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Reduction of Cu and nitrate leaching risk associated with EDDS-enhanced phytoextraction process by exogenous inoculation of plant growth promoting rhizobacteria

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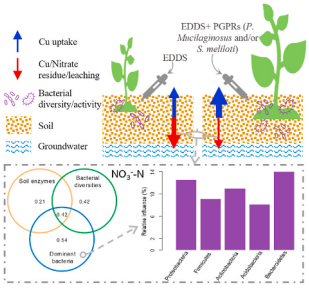
HIGHLIGHTS

- Short-term addition of EDDS increased Cu uptake but inhibited plant growth.
- The leaching risk of Cu and/or nitrate was exposed in the EDDS biodegradation.
- Soil Cu and NO_3^--N concentrations were reduced in the presence of PGPRs.
- Bacterial dominant taxa were the main contributors to NO_3^--N variation.
- Chelant-PGPRs system minimized environmental risks with enhanced phytoextraction.

GRAPHICAL ABSTRACT



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ABSTRACT

Biodegradable chelant *(S,S)–N,N′*-ethylenediaminedisuccinic acid (EDDS) has the more advantages of enhanced metal mobility, rapid degradation, environmental friendliness, and ammonium release. However, the risk of metal and/or nitrate residues and leaching within EDDS biodegradation remains as the bottleneck for the widespread application of EDDS-induced phytoremediation. This study aims to explore if the inoculation of plant growth-promoting rhizobacteria (PGPRs) can eliminate the risk associated with the short-term application of EDDS by investigating Cu phytoextraction and soil nitrate content. Results showed that EDDS application significantly increased the copper (Cu) concentration in shoots, soil total Cu, NH_4^+-N and NO_3^--N content, but decreased plant biomass. The inoculation of PGPRs in the soil showed a strong ability to increase plant biomass, Cu phytoextraction and soil NH_4^+-N content, and decrease soil Cu and NO_3^--N content. Moreover, bacterial dominant taxa were found to be the largest contributors to soil NH_4^+-N and NO_3^--N variation, and the abundance of denitrifying bacteria (Bacteroidetes and *Stenotrophomonas*) decreased in the treatment with PGPRs. The risk of residual Cu and nitrate leaching was reduced by the inoculation of PGPRs without significantly changing

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the stability of the bacterial community. These new findings indicate that the exogenous application of beneficial rhizobacteria can provide an effective strategy to reduce the risk in metal-contaminated soils of chelant-assisted phytoextraction.

1. Introduction

Non-ferrous metals mining and smelting activities have caused China's farmland to face varying degrees of heavy metal contamination (Ju et al., 2019; Zhou et al., 2020). Heavy metal (loid)s, including copper (Cu), can harm human health through skin contact, food chain, groundwater pollution, and other absorption pathways (Gul et al., 2021; Wang et al., 2021). Synthetic/natural chelate-assisted phytoextraction has received a lot of attention over the past 20 years and, to date, remains a key and effective technology for enhancing heavy metals dissolution, uptake and bioaccumulation of metals by plants (Gul et al., 2021; Khan et al., 2021; Xu et al., 2021). Compared with typical chelants, biodegradable chelant-assisted phytoextraction is more environmentally friendly, since they provide a short degradation cycle, and improve nutrient release (Epelde et al., 2008; Muehlbachova, 2011; Luo et al., 2015; Beiyuan et al., 2021). Recently, *(S,S)–N, N′*-ethylenediaminedisuccinic acid (EDDS) has been considered as an alternative biodegradable chelating agent to replace the typical chelant ethylenediaminetetraacetic acid (EDTA) (Ju et al., 2020b; Diarra et al., 2021; Xu et al., 2021). Studies have found that EDDS is degraded in the soil after a lag phase of 7–11 days (Tandy et al., 2006; Yang et al., 2013). After that, the presence of EDDS or metal-EDDS complexes may inhibit plant growth (Wei et al., 2007; Cestone et al., 2012) and impair soil biological activities (Yang et al., 2013; Fang et al., 2017). If plants fail to take up most of the released metals or metal-chelant complexes within a short period of time, the risk of leaching and contamination of subsurface and groundwater is increased (Nowack et al., 2006; Wang et al., 2012). Gul et al. (2021) have identified heavy metal leaching as well as contaminated groundwater as one of the challenges for chelant-enhanced phytoextraction. Park and Sung (2020) confirmed this side effect by showing in a recent study that the potential leaching factor value for Cu was greater than 3 after 7 days of 5 mmol kg^{-1} EDDS application. Additionally, EDDS has a stronger leaching effect in Cu than other chelants, i.e. EDTA and humic acid. Therefore, although EDDS is biodegradable, the possible metal leaching risk in EDDS-enhanced phytoextraction is still of concern in the short term (Wang et al., 2012; Park and Sung, 2020; Gul et al., 2021).

Most relevant studies only focus on the efficiency of EDDS-enhanced phytoextraction (Vamerali et al., 2015; Borker et al., 2020; Diarra et al., 2021; Xu et al., 2021), yet the awareness of other potential risks associated with the short-term application of EDDS is limited and unappreciated. An example of this is the release of nitrogen (N) element increased through EDDS short-term biodegradation (Fang et al., 2017; Beiyuan et al., 2021) may lead to excessive nitrate residues in the soil. Large amounts of residual nitrate, due to its high mobility (Prendergast-Miller et al., 2011), can easily increase the risk of leaching and thus contaminating groundwater. EDDS is known to be rich in nitrogen and carbon, which can provide available nutrients for soil microorganisms and/or plants growth after biodegradation. EDDS can be degraded into *N*-(2-aminoethyl) aspartic acid and then into ethylenediamine (Chen et al., 2010), to later reach the final products of EDDS degradation as ammonium (NH_4^+-N) and carbon dioxide (Beiyuan et al., 2021). With the help of soil microorganisms and enzymes, ammonium can be transformed to nitrate (NO_3^--N). Our previous study found that EDDS added to soil showed the strongest ability to promote soil N cycling in comparison with other chelating agents, and increased nitrate concentration in soil by up to 23.5 times (Fang et al., 2017). Both forms of N (NH_4^+-N and NO_3^--N) mentioned above contribute to increase plant biomass, and NO_3^--N is more readily absorbed by plants. In contraposition, high Cu levels reduce the uptake and accumulation of N, especially NO_3^--N, in plants by reducing the expression of nitrate transporters (Hippler et al., 2018; Huo et al., 2020). As for metals, there is strong evidence on the positive effect on Cu solubilization and increased Cu concentration levels in soil after EDDS treatment (Muehlbachova, 2011; Yang et al., 2013; Luo et al., 2015). This is confirmed by our previous study, where EDDS treatment reduced the biomass and nitrogen content of alfalfa growing in a Cu-contaminated soil (Ju et al., 2020b). Additionally, EDDS begins to degrade as soon as it is added to the soil, therefore the highest potential risk of Cu and nitrate leaching is during the first week after treatment. The study regarding the potential risk of nitrate leaching from EDDS treatment into the surrounding environment in a short period of time has not received much attention in the research field. This is important because by finding a simple tool that alleviate plant growth restriction under heavy metal stress could help to: firstly profit from the available N soil content by generating plant biomass; and second might provide an effective measure to mitigate the risk of nitrate leaching during EDDS-enhanced phytoextraction.

Nowadays, plant-associated beneficial rhizobacteria such as plant-growth-promoting rhizobacteria (PGPRs), as a novel phytobacterial strategy, has been used to promote plant growth in heavy metal contaminated soils and enhance phytoextraction of metal ions (Hayat et al., 2010; Pajuelo et al., 2011; Tