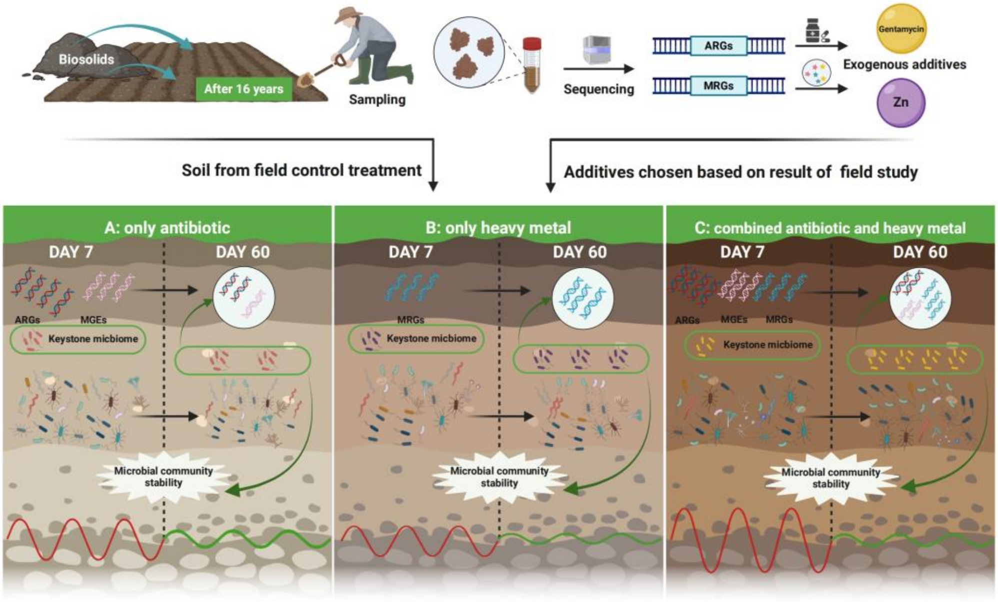

Figure 3-1: Chapter 3 graphical abstract.

1.Introduction

The application of biosolids has been recognized as an effective method for enhancing soil fertility and improving crop productivity (Elgarahy et al., 2024; Boudjabi et al., 2021). However, long-term application of biosolids poses a risk of accumulating heavy metals and antibiotics in the soil (Sun et al., 2024; Mossa et al., 2020). Soil microbial communities play essential roles in soil ecosystems, including nutrient cycling, organic matter decomposition, and plant growth regulation (Raza et al., 2023; Bhattacharyya et al., 2022). These communities are highly sensitive and vulnerable to changes in soil physicochemical properties, such as pH, and metal ion concentration, and antibiotic presence (Fu et al., 2023; Naz et al., 2022). Heavy metals such as zinc (Zn) and copper (Cu) can disrupt microbial cell membrane integrity and function, and antibiotics exert selective pressure on microorganisms, leading to the accumulation of antibiotic-resistant genes (ARGs) (Engin et al., 2023; Nguyen et al., 2019). These impacts can reduce soil microbial diversity and alter ecosystem functioning, ultimately impacting soil health. Although the individual impacts of heavy metals and antibiotics accumulation on soil microbial communities and ecosystem functions have been reported, the combined effects of both pollutants during the biosolids application in soil remain unclear. Understanding how heavy metals and antibiotics impact separately and together in soils, and how they influence soil microbial communities and their functions, is crucial for maintaining environmental and ecological health.

In soils exposed to both heavy metals and antibiotics, the combined effect of these pollutants may be more complex than their individual impact (Zhou et al., 2022; Rahman, 2020). Studies have shown that antibiotics and heavy metals can act synergistically in what is termed as a co-selection process (Murray et al., 2024; Vats et al., 2022). In this process, microorganisms that are resistant to one pollutant may also develop resistance to the other, leading to a broader spread of resistance genes (Engin et al., 2023; Imran et al., 2019; Yu et al., 2017). Due to the longer persistence of heavy metals in the environment, they might exert a more pronounced influence on microbial community shifts and resistance gene accumulation compared to antibiotics. This complexity is exacerbated by the fact that the accumulation of both heavy metals and antibiotics in the soil is often associated with the long-term persistence of resistance genes, which can be transferred through mobile genetic elements (MGEs) (Mazhar et al., 2021; Hu et al., 2017). The horizontal transfer of ARGs and metal resistance genes (MRGs) among microorganisms can significantly affect the diversity and stability of microbial communities, further complicating the long-term ecological impacts of biosolids application (Sun et al., 2025; Qin et al., 2022; Feng et al., 2021). These processes, when combined, may lead to a “resistance reservoir” in the soil, posing further risks to soil health and biodiversity. Understanding the mechanisms through which heavy metals and antibiotics jointly impact microbial communities is essential for predicting the ecological consequences of long-term biosolids application. Further, the primary driver of antibiotic resistance under co-occurrence of multiple selective pressures during long-term biosolids application remains unknown.

Core microorganisms persist across a wide range of environmental conditions and play fundamental roles in maintaining soil functions (Hartmann and Six, 2023; Hattori et al., 1976). In the context of heavy metal and antibiotic pollution, core microbes may help maintain ecosystem functions by tolerating and adapting to these stressors (Wang et al., 2019; Prabhakaran et al., 2016). These microorganisms may also contribute to the degradation of organic pollutants and the cycling of nutrients, such as nitrogen and carbon, in the presence of pollutants (Ahmad et al., 2022; Mbachu et al., 2020). Thus, core microbes could be critical in mitigating the impacts of heavy metal and antibiotic pollution on microbial community stability. They may act as key drivers in maintaining the structure and functionality of the microbial community in the face of such environmental stressors (Gillieatt and Coleman, 2024; Burdon et al., 2020). Understanding how these core microbes interact with heavy metals and antibiotics, and how their abundance and diversity change over time, could provide valuable insights into how to enhance the resilience of soil ecosystems subjected to prolonged pollution.

To provide a comprehensive understanding of how long-term biosolids application affects soil microbial communities and their functional stability, soil samples from a long-term field experiment that had been exposed to biosolids for 16 years were collected and the abundance of antibiotic and heavy metal resistance genes was determined. Based on this field experiment's results, a microcosm experiment was designed that involved the addition of both a heavy metal and an antibiotic whose resistance genes were found to be of the highest concentration to the soil applied with biosolids. This study hypothesizes that in the co-selection process, the heavy metal rather than the antibiotic plays a dominant role in driving resistance genes and microbial community changes. The other hypothesis is that changes in core microbiome could explain the observed changes in the abundance of resistance genes in response to the combined presence of the heavy metal and the antibiotic. The results of this study will provide a roadmap for making an informed decision on the optimal rate of application of biosolids while taking into consideration its potential to augment antibiotic resistance in the environment.

2.Materials and methods

2.1 Soil sampling and microcosm experimental design

As previously described by our group (Chapter 2-Sun et al., 2024), soil samples were collected from all five treatments (SW0–SW4) of a 16 years long-term field experiment with biosolids application rates of 0 t ha^{-1} (SW0), 4.5 t ha^{-1} (SW1), 9 t ha^{-1} (SW2), 18 t ha^{-1} (SW3), and 36 t ha^{-1} (SW4). SW0 served as the control without biosolids application. Gene abundances of ARGs and MRGs were first assessed in these field soils, and aminoglycoside- and zinc-resistance genes exhibited the highest relative abundance. Based on these results, SW0 soil was used for the microcosm experiment, in which gentamicin and $ZnSO_4$ were added exogenously to simulate biosolids-induced contamination. Based on commonly used doses in previous soil microcosm studies (DeVries et al., 2015; Sanchez-Cid et al., 2021), gentamicin sulfate was applied at concentrations of 5, 10, and 30 mg/kg. According to the Soil

Environmental Quality Risk Control Standard for Soil Contamination of Agricultural Land (GB 15618-2018), the risk screening value for zinc in soils with pH 6.5–7.5 is 250 mg/kg. As the pH of the SW0 soil measured in Chapter 2 was 7.1, $ZnSO_4$ was supplemented at concentrations of 125, 250, and 500 mg/kg to represent sub-threshold, threshold, and excessive levels relative to this risk screening benchmark. The experimental treatments were categorized as follows: treatment without antibiotic or heavy metal additions (Control); antibiotic-only treatments (A1, A2, and A3); heavy metal-only treatments (B1, B2, and B3); combined antibiotic and heavy metal treatments (C1, C2, and C3). A total of 180 bottles were used in the experiment, corresponding to 10 treatments with 18 replicates each.

2.2 Soil preconditioning and incubation

The air-dried SW0 soil was adjusted to 15 % gravimetric moisture content (≈ 60 % of field capacity) and pre-incubated at 25 °C in the dark prior to amendment, following established soil incubation protocols that optimize microbial activity and stability (Zheng et al., 2019). Following pre-incubation period, the soil was thoroughly mixed and evenly divided. Each incubation bottle contained 50 g of dry soil with 20% moisture content, and the bottles were wrapped in aluminum foil to maintain dark conditions. The incubation was conducted at 25°C for 60 days. Destructive sampling was performed at Day 1, Day 3, Day 7, Day 15, Day 30, and Day 60, with three aliquots removed per treatment at each time point. The collected soil samples were stored at -20 °C for the determination of microbial community and functioning. Microbial community composition analysis was conducted on soil samples collected at Day 1, Day 30, and Day 60. Metagenomic sequencing was performed on samples from Day 7 and Day 60.

2.3 Soil DNA extraction, sequencing and bioinformatic analysis

Total genomic DNA was extracted from 0.5 g of soil using the MagaBio Soil DNA Extraction Kit (Bioer Technology, China). To analyze the bacterial community, the V4 region of the 16S rRNA gene was amplified using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Walters et al., 2016). Fungal communities were targeted via the ITS region using primer pair ITS3F/ITS4R (Op De Beeck et al., 2014), while arbuscular mycorrhizal fungi (AMF) were detected using primer combinations AML1/AML2 and AMV4.5NF/AMDGR as described by Van Geel et al. (2014). All primers were synthesized by Invitrogen (Thermo Fisher Scientific). Sequencing was conducted on an Illumina Nova6000 platform, producing 250 bp paired-end reads (Guangdong Magigene Biotechnology Co., Ltd. Guangzhou, China)/ Quality control was performed on initial pair-end reads using Trimmomatic v0.39 (Bolger et al., 2014) to remove sequences with a quality score below 20. Subsequently, FLASH v1.2.7 combined the pair-end reads, followed by denoising and chimera filtering using the 'USEARCH' algorithm (Edgar, 2010). Reads shorter than 200 bp were excluded. Paired-end reads were demultiplexed based on unique sample-specific barcodes. After merging the reads, quality filtering and chimera removal were conducted using the DADA2 pipeline. Taxonomic assignments were performed using the SILVA v138 database for bacteria (Green et al., 2022), the UNITE v8.0 database for fungi (Eshaghi et al., 2021), and the Maarj*AM* database for AMF (Öpik et al., 2010).

Shotgun metagenomic libraries were prepared by Shanghai Biozeron Biological Technology Co., Ltd. using the TruSeq DNA Library Prep Kit (Illumina, catalog no. FC-121-2001). Library concentrations were quantified with a Qubit Fluorometer and High Sensitivity dsDNA Kit (Thermo Fisher Scientific). Sequencing was performed using a paired-end 150 bp (PE150) strategy on an Illumina NGS platform.

2.4 Statistical analysis

All data analyses were conducted using R (version 4.1.0). Soil microbial community stability was assessed using the average variation degree (AVD), which reflects the deviation of species' relative abundances from a normal distribution (Xun et al., 2021). A higher AVD indicates greater community variability, while a lower AVD suggests increased stability; therefore, community stability was expressed 1/AVD. Core microbiome identification was conducted using abundance–occupancy relationships, with neutral model fitting based on Sloan's approach (Shade and Stopnisek, 2019). Non-metric multidimensional scaling (NMDS) was employed to assess the effects of antibiotics and heavy metals additions on soil microbial communities and antibiotic gene communities across over time. Two-way ANOVAs were employed to evaluate the variations in microbial diversity and antibiotic gene abundances over time. To compare the relative abundances of antibiotic resistance genes (ARGs) among different treatments, Statistical Analysis of Metagenomic Profiles (STAMP) was used. STAMP enables the identification of statistically significant differences in functional gene profiles between groups while controlling false discovery. In this study, ARG annotation data derived from metagenomic sequencing were imported into STAMP, and pairwise comparisons were conducted between treatments (e.g., Control vs. antibiotics, Control vs. heavy metals, and combined treatments) using two-sided Welch's t-test. A Benjamini–Hochberg false discovery rate (FDR) correction was applied to control for multiple testing. Differences were considered statistically significant when adjusted p-values were below 0.05 and effect size (difference in proportion) exceeded a biologically relevant threshold (e.g., ≥1%). The results were visualized as extended error bar plots or bar graphs, showing both the direction and magnitude of gene abundance changes. This analysis allowed us to identify ARGs that were significantly enriched or suppressed under specific treatment conditions, which are discussed in detail in the following results section. Ordinary least squares (OLS) were employed to determine the relationship between core microbiome abundances and antibiotic gene abundances as well as microbial community stability. Random forest regression analysis was employed to predict the important microbial ASVs for antibiotic genes.

3.Results

3.1 Effects of biosolids amendments on ARGS and MRGs

Long-term biosolids application significantly affected abundances of both ARGs and MRGs in the agricultural field soils **(Fig. 3-2; Fig. 3-3)**. Specifically, ARGs abundance was significantly increased in the high biosolids application rates of SW3 and SW4 compared to Control **(Fig. 3-3A; Fig. 3-4)**. However, there was no significant influence on SW1 and SW2 treatment **(Fig. 3-3A)**. Among all genes, aminoglycoside antibiotic and MLSB were the most abundant classes of antibiotic resistance genes. The abundance of aminoglycoside antibiotics was significantly enriched in SW4 treatment. And MLSB abundance increased in both SW3 and SW4 treatment **(Fig. 3-3A)**. MRGs abundances were significantly increased in all biosolids treatments compared to Control **(Fig. 3-3B; Fig. 3-5)**. The resistance genes to Zn and Ni were the most abundant class among all MRGs, and their abundances significantly increased after biosolids application. In addition, biosolids application significantly increased the abundance of Cu resistance genes.

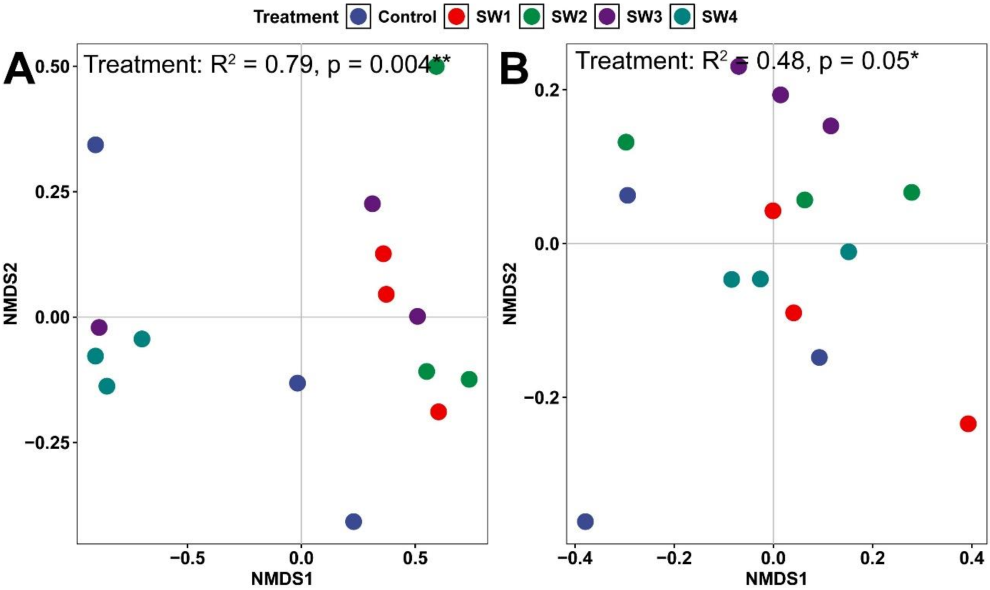

Figure 3-2: The effect of biosolids application on antibiotic resistance genes (A) and metal resistance genes (B) communities in soils.

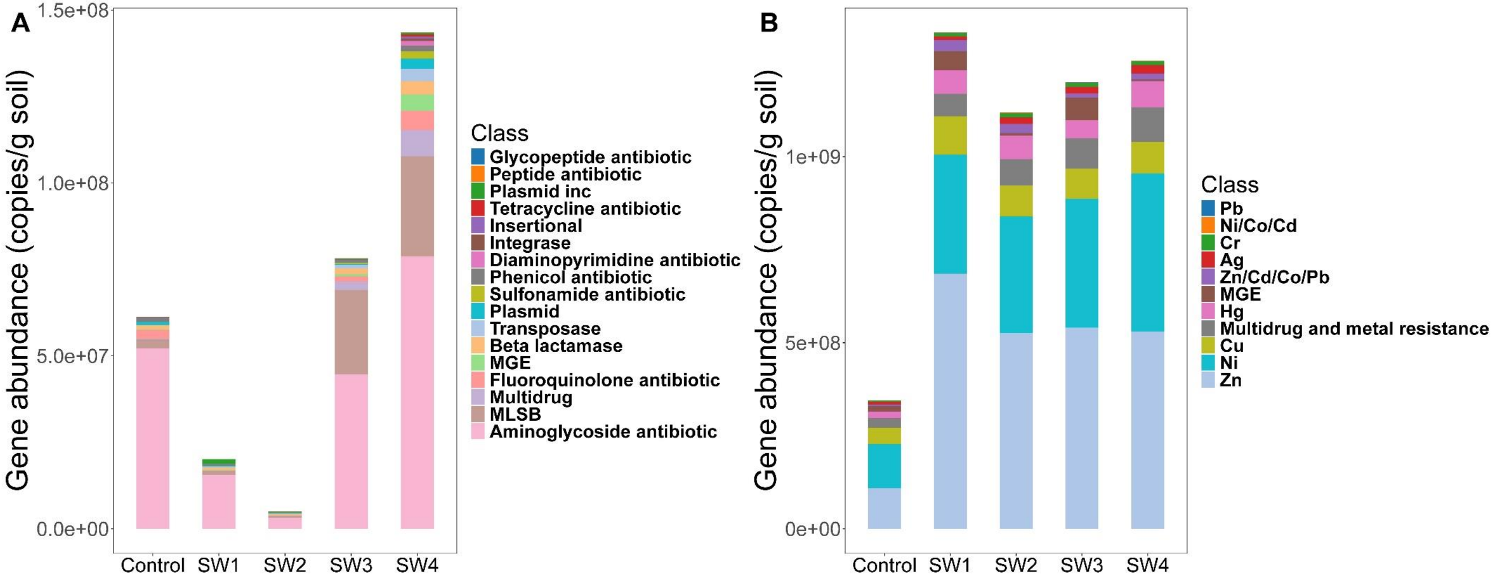

Figure 3-3: The effect of biosolids application on antibiotic resistance genes (A) and metal resistance genes (B) in soil.

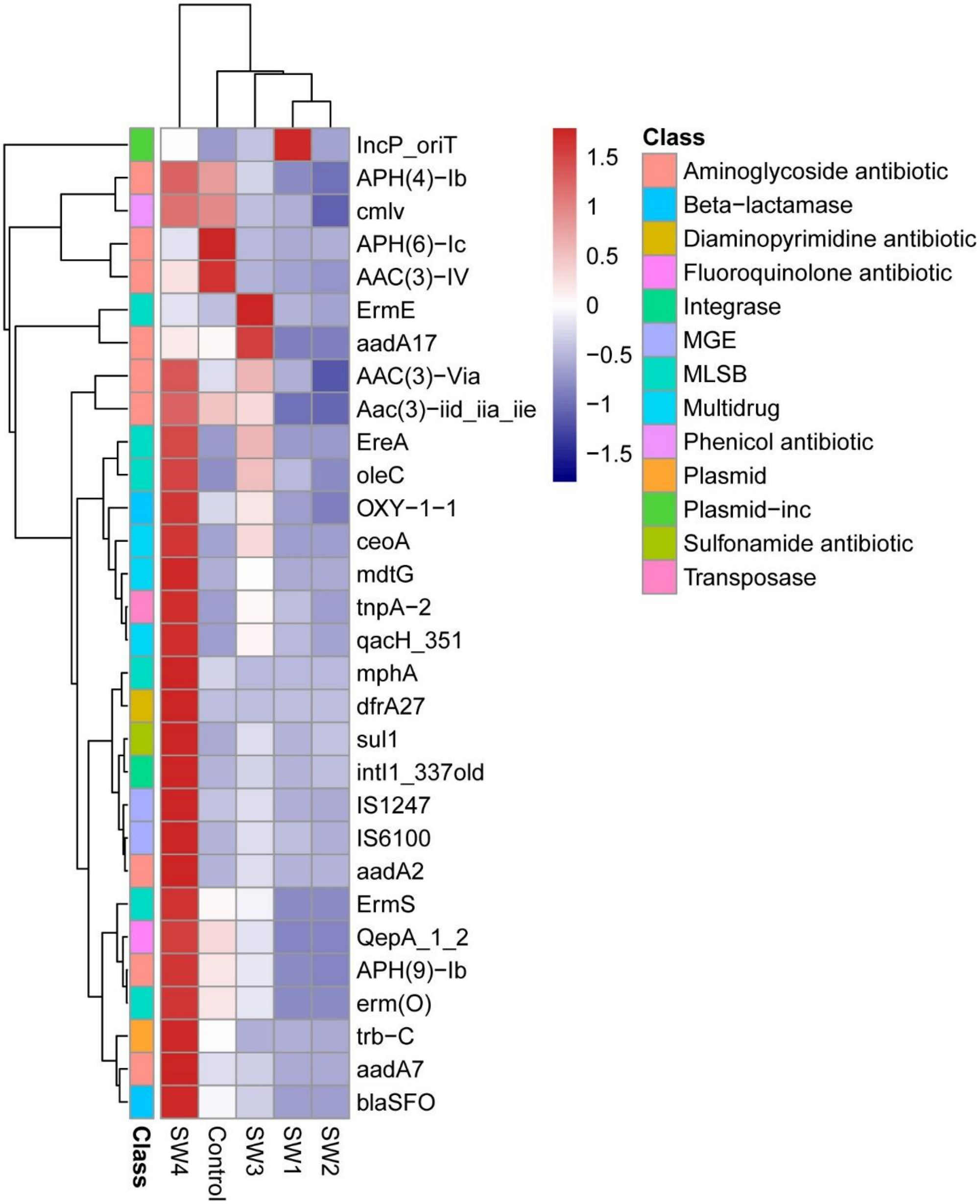

Figure 3-4: The effect of biosolids application on antibiotic resistance genes abundance.

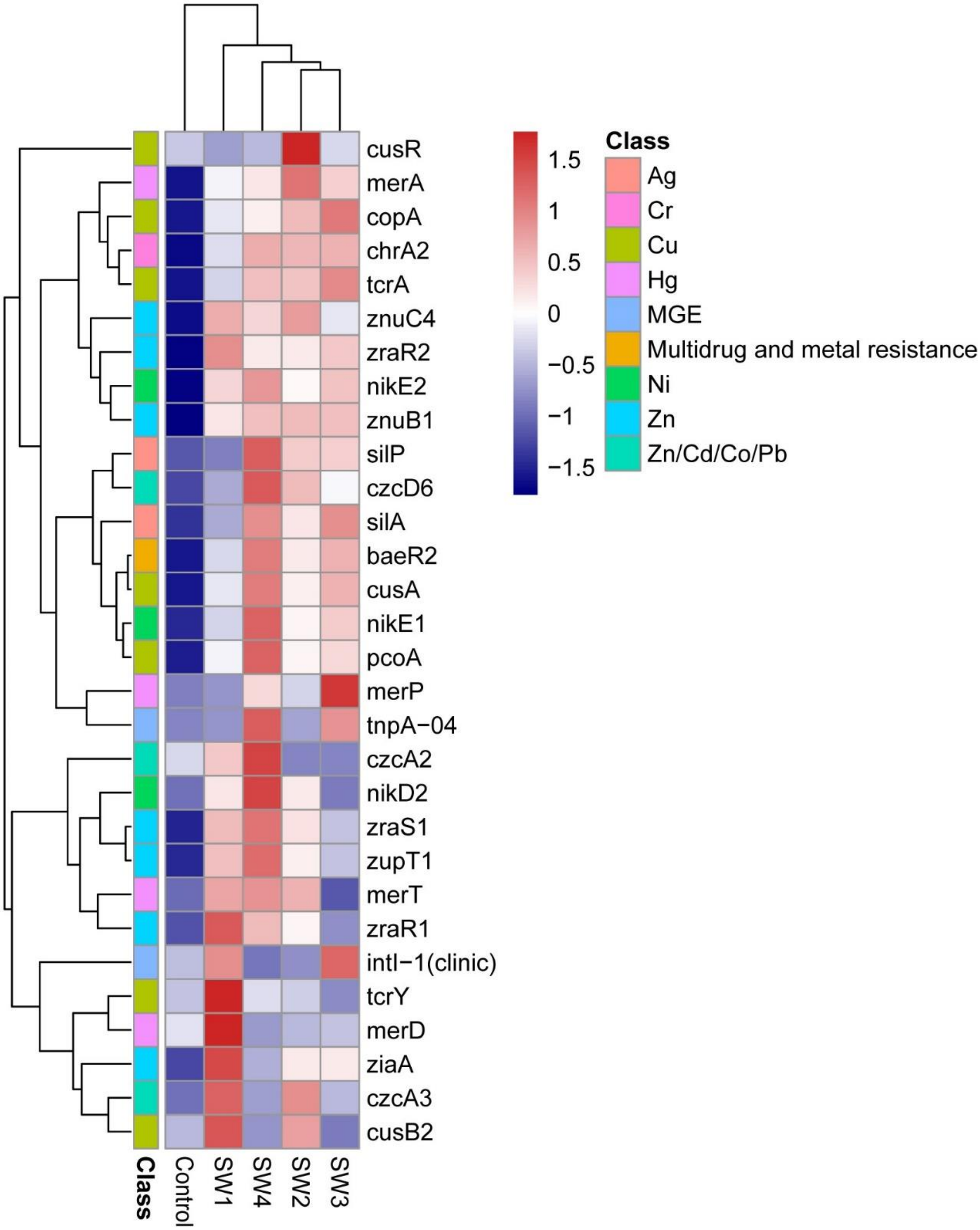

Figure 3-5: The effect of biosolids application on metal resistance genes abundance.

3.2 Effects of heavy metals and antibiotics on soil microbial community

$ZnSO_4$ and gentamicin sulfate were added to the soil to simulate biosolids-induced contamination and to explore their effects on soil microbial community. The result showed that the bacterial Shannon index was significantly different on Day 7 and Day 30 while fungal community was significantly different on Day 1 **(Fig. 3-6, Fig. 3-7)**. For the Day 7, antibiotic addition significantly decreased bacterial diversity while heavy metal addition showed no significant influence on bacterial diversity. Compared to Control, bacterial diversity decreased in C1 treatment while increased in C2 treatment. During Day 60, antibiotic addition significantly increased bacterial diversity while heavy metal addition showed no significant influence on bacterial diversity **(Fig. 3-6)**. As regards the fungal diversity, antibiotic addition significantly decreased fungal diversity while heavy metal addition and combined addition of both antibiotic and heavy metal showed no significant influence on fungal diversity on Day 1. Overall, both bacterial and fungal communities were significantly affected by the addition of heavy metal and antibiotic individually and by their combined addition at all days tested **(Fig. 3-8)**.

The results of metagenomic studies revealed that ARGs, MRGs and MGEs communities were significantly affected by antibiotic and heavy metal addition in both Day 7 and Day 60 samples **(Fig. 3-9)**. The ARG gene *CRD3-1* was found to be enriched after both antibiotic and heavy metal additions as well as their combination on Day 7 **(Fig. 3-10)**. Further, the ARGs *OXA-444*, *mdtF*, and *CRD3-1* were found to be enriched after antibiotic and heavy metal additions as well as their combination on Day 7 **(Fig. 3-10)**. As regards MRGs *tnpA* was found to be enriched in all three treatments compared to Control on Day 7, and *tnpA1* was found to be enriched after heavy metal only and combined addition on Day 7 **(Fig. 3-11)**. For MGEs, on Day 7, genes *merR*, *acr3*, *merE*, *co*pB, *merR1*, *dpsA*, *merA*, *actA*, *yfeB*, *nrsS*, *copP*, *yfeC*, *merB3, and co*pD were found to be enriched after antibiotic addition. The genes *merR*, *tupA*, *merE, and cmeB* were enriched after heavy metal addition, and *acn*, *copB*, *actA*, *tupA*, *merE* were enriched after combined addition **(Fig. 3-12)**. However, no genes were found to be enriched after only antibiotic addition on Day 60, while *ugd*, *CRD3-1, smeE*, and *OXA-444* were enriched after heavy metal addition **(Fig. 3-13)**. Most of these genes (*ugd, CRD3-1* and *smeE*) were also enriched during the combined addition of antibiotic and heavy metal on Day 60 **(Fig. 3-13)**. On Day 60, MRG *tnpAB* was found to be enriched in all three treatments compared to Control **(Fig. 3-14)**. On Day 60, MGEs *yfeD*, *copD*, *yfeB*, *yfeA*, *merD*, *mexI* and *nrsS* were found to be enriched after antibiotic addition. The MGEs *merT-P*, *arsH*, *yfeD*, *copD*, *tupA*, and *nrsS* were enriched after heavy and combined additions **(Fig. 3-15)**.

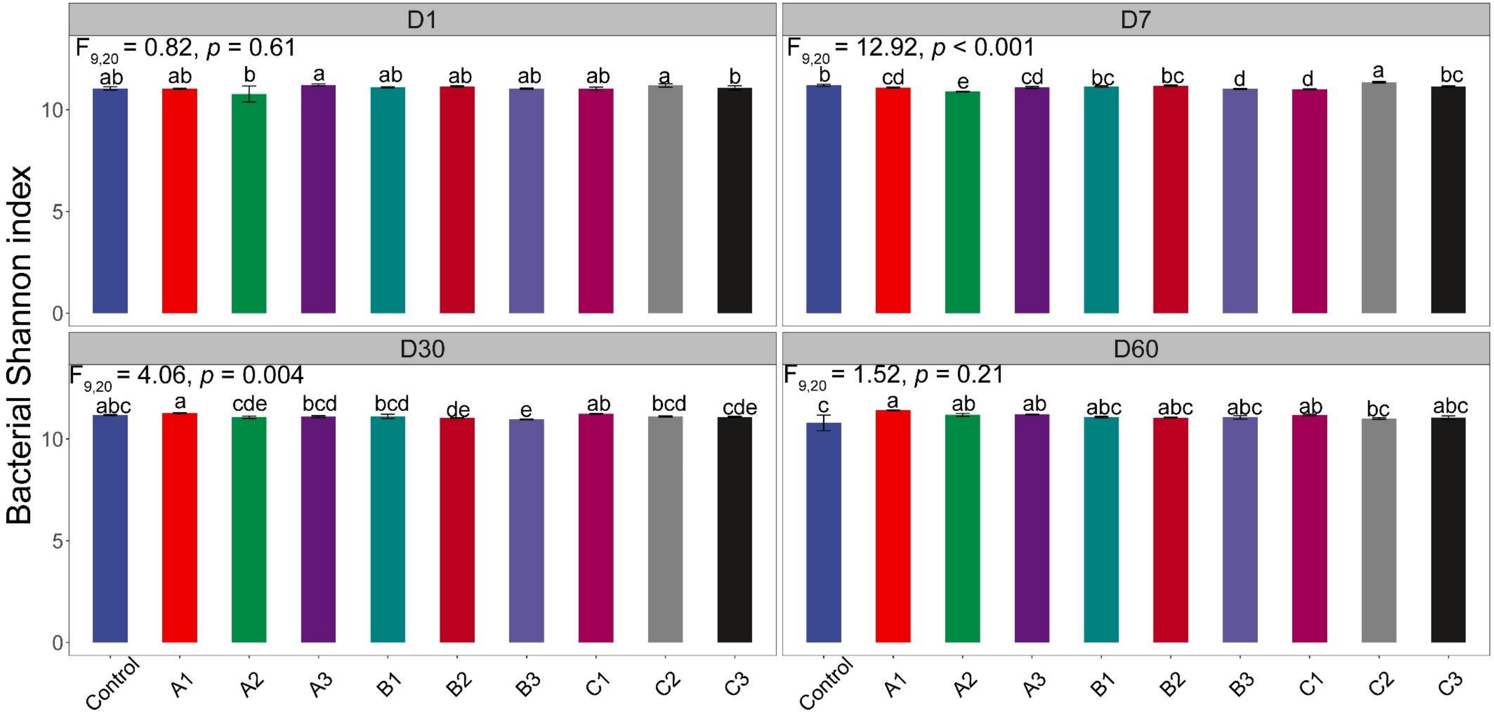

Figure 3-6: The effect of antibiotic, heavy metal and their combined additions on soil bacterial diversity at different incubation times.

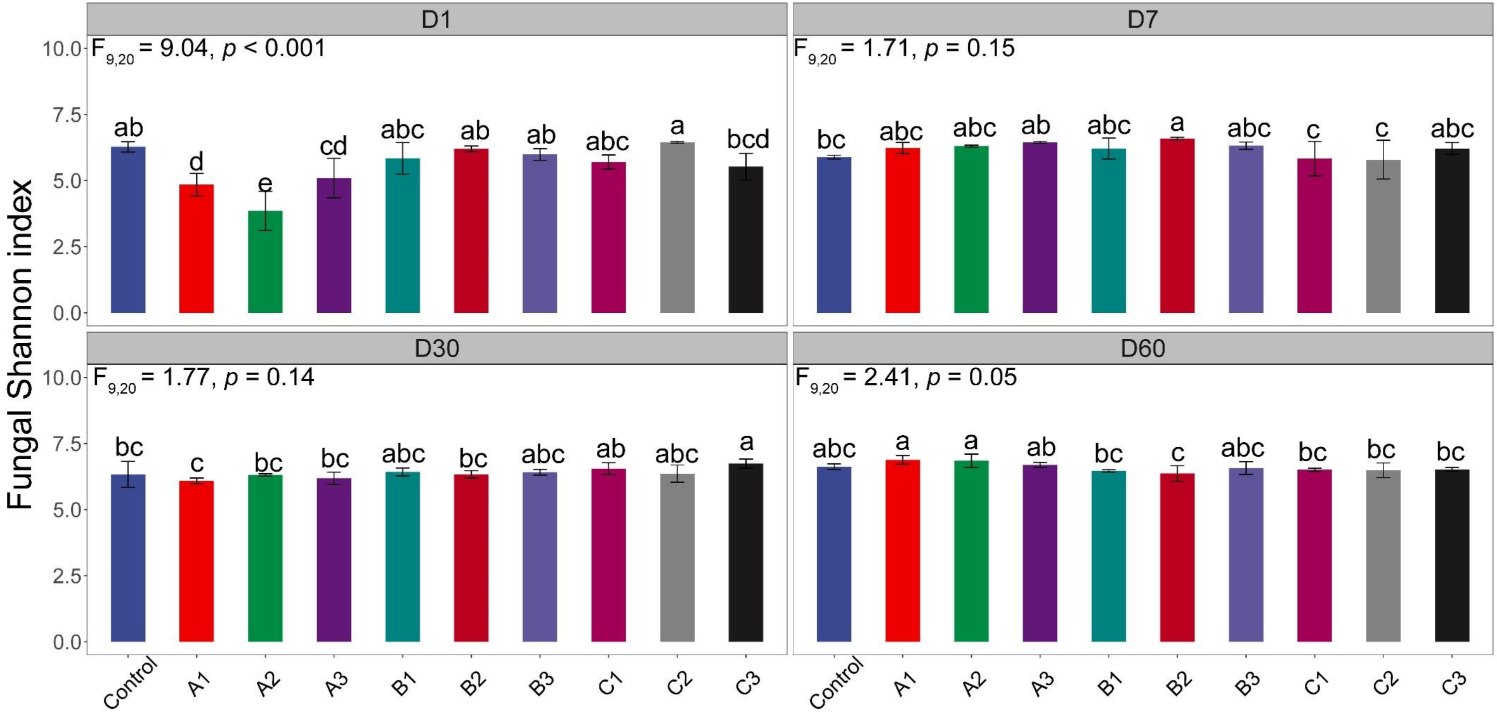

Figure 3-7: The effect of antibiotic, heavy metal and their combined additions on soil fungal diversity in different incubation times.

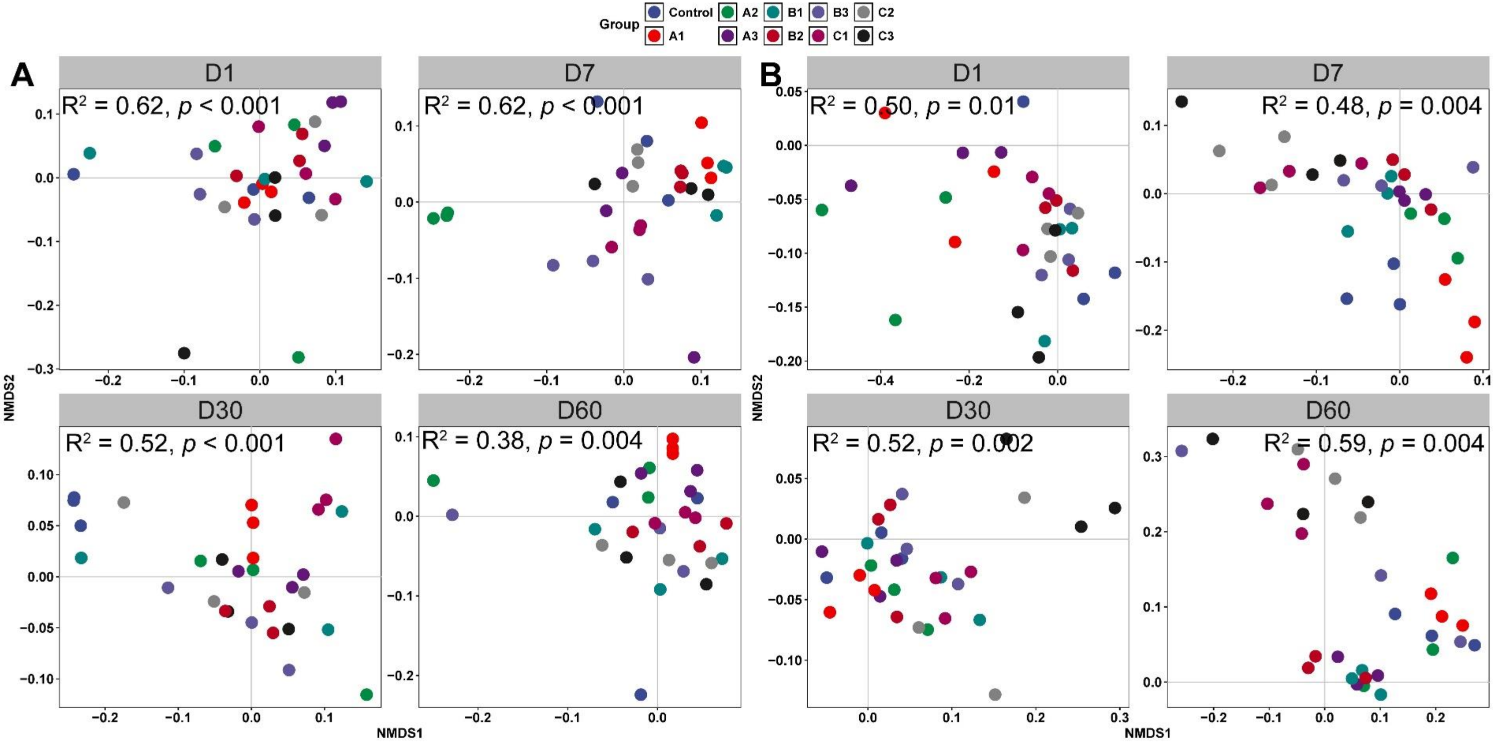

Figure 3-8: The effect of antibiotic, heavy metal and their combined additions on soil bacterial (A) and fungal (B) communities at Day 1, Day 7, Day 30 and Day 60.

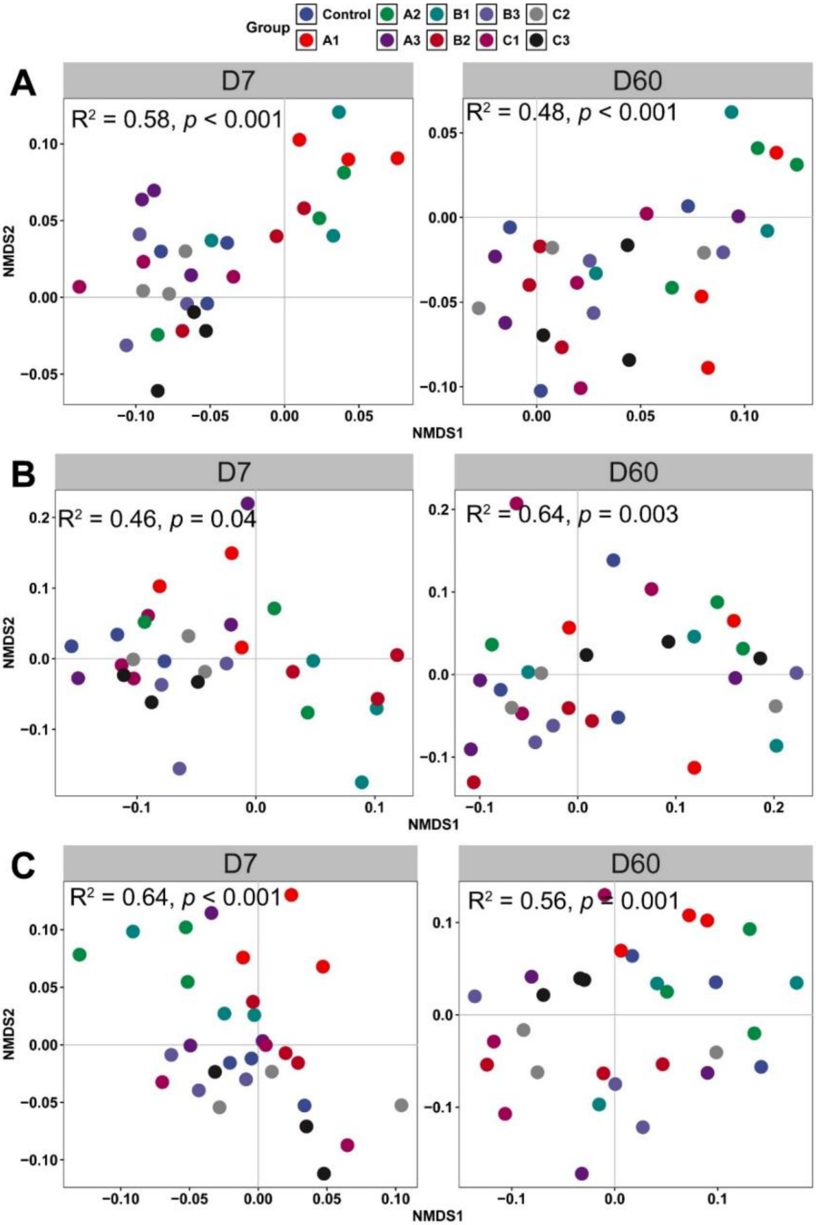

Figure 3-9: The effect of antibiotic, heavy metal and their combined additions on soil ARGs (A), MRGs (B) and MGEs (C) communities at Day 7 and Day 60.

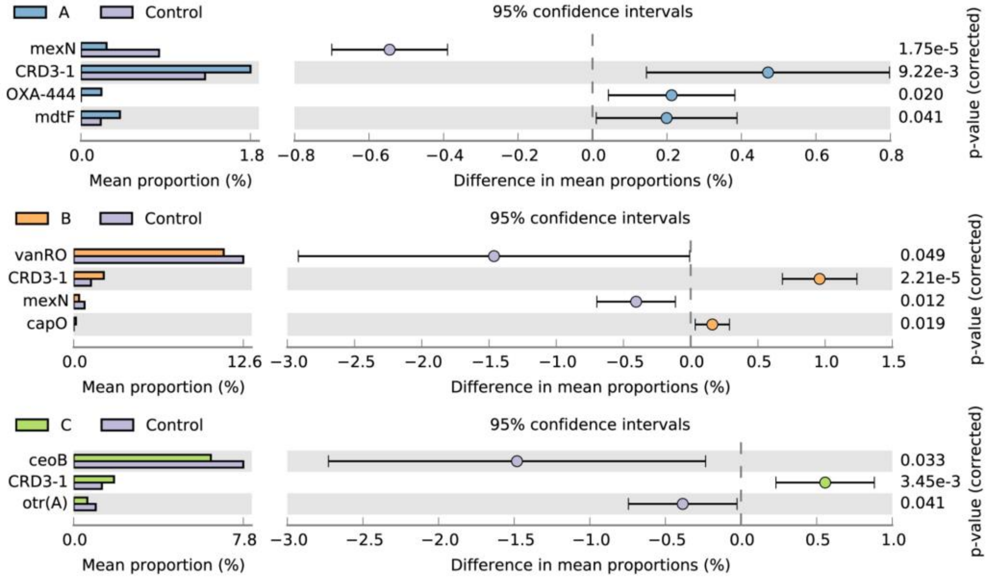

Figure 3-10: The effect of antibiotic, heavy metal and their combined additions on soil ARGs on Day 7.

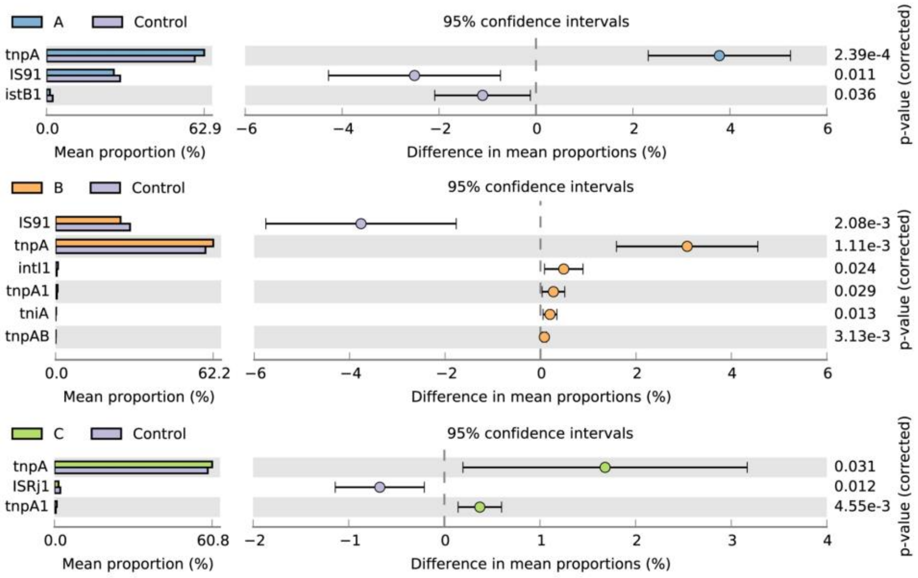

Figure 3-11: The effect of antibiotic, heavy metal and their combined additions on soil MRGs on Day 7.

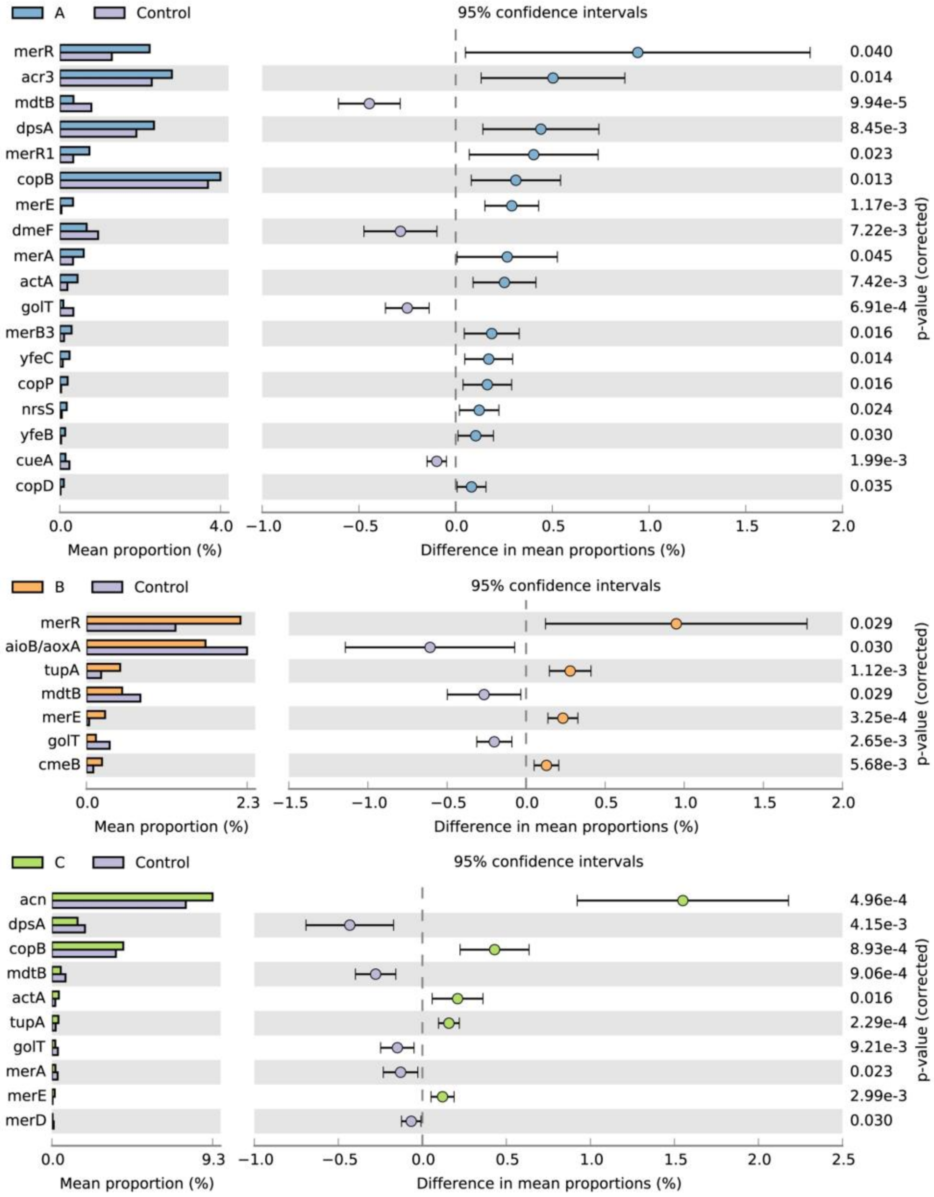

Figure 3-12: The effect of antibiotic, heavy metal and their combined additions on soil MGEs on Day 7.

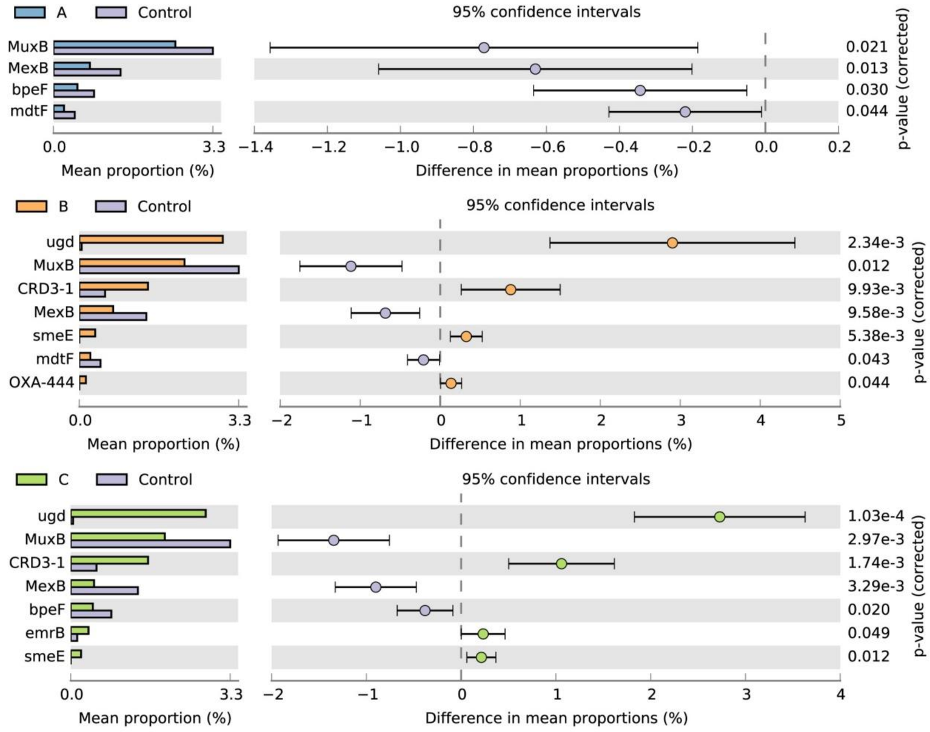

Figure 3-13: The effect of antibiotic addition on soil ARGs on Day 60.

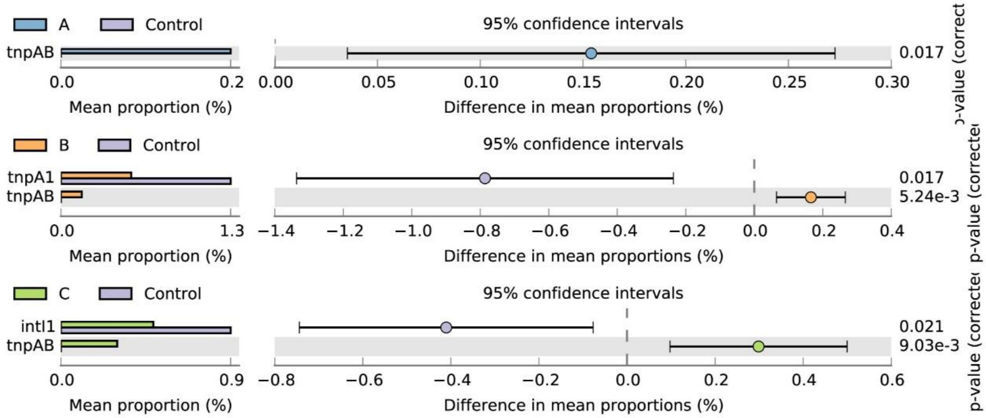

Figure 3-14: The effect of heavy metal addition on soil MRGs on Day 60.

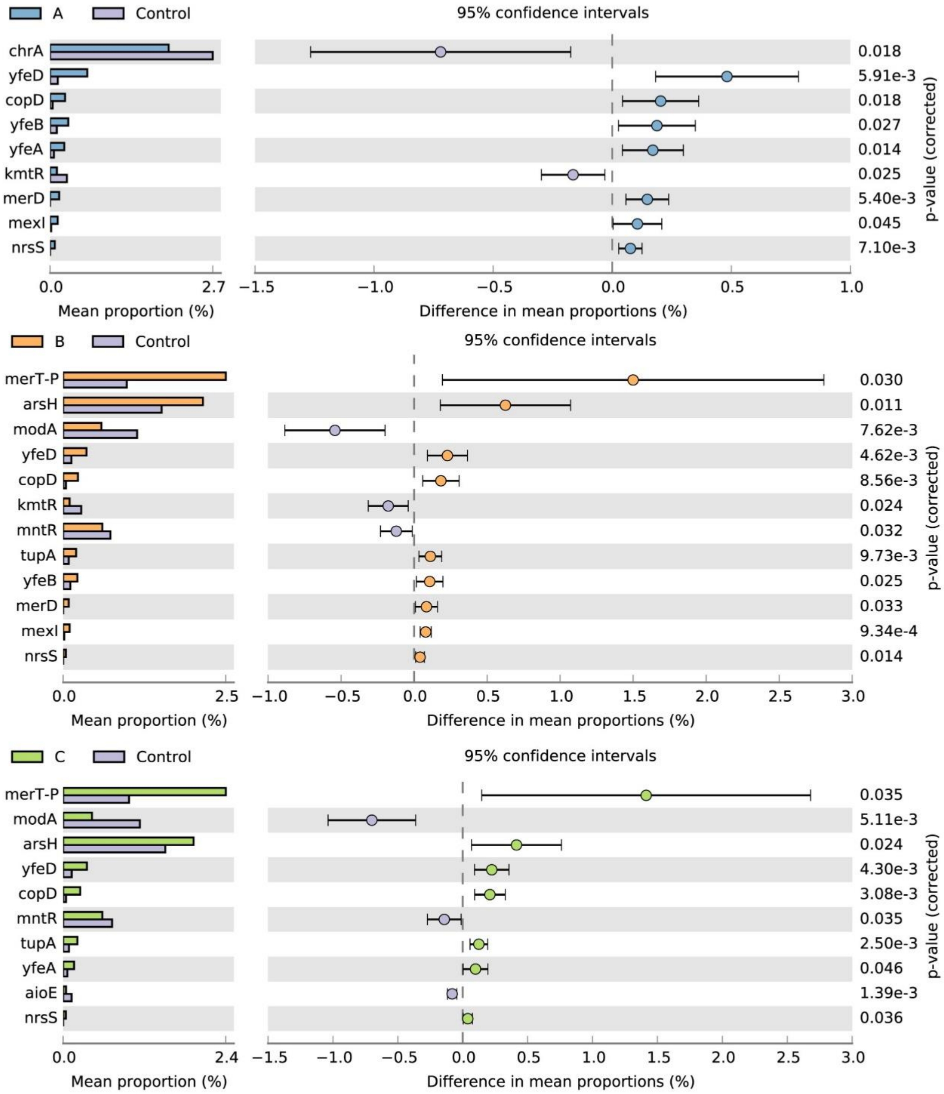

Figure 3-15: The effect of combined antibiotic and heavy metal addition on soil MGEs on Day 60.

3.3 The relationship between core microbiome and resistance gene abundance

There were 64 bacterial and 28 fungal OTUs identified as core microbiome across all soil samples **(Fig. 3-16A, B)**. Most core bacteria and fungi belonged to Acidobacteriota and Ascomycota **(Fig. 3-16C, D)**. The abundance of core bacteria was positively related to MRGs, MGEs and ARGs gene abundances **(Fig. 3-17A, C, E)**. The abundance of core fungi was positively related to both MGEs and ARGs gene abundances **(Fig. 3-17D, F)**. The result of random forest showed that core microbiomes explained 12%, 21% and 50% variations of ARGs, MRGs, and MGEs gene abundances **(Table 3-1, 3-2, 3-3)**. Bacterial OTU36 and OTU21, bacterial OTU44 and fungal OTU9, fungi OUT15 and OTU12, were the most important predictors of ARGs, MRGs, and MGEs gene abundances **(Table 3-1, 3-2, 3-3)**. Furthermore, it was found that there were positive correlations between core microbiome abundance and microbial stability **(Fig. 3-18)**.

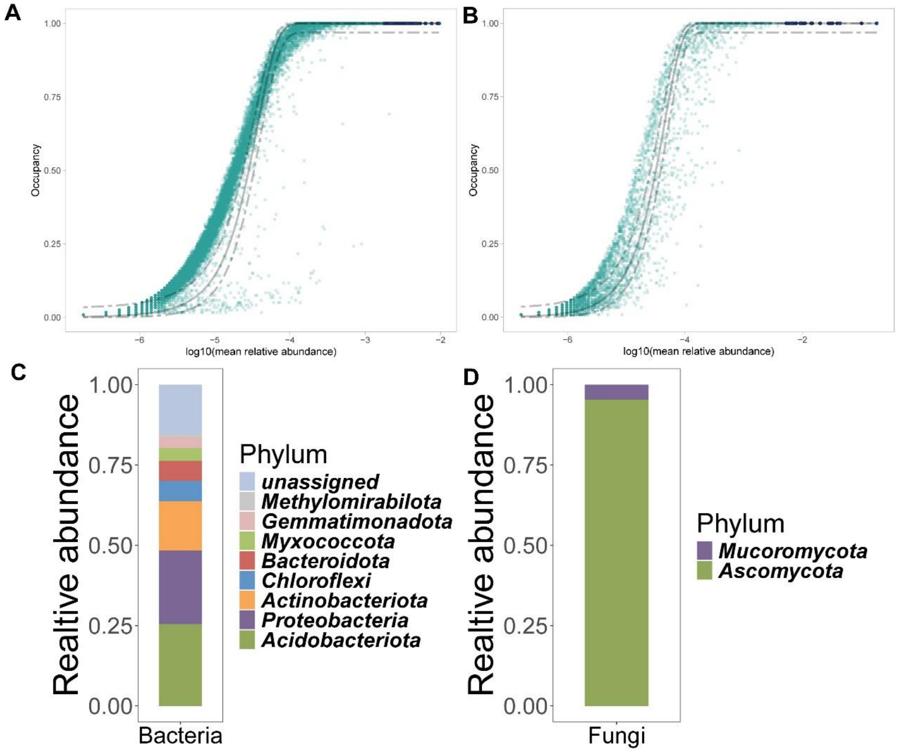

Figure 3-16: Abundance-occupancy distributions were used to identify core microbiome for bacteria (A) and fungi (B). Relative abundance of core microbiomes at genus level for bacteria (C) and fungi (D).

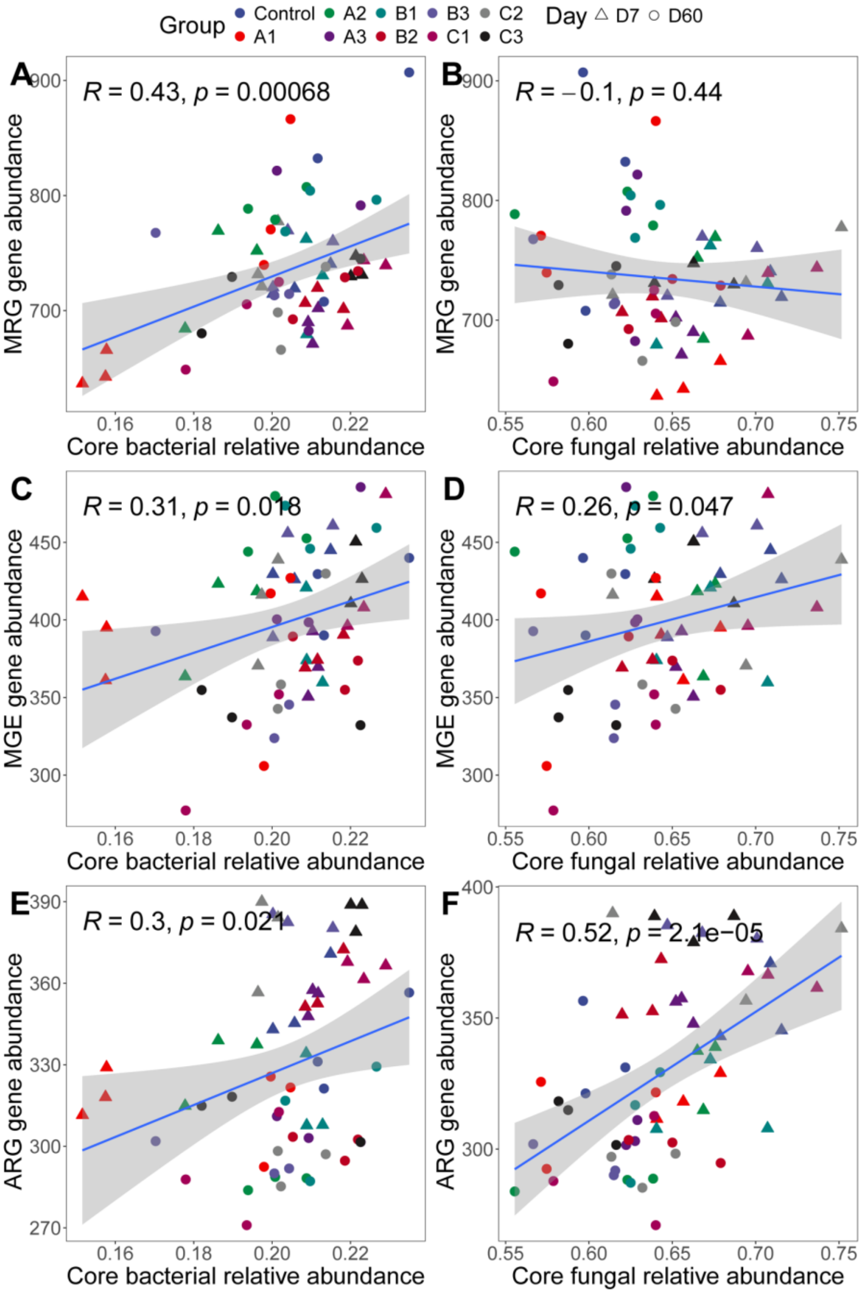

Figure 3-17: The relationship between core microbiome abundance and the abundance of MRGs, MGEs and ARGs.

Table 3-1: Important microbial predicators as predictors of ARGs abundance.

	%IncMSE	*p*	Phylum	Class	Order	Family	Genus
BOTU2	2.147906	0.049505	Actinobacteriota	Actinobacteria	Frankiales	Geodermatophilaceae	*Blastococcus*
BOTU33	2.284509	0.039604	Gemmatimonadota	AKAU4049	norank	norank	*norank*
BOTU34	2.349376	0.029703	Actinobacteriota	Actinobacteria	Propionibacteriales	Nocardioidaceae	*Aeromicrobium*
BOTU24	2.551005	0.049505	Actinobacteriota	MB_A2_108	norank	norank	*norank*
FOTU25	3.057342	0.049505	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Chaetomium*
FOTU1	3.125931	0.039604	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
BOTU12	3.157447	0.019802	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
BOTU40	3.801523	0.019802	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
FOTU26	6.497503	0.019802	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Chaetomium*
BOTU31	2.800369	0.009901	Acidobacteriota	Vicinamibacteria	Vicinamibacterales	Vicinamibacteraceae	*norank*
BOTU60	2.941457	0.009901	Acidobacteriota	Blastocatellia	Pyrinomonadales	Pyrinomonadaceae	*RB41*
BOTU47	4.066159	0.009901	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
BOTU14	4.732933	0.009901	Myxococcota	bacteriap25	norank	norank	*norank*
BOTU25	5.324896	0.009901	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
FOTU7	5.842301	0.009901	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
BOTU19	6.096104	0.009901	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
FOTU18	6.558762	0.009901	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
BOTU21	7.563134	0.009901	Chloroflexi	Chloroflexia	Thermomicrobiales	JG30_KF_CM45	*norank*
BOTU36	12.12324	0.009901	Gemmatimonadota	Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	*uncultured*

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***

Table 3-2: Important microbial predicators as predictors of MRG abundance.

	%IncMSE	*p*	Phylum	Class	Order	Family	Genus
BOTU18	2.472956	0.049505	Acidobacteriota	Vicinamibacteria	Vicinamibacterales	uncultured	*norank*
FOTU19	2.550776	0.029703	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Curvularia*
BOTU19	3.021307	0.029703	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
FOTU9	3.689331	0.039604	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Hagleromyces*
BOTU44	4.552806	0.029703	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	*Sphingomonas*
BOTU41	2.945111	0.009901	Acidobacteriota	Blastocatellia	Pyrinomonadales	Pyrinomonadaceae	*RB41*

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***

Table 3-3: Important microbial predicators as predictors of MGE abundance.

	%IncMSE	*p*	Phylum	Class	Order	Family	Genus
BOTU1	2.194836	0.029703	Bacteroidota	Bacteroidia	Chitinophagales	Chitinophagaceae	*Vibrionimonas*
FOTU18	2.799869	0.049505	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
FOTU7	2.817296	0.029703	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*
FOTU11	2.865789	0.019802	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Didymella*
FOTU3	3.126119	0.029703	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Chaetomium*
BOTU35	3.36783	0.019802	unassigned	unassigned	unassigned	unassigned	*unassigned*
FOTU23	4.504728	0.029703	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Chaetomium*
FOTU4	4.791187	0.019802	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Chaetomium*
BOTU15	4.796396	0.019802	Proteobacteria	Gammaproteobacteria	Burkholderiales	Comamonadaceae	*Variovorax*
FOTU12	5.138471	0.009901	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Chaetomium*
FOTU15	8.184549	0.009901	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Humicola*

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$**, and $p < 0.001$***

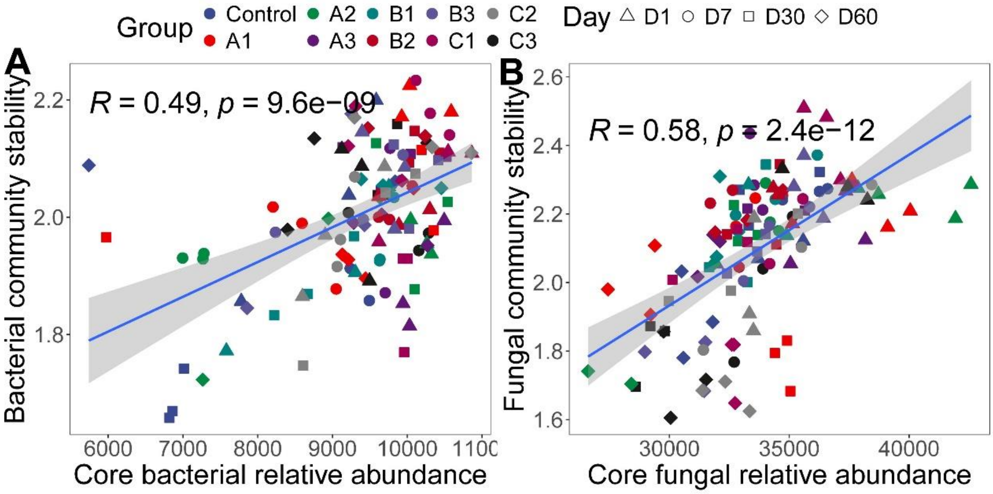

Figure 3-18: The relationship between core microbiome abundance and community stability.

4.Discussion

Biosolids application to agricultural soils as fertilizers is a recognized technique to increase soil fertility, nonetheless, biosolids have been reported to introduce heavy metals and antibiotics into the soil (Pritchard et al., 2010; Urbaniak et al., 2024). Consequently, the propagation of ARGs via biosolids application to agricultural soils is a serious threat. The results of the present study are consistent with studies previous studies on confirming that biosolids indeed spread ARGs (Qin et al., 2022; Yang et al., 2018). In this study, biosolids application also increased the presence of MGEs. MGEs are conduits of ARGs (Wu et al., 2022a), hence the simultaneous presence of both ARGs and MGEs is a matter of concern. Additionally, we report for the first time the increased abundance of MRGs, particularly those associated with Zn, Ni and Cu, in soils applied with biosolids. The co-selection of antibiotics and heavy metals resistance genes represents a critical challenge in contemporary microbial ecology and public health (Mazhar et al., 2021; Imran et al., 2019; Di Cesare et al., 2016b). While antibiotics have historically drawn the majority of attention as selective agents for antibiotic resistance genes, an increasing body of evidence suggests that the presence of heavy metals can significantly exacerbate this problem (Vats et al., 2022). Heavy metals not only exert their own selective pressures but may also co-select for antibiotic resistance genes through various mechanisms, including co-resistance (where resistance genes for metals and antibiotics reside on the same genetic elements) and cross-resistance (where a single resistance mechanism confers tolerance to multiple stressors) (Engin et al., 2023; Vats et al., 2022). This discussion focuses on three key aspects: (1) the dominant role of heavy metals, relative to antibiotics, in shaping both functional gene composition and microbial diversity under co-selection pressures; (2) the explanation of how changes in the core microbiome account for shifts in antibiotic resistance genes (ARGs) under combined antibiotic and heavy metal stress; and (3) the importance of the core microbiome in promoting overall community stability under these dual selection pressures.

4.1 Heavy metals as the dominant selective force under co-occurrence of multiple selective pressures

Heavy metal contamination can serve as a potent and sometimes dominant selective force driving microbial community structure and functional gene profiles even in the presence of other contaminants such as antibiotics. Unlike antibiotics, which can degrade over time or become diluted, many heavy metals persist indefinitely in the environment. For instance, metals such as copper, zinc, cadmium, and lead do not undergo microbial or abiotic degradation, hence, their concentrations can remain relatively stable or even accumulate over time, consequently applying continuous selective pressure on microbial communities (Shuaib et al., 2021; Prabhakaran et al., 2016). This characteristic persistence may give heavy metals a disproportionately large influence compared to antibiotics, whose activity may decline due to chemical breakdown or adsorption onto sediment particles (Nguyen et al., 2019).

Heavy metals can select microbial populations equipped with metal-resistance genes **(Fig. 3-10; Fig. 3-11, Fig. 3-12; Fig. 3-13; Fig. 3-14, Fig. 3-15)**, sometimes harbored on mobile genetic elements (MGEs) like plasmids or transposons (Das et al., 2016; Silver et al., 1996). These genetic elements frequently co-carry antibiotic resistance genes, thereby linking metal resistance to antibiotic resistance (Cai et al., 2023; Mosaka et al., 2023). Consequently, microbes possessing MGEs with metal and antibiotic resistance genes gain a fitness advantage in environments contaminated with both metals and antibiotics **(Fig. 3-10; Fig. 3-11, Fig. 3-12; Fig. 3-13; Fig. 3-14, Fig. 3-15)**. The prolonged presence of heavy metals ensures that these co-resistant microbes are continuously favored, thus amplifying the spread and persistence of antibiotic resistance genes even when antibiotic levels fluctuate or decline (Maurya et al., 2020). This phenomenon underscores the notion that heavy metals can serve as “primary” selective agents with antibiotics acting as “secondary” stressors, particularly in ecosystems with a longstanding history of heavy metal pollution (Vats et al., 2022). However, there are no studies determining the driver of ARGs in agricultural soils in the presence of both heavy metals and antibiotics. To understand this phenomenon, common heavy metal and antibiotic resistance genes that are present in high abundances in biosolids were identified. Accordingly, gentamicin and $ZnSO_4$ spiked-soils were studied to determine the key drivers of ARGs occurrence in agricultural soils with biosolids application. Our results showed that the composition of ARGs, MRGs, and MGEs was significantly altered following addition of the antibiotic and heavy metal on both Day 7 and Day 60, highlighting the persistent and multifaceted selective pressures exerted by these contaminants **(Fig. 3-9)**. Alarmingly, a broader set of resistance genes, including *CRD3-1*, *OXA-444*, *mdtF*, *vanRO*, *mexN*, *capO*, and *ceoB*, were enriched after both antibiotic and metal exposure on Day 7, suggesting a rapid initial response to combined stressors **(Fig. 3-10)**. Alarmingly, by Day 60, enrichment of ARGs was observed in soils treated with the heavy metal and and with both heavy metal and antibiotic **(Fig. 3-13)**. The ARGs *ugd*, *CRD3-1*, and *smeE* were elevated, while no ARGs were significantly enriched in treatments with antibiotic alone **(Fig. 3-13)**. This indicates that the selective pressure of the heavy metal was more pronounced than that of antibiotic. Similarly, several MRGs, particularly *tnpA* and its variants, were enriched across treatments on Day 7 and persisted under the heavy metal influence on Day 60 **(Fig. 3-11, Fig. 3-14)**. In line with this, MGEs such as *merR*, *tupA*, *copB*, and *nrsS* showed consistent enrichment under heavy metal and antibiotic alone treatments and in treatments with both heavy metal and antibiotic, particularly at Day 60 **(Fig. 3-12, Fig. 3-15)**. This reinforces the notion that metal contamination sustains the mobilization potential of resistance genes over time (Zhang et al., 2024a). Further, this study also shows the presence of increased abundances of the ARG CRD3 across all treatments. This ARG encodes a beta-lactamase that confer resistance to beta-lactam antibiotics (Ma et al., 2022). Taken together, these results empirically validate that heavy metals can act as persistent and dominant selective agents, not only shaping the community composition of ARGs and MRGs but also promoting their potential spread through MGEs in soils supplemented with biosolids.

Furthermore, exposure to heavy metals can induce stress responses that reshape the overall functional gene repertoire of the community **(Fig. 3-9)** (Ma et al., 2022; Zeng et al., 2020a). Genes encoding for metal-efflux pumps, metal-binding proteins, and detoxification enzymes become more prevalent, reflecting an increased selective pressure (Adhikary et al., 2024; Das et al., 2016). Numerous studies have confirmed that these shifts at the gene level can drive a broader restructuring of the microbial community (Fu et al., 2023; Ji et al., 2012). As microbial taxa adapt differentially to heavy metals, either by possessing inherent resistance mechanisms or by acquiring new ones via horizontal gene transfer community composition and diversity can change dramatically (Gillieatt et al., 2024). The removal or reduction of sensitive taxa may open ecological niches for more metal-tolerant or opportunistic species, thereby decreasing overall diversity and altering functional capacities (Sazykin et al., 2023; Wen et al., 2023). This disruption or realignment of community structure has significant ecological consequences, including shifts in nutrient cycling and interactions with higher trophic levels (Hempson et al., 2018; Jia et al., 2023). In short, heavy metals often occupy a primary role in driving selection pressures under co-selection scenarios. Their recalcitrant nature and capacity to co-select for antibiotic resistance underscore why heavy metals remain a pivotal factor in shaping both microbial functional genes and overall diversity.

4.2 Core microbiome changes explaining shifts in antibiotic resistance genes

In our study, we found that soil microbial diversity and community composition showed different temporal responses to the addition of heavy metals and antibiotics **(Fig. 3-6; Fig. 3-7, Fig. 3-8; Fig. 3-9)**. On Day 1, no significant change in bacterial diversity was observed, likely because microbial communities had not yet fully sensed or responded to the added stressors. This short-term insensitivity may be attributed to the intrinsic resistance of some microbial taxa, limited diffusion and bioavailability of Zn^{2+} and gentamicin in the soil matrix, or temporary physiological tolerance mechanisms such as efflux pumps and gene regulation buffering (Lazar et al., 2023). In contrast, fungal diversity showed an immediate response to antibiotic addition, suggesting that fungi may be more sensitive to chemical disturbance at early stages, possibly due to differences in cell structure, growth rate, or interactions with antibiotic-sensitive bacterial partners (Sun et al., 2024). By Day 7 and Day 30, the microbial responses became more pronounced. Bacterial diversity significantly decreased under antibiotic stress on Day 7, reflecting selective pressure against sensitive taxa and possible community restructuring (Schauberger et al., 2023). On Day 60, bacterial diversity was significantly higher under antibiotic treatment than the control, indicating possible community adaptation, enrichment of resistant taxa, or recovery driven by microbial succession (Philippot et al., 2021). These findings emphasize that microbial responses to pollutants are time-dependent and dynamic. In the short term, communities may resist disturbance due to structural buffering or microbial dormancy, but over time, persistent selective pressure can drive shifts in diversity and composition. The observed increase in diversity at later stages (e.g., Day 60) may also reflect ecological reassembly processes, where resistant or dormant taxa become dominant, or microbial interactions are reestablished under new equilibrium conditions (Philippot et al., 2021). Such delayed or compensatory responses are particularly relevant in complex environments like soil, where microbial adaptation is mediated by both genetic and environmental constraints.

Interestingly, in this study it was found that core microbiome could explain fluctuations in antibiotic resistance gene abundance **(Figure 3-17; Table 3-1; Table 3-2; Table 3-3)**. Core microbiome refers to a set of microbial taxa that are consistently found across similar habitats or conditions. Even under stress, these core taxa tend to remain relatively stable, maintaining essential ecosystem functions such as nutrient cycling, organic matter decomposition, and various symbiotic interactions (Neu et al., 2021; Berg et al., 2020). However, when faced with the concurrent stress of antibiotics and heavy metals, the structure of this core microbiome may shift in subtle yet significant ways (Rasheela et al., 2024).

One important mechanism underlying these shifts is the transfer of mobile genetic elements. In an environment with multiple selection pressures, horizontally transferable elements carrying multiple resistance determinants have a better chance of spreading within the community (Liu et al., 2024; Lin et al., 2021). Core microbiome members, often being the more dominant or competitively successful taxa, can serve as central “hubs” for genetic exchange (Coyte et al., 2015). This can lead to an increased abundance of ARGs in core taxa, either by new acquisition or by selective amplification of existing resistance genes. This is because core microbiome members are well-adapted to local environmental conditions, their survival and proliferation can effectively “lock in” the newly acquired resistance traits within the community (Wang et al., 2021). These generalist defense mechanisms can facilitate cross-resistance, thereby enhancing the core microbiome's capability to thrive in the presence of multiple types of stress (Murugaiyan et al., 2022). Over time, even moderate co-selection pressures can cause a gradual enrichment of these dual (or multiple) resistance traits within the stable taxa. For the core microbiome, random forest analysis identified their key roles in the variation in ARGs, MRGs, and MGEs, indicating their potential roles as biological drivers of resistance gene dynamics under co-contamination stress. For example, Chloroflexi and Gemmatimonadota emerged as top predictors of ARG shifts **(Table 3-1)**. These taxa are frequently detected in polluted or oligotrophic environments and are known for their resilience under environmental stress (Mujakić et al., 2022). Their enrichment has been previously reported to coincide with increased abundance of ARGs such as *OXA-444*, *mdtF*, and *ceoB* as seen in this study (Mujakić et al., 2022). The genera *Sphingomonas* and *RB41* were identified as primary predictors of MRGs **(Table 3-2)**. Species of *Sphingomonas* are widely recognized for their intrinsic resistance to heavy metals, including copper and arsenic, often mediated through efflux systems and metal detoxification pathways (Silver and Phung, 1996). *RB41* is frequently detected in metal-rich or acidic soils and participates in essential metal cycling processes (Garris et al., 2018). Fungal taxa *Chaetomium* and *Humicola*, both belonging to Chaetomiaceae, were strong predictors of MGE variation **(Table 3-3)**. These saprotrophic fungi contribute to organic matter decomposition and microbial turnover, potentially enhancing the conditions for horizontal gene transfer in the soil microbiome (Challacombe et al., 2019).

The result of these genetic and ecological processes is an observable shift in the suite of ARGs present in the community. Formerly rare or absent ARGs may become prominent if they are linked to metal resistance mechanisms that are strongly selected (Larsson and Flach, 2022; Sun et al., 2021). Meanwhile, certain ARGs that were prevalent but not co-linked to heavy metal resistance may diminish if they do not confer a competitive advantage under the new conditions (Ejileugha, 2022). Hence, by closely examining alterations in core microbiome composition, one can gain insights into the overall trajectory of antibiotic resistance in environments subject to multiple selection pressures. Furthermore, shifts in the core microbiome can serve as an indicator of broader ecological impacts (Neu et al., 2021). This is because core taxa often underpin key ecosystem functions, and changes in their composition might correspond to shifts in processes such as nitrogen cycling, carbon sequestration, or pollutant degradation (Hu et al., 2024). Elevated levels of certain metal and antibiotic-resistant core taxa could result in alterations to these ecosystem services (Li et al., 2021). Ultimately, better understanding of how the core microbiome responds to and drives ARGs shifts under co-selection will be critical for predicting the long-term stability and functional resilience of microbial ecosystems.

5.Conclusion

In conclusion, long-term biosolids application to agricultural soil that spanned 16 years significantly impacted the communities of antibiotic resistance genes and metal resistance genes, leading to increased abundance of these genes, particularly the aminoglycoside and zinc resistance genes. The microcosm experiment demonstrated that both heavy metals and antibiotics significantly affected soil microbial communities, with heavy metals acting as the dominant selective force under the co-occurrence of multiple selective pressures. Furthermore, the abundance of the core microbiome was positively correlated with the abundance of ARGs, MRGs, and MGEs, explaining variations in the antibiotic resistance genes. Additionally, the core microbiome abundance showed a positive relationship with microbial community stability. These findings underscore the importance of carefully managing biosolids applications to reduce the risk of resistance gene accumulation, which could pose long-term ecological challenges as well as significant threat. Moreover, the positive relationship between core microbiome abundance and microbial community stability highlights the potential role of microbial communities in enhancing soil resilience to environmental stressors. Hence, this study underscores the need for exercising caution while applying biosolids as fertilizers to agricultural soils due to the strong possibility of the presence of heavy metals functioning as key drivers in propagating ARGs and MRGs aided by MGEs.

Chapter 4

Arbuscular mycorrhizal fungi enhance soil microbial stability and mitigate resistance risks under long-term biosolids application

Sun Tao, Delaplace, P., Li, G., James, A., Pan, J., & Zhang, J. Novel insights into the effect of arbuscular mycorrhizal fungi inoculation in soils under long-term biosolids application: emphasis on antibiotic and metal resistance genes, and mobile genetic elements. Environmental Pollution, 2025, 125846. https://doi.org/10.1016/j.envpol.2025.125846

The microcosm experiment provided insight into the individual and combined impacts of pollutants on soil microbes under biosolids application. However, how to effectively mitigate these potential risks in practice remains an open question. Therefore, this chapter explores the role of AMF inoculation as a potential mitigation strategy using a pot experiment.

Abstract

The application of biosolids can improve soil fertility and crop productivity but also accompanies risks of heavy metals and antibiotics introduction. In the presence of heavy metals contamination, using arbuscular mycorrhizal fungi (AMF) is a promising strategy to enhance soil microbial community stability and plant tolerance resistance to heavy metals, and to reduce the spread of antibiotic resistance genes (ARGs). The present study investigated the impacts of AMF inoculation on soil and plant heavy metal contents, and soil microbial communities by pot experiments. The results showed that AMF inoculation significantly enhanced plant biomass, and reduced soil and plant heavy metals contents. While AMF inoculation did not alter bacterial and fungal community compositions, it increased bacterial diversity at higher biosolids concentrations. Notably, AMF inoculation enhanced microbial network complexity and increased keystone taxa abundance. Furthermore, several beneficial microorganisms with high resistance to heavy metals were enriched in AMF-inoculated soils. Metagenomic analysis revealed a reduction in the mobile genetic element (MGE) gene *IS91* in AMF-inoculated soils and an increase in heavy metal resistance genes compared to soils without AMF. The possibility of reduction in MGE-mediated spread of ARGs is one of the key findings of this study. As a caution, this study also detected enrichment of few ARGs in high biosolids-amended soils with AMF inoculation. Overall, AMF inoculation could be a valuable strategy in agriculture for mitigating the environmental risks associated with biosolids, heavy metals and antibiotic resistance, thereby promoting sustainable soil management and health.

Keywords: Metagenome, heavy metals, soil microbial community, *IS91* gene, keystone taxa.

The graphical abstract is displayed in **Figure 4-1**.

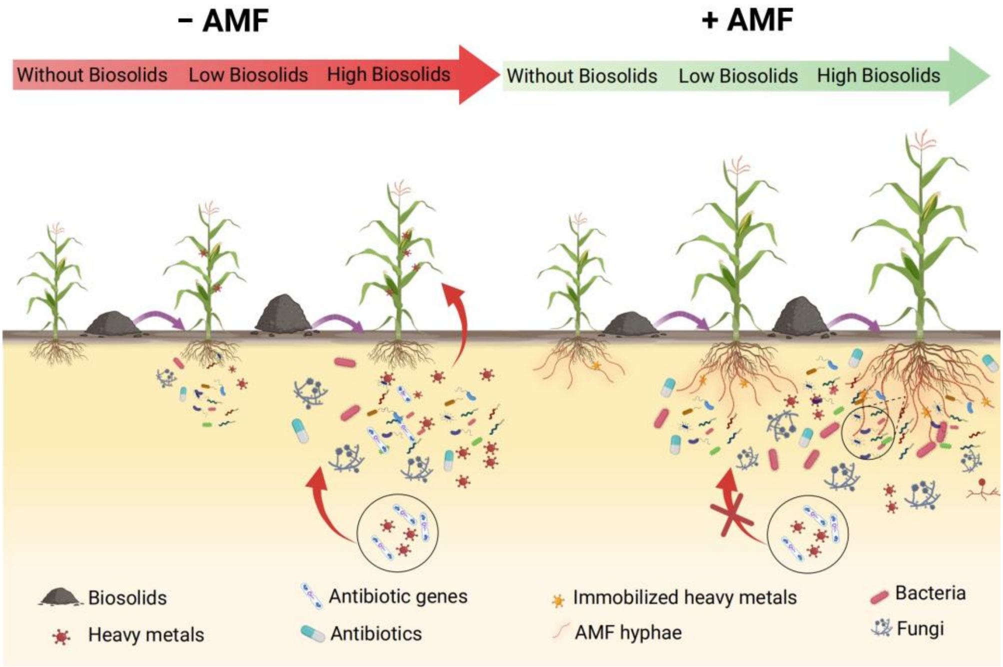

Figure 4-1: Chapter 4 graphical abstract.

1.Introduction

The solids that are separated out during wastewater purification, and are treated physically and chemically to obtain nutrient-rich organic materials are called biosolids (Kumar et al., 2017; US EPA). The application of biosolids in agriculture has been increasingly recognized for its potential to improve soil fertility and enhance crop productivity due to their rich organic content and essential nutrients (Sharma et al., 2017; Elgarahy et al., 2024). However, the use of biosolids also carries significant risks, particularly the introduction of heavy metals and antibiotics into the soil (Pritchard et al., 2010; Urbaniak et al., 2024). The deleterious effects of heavy metals in the environment are well-documented (Edo et al., 2024; Zhao et al., 2022), and the increasing overuse of antibiotics in domains like public health, agriculture, animal husbandry and allied sectors is transpiring as the most critical contributor to antibiotic pollution. Consequently, the spread of antibiotic resistance genes (ARGs) in the environment has become a significant concern due to its role in conferring antibiotic resistance to pathogenic bacteria overexposed to antibiotics (Nguyen et al., 2021; Ahmed et al., 2024). This issue poses serious risks to human and animal health, leading to increased mortality and substantial economic losses (Zainab et al., 2020). Additionally heavy metals can enhance ARGs and their trigger proliferation via a phenomenon known as co-selection (Maurya et al., 2020; Zhang et al., 2024a). The co-selection theory suggests that heavy metal contamination can lead to the simultaneous presence of metal resistance genes (MRGs) and ARGs through mechanisms such as co-resistance, cross-resistance and co-regulation, suggesting that the application of biosolids may create hotspots for both MRGs and ARGs (Naidoo, 2020; Zhang et al., 2024a). Additionally, mobile genetic elements (MGEs) such as transposons, integrons and plasmids play a crucial role in the proliferation and spread of ARGs via horizontal gene transfer among bacteria (Wu et al., 2022b). In a recent study, Zhang et al. (2024b) established a link between ARGs and MGEs in rivers having discharges of aquaculture and agriculture wastewater, and industrial and urban domestic wastewater. Considering the potential risks posed by heavy metals and ARGs in biosolids, it is essential to devise effective strategies to reduce the transfer of these contaminants from biosolids to soil and crops.

There are various physical and chemical approaches to remediate heavy metal contamination in soil, such as washing of soil, soil extraction and replacement, soil solidification, incineration, chemical leaching, chemical fixing etc. (Dhaliwal et al., 2020; Liu et al., 2022). However, the laboriousness, high cost, use of chemicals and formation of harmful by-products make most of these methods unsustainable. Some environmental-friendly techniques like the use of soil amendments, phytoremediation and application of specific microbial inoculants have been explored to reduce the mobility and bioavailability of heavy metals in soils (Shah and Daverey, 2020; Zheng et al., 2024). Among these, biological approaches have gained recognition for their effectiveness and minimal impact on soil and environmental health. Microorganisms use various tactics such as biosorption, bioaccumulation, biotransformation, precipitation, complexation, redox reactions, methylation and demethylation for their defense (Nivetha et al., 2023). These methods result in speciation and reduced bioavailability of heavy metals in the environment. Over the last few decades several bacteria have been identified for their potential to remediate heavy metal contaminated soils (Roy et al., 2024). However, the curb of ARGs is the environment has proven to be more challenging (Visca et al., 2024) owing to their biological nature and ability to adapt.

Recently, Dou et al. (2024) reported the in-situ remediation of Cd and acid rain co-contaminated soil using a bacterium-fungus combination. Notably, among fungi, arbuscular mycorrhizal fungi (AMF) stand out for their dual role in promoting plant growth and enhancing plant resistance to heavy metals while simultaneously reducing the spread of ARGs (Wahab et al., 2023). AMF are highly beneficial due to their ability to establish mutualistic associations with the roots of approximately 90% of terrestrial plant species, including a wide range of agricultural crops (Diagne et al., 2020). This symbiosis is particularly advantageous for plants, as AMF can significantly enhance the uptake of essential nutrients, especially phosphorus, from the soil (Khaliq et al., 2022). Extensive research has demonstrated that the symbiotic structures of mycorrhizae play a role in the sequestration and compartmentalization of heavy metals, which in turn reduces their movement from plant roots to above-ground tissues (Dhalaria et al., 2020). Moreover, AMF hyphal exudates are rich in carbohydrates, which can recruit certain microorganisms to colonize the hyphal surface, forming what is known as the hyphosphere microbiome (Zhang et al., 2022b). These hyphosphere microbial communities are closely associated with AMF and mutually influence each other's growth, activity and function (Zhang et al., 2024c). AMF have also been shown to reduce the bioavailability of heavy metals like cadmium (Cd) (Boorboori and Zhang, 2022) and control the spread of ARGs (Hu et al., 2022). To the best of our knowledge, no studies have yet examined the simultaneous effects of AMF on both heavy metal toxicity and the dissemination of ARGs. Therefore, understanding the mechanisms by which AMF regulate soil heavy metal toxicity and reduces the spread of antibiotic resistance is crucial for the effective and safe application of biosolids. Further, it remains unclear whether AMF can collaborate with other soil microorganisms to mitigate the toxicity of heavy metals and ARGs. Hence, it is crucial to also explore the synergistic effects of AMF and functional microorganisms in alleviating the pollution caused by the application of biosolids, which introduces antibiotics and heavy metals into the environment.

In this context, the objectives of the current study were to investigate the effects of AMF inoculation on changes in heavy metal concentrations, microbial communities, metal resistance genes, mobile genetic elements, and antibiotic resistance genes in soil with long-term biosolids application. This study hypothesizes that (i) AMF inoculation could reduce the content of heavy metals in soil and plants; (ii) AMF inoculation, in the presence of heavy metals, could improve the stability of the microbial community by recruiting beneficial microorganisms; (iii) inoculated AMF could work with the indigenous microbial population to enhance resistance to heavy metals and reduce the presence of ARGs and MRGs.

2.Materials and methods

2.1 Pot experiment design

The pot experiment was carried out in a glasshouse at the Chinese Academy of Agricultural Sciences between April and June 2023. The maize variety Zhengdan958 (*Zea mays L.*) was selected as the host plant. Soils from treatments with biosolids application rates of 0, 4.5, and 36 t ha^{-1} are designated as C (control, without biosolids), L (low biosolids concentration), and H (high biosolids concentration), respectively. The biosolids used in this study were air-dried municipal sewage products obtained from Beijing Drainage Group Co., Ltd., applied without sterilization in October 2021. The experiment followed a 3 × 2 × 2 full factorial design, with three biosolids application levels in the hyphal chamber (HC) (C, L, and H), two mycorrhizal treatments in the root chamber (RC) (NM: non-AMF inoculated and AM: AMF inoculated), and two microbial conditions in the HC (sterilized vs. unsterilized). The unsterilized soils contained indigenous microbial communities, while the sterilized soils were treated with γ-irradiation at 25 kGy using ^{60}Co to inactivate all microorganisms. Each of the 12 treatment combinations was replicated four times, resulting in a total of 48 pots. Maize seeds were sterilized by soaking in a 10% (v/v) H_2O_2 solution for 30 minutes, then thoroughly rinsed with deionized water before being germinated in a sterile tray for 48 hours in darkness. Three seedlings were initially planted per pot, and after one week, they were thinned to one plant per pot.

The dimensions of each pot were 11.5 cm long (L), 10 cm wide (W), and 15 cm high (H), and was filled with 1.8 kg of soil. The microcosms consisted of three physically separated chambers: a root chamber for plant growth (RC, 3.5 × 10 × 15 cm), a buffer chamber (BC, 2 × 10 × 15 cm), and a hyphal chamber for hyphal development (HC, 6 × 10 × 15 cm), separated by 30 µm nylon mesh (Li et al., 2023) **(Fig. 4-2)**. The buffer chamber (BC) was positioned in the center, with the root chamber (RC) and hyphal chamber (HC) on either side. Maize was grown in the RC, allowing only AMF hyphae to extend through the nylon mesh into both the BC and HC. The maize roots were restricted by the nylon mesh and could not pass through, and the BC acted as a barrier to limit microbial diffusion from the non-sterilized HC into the RC. Additionally, it prevented colonization of the plants by indigenous AM fungi present in the HC.

Soil composition differed among compartments to serve their functional roles. The RC and BC were both filled with control soil (biosolids-free, non-sterilized) to ensure consistent plant growth and exclude confounding microbial inputs. In contrast, the HC received either sterilized or unsterilized soils corresponding to the biosolids treatments (C, L, or H), depending on the assigned combination. This allowed the manipulation of microbial communities in the HC while maintaining constant plant and root-zone conditions.

Sampling and analysis were conducted on distinct compartments. The hyphal chamber (HC) was sampled for microbial community analysis, including DNA extraction, metagenomic sequencing, and quantification of antibiotic resistance genes (ARGs), metal resistance genes (MRGs), and mobile genetic elements (MGEs). The root chamber (RC) was sampled for plant performance measurements, including shoot and root biomass, nutrient uptake, and AMF colonization. The buffer chamber (BC) was not sampled but acted as a strict physical barrier to prevent microbial and root cross-contamination. Although soil sterilization is a commonly used method to control microbial background, it can introduce biases, such as altering soil physical and chemical properties, releasing nutrients through microbial lysis, or modifying microbial recolonization dynamics. These limitations are further addressed in the general discussion to contextualize potential impacts on experimental outcomes.

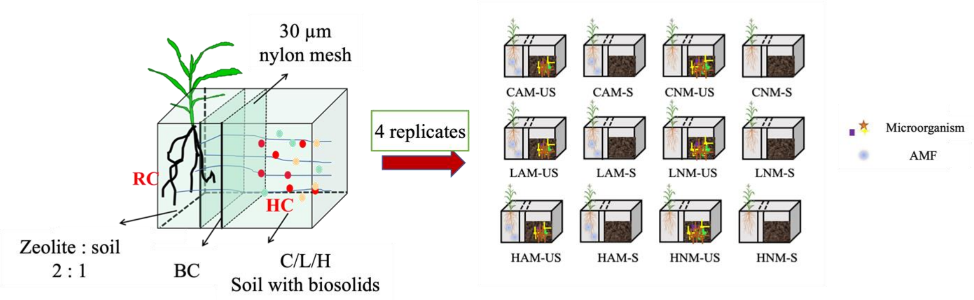

Figure 4-2: Schematic representation of pot experiment setup.

2.2 Plant growth substrate and AMF inoculum

The substrate consisted of a mixture of zeolite and low-phosphorus soil (2:1, w/w), which underwent γ-irradiation sterilization at 25 kGy using ^{60}Co to inactivate all microorganisms (Qin et al., 2020). The soil used in the root chamber (RC) and buffer chamber (BC) was sourced from an uncultivated arable field at the Shangzhuang Experimental Station (40◦08′N, 116◦10′E) in Beijing, China. The basic soil properties were: pH 8.19 (in water), organic carbon (SOC) 11.5 g kg^{-1}, available phosphorus (Olsen-P) 4.18 mg kg^{-1}, inorganic nitrogen 19.9 mg kg^{-1} and available potassium (AK) 82.4 mg kg^{-1}. Before the start of the experiment, each kilogram of soil was supplemented with 200 mg of nitrogen, 10 mg of phosphorus, and 200 mg of potassium in the forms of $(NH_2)_2CO$, KH_2PO_4, and K_2SO_4, respectively.

In our previous study, the relative abundance data indicated *Glomeraceae* as the most dominant family across all treatments (Sun et al., 2024). Given this predominance and its well-documented symbiotic efficiency with maize (Zhu et al., 2020), the root chamber was subsequently inoculated with the AMF *Funneliformis mosseae* (HK01), a member of the Glomeraceae family. The AMF inoculum of Glomeromycota was obtained from the Academy of Agricultural and Forestry Sciences in Beijing. The inoculum contained approximately 50 spores per gram, along with mycelium and fine root segments, propagated in a 1:1 (w/w) mixture of soil and sand, using *Zea mays L.* as the host plant grown for four months in a greenhouse. In the pot experiment, each AM treatment group received 8 % (w/w) AMF as inoculum in the root chamber.

2.3 Harvest and sample analysis

After two months' growth, the plants were harvested. In brief, maize shoots were clipped and dried to measure aboveground biomass. Maize roots were stored at -20 °C for assessment of AM colonization and hyphal length density **(Table 5-1)**. Arbuscular mycorrhizal hyphae and mycorrhizal colonization were measured using the gridline-intercept method as described Abbott et al. (1984) and McGonigle et al. (1990), respectively. The heavy metal contents of both the HC soil and plant samples were determined using inductively coupled plasma optical emission spectrometry (ICP-OES).

Table 4-1: Intensity of arbuscular mycorrhizal colonization (M%) and frequency of arbuscules (A%) in maize roots and hyphae length density (HLD) in soil under different treatments.

Treatment	M	A	HLD
CAM-S	18.79 ± 10.55b	19.39 ± 10.03b	1.44 ± 0.07e
LAM-S	41.05 ± 13.88a	41.05 ± 13.88a	3.67 ± 0.16a
HAM-S	7.93 ± 1.51b	8.29 ± 1.46b	2.37 ± 0.03c
CAM-US	19.72 ± 4.8b	20.53 ± 5.66b	2.02 ± 0.06d
LAM-US	41.84 ± 9.27a	45.01 ± 5.63a	3.21 ± 0.02b
HAM-US	17.75 ± 8.51b	19.32 ± 10.79b	1.07 ± 0.04f

2.4 Microbial DNA extraction, PCR and Illumina sequencing

Total soil DNA was extracted from 0.5 g of soil. PCR amplification targeted the V4 region for bacteria with the primer pair 515F/806R and the ITS region for fungi with primers ITS3F/ITS4R, respectively (Walters et al., 2016; Op De Beeck et al., 2014). Primers were synthesized by Invitrogen™ (Thermo Fisher Scientific Inc.). Sequencing reads were demultiplexed by unique barcodes, then merged, denoised, and filtered for chimeras using the DADA2 pipeline. Bacterial taxonomy was assigned using the SILVA database (Green et al., 2022), while fungal taxonomy was determined with the UNITE database (Eshaghi et al., 2021). For a more detailed description of bioinformatics procedures, please refer to Supplementary Materials, which provide comprehensive information.

Metagenomic shotgun sequencing libraries were generated and sequenced by Shanghai Biozeron Biological Technology Co., Ltd. For each sample, the TruSeq DNA Library Preparation Kit (catalog no: FC-121-2001, Illumina, USA) was utilized to create the sequencing libraries. The concentration of the constructed libraries was determined using the High Sensitivity Double Stranded DNA Kit on a Qubit

Fluorometer (Thermo Fisher Scientific Inc.). Sequencing of all samples was carried out on an NGS platform in paired-end 150 bp (PE150) mode.

2.5 Statistical analysis

All data analyses were performed using R software (version 4.2.0). Two-way ANOVAs were employed to evaluate the variations in microbial diversity, plant biomass, heavy metal contents. Non-metric multidimensional scaling (NMDS) was employed to assess the effects of AMF inoculation on soil microbial communities across different biosolids application rates. A microbial co-occurrence network was constructed using the 'microeco' package by combing bacterial and fungal taxa using a threshold of 0.01%. The 'igraph' package was then utilized for analyzing network properties. Pairwise comparisons of predicted gene abundance between soils with and without AMF inoculation were performed using STAMP.

3.Results

3.1 Effect of AMF on plant biomass, and soil and plant heavy metals contents

Biosolids application significantly increased maize biomass by 47.77% in the treatment with AMF inoculation compared to no-AMF treatment (AMF: F= 68.33, $p < 0.001$; Biosolids: F= 31.81, $p < 0.001$) **(Fig. 4-3A; Table 4-2)**. Soil and plant heavy metals contents were also significantly affected by the AMF inoculation and biosolids application rates **(Fig. 4-3B-F; Fig 4-4; Table 4-2)**. In the soil with high application of biosolids, AMF inoculation significantly reduced Cu and Zn content by 6.96% and 5.58%, respectively. Moreover, there was a significant reduction in Zn and Hg contents by 4.69% and 31.56% in the soil with AMF inoculation, especially in unsterilized soil **(Fig. 4-3C, F)**. In case of heavy metals accumulation in plant, there was a significant decrease in root Zn content by 31.51% in unsterilized soil **(Fig. 4-4 A, B)**; in case of sterilized soil, there was reduction of root Cu and Zn by 4.30% and 6.10% at high biosolids application with AMF inoculation **(Fig. 4-4A-B; Table 4-2)**. Overall, there was a reduction in heavy metal contents in the roots with biosolids application and AMF inoculation. Notwithstanding the accumulation of heavy metals in roots, for maize leaf, a significant decrease in leaf Cu (AMF: F= 0.66, p= 0.43; Biosolids: F= 3.43, p= 0.05), Zn (AMF: F= 14.58, $p < 0.001$; Biosolids: F= 6.37, p= 0.006) and Cd (AMF: F= 7.32, p= 0.01; Biosolids: F= 6.31, p= 0.006) contents by 63.61%, 16.66% and 18.41% in the treatment with application of biosolids and AMF inoculation was observed **(Fig. 4-4D-F)**.

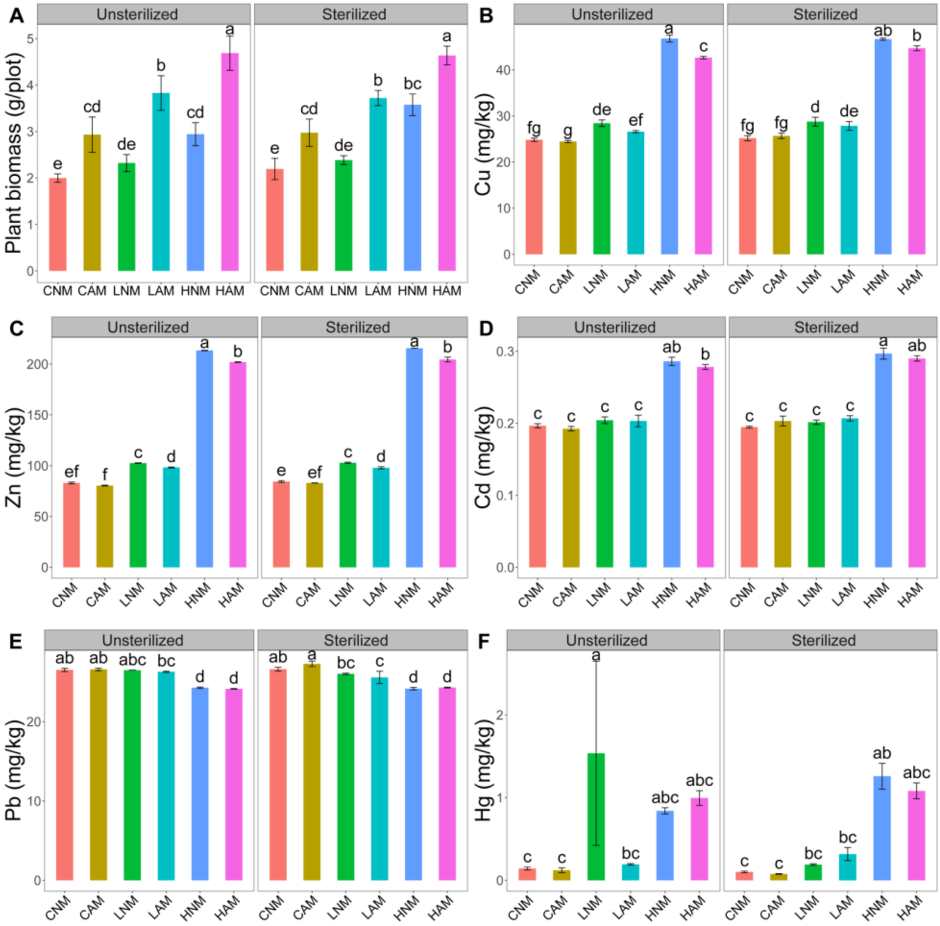

Figure 4-3: The effect of AMF inoculation on plant biomass (A), and soil heavy metals contents (B, C, D, E, F) in soils with different biosolids application rates.

Table 4-2: Results of three-way ANOVA investigating the effects of AMF inoculation, soil microorganisms, and biosolids application on plant biomass and heavy metal contents in plants and soil.

		AMF	Biosolids	Microbiome	AMF*Biosolids	AMF*Microbiome	Biosolids*Microbiome	AMF*Biosolids* Microbiome	Residuals
Plant biomass	Df	1	2	1	2	1	2	2	6
	F	68.33	31.81	0.76	1.55	1.29	0.38	0.34	
	p	< 0.001***	< 0.001***	0.39	0.23	0.26	0.69	0.71	
Soil Cu	Df	1	2	1	2	1	2	2	6
	F	12.67	1030.23	4.6	5.29	2.39	0.06	0.38	
	p	0.002**	< 0.001***	0.04*	0.01*	0.14	0.94	0.69	
Soil Zn	Df	1	2	1	2	1	2	2	6
	F	53.09	8922.53	8.48	11.88	1.08	3.51	1.45	
	p	< 0.001***	< 0.001***	0.007**	< 0.001***	0.31	0.05*	0.25	
Soil Cd	Df	1	2	1	2	1	2	2	6
	F	0.09	315.51	2.65	0.91	1.02	0.91	0.26	
	p	0.77	< 0.001***	0.12	0.41	0.322	0.41	0.77	
Soil Pb	Df	1	2	1	2	1	2	2	6
	F	0.006	68.92	0.1	1.17	0.34	2.44	0.45	
	p	< 0.001***	< 0.001***	0.75	0.32	0.56	0.1	0.64	
Soil Hg	Df	1	2	1	2	1	2	2	6
	F	0.96	6.07	0.37	0.81	0.74	1.34	1.6	
	p	0.33	0.007**	0.54	0.45	0.39	0.27	0.22	
Root Cu	Df	1	2	1	2	1	2	2	6
	F	1.39	4.55	0.12	0.53	2.46	1.63	1.63	
	p	0.25	0.02 *	0.74	0.59	0.13	0.22	0.22	

	Df	1	2	1	2	1	2	2	6
Root Zn	F	14.4	5.8	0.46	0.43	1.56	2.67	0.61	
	p	< 0.001***	0.008**	0.5	0.66	0.22	0.09	0.55	
	Df	1	2	1	2	1	2	2	6
Root Cd	F	0.66	3.43	0.97	0.22	3.87	0.98	0.74	
	p	0.43	0.05*	0.34	0.8	0.06	0.39	0.49	
	Df	1	2	1	2	1	2	2	6
Leaf Cu	F	1.39	0.96	0.89	0.96	0.77	0.96	0.96	
	p	0.25	0.4	0.35	0.4	0.39	0.4	0.4	
	Df	1	2	1	2	1	2	2	6
Leaf Zn	F	14.58	6.37	0.22	0.12	0.96	0.52	0.08	
	p	< 0.001***	0.006**	0.64	0.89	0.34	0.6	0.92	
	Df	1	2	1	2	1	2	2	6
Leaf Cd	F	7.32	6.31	6.55	0.16	1.81	0.05	0.5	
	p	0.01*	0.006**	0.2*	0.85	0.19	0.95	0.61	

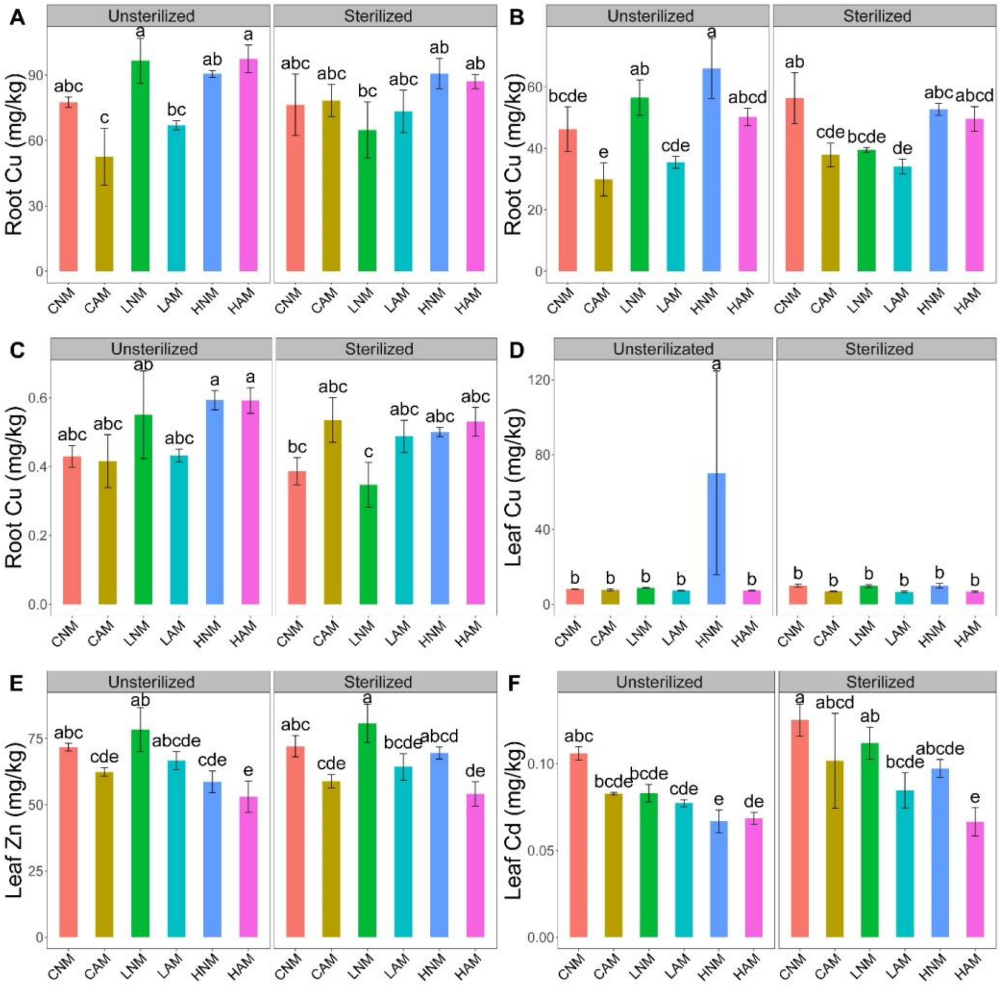

Figure 4-4: The effect of AMF inoculation on plant root (A-C) and leaf (D-F) heavy metals contents under different levels of biosolids application.

3.2 Effect of AMF on soil microbial richness and community compositions

Although there was no significant influence of AMF and biosolids application rates on the bacterial Shannon index (AMF: F= 0.37, *p*= 0.56; Biosolids: F= 0.32, *p*= 0.73), there were interactive effects of AMF and biosolids application on the bacterial Shannon index (AMF*Biosolids: F= 3.93, *p*= 0.05) **(Fig. 4-5A; Table 4-3)**. A significant increase (1.97%) in bacterial Shannon index in the treatment having high application rates of biosolids with AMF inoculation was observed **(Fig. 4-5A)**. However, there was no significant influence of AMF inoculation and biosolids application on the fungal Shannon index (AMF: F= 0.05, p= 0.83; Biosolids: F= 0.64, p= 0.55; AMF*Biosolids: F= 2.52, p= 0.12) **(Fig. 4-5B; Table 4-3)**. Although there were overall no vast significant effects of AMF on soil bacterial and fungal communities, NMDS analyses revealed distinct gradual influences with the biosolids application rates' increase (Bacterial community: AMF: F= 1.24, p= 0.22; Biosolids: F= 3.92, $p < 0.001$; AMF*Biosolids: F= 1.30, p= 0.16) (Fungal community: AMF: F=0.04, p= 0.49; Biosolids: F= 3.31, $p < 0.001$; AMF*Biosolids: F= 0.11, p= 0.53) **(Fig. 4-5C and 4-5D)**.

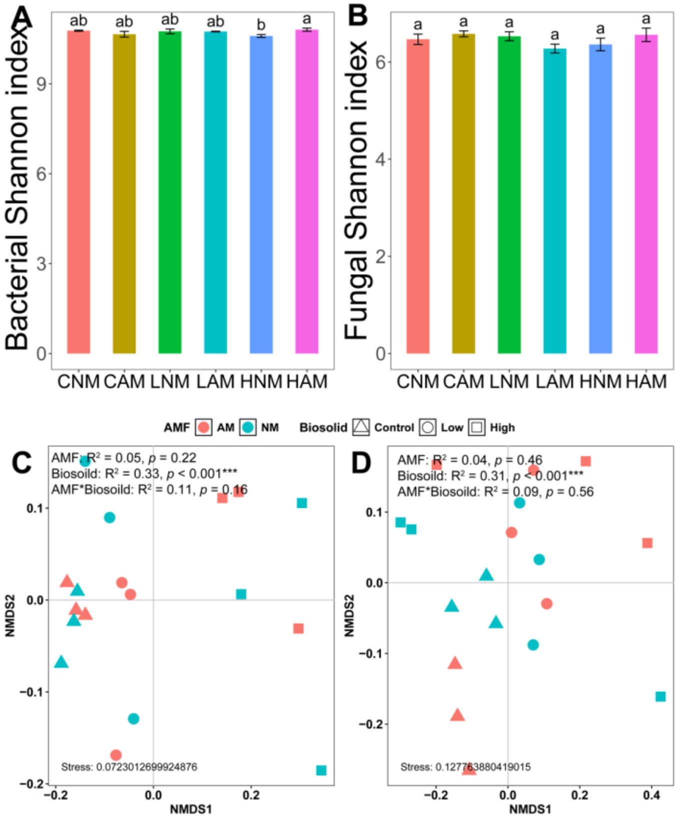

Figure 4-5: Graphical representation of variation in the Shannon indexes of soil bacterial (A) and fungal (B) communities after AMF inoculation at different application concentrations of biosolids, and the effect of AMF inoculation on soil bacterial (C) and fungal (D) community compositions in soil at different concentrations of biosolids application.

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$** and $p < 0.001$***.

Table 4-3: Results of two-way ANOVA investigating the effects of AMF inoculation and biosolids application on soil bacterial and fungal Shannon index.

		AMF	Biosolids	AMF*Biosolids	Residuals
Bacterial Shannon index	Df	1	2	2	12
	F	0.37	0.32	3.93	
	p	0.56	0.73	0.05*	
Fungal Shannon index	Df	1	2	2	12
	F	0.05	0.64	2.52	
	p	0.83	0.55	0.12	

A bacteria-fungi co-occurrence network was constructed to test the effects of AMF inoculations on microbial interactions **(Fig. 4-6A)**. There were 7 keystone taxa (4 bacterial and 3 fungal amplicon sequencing variants, ASVs) found in the network **(Fig. 4-6B)**. The abundance of bacterial ASV105, ASV20, ASV81 and ASV 460, and fungal ASV 31 were significantly increased with AMF inoculation **(Fig. 4-7)**. In addition, a significant increase in network complexity (node number, edge number, average degree, and modularity) was also observed in the soil with AMF inoculation **(Fig. 4-6C, D, E, F)**.

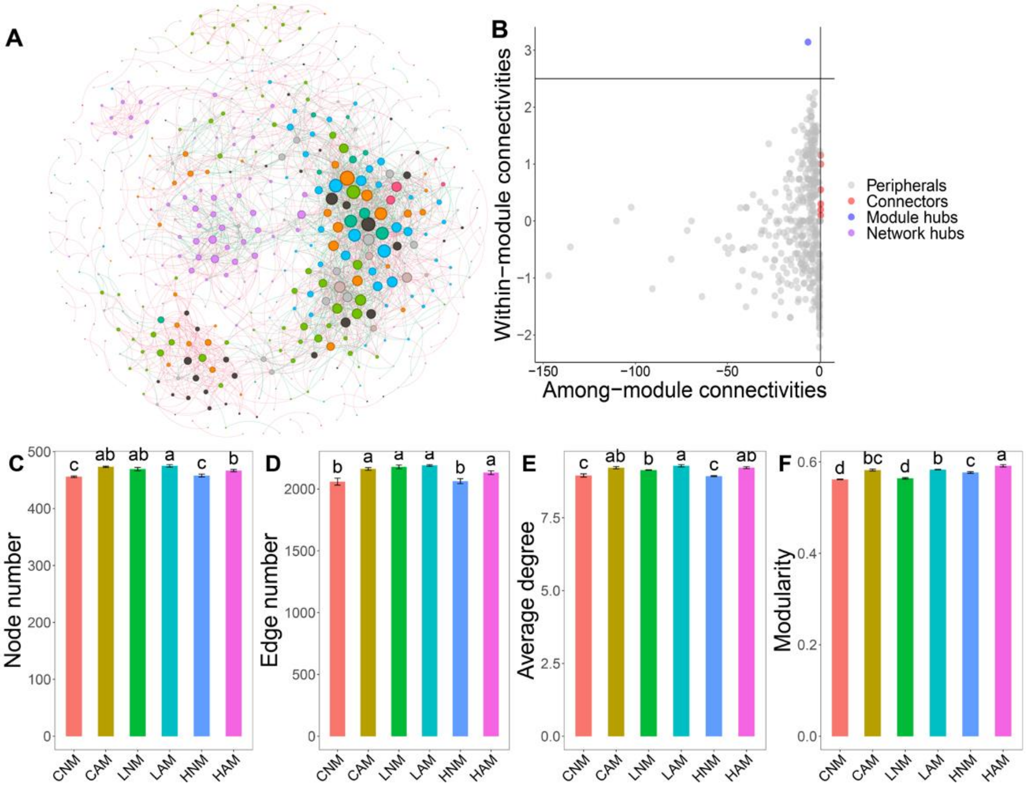

Figure 4-6: Graphical representation of co-occurrence network of bacterial and fungal interactions (A), the distribution network of keystone taxa (B), and the effect of AMF inoculation on network complexity (C, D, E, F) in soils with different biosolids application rates.

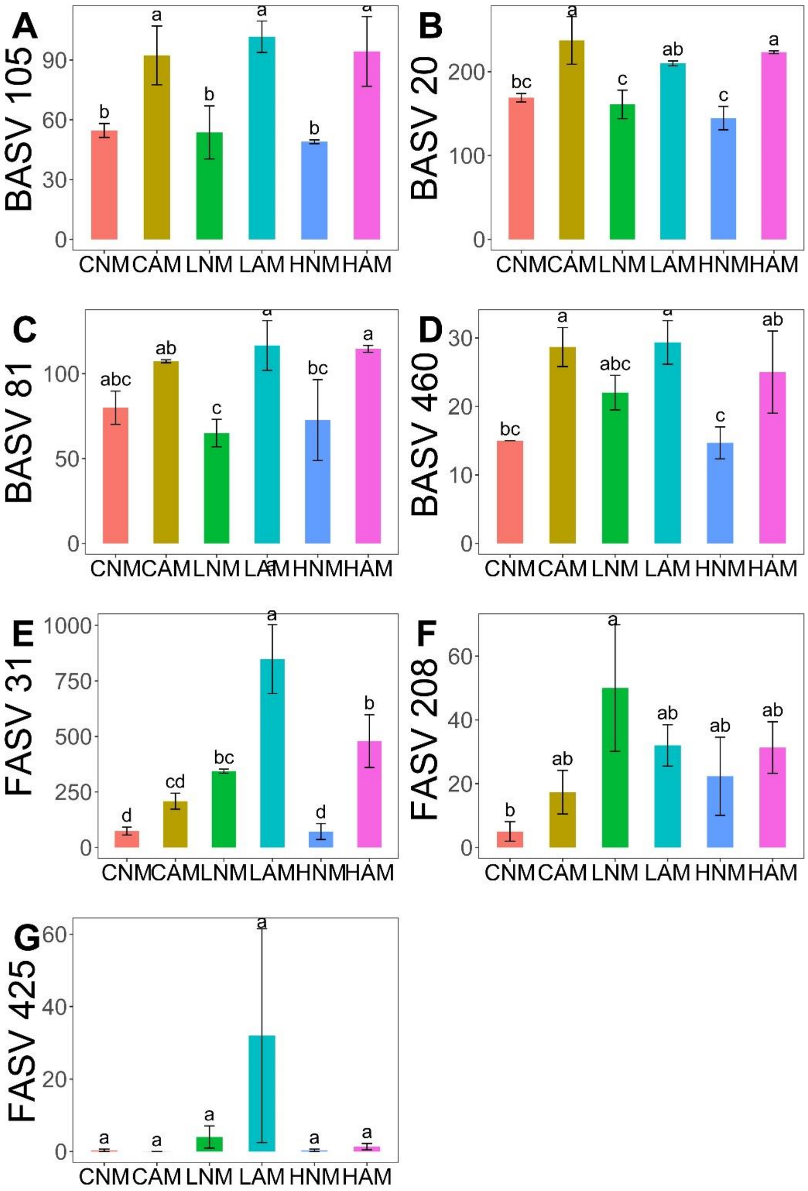

Figure 4-7: The effect of AMF inoculation on the abundance of keystone taxa abundance under different levels of biosolids application.

Metagenomics revealed that several microbial taxa were enriched and depleted in soils with AMF inoculation and without AMF inoculation **(Fig. 4-8, and Fig. 4-9, 4-10)**. In all application rates of biosolids, there were more taxa enriched in soil with AMF inoculation compared to the soil without AMF inoculation **(Fig. 4-8, and Fig.**

4-9, 4-10). The microbial taxa *Gemmatimonadetes*, *Planctomycetia*, *Oomycota* and *Binatia* were found enriched in all soils with AMF inoculation. There was also a significant increase of *Gammaproteobacteria MHYJ01*, *Blastocatellia*, *Verrucomicrobiae*, *Methylomirabilia*, *Terriglobia*, *Bacilli*, *Polyangia*, *Clostridia*, and *Doudnabacteria* abundance observed in soil with AMF under control and low biosolids application rates. Moreover, the *UBA2214*, *Chlorophyceae*, *Mucoromycetes*, and *Dothideomycetes* were also found enriched in soil with AMF inoculation and biosolids application.

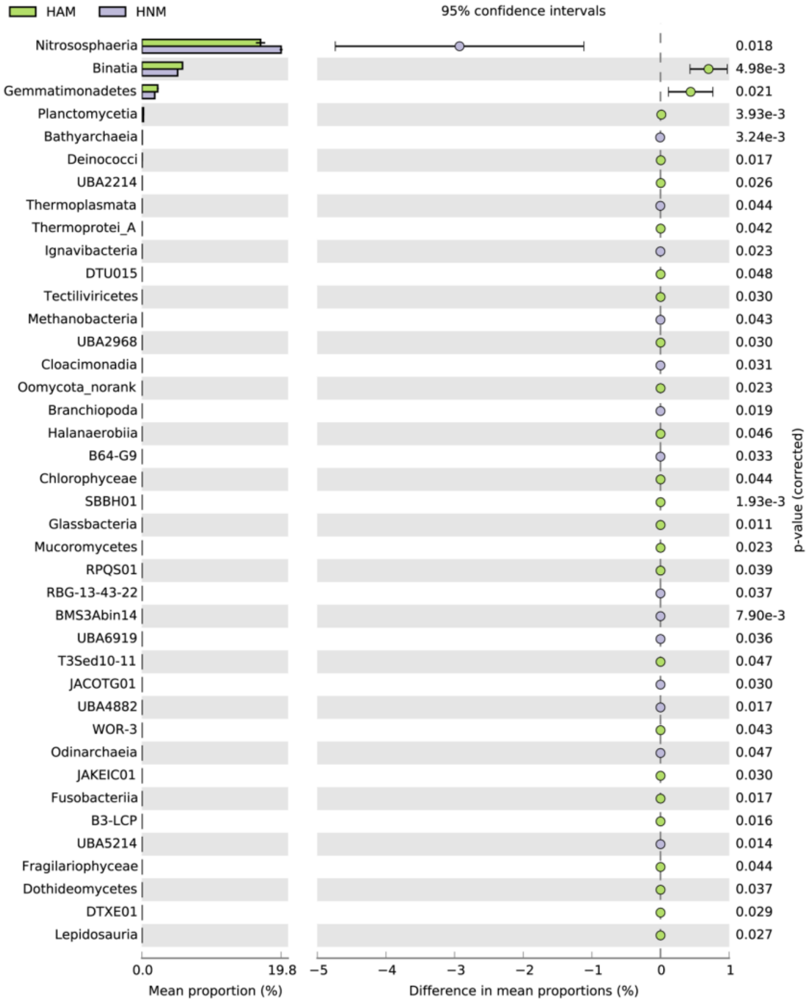

Figure 4-8: Graphical representation of comparison of microbial taxa with AMF inoculation and without AMF inoculation at high biosolids application rate, determined using 95 % confidence intervals for the difference in mean proportions.

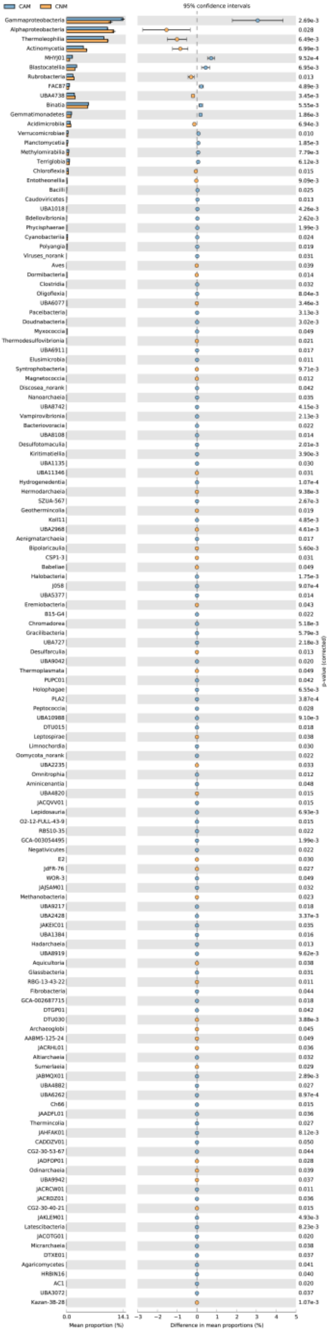

Figure 4-9: Comparison of microbial taxa with AMF inoculation and without AMF inoculation in control treatment, visualized using 95 % confidence intervals for the difference in mean proportions.

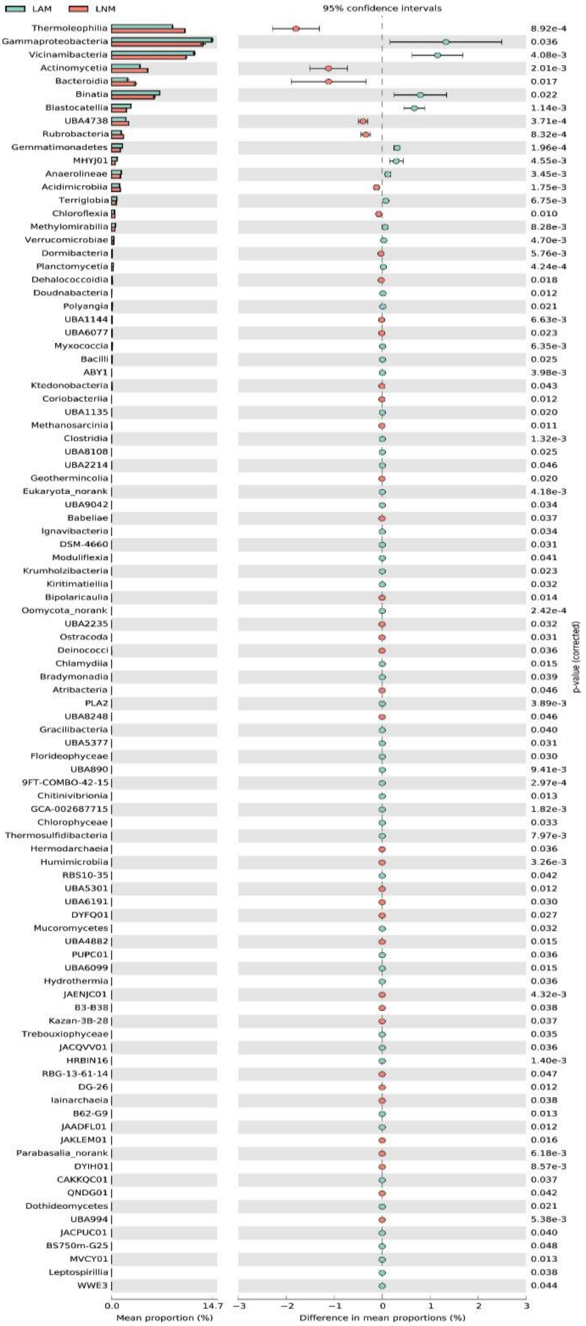

Figure 4-10: Comparison of microbial taxa with AMF inoculation and without AMF inoculation in low levels of biosolids application, visualized using 95% confidence intervals for the difference in mean proportions.

3.3 Effect of AMF inoculation on ARGs, MRGs and MGEs

In the present study, variations in antibiotic resistance genes (ARGs), mobile genetic elements (MGEs) and heavy metal resistance genes (MRGs) could be observed vis-à-vis biosolids concentrations in AMF treatments **(Fig. 4-11, 4-12, 4-13)**. Regarding ARGs, *vanRO* and *mtrA* gene abundances were more enriched in soil without AMF inoculation in the control treatment **(Fig. 4-11)**. These genes were not enriched in soil with AMF inoculation in the control treatment **(Fig. 4-11)**. In contrast, in the soil with biosolids application, there was a severe enrichment of ARGs in soil with AMF inoculation while not in soil without AMF inoculation. Gene *ceoB* was found to be enriched in all soils with biosolids application. Moreover, *bcrA* and *MuxB* were also enriched in soils with low biosolids application **(Fig. 4-11)**.

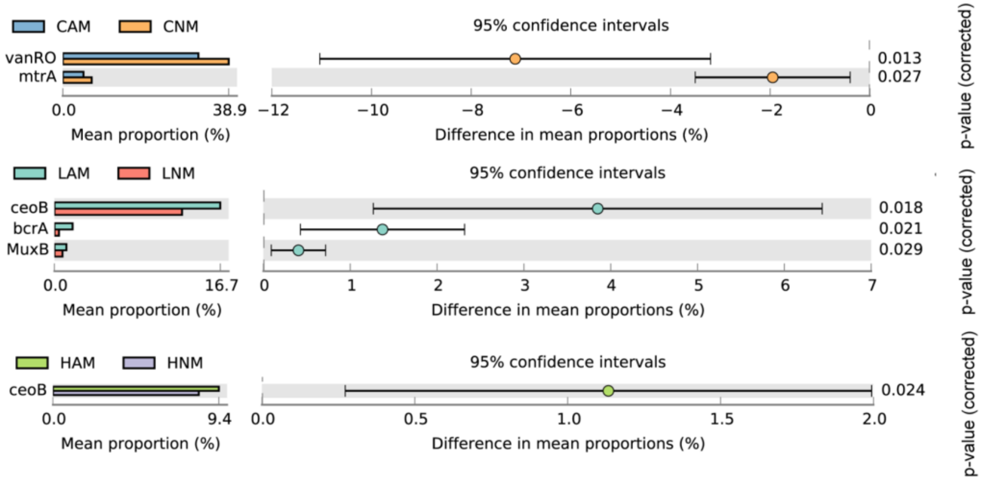

Figure 4-11: The effect of AMF inoculation on ARGs in soils with different biosolids concentrations.

In case of MRGs, the genes *ruvB*, *dspA*, *mntR* and *chrC*, *bfrA*, and *cueA* were found to be enriched in soil with AMF inoculation in control, low and high application of biosolids, respectively **(Fig. 4-12)**. In contrast, the genes *merT-P*, *actP*, *ctpV*, *chrA*, and *arsC*, *acn*, *ctpV*, *brfA*, and *dmeF* were enriched in soil without AMF inoculations under control, low and high application of biosolids, respectively **(Fig. 4-12)**. For MGEs, *IS91* was observed to be enriched in soils without AMF inoculation while *int3* was found to be enriched in soils with AMF inoculation **(Fig. 4-13)**.

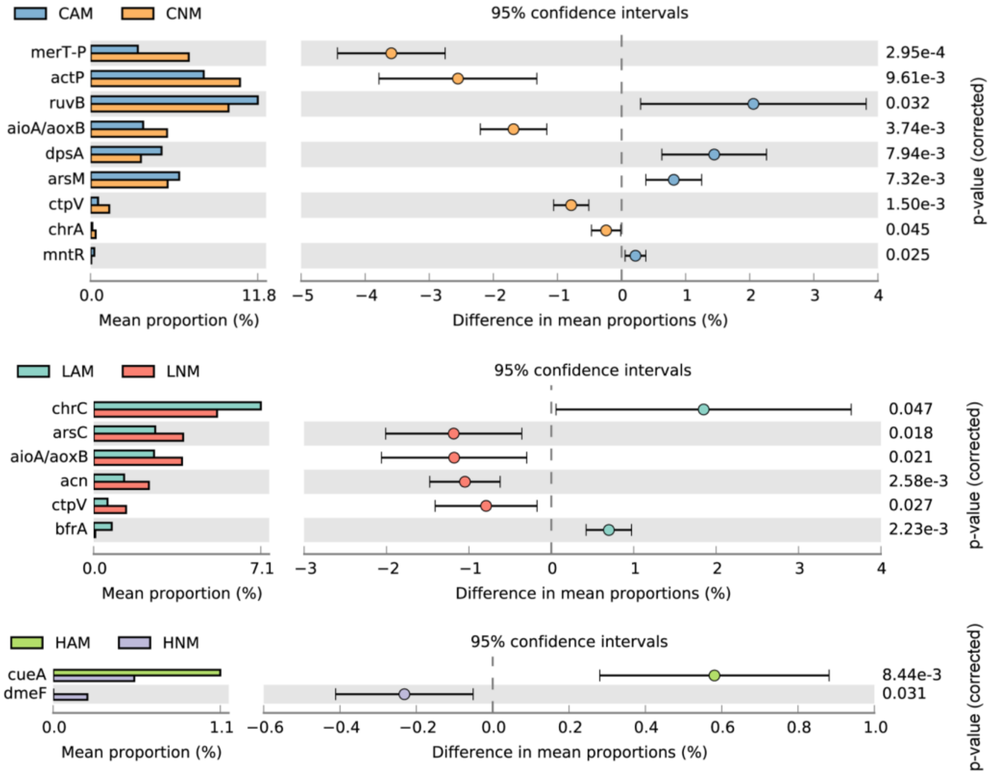

Figure 4-12: The effect of AMF inoculation on MRGs in soils with different biosolids concentrations.

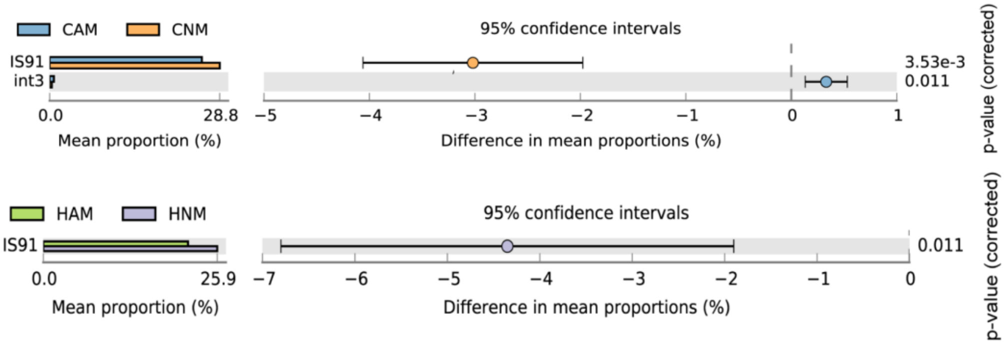

Figure 4-13: The effect of AMF inoculation on MGEs in soils with different biosolids concentrations.

4.Discussion

Heavy metals have been previously reported to exert significant toxic effects on soil microecology (Wu et al., 2018; Sun et al., 2024). In the present study, it was found that AMF inoculation significantly reduced the soil heavy metals content, particularly Cu and Zn **(Fig. 4-3)**, which have been found to be the two major heavy metals present in the current study area (Sun et al., 2024). Previous studies have found that AM inoculation is a crucial and sustainable approach that boosts the ability of plants to extract and sequester pollutants from the soil (Jeffries et al., 2003; Ahmad et al., 2018; Tiwari et al., 2022). Notably, in this study it was found that the reduction of plant heavy metal content was significantly higher in soils with the presence of microbes than in sterilized soils **(Fig. 4-3)**. This indicated that AMF can mitigate the toxic effects of heavy metals on soil microbial communities and may form a symbiotic association with native soil microbes to sequester heavy metals in the soil, thereby reducing their accumulation in plants. The harmful effects of heavy metals on microbial communities occur through several mechanisms, including acting as redox catalysts in the production of reactive oxygen species, disrupting enzyme functions, affecting ion regulation, and interfering with DNA and protein synthesis, thereby decreasing bacterial survivability and community diversity (Hobman and Crossman, 2015; Hong et al., 2019). In the present study, an increase in soil bacterial richness with AMF inoculation in high biosolids input conditions was observed **(Fig. 4-5)**, indicating that AMF could mitigate the deleterious effects of heavy metals on soil bacterial diversity. AMF have the potential to sequester heavy metals in soil, and they can also chelate heavy metals within their colonies (Tiwari et al., 2022; Dhalaria et al., 2020; Huang et al., 2018), reducing their bioavailability. In addition, AMF can detoxify heavy metals by directly adsorbing the metals on the fungal surface and immobilizing them in the soil through the secretion of the low-molecular weight glycoprotein, glomalin (Dhalaria et al., 2020; Tiwari et al., 2022). Furthermore, it was reported that AMF can mitigate the toxic effects of heavy metals on bacteria by enhancing soil enzyme activity as well (Riaz et al., 2021).

Edges, nodes, and average degree are widely recognized indices used to describe network properties and understand interactions between microbial groups. Microbial network complexity is frequently utilized to model these networks and evaluate their responses to stimulation (Zhai et al., 2024). This is established on Macarthur's concept that the complexity of ecosystems begets their stability (Macarthur, 1955). In the present study, AMF inoculation significantly improved the complexity and stability of the entire microbial network in soil **(Fig. 4-6)**. Besides mitigating the toxicity of heavy metals on microbes, AMF also enhanced maize biomass and ameliorated heavy metal toxicity on the plants. The increase in biomass was possibly due to the improved plant nutrient uptake. It has been previously reported that AMF not only boosted plant growth but also increased the mobility of nutrients (Mitra et al., 2023). As regards to heavy metal toxicity, AMF have been reported to reduce the translocation of heavy metals to shoots and retain them in the roots enabling metal tolerance in plants (Riaz et al., 2021). This was clearly observed in the present study with reduced presence of heavy metals in maize shoots as compared to the roots. Further, the hyphae of AMF contribute to strengthening soil aggregates and enhancing soil structure, which increases water retention, reduces erosion, and creates a more favorable environment for diverse microbial communities that can robustly support plant growth (Fall et al., 2022; Gujre et al., 2021). Additionally, AMF secretes a variety of organic compounds such as sugars and fatty acids into the rhizosphere which can promote the growth of specific beneficial microbes that contribute to plant health and soil fertility (Basu et al., 2018). Overall, AMF can potentially create a mutualistic symbiosis between the native microbes and plants (Khaliq et al., 2022), enhancing plant vitality even in the presence of heavy metal stress. In this study, AMF incubation significantly increased the abundance of *Gemmatimonadetes*, *Blastocatellia* and *Gammaproteobacteria* **(Fig. 4-8)**. *Gemmatimonadetes* and *Blastocatellia* are known for their ability to survive in oligotrophic (low-nutrient) conditions and extreme environments, which often include environments contaminated with heavy metals (Wang et al., 2019; Alekseev et al., 2020). These microbes can tackle heavy metals through mechanisms such as biosorption, bioaccumulation, and possibly also transform the metals into less toxic forms (George and Wan, 2020). Gammaproteobacteria is a large and diverse class of bacteria that include many well-known genera such as *Pseudomonas* and *Escherichia* (Gao et al., 2009; Williams et al., 2010). These bacteria are often highly adaptable and can resist heavy metals stress through various mechanisms, including secretion of extracellular polysaccharides and biosurfactants, and the use of efflux pumps to remove toxic metals from the cytoplasm (Chellaiah, 2018; Mathivanan et al., 2021; Fakhar et al., 2022).

Keystone taxa are the species or groups of organisms within an ecosystem that have a disproportionately large impact on their environment relative to their abundance. In the present study, seven keystone taxa (4 bacteria and 3 fungi), were enriched in soil with AMF inoculation **(Fig. 4-6)**. Keystone taxa have been found to play crucial roles in maintaining the structure and function of the ecosystem, enhancing stability and resilience of microbial communities, influencing nutrient cycling, soil structure, and improving the overall health of plants and microorganisms (Hu et al., 2024; Xiao et al., 2021). Additionally, keystone taxa possess the ability to detoxify heavy metals by either transforming them into less harmful forms or by immobilizing them, thereby reducing their bioavailability in the soil (Yu et al., 2021). Further, the several keystone genera identified in the present study have been previously reported to bioremediate heavy metals. *Sphingomonas* spp. are known for their robust bioremediation capabilities of heavy metals via biosorption, secretion of extracellular polymeric substances (EPS) and bioreduction (Reddy et al., 2024). These processes help remove heavy metals from the environment or convert them into less toxic forms (Mishra et al., 2022). *Skermanella* exhibits significant tolerance to heavy metals and may process them through mechanisms like biosorption and intracellular sequestration (George and Wan, 2020). *Cellulomonas* sp. can thrive in heavy metal-contaminated environments and can sustain heavy metals stress via biosorption and EPS production (Gupta and Diwan, 2017). *Apiosordaria* sp. can tolerate heavy metals through biosorption and EPS production, and are often used in bioremediation studies owing to their potential to grow in heavy metal-contaminated environments and reduce the toxicity of these metals (Sun et al., 2014). *Myrmecridium* sp. show tolerance to heavy metals and can process them through biosorption and sequestration mechanisms, which can help in the stabilization and detoxification of heavy metals in the environment (Lotlikar et al., 2019).

The presence of ARGs in municipal solid wastes is a serious concern owing to the practice of applying biosolids as fertilizers in agriculture (Sorinolu et al., 2021). MGEs and MRGs are known to aid the diffuse contamination of ARGs (Zhang et al., 2024b; Tan et al., 2024). Metagenomic analysis in the current study revealed a reduction in the IS91 gene of the MGE gene pool in the AMF-inoculated soil **(Fig. 4-13)**. *IS91* family includes insertion sequences which are essentially a type of transposable element, and it could be involved in the horizontal transfer of genes, including those responsible for antibiotic resistance or other adaptive traits (Yang et al., 2020; Pan et al., 2021). The reduction of the *IS91* gene in AMF-inoculated soil indicated that AMF could play a critical role in stabilizing the gene pool of the soil microbial community, potentially reducing the spread of MGEs that can spread undesirable traits like antibiotic resistance. This technique holds potential in mitigating MGE-mediated spread of ARGs in agricultural fields treated with biosolids and municipal solid wastes. MRGs such as Cu-resistance-related *cueA*, multimetal(loid)-resistance-related *ruvB*, Fe-resistance-related *dpsA* and *bfrA*, As-resistance-related *arsM* and Mn-resistance- related *mntR* were enriched in soil with AMF inoculation **(Fig. 4-12)**. The gene *cueA* typically encodes a copper efflux protein that helps the cell pump out excess copper ions from the cytoplasm, thereby protecting the cell from copper toxicity (Rademacher and Masepohl, 2012). *RuvB* is a gene that confers multimetal resistance, enabling it to defend against toxic, redox-active elements like Cr, Se, and Fe (Huang et al., 2023). The Fe-related *dpsA* gene encodes a protein that protects chromosomal DNA from oxidative stress, with its expression being upregulated in response to nutrient limitations, particularly nitrogen or phosphorus deficiency (Michel et al., 2003; Huang et al., 2023). Gene *brfA* encodes a bacterioferritin, an iron-storage protein that helps maintain iron homeostasis by storing excess iron and releasing it when needed (Monfeli and Beeson, 2007; Abreu et al., 2019). The *arsM* gene encodes arsenite methyltransferase, which detoxifies arsenic by methylating arsenite into less toxic, more easily volatilized forms (Chen et al., 2014b). The *mntR* gene encodes a regulatory protein involved in manganese homeostasis, typically controlling the expression of genes related to manganese uptake and detoxification (Pittman, 2005). These observations provide novel insights on the modulation of native soil microbial communities when in the presence of inoculated AMF. Further, these observations are in concordance with one of the previously mentioned results of the current study, where an increase in microbial riches with AMF inoculation despite the presence of heavy metals was observed. Hence, it is possible to conjecture that AMF inoculation enriched metal-resistant microbes in the soil. Inoculation with AMF can enhance soil microbial communities' resistance to heavy metals by enriching beneficial microorganisms and keystone taxa while reducing the presence of MGEs and consequently, reducing the spread of ARGs.

Overall, stable microbial population aids the maintenance of soil health and plant vitality.

Unanticipatedly, in this study, it was also found that some ARGs such as *ceoB*, *bcrA*, and *MuxB* were enriched in the soil with AMF inoculation **(Fig. 4-11)**. The gene *ceoB* is part of an efflux pump system often found in bacteria, particularly within the ceo operon (Nair et al., 2004). This system is usually involved in conferring resistance to multiple antibiotics, particularly aminoglycosides, and other toxic compounds by actively pumping them out of the cell (Li et al., 2015). The gene *bcrA* is associated with resistance to bacitracin. It encodes a protein that is part of an ATP-binding cassette transporter system which can expel bacitracin from bacterial cells, thereby conferring resistance to the antibiotic (Ahmad et al., 2020). *MuxB* is part of a multidrug efflux pump system in bacteria, commonly associated with resistance to a variety of antibiotics, including β-lactams and other broad-spectrum antibiotics (Sun et al., 2014). Efflux pumps like *MuxB* can contribute to multidrug resistance by actively expelling a wide range of antibiotics out of the cell. However, in the present study, some ARGs such as *vanRO* and *mtrA* showed reduced presence when the soils were treated with only AMF inoculation and without the biosolids. The gene *vanRO* confers resistance to glycopeptides and *mtrA* confers multidrug resistance (Osiris et al., 2024). These observations are timely and serve to caution the scientific community and stakeholders to further thoroughly investigate the presence and spread of ARGs in soils amended with biosolids. Additionally, more studies are warranted for the detection of the presence of other ARGs when biosolids-amended soils are inoculated with AMF. From the present study it is evident that the effect of AMF inoculation vis-à-vis the increase of ARGs is incumbent of the type of ARG.

5.Conclusion

In conclusion, AMF inoculation significantly increased plant biomass, and reduced soil and plant heavy metals contents, particularly of Cu and Zn, as well as reduced the potential spread of antibiotic resistance genes via mobile genetic elements. The influence of AMF on heavy metals was stronger in the unsterilized soil with the indigenous microbial communities compared to the sterilized soil, which indicated the development of a symbiotic relationship between the inoculated AMF and the indigenous soil microbial communities. Although there was no significant influence of AMF inoculation on the diversity of indigenous bacterial and fungal communities, there was an interactive effect of AMF and biosolids application rates, particularly in terms of bacterial diversity in treatments with AMF inoculation in soils with high biosolids application rates. Further, AMF inoculation significantly increased microbial network complexity and keystone taxa abundance potentially aiding soil health and consequently, plant vigour. Moreover, it was also found that several beneficial microorganisms that have high resistance to heavy metals were enriched in soil with AMF inoculations. Metagenomics showed *IS91* gene was reduced in the AMF-inoculated soil, indicating that AMF could potentially reduce the spread of antibiotic resistance genes via mobile genetic elements. As far as we are aware, this is the first study to identify a reduction in the MGE *IS91* in biosolids-treated soil following AMF inoculation. This technique holds potential in mitigating MGE-mediated spread of ARGs in agricultural fields treated with biosolids or MSWs. To serve as a caution, this study revealed a higher abundance of antibiotic resistance genes in soils with AMF inoculation and biosolids amendment compared to soils without AMF inoculations and but with biosolids amendment. Overall, our findings suggest that integrating AMF inoculation into agricultural practices could be a useful strategy for managing soil health and mitigating the risks associated with heavy metals and mobile genetic elements-mediated spread of antibiotic resistance in the environment. However, further vigilance is required regarding the enrichment of antibiotic resistance genes. This study highlights the potential role of AMF in promoting sustainable soil and plant health while potentially reducing the deleterious effects of heavy metal contamination in the environment.

Chapter 5

Soil multifunctionality and health improved by long-term biosolids-amended and AMF-treated soils

Sun Tao, Delaplace, P., Li, G., James, A., Pan, J., & Zhang, J. Arbuscular mycorrhizal fungi improve soil health and multifunctionality in agricultural fields treated long-term with biosolids. Under Review.

The pot experiment examined microbial regulation as a strategy to alleviate risks associated with biosolids application. Nonetheless, a broader evaluation is required to determine whether such interventions can enhance soil health and ecosystem functioning. Accordingly, this chapter integrates data from both field and pot experiments to assess soil multifunctionality and to develop an index-based evaluation framework.

Abstract

The soil fertility and nutrient cycling can be enhanced with the application of biosolids but it can also pose risks associated with inadvertent introduction of heavy metals and pharmaceuticals. The combination of these effects on soil health and multifunctionality remain unclear. Therefore, understanding the impact of biosolids application on soil health and multifunctionality is essential to use it as a strategy for sustainable agriculture. We sampled soil from an agricultural field under long-term (16 years) biosolids application at varying rates, ranging from 4.5 to 36 t dry biosolids ha^{-1} annually. Bacterial, fungal and arbuscular mycorrhizal (AM) fungal communities were determined, and soil health index and multifunctionality quantified. The results of the field experiment showed that biosolids application significantly increased soil health index and multifunctionality. Soil bacterial and AM fungal diversity were positively correlated to soil multifunctionality. The abundance of bacterial and AM fungal keystone taxa were positively correlated to soil health index and multifunctionality. Additionally, to corroborate these results, pot experiments were conducted to test the effect of AM fungal inoculation on soil microbial diversity and multifunctionality. Here also, AM fungal inoculation significantly increased soil multifunctionality. In addition, the results of metagenomic analyses showed that AM fungal inoculation significantly increased soil carbon, nitrogen, phosphorus and sulfur gene abundance, which was positively correlated to soil multifunctionality. Overall, the findings underscore the benefits of judicious application of biosolids combined with AM fungal bioaugmentation as a viable strategy for sustainable agriculture.

Keywords: Biosolids, soil health, soil multifunctionality, arbuscular mycorrhizal fungi.

The graphical abstract is displayed in **Figure 5-1**.

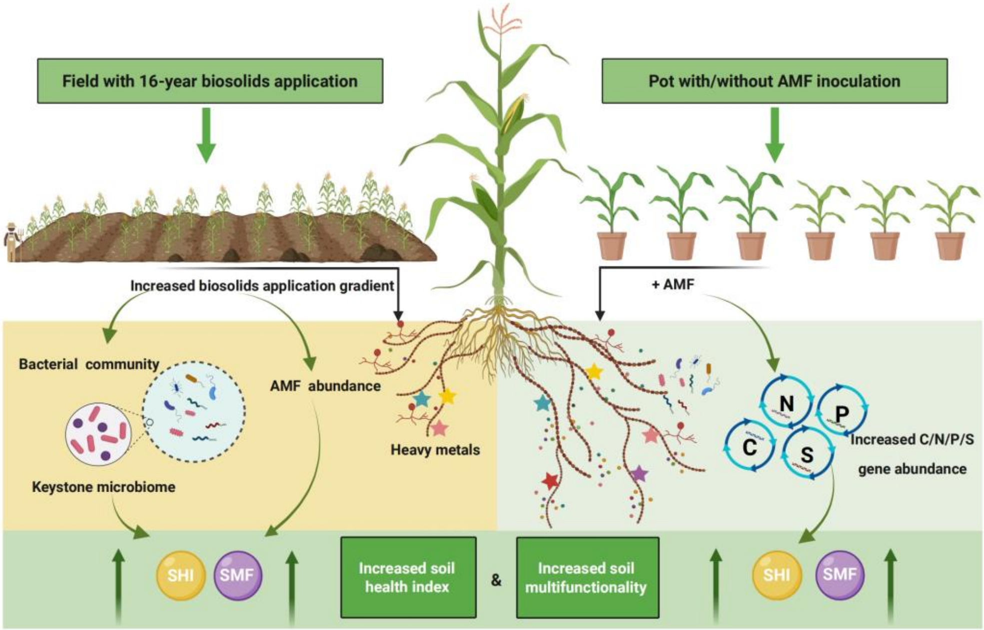

Figure 5-1: Chapter 5 graphical abstract.

1.Introduction

Soil health is fundamental to supporting plant and animal productivity as well as multiple ecosystem functions (Jia et al., 2025; Teague and Kreuter, 2020). Healthy soil supports high-yield crop production while maintaining optimal cycling of nutrients, including carbon storage and utilization (Adewara et al., 2024; Pandao et al., 2024). Therefore, enhancing soil health and multifunctionality is essential for maintaining sustainable agricultural systems and ensuring long-term food security. Biosolids application is increasingly recognized as a potential resource for sustainable agriculture, as it is rich in organic matter and essential nutrients that can improve soil fertility and structure, and promote high-yield crop production (Elgarahy et al., 2024; Marchuk et al., 2023; Kumar et al., 2017). However, the occurrence of heavy metals, such as cadmium and lead, and organic contaminants, such as pharmaceutical residues and microplastics in biosolids may pose long-term environmental risks (Popoola et al., 2023; Mohajerani et al., 2020). Hence, the overall impact of biosolids on soil health remains unclear. Understanding the effects of biosolids application on soil health and multifunctionality is essential for optimizing its use in sustainable agriculture while minimizing potential environmental risks.

Soil microbial communities are invaluable for soil biota and partake in several crucial functions, such as primary production, nutrient cycling and carbon storage (Jia et al., 2025). Several studies have found that soil multifunctionality, in both natural and agricultural ecosystems, is critically linked to microbial diversity (Jiao et al., 2022; Han et al., 2021; Luo et al., 2018; Delgado-Baquerizo et al., 2016). However, the benefits of microbial diversity on soil multifunctionality are mediated by a combination of natural factors and management practices (Jia et al., 2023; Jia et al., 2024; Dong et al., 2022). While biosolids have the potential to improve soil fertility and microbial diversity, they may also introduce pollutants that undermine soil microbiota and multifunctionality (Zhang et al., 2024d; Zheng et al., 2021). Soil microbes are highly sensitive to changes in their microenvironment, and heavy metals found in biosolids may alter the structure of microbial communities, inhibit the growth of some microbes, and affect their ecological functions (Sun et al., 2024; Shuaib et al., 2021; Pérez-de-Mora et al., 2006). For instance, Cu and Pb could disrupt microbial growth, alter community composition, and inhibit key microbial functions necessary for nutrient cycling and soil health (Sun et al., 2024). In addition to heavy metals, pollutants such as pharmaceutical residues and microplastics found in biosolids may also have long-lasting effects on soil microbial communities, reducing microbial diversity and impacting soil biological functions (Hale et al., 2022). Furthermore, it may also promote the selection of antibiotic resistance genes (ARGs) in the microbiome, raising concerns about the spread of antimicrobial resistance (Hung et al., 2022; Law et al., 2021). Given these conflicting effects, it is crucial to investigate the impact of biosolids application on soil microbial communities and multifunctionality, particularly regarding how to balance the benefits and risks to ensure its sustainable use in agriculture.

Microorganisms enable soil multifunctionality (SMF) in a context-dependent manner based on the prevailing edaphic factors (Jia et al. 2024). SMF has been shown to be positively influenced by both bacteria and fungi (Luo et al. 2018), however, their influence may be different in soils with long-term biosolids application as they are highly dependent on factors such as soil type, nutrient availability, moisture, and the presence of contaminants (Morgan et al., 2024; Mohapatra et al., 2016). Soil bacteria are often more sensitive to changes in nutrient availability, pH, and the presence of contaminants (Wu et al., 2017; Oliveira et al., 2006). In biosolids-amended soils, bacteria can contribute significantly to nutrient cycling, organic matter decomposition, and soil structure improvement (Elgarahy et al., 2024; Ploughe et al., 2021). However, some bacterial species may also be negatively affected by pollutants such as heavy metals in biosolids, leading to altered microbial communities and reduced soil function (Sun et al., 2024; Olaniran et al., 2013). Soil fungi, including arbuscular mycorrhizal fungi (AMF), are essential for nutrient cycling, and contribute to soil structure by forming mycelial networks that improve soil aggregation and water retention (Wang et al., 2022b; Hooker et al., 1995). In the context of biosolids application, fungi might be more resilient to some pollutants than bacteria and can help buffer the effects of pollutants like heavy metals. Fungi also support the growth of beneficial microbial communities by providing nutrients to plants and other microbes in exchange for carbohydrates (Devi et al., 2020). AMF, in particular, have been shown to alleviate heavy metal toxicity by sequestering metals in their hyphal structures, thus mitigating their harmful effects on plants and soil microbes (Riaz et al., 2021). The potential of AMF to sequester and neutralize heavy metals in their hyphal tissues helps mitigate the negative effects of pollutants in biosolids-amended soils (Sun et al., 2025), which might make them even more crucial for promoting long-term soil health and multifunctionality. Moreover, AMF can engage with bacteria to enhance soil functionality, facilitating nutrient cycling and promoting microbial diversity (Fall et al., 2022). Nonetheless, it is still unknown how biosolids application shifts the relationship between soil microbiota and SMF, and which microbial taxa may play critical roles in driving SMF.

To explore the effects of biosolids application on the interdependence of soil multifunctionality and soil microbial diversity, we collected and analyzed soil samples from agricultural field that has had 16 years of biosolids application annually. Further, pot experiments were conducted to identify the effect of AMF bioaugmentation on long-term biosolids applied soil. The soil microbial community structure was determined and soil health index and soil multifunctionality quantified. We hypothesize that (1) appropriate application rates of biosolids can enhance soil health index and soil multifunctionality while the overuse of biosolids can reduce both parameters, (2) AMF could interact with other microbes in the soil and alleviate the harmful impacts of heavy metals introduced to soil by biosolids, promoting soil multifunctionality.

2.Materials and methods

2.1 Site description and sampling

The soil samples were obtained from a 16-year long-term field experiment (Chapter 2). The details about the crop rotations, soil type and climatic conditions of this field are available in Chapter 2. The field experiment involved a gradient of biosolids application of 0 t (Control), 4.5 t (SW1), 9 t (SW2), 18 t (SW3), and 36 t (SW4) dry biosolids ha^{-1} annually. In the year 2022, month of June, soil samples (0–20 cm) were collected from the fields undergoing the biosolids treatment. After removing surface residues, five soil cores per plot were combined into a composite sample. Soil samples were placed in a container closed with dry ice and promptly sent to the laboratory. Upon arrival, they passed through a 2 mm mesh sieve and divided into three subsamples. The first part was stored at 4 °C to study soil biological indicators, and second part was air-dried and marked for soil physicochemical properties analyses. The rest was kept at –20 °C for DNA sequencing. Additional details regarding the long-term field experiment and soil properties are available in our previous study (Chapter 3-Sun et al., 2024).

2.2 Pot experiment setup and sampling

Following the wheat harvest in the field, three treatments were selected for the pot experiment: C (Control), L (SW1: low biosolids application), and H (SW4: high biosolids application). The host plant was maize (*Zea mays L.)* cv. Zhengdan958. The study employed a full factorial design of 3 × 2 × 2, involving three biosolids application rates in the hyphal chamber (C, L, H) and two mycorrhizal treatments (AMF-inoculated and non-inoculated) applied to the root chamber. Additionally, the hyphal chamber under each biosolids application rate was further classified based on soil sterilization status, distinguishing between soils with native microbiota (unsterilized) and those subjected to sterilization. There were twelve treatments with 4 replicats, totaling 48 pots. The pot experiment was conducted in a greenhouse from April to June 2023 to evaluate the role of AMF in biosolids-amended soils, following the setup detailed in Sun et al. (2025) (Chapter 4). Briefly, a compartmentalized root-hyphae system was used, where maize was grown in the root chamber (RC), while the hyphal chamber (HC), separated by a 30 µm nylon mesh, allowed AMF hyphal extension without root penetration. After eight weeks of growth, the maize plants were harvested. The aboveground biomass was clipped for biomass measurement. The top 1 cm of soil in the hyphal chamber was removed to eliminate any surface contamination. The remaining soil was sieved through a 2 mm mesh and divided into three subsamples for subsequent analyses. The first part was stored at 4 °C to study soil biological indicators and second part was air-dried and marked for soil physicochemical properties analyses. The rest was kept at –20 °C for DNA sequencing.

2.3 Soil properties measurement

A penetrometer was used to measure soil physical indicators including surface hardness (SurfHard: 0–10 cm soil depth) and subsurface hardness (SubHard: 10–20 cm soil depth) in the field (Zhang et al., 2023). For soil chemical indicators, pH, EC, NH_4^+-N, NO_3^--N, Olsen P, available K, Fe, Mn, Zn, and Mg, organic carbon (SOC), permanganate oxidizable carbon (POXC), total phosphorus (TP) and total potassium were determined based on the protocol described in Zhang et al. (2023). Heavy metals including Cu, Zn, Cd, Pb, Cr, Ni, As, and Hg were quantified as described in Sun et al., (2024).

For biological indicators, soil proteins were measured spectrophotometrically as described in Wright and Upadhyaya (1996). Soil respiration and N_2O emission were measured with a gas chromatograph. Soil extracellular enzyme activities including α-1,4 Glucosidase (AG: sugar degradation), β-Cellubiosidase (BC: cellulose degradation), β-1,4 Glucosidase (BG: sugar degradation), β-1,4 Xylosidase (BX: hemicellulose degradation), β-1,4- Nacetylglucosaminidase (NAG: chitin degradation), leucine-aminopeptidas (LAP: nitrogen mineralization), acid phosphatase (ACP: phosphorus mineralization) and sulfatase (SUL: sulfate hydrolysis) were measured as described in Bell et al. (2013). The glomalin-related soil protein contents including total glomalin-related soil protein (TEG) and easily extractable glomalin (EEG) were determined following the method described by Wright et al. (1998).

2.4 Soil DNA extraction, sequencing and bioinformatic analysis

For both field and pot experiment, the MagaBio Kit from Bioer Technology, China was used for total DNA extraction from 0.5 grams of soil. The primers used for targeting the V4 region of bacteria for PCR amplification was from Walters et al. (2016). The primers used for amplifying the ITS region of fungi was from Op De Beeck et al. (2014), and the primers for a specific region of AM fungi were from Van Geel et al. (2014). The primers were synthesized by Invitrogen™ (Thermo Fisher Scientific Inc.). The sequencing reads were processed according to our previously published study (Sun et al., 2024). Taxonomic assignment was done with the SILVA v138, UNITE v8.0 and MaarjAM for bacteria, fungi and AM fungi, respectively (Green et al., 2022; Eshaghi et al., 2021; Öpik et al., 2010).

For the pot experiments, Shanghai Biozeron Biological Technology Co., Ltd generated and sequenced metagenomic shotgun sequencing libraries. For each sample, the sequencing libraries were created using the TruSeq DNA Library Preparation Kit (catalog no: FC-121-2001, Illumina, USA). An NGS platform in paired-end 150 bp (PE150) mode was used for sequencing of all the samples.

2.5 Soil health scoring and calculating and multifunctionality assessment

For soil multifunctionality, maximum method ($f(x) = x_i/x_{max}$) was used to normalize the soil extracellular enzyme activities and soil respiration to a range of 0–1. All the normalized values were averaged to quantify soil multifunctionality index (Maestre et al., 2012).

For the soil health index assessment, firstly, a total dataset including TN, pH, AP, TP, AK, TK, SOC, EC, EEG, TEG, NH_4^+-N, NO_3^--N, Mg, Na, Ca, POXC, CO_2, N_2O, Cr, Cd, Pb, Hg, Ni, Cu, Zn, As, SurfHard, SubHard and protein was created. Then a minimum dataset (MDS) was established by selecting representative parameters from the total datasets (Li et al., 2022). Indicator redundancy was assessed using Pearson correlation analysis, followed by principal component analysis (PCA) to reduce dimensionality (Andrés-Abellán et al., 2019). Principal components (PCs) with eigenvalues exceeding 1.0 were retained for further analysis due to their significant explanatory power (Yu et al., 2018). Within each significant PC, indicators exhibiting factor loadings ≥0.5 were initially grouped (Zhang et al., 2016b). In cases where indicators exhibited high loadings across multiple PCs, they were assigned to the group with the highest factor loading to avoid redundancy.

Vector norm values were computed to quantitatively evaluate each indicator's contribution to soil quality representation (Shao et al., 2020). Indicators were pre-selected when their vector norm values reached at least 90% of the maximum within each respective group (Huang et al., 2021). If two pre-selected indicators showed a Pearson correlation coefficient above 0.5, only the indicator with the higher norm value was retained for inclusion in the MDS; otherwise, both were included. Finally, total nitrogen, soil pH, moisture, total phosphorus, exchangeable Calcium and heavy metal of Ni were selected to create MDS.

Then selected indicators were standardized into dimensionless values ranging from 0 to 1, linear, non-linear, and comprehensive assessment of soil health (CASH) scoring approaches were applied (Mahajan et al., 2020). Linear scoring included three models: "more is better," "less is better" (applicable to sand and silt), and "optimal range" (applied specifically to soil pH, optimal between 5.5–6.5) (Huang et al., 2021). Equations (1) and (2) were used based on the indicator characteristic, with Eq. (2) employed for metrics favoring higher values or below optimal ranges, and Eq. (3) for metrics favoring lower values or above optimal ranges (Li et al., 2022).

$$S_L = \frac{x}{x_{max}} \tag{1}$$

$$S_L = \frac{x_{min}}{x} \tag{2}$$

$$S_{NL} = \frac{a}{1+(x/x_{\mu})^{b}} \tag{3}$$

The non-linear method was implemented according to Eq. (3): where is the non-linear score, is the maximum score (set at 1), x_{μ} represents the indicator value, is the mean value, and is the slope, defined as −2.5 for "more is better" and 2.5 for "less is better" scenarios (Li et al., 2022).

The calculated indicator scores were combined to produce a comprehensive Soil Health Index (SHI) using both additive (Eq. 4) and weighted additive (Eq. 5) approaches:

$$SHI_A = \sum_{i=1}^{n} S_i/n \tag{4}$$

$$SHI_w = \sum_{i=1}^{n} (w_i \times S_i) \tag{5}$$

Where SHI_A and SHI_W denote the additive and weighted additive soil health indexes, respectively; S_i represents the transformed indicator scores derived from linear or nonlinear methods; n is the total number of indicators within the minimal data set (MDS); W_i and indicates the normalized weight of each indicator, calculated based on the explained variance of corresponding principal components (PCs).

This study evaluated and compared four distinct SHIs, generated by integrating different scoring and aggregation methods: linear scoring with additive aggregation (SHI-LA), linear scoring with weighted additive aggregation (SHI-LWA), nonlinear scoring with additive aggregation (SHI-NLA), and nonlinear scoring with weighted additive aggregation (SHI-NLWA). Higher values of SHI indicated improved soil functionality and lower contamination or degradation levels.

2.6 Statistical analysis

The R (version 4.1.0) was used to analyze the data. One-way ANOVAs were employed to evaluate the difference in soil multifunctionality and soil health index among different treatments. The 'microeco' package was used to generate the soil microbial co-occurrence networks (Liu et al., 2021), applying robust correlations (Spearman's $\rho > 0.60$) and FDR-corrected p-values < 0.01 for network construction. Subsequently, the network properties were analyzed using the 'igraph' package. Partial least squares path modeling (PLS-PM) was used to explore how soil properties (pH and SOC) influence the links between microbial diversity and community composition (including bacteria, fungi, and AM fungi) and multifunctionality as well as soil health index by R package 'plspm'. Model performance was evaluated using the goodness-of-fit index.

3.Results

3.1 Effects of biosolids amendments on soil multifunctionality and soil health indices

Biosolids amendments significantly influenced soil multifunctionality and soil health indices **(Fig. 5-2)**. Soil multifunctionality was significantly higher in treatments with higher biosolids application rates, particularly in SW3 and SW4 **(Fig. 5-2A)**. The enzyme activities of ACP, SUL, AG, BG, BC, BX were highest in the SW3 treatment **(Table 5-1)**, and the activities of LAP and NAG were highest in SW4 treatment **(Table 5-1)**. For soil health indices, soil parameters including pH, TN, TP, Ca, Ni and N_2O emission were selected to quantify soil health index. Among the different treatments, there was no significant difference in soil pH and N_2O emissions. Soil TN and TP significantly increased after biosolids applications, particularly in SW3 and SW4. Soil Ca content significantly increased in SW4 while no significant difference among other treatments were observed. Biosolids application led to substantial improvements in different soil properties **(Table 5-1)**. SHI-LA significantly increased in all biosolids-treated soils, with no significant differences among treatments **(Fig. 5-2B)**. In contrast, SHI-LWA exhibited a significant increase in SW3 and SW4 **(Fig. 5-2C)**. In terms of SHI-NLA, a significant improvement was observed in SW3 and SW4, with SW4 showing the highest values **(Fig. 5-2D)**. SW1 and SW2 exhibited moderate increases but were not significantly different from the control. SHI-NWLA displayed the most pronounced variations, with a clear increase from SW1 to SW4 **(Fig. 5-2E)**. The lowest values were found in control, while SW3 and SW4 significantly outperformed SW1 and SW2. The correlation matrix provides insights into the relationships between SMF and various soil health indices **(Fig. 5-3)**. SMF exhibited significant positive correlations with all SHI **(Fig. 5-3)**.

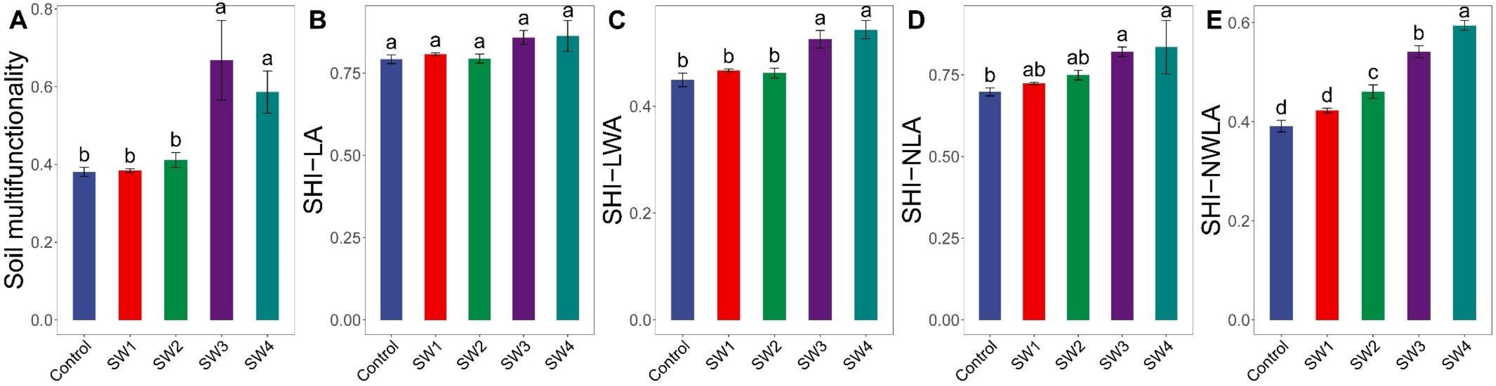

Figure 5-2: The effect of biosolids application on soil multifunctionality (A), and soil health index (B, C, D, F) with varying rates of biosolids application.

Table 5-1: The effect of biosolids application on soil physical, chemical and biological parameters in field experiment.

	Control	SW1	SW2	SW3	SW4
Hardness (kPa)	1552.50 ± 297.45a	1673.17 ± 319.12a	1742.33 ± 100.91a	1845.5 ± 532.82a	1564 ± 195.76a
TN (g/kg)	1.06 ± 0.06d	1.3 ± 0.05d	1.67 ± 0.07c	2.44 ± 0.08b	3.42 ± 0.18a
pH	7.13 ± 0.04a	7.14 ± 0.02a	7.15 ± 0.01a	7.08 ± 0.05a	7.12 ± 0.04a
AP (mg/kg)	52.71 ± 13.54c	71.09 ± 4.88bc	97.43 ± 6.77b	191.48 ± 5a	217.12 ± 12.46a
TP (g/kg)	1.41 ± 0.11a	1.46 ± 0.1a	1.67 ± 0.13a	1.48 ± 0.08a	1.28 ± 0.33a
AK (mg/kg)	289.61 ± 44.16b	199.82 ± 4.4b	306.91 ± 38.26b	296.41 ± 45.59b	606.45 ± 107.62a
TK (g/kg)	19.91 ± 0.42a	19.05 ± 0.53a	21.24 ± 0.57a	21.89 ± 1.3a	17.77 ± 3.67a
SOM (g/kg)	18.83 ± 1.12e	25.30 ± 1.64d	32.67 ± 1.47c	40.91 ± 2.46b	58.95 ± 0.89a
EC (us/cm)	138.40 ± 11.67a	140.27 ± 2.92a	134.03 ± 3.96a	193.23 ± 37.12a	183.37 ± 22.63a
NH_4^+-N (mg/kg)	21.96 ± 1.52a	22.24 ± 0.69a	22.09 ± 0.23a	19.96 ± 2.07ab	17.68 ± 0.29b
NO_3^--N (mg/kg)	11.06 ± 3.38c	14.36 ± 1.09bc	15.4 ± 0.8bc	32.66 ± 9.34ab	37.81 ± 9.01a
Mg (mg/kg))	436.11 ± 11.46b	418.67 ± 4.5b	422.34 ± 6.07b	440.44 ± 32.43b	500.52 ± 16.54a
Na (mg/kg))	131.87 ± 18.45a	132.3 ± 5.61a	91.23 ± 13.88ab	96.6 ± 16.67ab	75.53 ± 3.6b
Ca(mg/kg)	3819.01 ± 164.3ab	3844.08 ± 113.15ab	3676.27 ± 36.99b	3720.44 ± 184.76b	4222.01 ± 19.71a
POXC (g/kg)	0.2 ± 0.03b	0.19 ± 0.02b	0.18 ± 0.03b	0.25 ± 0.01ab	0.33 ± 0.04a
Cr (mg/kg)	64.72 ± 1.66ab	60.8 ± 2.33b	64.22 ± 1.59ab	65.29 ± 0.98ab	68.1 ± 0.48a
Ni (mg/kg)	28.37 ± 0.7a	27.73 ± 1.28a	28.88 ± 0.64a	29.26 ± 0.46a	29.68 ± 0.16a
Cu (mg/kg)	27.13 ± 1.18d	29.58 ± 0.77d	35.09 ± 0.67c	43.52 ± 1.46b	57.34 ± 1.29a
Zn (mg/kg)	102.77 ± 8.81d	109.45 ± 6.7d	141.65 ± 5.44c	198.16 ± 8.61b	281.88 ± 7.38a
As (mg/kg)	15.93 ± 0.36a	15.87 ± 0.69a	15.96 ± 0.57a	16.64 ± 0.29a	16.89 ± 0.21a
Cd (mg/kg)	0.23 ± 0.01d	0.24 ± 0.01d	0.28 ± 0.01c	0.32 ± 0.01b	0.39 ± 0.02a

Pb (mg/kg)	23.71 ± 0.58c	23.28 ± 0.35c	25.07 ± 0.13b	25.93 ± 0.62b	28.33 ± 0.23a
Hg (mg/kg)	148.22 ± 66.79d	198.82 ± 23.55d	607.36 ± 79.27c	916.99 ± 98.83b	1414.89 ± 58.7a
Protein (mg g^{-1})	1.45 ± 0.14d	1.98 ± 0.3d	3.43 ± 0.25c	5.39 ± 0.42b	7.23 ± 0.37a
EEG	0.79 ± 0.07e	0.98 ± 0.04d	1.25 ± 0.04c	1.58 ± 0.04b	1.86 ± 0.06a
TEG	2.66 ± 0.24e	3.78 ± 0.11d	4.5 ± 0.28c	5.09 ± 0.1b	6.67 ± 0.14a
Soil respiration (mg CO_2-C kg^{-1} d^{-1})	14.01 ± 3.80a	14.25 ± 0.35a	18.36 ± 0.85a	17.91 ± 2.23a	18.62 ± 0.74a
N_2O (ug N kg^{-1} d)	0.58 ± 0.02a	0.56 ± 0.01a	0.6 ± 0.04a	0.57 ± 0.01a	0.56 ± 0.01a
ACP (nmol h^{-1} g^{-1})	33.76 ± 1.32b	32.75 ± 1b	29.17 ± 2.55b	65.58 ± 15.64a	44.26 ± 6.95ab
SUL (nmol h^{-1} g^{-1})	8.44 ± 0.26b	7.89 ± 0.18b	6.63 ± 0.45b	10.91 ± 1.39a	8.83 ± 0.31ab
AG (nmol h^{-1} g^{-1})	7.09 ± 1.99ab	3.74 ± 0.44b	4.97 ± 1.14b	15.78 ± 6.28a	8.49 ± 1.13ab
BG (nmol h^{-1} g^{-1})	6.17 ± 1.17b	16.44 ± 1.41b	14.94 ± 2.94b	41.38 ± 13.4a	24.14 ± 3.73ab
BC (nmol h^{-1} g^{-1})	5.45 ± 0.57b	4.54 ± 0.05b	4.29 ± 0.22b	10.07 ± 2.17a	6.03 ± 1.35b
BX (nmol h^{-1} g^{-1})	6.60 ± 1.5ab	4.70 ± 0.11b	4.54 ± 0.71b	13.35 ± 4.38a	7.44 ± 0.76ab
NAG (nmol h^{-1} g^{-1})	1.25 ± 0.16a	1.29 ± 0.24a	1.64 ± 0.09a	4.20 ± 0.89a	6.72 ± 3.65a
LAP (nmol h^{-1} g^{-1})	202.45 ± 45.77b	185.81 ± 14.37ab	266.03 ± 66.28a	84.15 ± 7.68ab	153.8 ± 30.7ab

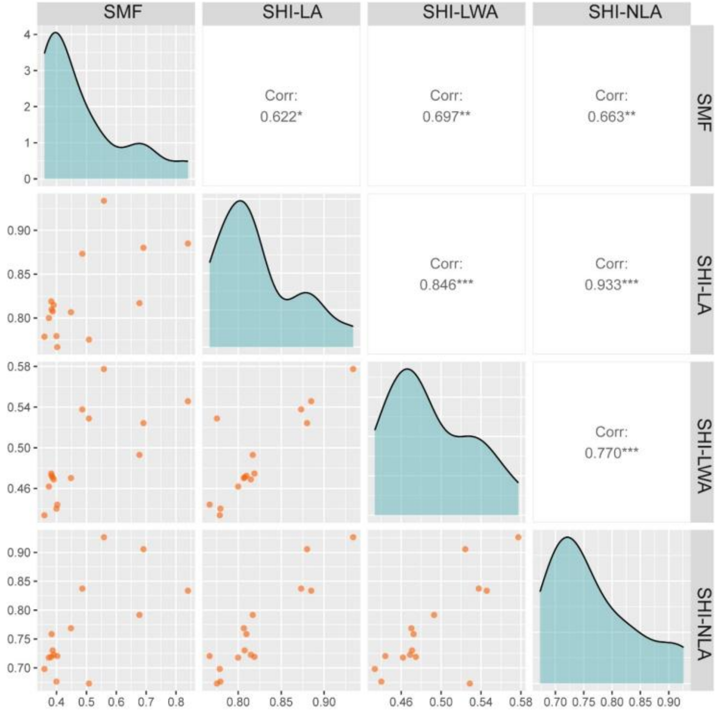

Figure 5-3: The relationships between soil multifunctionality and different soil health indices.

Note: Significance is indicated by $p < 0.05$*, $p < 0.01$** and $p < 0.001$***.

3.2 Effects of soil microbial diversity on soil multifunctionality

There was significant positive correlation of SMF with bacterial and AM fungal richness but not to fungal richness **(Fig. 5-4)**. As evident in the Mantel test, soil bacterial and AM fungal community dissimilarity were positively correlated to multifunctionality dissimilarity. Soil fungal community dissimilarity did not exhibit a significant correlation with multifunctionality dissimilarity **(Fig. 5-4)**.

A co-occurrence network for bacteria and fungi was made to assess the impact of biosolids amendments on their interactions **(Fig. 5-5A)**. The network analysis revealed a total of 18 keystone taxa, comprising 7 bacterial, 5 fungal and 6 AM fungal OTUs **(Fig. 5-5B)**. As presented in **Table 5-2**, Glomerales dominated the AM fungal OTUs, while Acidobacteriota and Proteobacteria were the major bacterial OTUs. Ascomycota and Glomeromycota dominated the fungal OTUs. Among the keystone, only AM fungal OTU64 and bacterial OTU372 and OTU40 were positively related to SMF and soil health index.

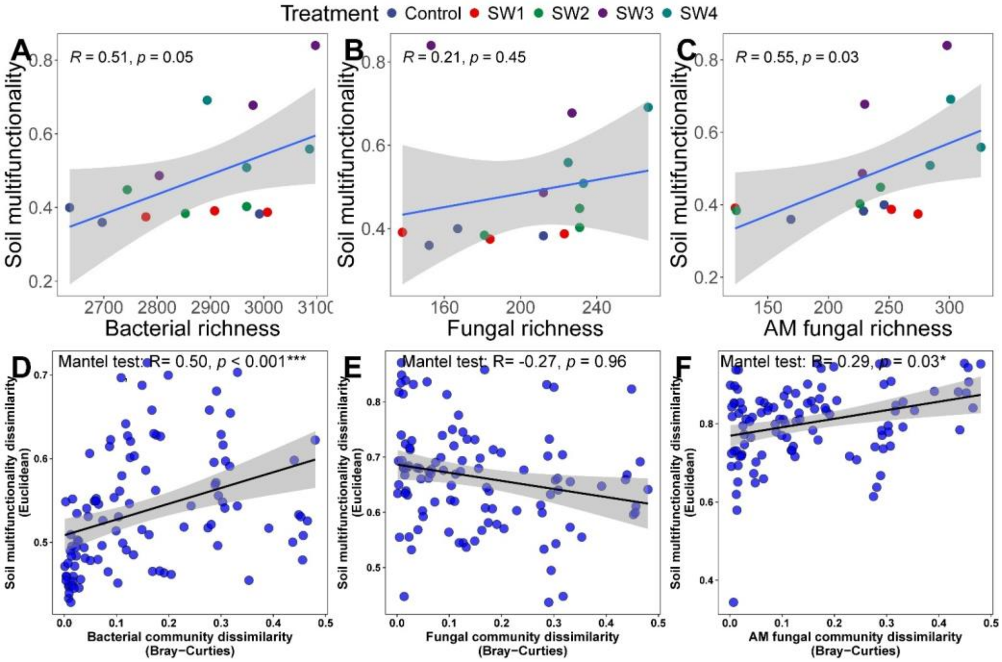

Figure 5-4: The relationship between soil bacterial, fungal and AM fungal richness and community dissimilarity with multifunctionality.

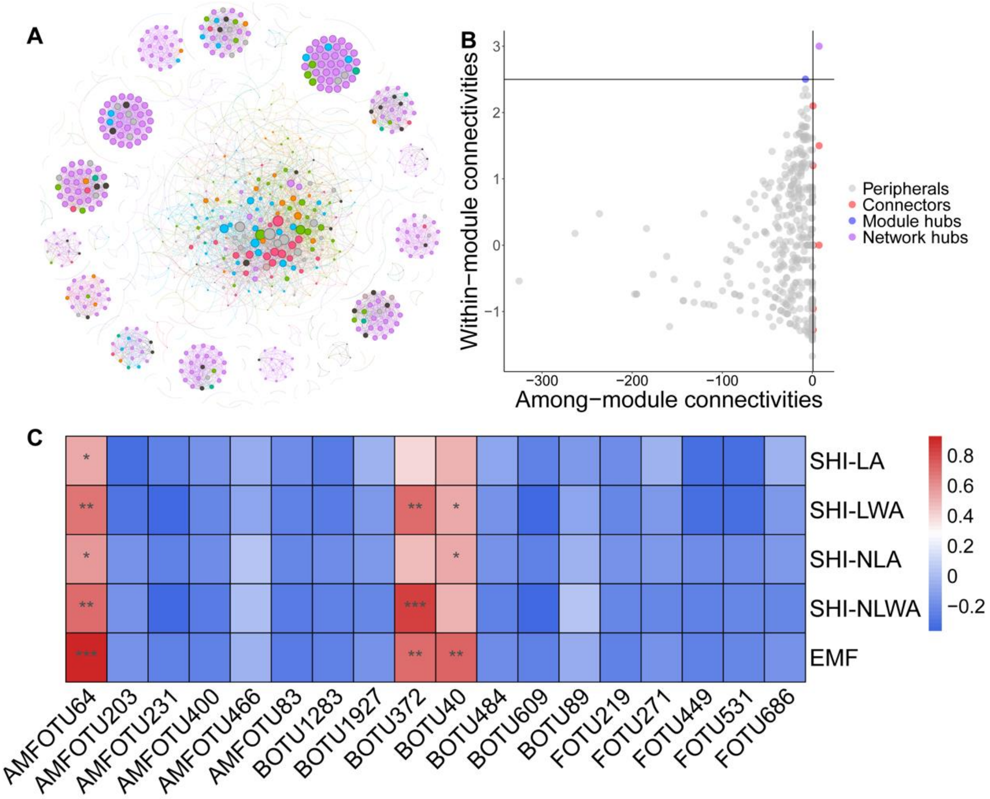

Figure 5-5: Co-occurrence network of microbial interactions (A), keystone taxa distribution network (B), and relationship between keystone taxa abundance and soil multifunctionality, soil health index in soils with varying rates of biosolids application.

Note: Significance is according to $p < 0.05$*, $p < 0.01$** and $p < 0.001$***.

Table 5-2: The distribution of network keystone taxa.

ASVID	Type	Phylum	Order
AMFOTU_64	Network hubs	*Glomeromycota*	*Glomerales*
AMFOTU_231	Connectors	*Glomeromycota*	*Glomerales*
AMFOTU_400	Connectors	*Glomeromycota*	*unassigned*
AMFOTU_466	Connectors	*Glomeromycota*	*unassigned*
AMFOTU_203	Connectors	*Glomeromycota*	*Glomerales*
AMFOTU_83	Connectors	*Glomeromycota*	*Glomerales*
BOTU_1283	Connectors	*Acidobacteriota*	*Subgroup_7*
BOTU_1927	Connectors	*Acidobacteriota*	*Vicinamibacterales*
BOTU_372	Module hubs	*Proteobacteria*	*Rhizobiales*
BOTU_40	Connectors	*Proteobacteria*	*Burkholderiales*
BOTU_484	Connectors	*Proteobacteria*	*Pseudomonadales*
BOTU_609	Connectors	*Proteobacteria*	*Burkholderiales*
BOTU_89	Connectors	*Proteobacteria*	*Burkholderiales*
FOTU_219	Connectors	*Ascomycota*	*Magnaporthales*
FOTU_271	Connectors	*Glomeromycota*	*Glomerales*
FOTU_449	Connectors	*Ascomycota*	*Sordariales*
FOTU_531	Connectors	*Ascomycota*	*Pleosporales*
FOTU_686	Connectors	*Glomeromycota*	*Glomerales*

The result of PLS-PM showed soil factors including pH and SOC had positive effects of AM fungal, bacterial diversity and community composition while had negative effects on fungal diversity and community composition. Bacteria and AMF had positive effects on SMF which further contributed to soil health index. In addition, we found AMF had indirectly positive effect on SMF **(Fig. 5-6)**.

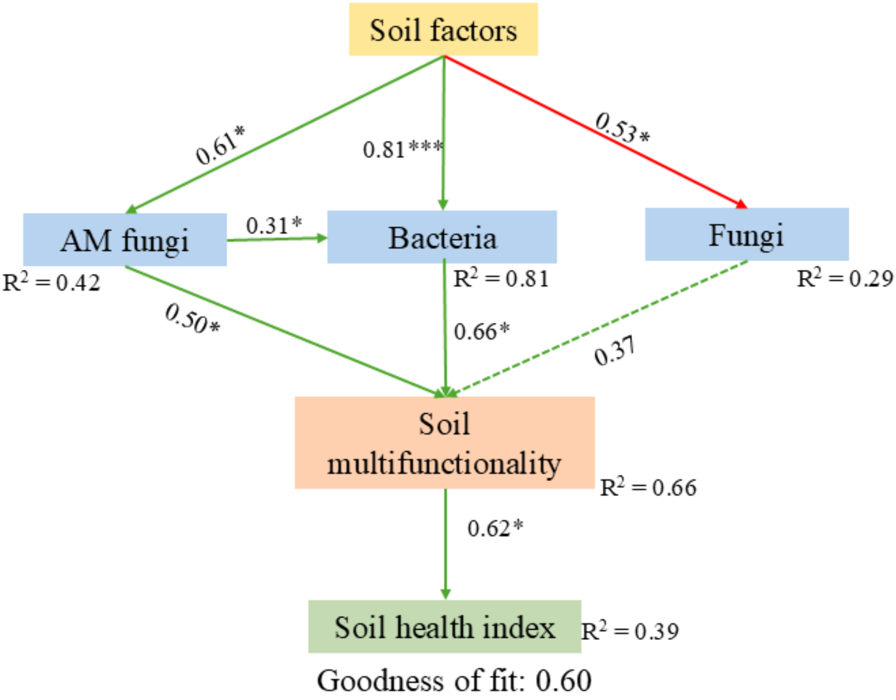

Figure 5-6: Partial least squares path analysis for the effects soil parameters on the relationship between soil microbial diversity and soil multifunctionality as well as soil health index.

Note: Significance is according to $p < 0.05$*, $p < 0.01$** and $p < 0.001$***. Green and red line indicate positive and negative relationships, respectively. Continuous and dashed lines indicate significant and nonsignificant relationships, respectively. R^2 denotes the proportion of variance explained.

3.3 Soil multifunctionality influenced by AMF inoculation

The pot experiment demonstrated that AMF inoculation, particularly under high biosolids application, significantly reduced Cu and Zn concentrations in the soil **(Table 5-3)**. Moreover, soil multifunctionality and plant biomass were also significantly influenced by biosolids application rates and AMF inoculation **(Fig. 5-7A; Fig. 5-8)**. The LAM and HAM treatments exhibited significantly higher soil multifunctionality compared to the CAM and CNM treatments, suggesting that AMF inoculation positively influenced soil health and ecosystem functions. Functional genes of carbon **(Fig. 5-7B)**, nitrogen **(Fig. 5-7C)**, and sulfur **(Fig. 5-7E)** cycling gene abundances did not show significant variation across treatments. However, AMF inoculation significantly increased phosphorus cycling gene abundance in no-biosolids application soil **(Fig. 5-7D)**. Furthermore, correlation analyses demonstrated significant positive correlations between soil multifunctionality and maize biomass (**Fig. 5-7F**; R = 0.49, *p* = 0.0025), carbon cycling genes (**Fig. 5-7G**; R = 0.59, *p* = 0.011), nitrogen cycling genes (**Fig. 5-7H**; R = 0.52, p = 0.0219), phosphorus cycling genes (**Fig. 5-7I**; R = 0.52, *p* = 0.013), and sulfur cycling genes (**Fig. 5-7J**; R = 0.17, *p* = 0.0022).

Table 5-3: The effect of AMF inoculation on soil heavy metals contents in soils with different biosolids application rates.

Biosolids	AMF	Microbiome	Cu (mg/kg)	Zn (mg/kg)	Cd (mg/kg)	Pd (mg/kg)	Hg (mg/kg)
Control	AM	Sterilized	25.68 ± 0.6fg	82.95 ± 0.18ef	0.2 ± 0.01c	27.29 ± 0.34a	0.08 ± 0.01c
Control	AM	Unsterilized	24.44 ± 0.25g	80.44 ± 0.41f	0.19 ± 0c	26.59 ± 0.16ab	0.12 ± 0.03c
Control	NM	Sterilized	25.17 ± 0.53fg	84.42 ± 0.96e	0.19 ± 0c	26.62 ± 0.23ab	0.1 ± 0.01c
Control	NM	Unsterilized	24.83 ± 0.35fg	83 ± 0.89ef	0.2 ± 0c	26.53 ± 0.21ab	0.14 ± 0.02c
Low	AM	Sterilized	27.86 ± 0.95de	97.89 ± 1.1d	0.21 ± 0c	25.58 ± 0.78c	0.32 ± 0.08bc
Low	AM	Unsterilized	26.61 ± 0.26ef	98.25 ± 0.48d	0.2 ± 0.01c	26.29 ± 0.07bc	0.19 ± 0.01bc
Low	NM	Sterilized	28.78 ± 0.95d	102.82 ± 0.59c	0.2 ± 0c	26.02 ± 0.1bc	0.19 ± 0.01bc
Low	NM	Unsterilized	28.43 ± 0.72de	102.5 ± 0.33c	0.2 ± 0c	26.49 ± 0.01abc	1.54 ± 1.11a
High	AM	Sterilized	44.7 ± 0.53b	204.46 ± 2.31b	0.29 ± 0ab	24.3 ± 0.06d	1.08 ± 0.1abc
High	AM	Unsterilized	42.66 ± 0.29c	201.93 ± 0.32b	0.28 ± 0b	24.14 ± 0.04d	1 ± 0.09abc
High	NM	Sterilized	46.66 ± 0.22ab	215.62 ± 0.06a	0.3 ± 0.01a	24.16 ± 0.16d	1.26 ± 0.16ab
High	NM	Unsterilized	46.78 ± 0.76a	213.44 ± 0.21a	0.29 ± 0.01ab	24.28 ± 0.09d	0.84 ± 0.04abc

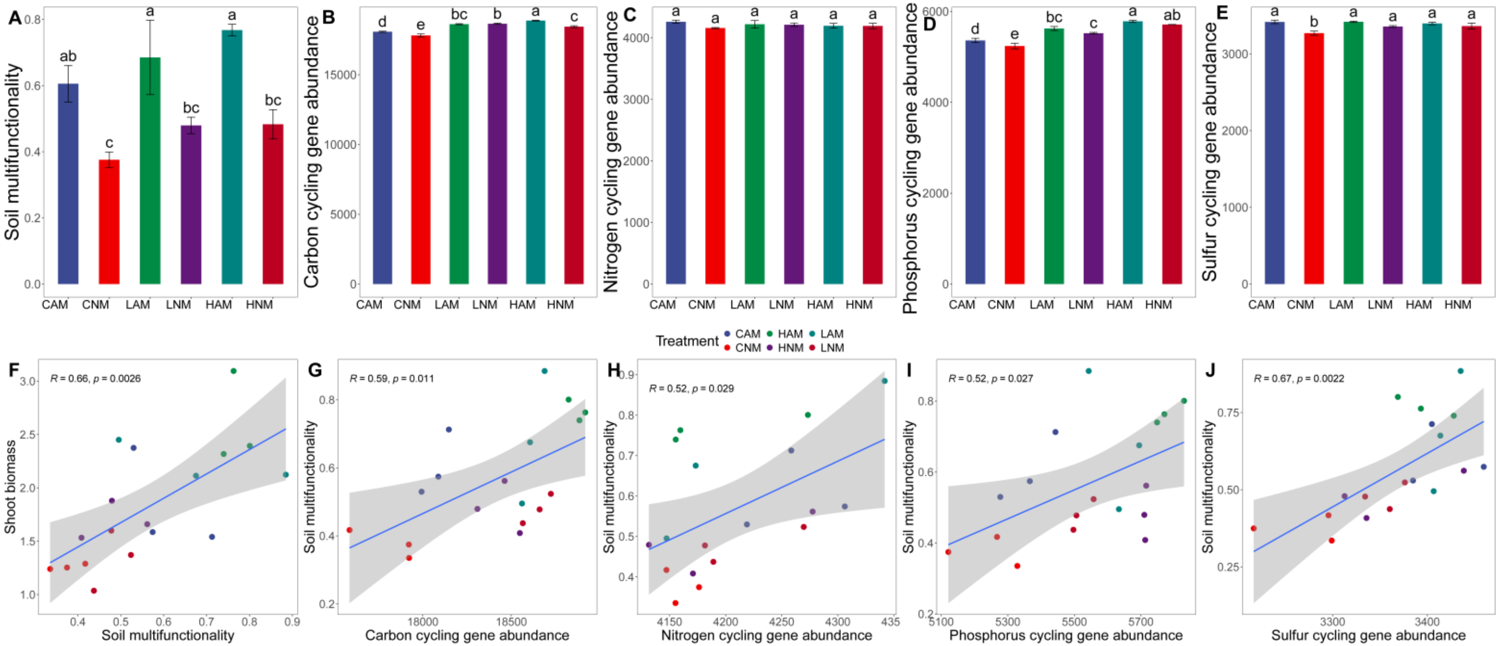

Figure 5-7: The AMF inoculation effect on soil multifunctionality, carbon, nitrogen, phosphorus and sulfur in soils with varying rates of biosolids application.

Note: Lowercase letters indicate significant differences ($p < 0.05$) between the treatments.

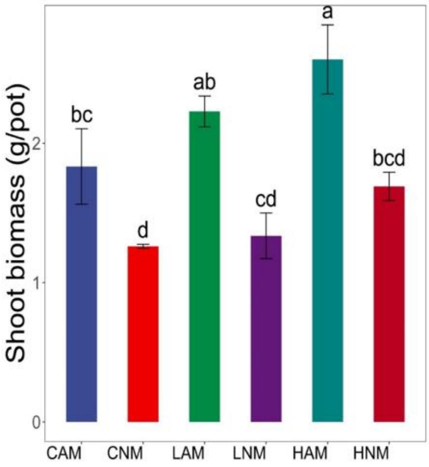

Figure 5-8: The effect of AMF inoculation on shoot biomass in different biosolids application rates in pot experiment.

Gene abundances associated with C, N, P, and S cycling exhibited distinct variation among treatments. As evident in **Fig. 5-9**, genes involved in carbon fixation and decomposition showed increased abundance in HAM and LAM treatments in comparison to the control. Key genes associated with nitrification, organic nitrogen metabolism, and denitrification were significantly enriched in AMF inoculated soils **(Fig. 5-9)**. The highest expression levels were observed in the HAM and LAM treatments, indicating that biosolids application and AMF inoculation promoted nitrogen transformation processes. Notably, genes related to dissimilatory nitrate reduction (DNRA) were reduced in LNM and HNM treatments Regarding the phosphorus cycle, genes associated with inorganic phosphorus solubilization, and organic phosphorus mineralization were more abundant in the HAM and LAM treatments **(Fig. 5-9)**. For the sulfur cycle, genes associated with sulfide oxidation, dissimilatory sulfate reduction, and sulfur disproportionation exhibited higher abundance in HAM and LAM treatments than in the control **(Fig. 5-9)**. Overall, AMF inoculation enhanced microbial functional potential for key biogeochemical cycling processes, including carbon degradation, nitrogen transformation, phosphorus solubilization, and sulfur reduction in biosolids application soils.

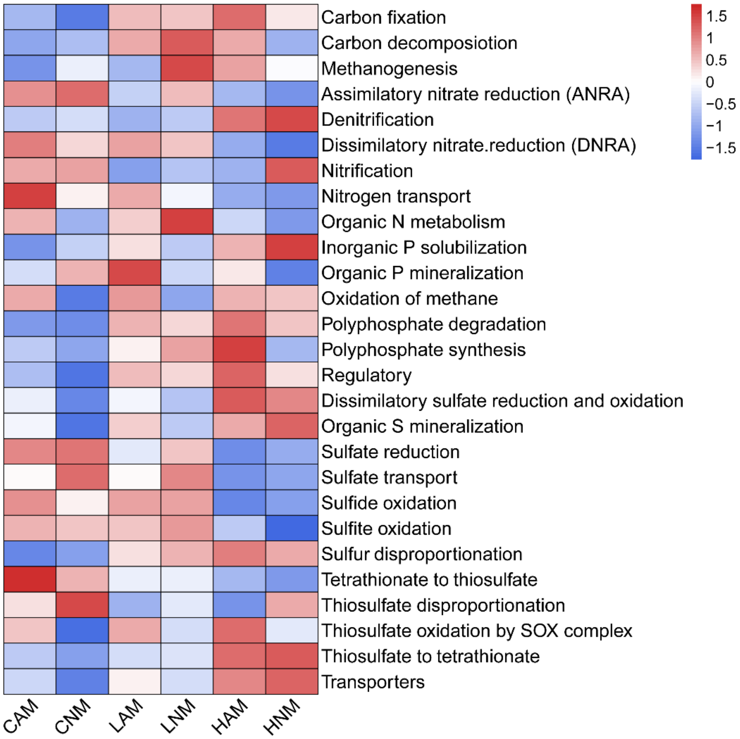

Figure 5-9: AMF inoculation effects on soil carbon, nitrogen, phosphorus, and sulfur cycling gene abundance in soils treated with varying biosolids concentrations.

4.Discussion

4.1 Appropriate application rates of biosolids enhance soil health and multifunctionality

Biosolids containing abundant organic matter and essential nutrients such as nitrogen, phosphorus, and potassium, have gained attention as a potential soil amendment with the growing emphasis on sustainable agricultural practices (Elgarahy et al., 2024). In our study, we found that long-term application of biosolids significantly improved soil health and multifunctionality. Biosolids application has been previously reported to improve soil organic matter, and soil structure by increasing aggregation, water retention and aeration (Elgarahy et al., 2024; Lu et al., 2012). This is particularly beneficial for soils with poor physical properties, such as sandy or compacted soils. The improved soil structure can enhance water infiltration and regulate water flow, thereby reducing erosion. Additionally, the increased organic matter content contributes to carbon sequestration, mitigating climate change (Wijesekara et al., 2021; Wijesekara et al., 2017). The nutrients in biosolids, such as nitrogen and phosphorus, provide essential elements for plant growth, reducing the need for synthetic fertilizers (Rigby et al., 2016; Lu et al., 2012). This can not only promote plant yield but also minimizes the environmental impact associated with fertilizer runoff (Rigby et al., 2016; Pritchard et al., 2010), and negate the costs incurred on fertilizer applications in agricultural fields. Furthermore, biosolids application can increase soil microbial diversity and activity, which play a crucial role in promoting nutrient cycling and organic matter decomposition (Pathma et al., 2012). Enhanced soil microbial activity can lead to improved soil fertility and resilience, contributing to the overall health of the soil ecosystem.

While the benefits of biosolids application are evident, excessive use can lead to soil degradation (Gianico et al., 2021). In our study, it was found that soil health was reduced in treatment SW4. This could be due to the heavy metals accumulation as they are often present in biosolids (Sun et al., 2024). Over time, these metals can build up in the soil, posing risks to plant health, soil organisms, and ultimately human health through the food chain. Heavy metals can disrupt soil microbial communities, reducing their diversity and activity (Abdu et al., 2017). This can negatively impact nutrient cycling and organic matter decomposition, compromising soil health (Alengebawy et al., 2021; Cardoso et al., 2013). Additionally, heavy metals can be toxic to plants, inhibiting root growth and reducing crop yields (Bharti et al., 2022). In severe cases, heavy metal contamination can render soils unsuitable for agricultural use (Wu et al., 2022b; Kumar et al., 2019). Therefore, it is crucial to determine the optimal application rates and monitor soil conditions to maximize the benefits of biosolids while minimizing potential risks.

In this study, total nitrogen, soil pH, moisture, total phosphorus, exchangeable calcium, and nickel (Ni) content were selected to construct a minimum data set (MDS) for soil health evaluation. These indicators were chosen based on their representativeness and sensitivity to biosolids application. Total nitrogen and total phosphorus reflect the nutrient status and fertility potential of the soil, which are directly influenced by biosolids inputs. Soil pH and moisture are key environmental regulators that shape microbial activity, nutrient availability, and plant growth. Exchangeable calcium is not only a vital nutrient but also contributes to soil structural stability and buffering capacity, especially in calcareous soils. Nickel, as a representative heavy metal, captures the potential ecological risks associated with long-term biosolid amendment. The inclusion of both fertility-related and pollution-sensitive indicators ensures a comprehensive and balanced assessment of soil health and multifunctionality under biosolids application. This integrated approach allows us to better understand the trade-offs between productivity enhancement and environmental safety, and supports more informed decision-making in sustainable land management (Zhang et al., 2023).

4.2 Bacteria and AMF as key drivers of multifunctionality in soils treated long-term with biosolids

In our study, we found that bacterial and AM fungal diversity were positively related to soil multifunctionality. Bacteria are among the most abundant and diverse microorganisms in soil, and their activity is essential for maintaining soil health. In soils amended with biosolids, bacteria play a key role in decomposing organic matter and releasing nutrients such as nitrogen and phosphorus (Ahmad et al., 2019; Zhu et al., 2019). This process not only enhances soil fertility but also supports plant growth. Moreover, certain bacterial species have the ability to immobilize heavy metals, reducing their bioavailability and toxicity. For example, Pseudomonas and Azotobacter can produce extracellular polymeric substances that bind heavy metals, intercepting them from entering the soil solution (Zeng et al., 2020b; Gupta and Diwan, 2017). Moreover, Streptomyces rimosus can transform heavy metals into less toxic forms via processes such as oxidation, reduction, or methylation (Etesami et al., 2018; Das et al., 2016). These mechanisms contribute to the resilience of bacterial communities in biosolids-amended soils, allowing them to maintain their functions despite the presence of heavy metals. Further, AMF can form symbiotic relationships with plant roots, extending their hyphae into the soil to access nutrients and water for plants. In return, the fungi receive carbohydrates from the plants (Wahab et al., 2023; Wipf et al., 2019). This mutualistic relationship is particularly beneficial in biosolids-amended soils, where AMF can enhance plant uptake of nutrients such as phosphorus, which is often present in biosolids in forms that are not readily available to plants. AMF also plays a crucial role in mitigating the effects of heavy metals. The hyphae of AMF can bind heavy metals, reducing their translocation to plant roots (Riaz et al., 2021; Bhantana et al., 2021). Additionally, AMF can improve plant tolerance to heavy metals by enhancing antioxidant enzyme activity and reducing oxidative stress (Riaz et al., 2021; Dhalaria et al., 2020). These mechanisms make AMF an important component of soil microbial communities in biosolids-amended soils, contributing to soil multifunctionality and plant health.

Another concern associated with biosolids application is the presence of antibiotics, which can enter the soil through the application of biosolids derived from wastewater treatment plants (Elgarahy et al., 2024; Clarke et al., 2011). Antibiotics can disrupt soil microbial communities, reducing their diversity and activity (Cycoń et al., 2019; Caracciolo et al., 2015). However, both bacteria and AMF have shown resilience to antibiotics, allowing them to maintain their functions in biosolids-amended soils (Sun et al., 2025; Santaella et al., 2020). For example, some bacteria possess antibiotic resistance genes, enabling them to survive in the presence of antibiotics (Jian et al., 2021). These bacteria can continue to perform essential functions such as nutrient cycling and organic matter decomposition. Similarly, AMF have been shown to tolerate certain antibiotics, maintaining their symbiotic relationships with plants and contributing to soil health (Wahab et al., 2023; Fall et al., 2022). Therefore, bacteria and AMF are key drivers of multifunctionality in soils with long-term biosolids application. Their roles in nutrient cycling, heavy metal immobilization, and plant growth promotion are essential for maintaining soil health and productivity.

4.3 AMF mitigation of heavy metal stress and enhancement of soil multifunctionality in biosolids-amended soils

The long-term application of biosolids in agricultural soils can lead to the accumulation of heavy metals, posing a threat to soil health and plant growth (Gianico et al., 2021; Kumar et al., 2017). Bioaugmentation with AMF serves as a sustainable measure to increase plant resistance to heavy metal stress while suppressing the environmental spread of these contaminants (Sun et al., 2025). In this study, AMF incubation could mitigate heavy metal stress and enhance plant biomass and soil multifunctionality in biosolids-amended soils **(Fig. 5-7; Fig. 5-8; Table 5-3)**. AMF plays a crucial role in reducing the bioavailability and toxicity of heavy metals in biosolids-amended soils. One of the primary mechanisms is the binding of heavy metals to fungal cell walls and hyphae (Safronova et al., 2011). The chitin and glomalin in AMF cell walls have a high affinity for heavy metals, effectively immobilizing them and preventing their uptake by plant roots (Sun et al., 2023; Riaz et al., 2021). Moreover, AMF modulate the chemical speciation of heavy metals in the soil, for instance, through the secretion of organic acids that reduce metal solubility and biological availability (Wang et al., 2022c). Furthermore, AMF can enhance plant tolerance to heavy metals through several mechanisms. Firstly, they improve nutrient uptake, particularly phosphorus, which is essential for plant growth and stress tolerance (Bhantana et al., 2021; Begum et al., 2019). Secondly, AMF can induce the production of antioxidant enzymes in plants, such as superoxide dismutase and catalase (Zou et al., 2021), which could neutralize reactive oxygen species (ROS) generated by heavy metal stress, reducing oxidative damage to plant cells (Rizwan et al., 2018; Liang et al., 2017).

In addition, AMF could contribute to soil multifunctionality by enhancing nutrient cycling and improving soil structure, promoting plant growth (Bhantana et al., 2021). The hyphal networks of AMF extend into the soil, increasing the surface area for nutrient absorption and facilitating the transfer of nutrients to plants (Riaz et al., 2021; Wipf et al., 2019). This process enhances soil fertility and supports sustainable agricultural production. Furthermore, AMF could improve soil aggregation by producing glomalin, which could enhance water infiltration, reduce erosion, and increase the resilience of soils to environmental stresses (Hartmann and Six, 2023; Keesstra et al., 2018). Therefore, AMF plays a vital role in mitigating heavy metal stress and enhancing soil multifunctionality in biosolids-amended soils. By leveraging the benefits of AMF, it is possible to maximize the positive impacts of biosolids application while minimizing the risks associated with heavy metal contamination. Future research should focus on optimizing AMF inoculation strategies and understanding their interactions with other soil microorganisms to further enhance soil health and productivity.

5.Conclusion

Overall, long-term application of biosolids significantly enhances soil health and multifunctionality. Soil AM fungal and bacterial diversity and communities showed positive correlations with soil multifunctionality. The abundance of AM fungal and bacterial keystone taxa was positively related to soil multifunctionality. The pot experiments corroborated with the results observed for the analyses of soil from the field, and showed that AM fungal inoculation significantly increased soil multifunctionality and the potential for carbon, nitrogen, phosphorus, and sulfur cycling. The carbon, nitrogen, phosphorus and sulfur cycling genes were positively related to multifunctionality. These findings highlight the potential of combining biosolids application with AM fungal bioaugmentation as a sustainable agricultural practice to improve soil fertility and ecosystem functioning. However, careful management with respect to application rates is essential to mitigate potential risks associated with heavy metals and antibiotics that may be present in biosolids. This study provides a foundation for optimizing biosolids use in agriculture while maintaining soil health and multifunctionality for long-term sustainability.

Chapter 6

General discussion, conclusion, and perspectives

1. Discussion

1.1 Balancing the benefits and risks of sewage sludge-derived biosolids in sustainable soil management

Long-term application of biosolids offers a practical route for recycling valuable nutrients into agricultural soils while improving soil fertility, structure, and microbial diversity. By supplying slowly released nitrogen, phosphorus, and organic carbon, biosolids can enhance aggregate stability, water retention, and cation exchange capacity, resulting in higher crop yields and greater overall resilience. Consequently, biosolids serve as a viable alternative or supplement to synthetic fertilizers, reducing production costs and harnessing municipal waste to bolster soil health.

Biosolids containing abundant organic matter and essential nutrients such as nitrogen, phosphorus, and potassium have gained particular interest as sustainable soil amendments (Elgarahy et al., 2024). Our results showed long-term application of biosolids can significantly improve soil health and multifunctionality, in part by boosting soil organic matter, aggregation, water retention, and aeration (Elgarahy et al., 2024; Lu et al., 2012). These changes are particularly advantageous for sandy or compacted soils with poor physical properties; enhanced soil structure increases water infiltration and reduces erosion, and the additional organic matter contributes to carbon sequestration, thereby helping to mitigate climate change (Wijesekara et al., 2021; Wijesekara et al., 2017). Moreover, the macronutrients in biosolids, such as nitrogen and phosphorus, reduce the need for synthetic fertilizers (Rigby et al., 2016; Lu et al., 2012) and consequently lower both fertilizer costs and the environmental impact of nutrient runoff (Rigby et al., 2016; Pritchard et al., 2010). Soil microbial diversity and activity typically rise following biosolids application, stimulating nutrient cycling and organic matter breakdown (Pathma et al., 2012) while reinforcing soil fertility and resilience.

Despite these clear benefits, biosolids also pose certain risks, particularly through the gradual buildup of heavy metals and antibiotic residues that persist following wastewater treatment. Metals such as copper, zinc, nickel, and chromium do not degrade over time. Under repeated biosolid applications, their concentrations can steadily increase, potentially reaching ecotoxic or phytotoxic levels. Heavy metals can disrupt soil organisms by inducing oxidative stress or hindering critical enzymes, resulting in diminished microbial activity and altered community composition (Abdu et al., 2017; Bharti et al., 2022). Excessive metal accumulation may also inhibit root growth and reduce crop yields, and in severe instances compromise soil suitability for agriculture (Wu et al., 2022b; Kumar et al., 2019). Similarly, antibiotics excreted by humans or livestock may remain in biosolids, exerting subtle selective pressures on soil bacteria and potentially accelerating the spread of antibiotic resistance.

Accordingly, optimizing biosolids application rates and closely monitoring soil conditions are essential to balance these benefits and risks. Although the moderate use of biosolids can improve soil health and minimize the environmental footprint of agriculture, excessive or unmanaged application may degrade soil quality by elevating heavy metal loads and threatening crop viability. Properly calibrating application levels, assessing the sites baseline conditions, and tracking any changes in metal concentrations can help maximize the agronomic advantages of biosolids while mitigating the long-term ecological and public health concerns associated with heavy metal and antibiotic residues. In addition, responsible biosolids management can mitigate many risks. Strict regulatory standards cap heavy metal loadings, while advanced treatment processes—such as extended composting or chemical oxidation—lower contaminants. Additionally, well-monitored application rates prevent overaccumulation of metals and reduce the likelihood of antibiotic-driven microbial shifts. Practices like rotating biosolid-amended fields, adjusting soil pH, or combining biosolids with amendments (e.g., biochar) can further lock metals into stable complexes or degrade residual antibiotics. Compared with these methods, biological techniques, rooted in the use of microorganisms, fungi, or plants, stand out from conventional chemical and physical methods due to their environmental benefits. As they rely on naturally occurring or selected organisms to degrade or immobilize contaminants, thus minimizing secondary pollution. Furthermore, biological approaches can sustain agricultural productivity and ecosystem health over longer periods. Therefore, a fuller understanding of the biological processes at play remains essential, particularly given the complexity of soil microbiology.

1.2 Co-selection of heavy metals and antibiotics: microbial community shifts and resistance gene dynamics

Long-term biosolid applications can trigger intricate ecological processes in soil, particularly when heavy metals and antibiotics occur in tandem. While each contaminant alone can alter microbial communities and promote resistance, their combined presence often magnifies these effects through co-selection. Heavy metals persist indefinitely in soil matrices, binding to clay particles and organic matter, and they continue to impose selective pressures on resident microorganisms regardless of environmental shifts. Even minor changes in soil pH or redox conditions can modify metal speciation, influencing bioavailability and amplifying toxic effects. Antibiotics, by contrast, can degrade or absorb under certain circumstances, yet low, sub-inhibitory concentrations frequently persist in soil, repeatedly stressing microbial populations. When both metals and antibiotics overlap, the environment favors microbes tolerant of metals and those carrying antibiotic resistance genes—particularly if such traits are localized on the same mobile genetic elements (MGEs).

Co-selection exerts profound impacts on microbial community composition and functioning. Initially, biosolids' organic matter can stimulate microbial growth, but as metals and antibiotics accumulate, the environment shifts in favor of organisms adept at detoxification. Sensitive taxa—like specialized decomposers or beneficial plant symbionts—may be outcompeted or succumb to toxicity. This transition can reduce functional redundancy, meaning fewer species remain to conduct critical tasks such as cellulose breakdown, nitrification, or phosphorus solubilization. Although these processes do not necessarily halt, they often become less efficient and more vulnerable to disturbances. Therefore, understanding how microbial communities or functional genes shift during co-selection is pivotal for devising effective strategies against heavy metal and antibiotic contamination. Pinpointing the organisms or gene sets that thrive under high-metal, high-antibiotic conditions allow researchers and policymakers to implement more targeted interventions. Moreover, determining whether antibiotics or heavy metals act as the stronger selective agent further refines the focus of remediation efforts. Understanding which factor dominates enables more nuanced strategies to curb co-selection, preserve soil functionality, and protect environmental health.

In present study, we found that heavy metal contamination can serve as a powerful and enduring pressure on soil microbial communities and their genetic repertoire. Unlike antibiotics, which can decay or diminish, metals such as copper, zinc, cadmium, and lead remain in the environment indefinitely. This persistence means they continuously shape microbial population dynamics, often exerting greater influence than antibiotics, particularly in ecosystems long subjected to metal inputs. Microorganisms that harbor metal-resistance genes—frequently located on mobile genetic elements like plasmids or transposons—gain a decisive advantage in such habitats. Because these same elements may co-carry antibiotic resistance genes, metal presence effectively maintains conditions under which both metal- and antibiotic-resistant strains thrive. Consequently, even if antibiotic levels decrease, the ongoing selective effect of metals keeps co-resistant populations active, thereby promoting the further spread and fixation of resistance traits. In essence, heavy metals can act as a "primary" selective agent, with antibiotics functioning more like an additional or "secondary" layer of stress.

Consistent with the incubation experiment, long-term field study showed that heavy metals are main drivers of microbial community assembly processes. However, soil microbial communities showed distinct responses to heavy metals and nutrient addition. Long-term application of biosolids can markedly influence soil physicochemical properties, including pH, nutrient concentrations, and organic matter content. By altering these parameters, biosolids reshape soil microbial communities—bacteria, fungi, and arbuscular mycorrhizal fungi (AMF) in varied ways. While additional nutrients and organic carbon generally promote microbial diversity, the concurrent introduction of heavy metals complicates these dynamics. Heavy metals such as chromium (Cr), nickel (Ni), and arsenic (As) can be detrimental, posing risks to both the ecosystem and public health. Microbes differ in their sensitivity to heavy metals, with certain groups more vulnerable to metal-induced stress than others.

In many cases, bacterial diversity correlates more strongly with nutrient availability, particularly soil organic matter and dissolved organic carbon (DOC). These readily usable resources support bacterial growth. By contrast, fungi and AMF often display heightened sensitivity to heavy metals like Cr and Ni, leading to reductions in fungal richness and shifts in community composition. Fungal species, especially those forming symbiotic relationships with plants, contribute significantly to nutrient uptake—sometimes including metals. Consequently, high metal loads can affect both their population dynamics and their ecological functions. Studies reveal that some fungal phyla (e.g., *Rozellomycota*, *Blastocladiomycota*, and even certain *Ascomycota* and *Basidiomycota*) exhibit robust metal tolerance, allowing them to outcompete more sensitive taxa.

Ecologically, different processes govern the assembly of these microbial groups under biosolids amendments. In soils receiving long-term biosolids, bacteria often display a substantial element of stochastic processes—random drift shaping community turnover—particularly in the absence of severe metal pressures. However, under conditions where metals such as Cd or Zn become pronounced, deterministic factors, driven by selective pressure, come to the fore. Heavy-metal stress can reduce bacterial diversity by eliminating sensitive strains, while favoring those that possess metal-resistance traits. DOC, on the other hand, can mitigate some of these pressures, providing enough energy and carbon to maintain bacterial proliferation. Fungal communities, including AMF, typically show stronger deterministic processes in response to heavy metals, which act as a powerful filter. Over time, fungi that thrive in high-metal environments—due to genetic traits enhancing metal tolerance—dominate. For example, homogeneous selection describes how metal-adapted fungi progressively replace less tolerant groups, stabilizing community composition. Moreover, dispersal limitation may become pronounced when metals disrupt microbial dispersal or colonization pathways, particularly for AMF whose hyphal networks are critical for nutrient exchange with plants.

Hence, while biosolids deliver nutrients that benefit microbial life, the simultaneous input of heavy metals triggers contrasting responses among bacteria, fungi, and AMF. Bacterial communities often show high turnover rates modulated by both random drift and selective pressures, while fungal and AM fungal communities display more deterministic patterns. Heavy-metal tolerance plays a decisive role for fungal phyla, influencing their relative abundance and ecological function. Overall, understanding these distinct responses is essential for managing biosolids application: balancing nutrient advantages against the ecological impacts of metal accumulation, while recognizing that deterministic processes can drive shifts in fungi and AMF, whereas bacterial assemblies may initially rely more on stochastic mechanisms, eventually transitioning to selective forces as metal levels intensify.

Interestingly, we found that core microbiomes can explain fluctuations in antibiotic resistance gene abundance and support community stability. The core microbiome, comprising microbial taxa that remains relatively constant across diverse conditions, underpins key functions such as nutrient cycling, decomposition, and symbiotic interactions. Yet when subjected to the dual pressures of heavy metals and antibiotics, even this stable subset can undergo significant shifts that illuminate broader trends in antibiotic resistance. A key driver of these transformations is the spread of mobile genetic elements carrying multiple resistance determinants. Dominant core taxa, often well-adapted to local environmental conditions, may act as principal hubs for horizontal gene transfer. Once core microbes acquire plasmids or transposons encoding both metal- and antibiotic-resistance, they gain a pronounced fitness advantage under co-selective stresses. Moreover, many of these foundational taxa already possess wide-ranging defense mechanisms—like efflux systems—capable of exporting not only metals but also various antimicrobial compounds. Consequently, sustained metal exposure can gradually promote multi-resistance within the core community, even when antibiotic concentrations fluctuate. These genetic and ecological modifications lead to observable shifts in the composition and prevalence of ARGs. Genes linked to metal resistance tend to increase if they simultaneously confer protection against antibiotics, while other ARGs unconnected to metal tolerance may recede. This reorganization affects the performance of critical soil processes, potentially altering carbon sequestration, nitrogen mineralization, or the degradation of pollutants. Over the long term, fluctuations in core community composition may signal changing ecosystem stability or resilience to disturbance. Accordingly, investigating the response of core microbiome members under co-selection scenarios is vital to predicting how soil systems will evolve, identifying potential feedback loops between metal and antibiotic resistance, and guiding more effective interventions aimed at preserving soil functions and mitigating the proliferation of detrimental resistance genes.

1.3 Arbuscular mycorrhizal fungi mitigate heavy metal toxicity and influence antibiotic resistance in biosolids-amended soils

Arbuscular mycorrhizal fungi (AMF) are beneficial organisms that are associated with the roots of roughly 90% of terrestrial plant species, including most crops (Diagne et al., 2020). This symbiosis greatly benefits plants by enhancing nutrient uptake, particularly phosphorus from the soil (Khaliq et al., 2022). Multiple studies indicate that the structural components of these mycorrhizae help sequester and compartmentalize heavy metals, thereby limiting their transfer from roots to above-ground tissues (Dhalaria et al., 2020). In the present study, we found that inoculating soils with arbuscular mycorrhizal fungi (AMF) reduced soil heavy metal concentrations, notably copper (Cu) and zinc (Zn), identified as the primary metals in our research area (Sun et al., 2024). Previous findings corroborate that AMF inoculation is a sustainable, effective strategy to enhance plant uptake of pollutants and reduce metal accumulation in soil (Jeffries et al., 2003; Ahmad et al., 2018; Tiwari et al., 2022). Interestingly, our data suggests that the magnitude of metal reduction was greater in non-sterilized soils—implying that the interactions between AMF and native microbial communities can synergistically decrease the accumulation of heavy metals in plants. Several mechanisms explain why heavy metals negatively affect microbial communities. Metals can catalyze the production of reactive oxygen species (ROS), interfere with ion regulation, and damage proteins or nucleic acids (Hobman and Crossman, 2015; Hong et al., 2019). Nonetheless, the addition of AMF improved soil bacterial richness, even under high biosolid inputs. We propose that AMF colonies not only capture metals in fungal tissues (Tiwari et al., 2022; Dhalaria et al., 2020) but also chelate them through secreted compounds like glomalin, decreasing their bioavailability. Detoxification mechanisms driven by AMF, such as adsorbing metals on fungal surfaces, further curtail toxicity for other microbes (Dhalaria et al., 2020; Tiwari et al., 2022). Enhanced enzyme activities, spurred by AMF, also help soil bacterial populations cope with metals (Riaz et al., 2021).

Beyond reducing metal toxicity, AMF contributed to a more complex and stable soil microbial network, as indicated by higher network connectivity and stability indices (Zhai et al., 2024). Such increased complexity aligns with the principle that biodiversity and intricate interactions bolster ecosystem resilience (Macarthur, 1955). Concomitantly, we observed improvements in plant growth: AMF not only facilitated nutrient mobility (Mitra et al., 2023) but decreased the metal translocation from roots to shoots (Riaz et al., 2021). Our results affirmed that plants in AMF-treated soil had lower shoot metal concentrations, reflecting more effective metal sequestration in roots or soil compartments. This aligns with the well-documented roles of AMF in strengthening soil aggregates, improving water retention, and releasing organic compounds that foster beneficial bacterial proliferation (Fall et al., 2022; Basu et al., 2018; Khaliq et al., 2022). Consequently, *Gemmatimonadetes*, *Blastocatellia*, and *Gammaproteobacteria* increased notably in AMF-inoculated treatments, consistent with the capacity of these microbial groups to survive oligotrophic and metal-rich conditions (Wang et al., 2019; Alekseev et al., 2020; Gao et al., 2009).

A critical insight involved identifying keystone taxa—those exerting disproportionately large ecological impacts relative to their abundance. Our data revealed that four bacterial and three fungal keystone taxa were enriched following AMF inoculation. Our incubation experiments showed these core microbiomes supported microbial community stability. Keystone groups can detoxify metals through biosorption, bioaccumulation, or metal transformation (Yu et al., 2021), implying that AMF fosters a habitat conducive to these specialized organisms. For instance, *Sphingomonas* is reported to remove metals via extracellular polymeric substances and bioreduction (Reddy et al., 2024), while *Skermanella* and *Cellulomonas* exhibit tolerance and biosorption capabilities (George and Wan, 2020; Gupta and Diwan, 2017). Collectively, these keystone microbes help stabilize soils against metal-induced stress, strengthening overall microbial function and community stability.

Another pressing concern lies in antibiotic resistance genes (ARGs) that may accompany municipal solid wastes and biosolids (Sorinolu et al., 2021). Mobile genetic elements (MGEs) and metal resistance genes (MRGs) frequently exacerbate the spread of ARGs (Zhang et al., 2024a; Tan et al., 2024). In this study, metagenomic analysis indicated that AMF-treated soils exhibited a drop in the abundance of *IS91*, an insertion sequence linked to horizontal gene transfer of resistance traits (Yang et al., 2020; Pan et al., 2021). To our knowledge, this is the first published evidence that AMF inoculation can diminish *IS91* levels in soils amended with biosolids, underscoring AMF’s potential to restrict the proliferation of undesirable genetic elements. We also noted an enrichment of specific MRGs, including *cueA*, *ruvB*, *dpsA*, *bfrA*, *arsM*, and *mntR* in AMF-treated soils. These genes are implicated in metal tolerance or detoxification (Rademacher and Masepohl, 2012; Huang et al., 2023), aligning with our observation that AMF fosters a niche for metal-resistant microbes. Enhanced microbial diversity often translates to more robust functional gene repertoires, thereby improving soil health and plant vitality. However, it is crucial to consider that an enriched presence of metal-resistant microbes might concurrently harbor ARGs—an association that demands ongoing scrutiny. Interestingly, we also detected higher abundances of certain ARGs (*ceoB*, *bcrA*, and *MuxB*) in AMF-inoculated soils, though others like *vanRO* and *mtrA* declined when AMF alone was present. *ceoB* encodes efflux pumps tied to aminoglycoside resistance (Li et al., 2015), *bcrA* confers bacitracin resistance (Ahmad et al., 2020), and *MuxB* contributes to multidrug efflux (Sun et al., 2014). Such patterns reveal the nuanced impact of AMF on ARG distributions. While AMF can stabilize microbial communities and reduce MGE spread, the net changes in resistance gene prevalence may hinge on the ARG type or the interplay between microbes and environmental conditions. Our results highlight the importance of monitoring diverse ARG classes, as certain genetic pathways may be favored by improved nutrient availability or shifts in community structure, irrespective of heavy metal mitigation.

Our study underscores the pivotal role of AMF in mitigating heavy metal toxicity, reshaping the soil microbiome, and influencing antibiotic resistance dynamics. By creating a more hospitable environment through metal sequestration and improved soil structure, AMF promotes both plant health and microbial network complexity. Although AMF inoculation appears to lower certain ARGs and MGEs, some genes persist or even increase, reflecting the multifaceted nature of microbial ecosystems under metal stress. As biosolids application remain a viable strategy for soil enrichment, harnessing AMF's ecological functions can help optimize soil fertility and resilience, provided we remain vigilant about the subtle, ARG-related consequences that may arise under these evolving soil conditions.

1.4 Arbuscular mycorrhizal fungi improve soil health and multifunctionality in agricultural fields treated long-term with biosolids

We found that AMF supported soil multifunctionality by promoting nutrient turnover and enhancing soil structure (Bhantana et al., 2021). Their extensive hyphal networks enlarge the root's functional surface area, facilitating nutrient flow to plants (Riaz et al., 2021; Wipf et al., 2019). Simultaneously, glomalin production improves soil aggregation, aiding water infiltration and erosion control while reinforcing soil resilience to environmental challenges (Hartmann and Six, 2023; Keesstra et al., 2018). AM fungal diversity emerged as a crucial driver of soil multifunctionality in biosolids-amended systems. By forming extensive symbiotic networks with plant roots, AMF enhance nutrient availability, particularly phosphorus in a manner that is highly beneficial under conditions where biosolids supply ample but sometimes immobilized nutrients. In exchange for plant-derived carbon, these fungi facilitate nutrient transfer, reduce the adverse effects of heavy metals, and bolster overall plant vigor. This protective effect is partly due to the capacity of AMF hyphae to bind metals, limiting their movement to above-ground plant tissues, as well as to stimulate antioxidant pathways in plants, alleviating oxidative stress linked to metal toxicity.

Another concern with biosolid use is antibiotic residue, which can disrupt soil microbiomes and compromise essential functions. Yet AMF have shown notable tolerance to some antibiotics, allowing them to maintain key ecological roles such as nutrient cycling and soil structure improvement. Their hyphal networks promote water infiltration, reduce erosion, and enhance root growth, all of which help sustain plant productivity and resilience against contaminants. Consequently, AMF are pivotal to preserving and reinforcing multifunctionality in soils subject to continuous biosolids application, highlighting the importance of practices that protect and promote AMF colonization in long-term agricultural management. Harnessing AMF's advantages enables biosolid application to deliver greater agronomic benefits while mitigating heavy metal risks. Going forward, refining inoculation strategies and elucidating AMF interactions with other soil microorganisms will be pivotal for advancing soil health and productivity under long-term biosolids management.

1.5 Implications, limitations and future directions

This study provides important insights into the complex trade-offs associated with long-term biosolid application in agricultural soils, highlighting both the agronomic benefits and potential ecological risks. From a stakeholder perspective, the findings underscore that biosolids represent a cost-effective and nutrient-rich amendment, capable of improving soil structure, microbial activity, and overall multifunctionality. At the farm management level, biosolids can reduce dependency on synthetic fertilizers and enhance crop performance. However, the risks associated with heavy metal accumulation and antibiotic resistance gene (ARG) propagation call for carefully calibrated application rates and regular monitoring of soil indicators. Outreach efforts targeting farmers, extension agents, and local authorities are essential to raise awareness about best practices and potential hazards. Moreover, training programs and policy tools should support site-specific decision-making, considering baseline soil health, biosolid composition, and crop requirements.

Our experimental design, while effective in disentangling the effects of AMF and biosolids under controlled conditions, has limitations. Pot experiments inherently constrain root and microbial interactions, may exaggerate border effects, and cannot fully simulate the spatial heterogeneity, weather variability, or biological complexity of field conditions. Additionally, sterilized soil treatments, although useful for mechanistic interpretation, may introduce artifacts by disrupting native microbial assemblages or altering nutrient availability. Future studies should validate our findings under long-term, field-scale settings, preferably across diverse agroecosystems with varying soil textures, climate conditions, and management histories.

From a broader perspective, this research points toward the need for integrated soil management strategies that combine biosolids with biological inoculants (e.g., AMF), metal-immobilizing materials (e.g., biochar), or pH-adjusting amendments (e.g., lime) to optimize nutrient supply while minimizing pollutant risks. The synergistic interaction between AMF and native microbes, in particular, offers a promising direction for nature-based remediation and resilience-building. The emerging role of biosolids in carbon farming also deserves attention: through enhanced organic matter inputs, biosolids can contribute to soil carbon sequestration, improve aggregate stability, and potentially mitigate greenhouse gas emissions. However, the net climate benefit of biosolid application should be quantified via life-cycle assessments that include transport, processing, and field-level emissions.

Importantly, we recommend that regulatory frameworks evolve beyond total metal concentration limits. Given the role of heavy metals in co-selecting for ARGs via mobile genetic elements, bioavailability-adjusted thresholds, ARG and MGE surveillance, and function-based soil health indicators should be integrated into future biosolid management guidelines. Our use of a minimum data set (MDS)—including soil nutrients, pH, moisture, and toxic element concentrations—demonstrates a reproducible and balanced approach for evaluating soil health impacts of biosolid amendments.

Comparatively, while biosolids provide more nutrients than traditional compost and stronger structural improvements than manure, they also pose higher risks related to contaminants. Combining biosolids with other amendments may reduce such risks while retaining functional benefits. In this context, understanding the distinction between essential elements (e.g., Cu, Zn, required in trace amounts) and non-essential or toxic elements (e.g., Pb, Cd, As) is critical for nutrient management. Even essential metals may become harmful if over-accumulated due to repeated application.

Finally, cost considerations must be integrated into decision-making. While biosolids reduce fertilizer expenditures, hidden costs associated with transportation, monitoring, public acceptance, and possible remediation of polluted soils must be considered. Therefore, future work should include cost-benefit analyses across environmental, agronomic, and public health dimensions to support evidence-based policy and field-level implementation.

Altogether, this study emphasizes the value of microbiome-aware, multi-indicator soil assessment frameworks in biosolids management. By integrating ecological, agronomic, and regulatory perspectives, such frameworks can guide sustainable practices that harness the benefits of biosolids while mitigating long-term environmental risks.

2. Conclusion

Our study systematically evaluated the ecological impacts and potential management strategies associated with the long-term application of biosolids in agricultural soils. We demonstrated that biosolids application substantially influenced soil physicochemical properties, heavy metal accumulation, and microbial community structure and dynamics across a series of field experiments, microcosm assays, and pot experiments. The field study showed biosolids application significantly altered bacterial, fungal, and arbuscular mycorrhizal fungal community compositions. Notably, soil microbial communities exhibited distinctive responses to biosolids applications. While both soil organic matter (SOM) and heavy metals (Cu, Pb, Cd, Zn, and Hg) influenced bacterial and arbuscular mycorrhizal fungal communities, only the fungal community responded to heavy metals (Cr, Ni, and As). Bacterial community assembly was predominantly governed by stochastic processes, whereas fungal and AM fungal communities were shaped by deterministic processes. Heavy metals emerged as key drivers of soil microbial community assembly processes.

Furthermore, long-term biosolids application led to a marked increase in antibiotic resistance genes and metal resistance genes, especially aminoglycoside and zinc resistance genes. Microcosm experiments simulating co-selection pressures from heavy metals and antibiotics revealed that heavy metals acted as the dominant selective force affecting soil microbial communities and resistance genes. The abundance of core microbiomes was positively correlated with ARGs, MRGs, and mobile genetic elements, and played a crucial role in enhancing microbial community stability under environmental stresses.

In addition, pot experiments demonstrated that AMF inoculation significantly improved plant biomass and reduced soil and plant heavy metal contents, especially for copper and zinc. Although AMF inoculation did not drastically change microbial community composition, it increased microbial network complexity and enriched beneficial heavy metal-resistant microorganisms. Importantly, AMF inoculation reduced the abundance of the mobile genetic element *IS91*, suggesting its potential to mitigate the MGE-mediated spread of ARGs in biosolids-treated soils.

Finally, integrated analyses of field and pot experiments revealed that long-term biosolids application significantly enhanced soil health and multifunctionality, with positive correlations observed between soil multifunctionality and the diversity of AM fungi and bacteria. AMF inoculation further boosted multifunctionality and functional gene abundance related to carbon, nitrogen, phosphorus, and sulfur cycling.

Overall, these findings underscore the dual role of biosolids application in improving soil fertility while posing ecological risks and highlight the potential of combining biosolids management with AMF inoculation as a viable strategy to enhance soil health, mitigate environmental risks, and promote sustainable agricultural development. However, careful attention to biosolids application rates and long-term impacts remains crucial to ensure environmental and agricultural sustainability.

3. Perspectives

As global agriculture seeks to reconcile productivity with sustainability, biosolids emerge as a promising yet complex tool. Their nutrient-rich composition and capacity to enhance soil structure, organic matter, and microbial diversity position them as viable alternatives to synthetic fertilizers. However, the persistent risks posed by heavy metals and antibiotic residues—often introduced unintentionally via biosolids—demand integrated, science-based management approaches. This study contributes critical insight into these trade-offs, showing that long-term biosolid use can shift microbial community dynamics, enrich resistance genes, and impact soil ecosystem functions.

Importantly, the research highlights the underexplored role of arbuscular mycorrhizal fungi (AMF) as a biological buffer. AMF not only reduce plant heavy metal uptake and support microbial diversity but also curb the spread of mobile genetic elements linked to antibiotic resistance. Their capacity to build complex and stable microbial networks suggests that AMF inoculation may serve as a nature-based solution to mitigate biosolids-induced risks without compromising soil fertility.

Looking forward, the integration of biological inoculants, soil health indicators, and functional gene monitoring should become standard in biosolids management. Future regulatory frameworks must shift from total metal content thresholds to include bioavailability assessments and genetic risk indicators (e.g., ARGs, MGEs). Moreover, long-term, multi-site field trials across varied agroecosystems are urgently needed to validate these findings at scale. Finally, policy and outreach must evolve to balance agronomic benefits with public health concerns, including investment in farmer training, economic incentive structures, and life-cycle-based evaluations of biosolids use.

Ultimately, this study underscores the need for a microbiome-aware, resilience-oriented paradigm in soil management—one that not only recycles nutrients but actively safeguards microbial integrity and ecosystem functionality in the Anthropocene.

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Appendix-Publications

1. **Sun Tao**, Delaplace, P., Li, G., James, A., Pan, J., & Zhang, J. Novel insights into the effect of arbuscular mycorrhizal fungi inoculation in soils under long-term biosolids application: emphasis on antibiotic and metal resistance genes, and mobile genetic elements. *Environmental Pollution*, 2025, 125846.

2. **Sun Tao**, Li, G., Mazarji, M., Delaplace, P., Yang, X., Zhang, J., & Pan, J. Heavy metals drive microbial community assembly process in farmland with long-term biosolids application. *Journal of Hazardous Materials*, 2024, 468, 133845.

3. Qiao, X., **Sun Tao**, Lei, J., Xiao, L., Xue, L., Zhang, H., ... & Bei, S. Arbuscular mycorrhizal fungi contribute to wheat yield in an agroforestry system with different tree ages. *Frontiers in Microbiology*, 2022, 13, 1024128. (Co-First Author)

4. Zhang, H., Degré, A., De Clerck, C., Li, S., Lian, J., Peng, Y., **Sun Tao**, ... & Zhang, J. Changes in bacterial community structure and carbon metabolism in sandy soil under the long-term application of chitin-rich organic material and attapulgite. *Applied Soil Ecology*, 2024, 194, 105161.

5. Wang, M., Hao, C., Li, S., **Sun Tao**, Shi, X., Zhao, W., & Xue, B. Assessment of heavy metal pollution in surface farmland soils of the Fengfeng coal mine subsidence area based on a standardized method. *Journal of North China Institute of Science and Technology*, 2018, 15(4), 93-98.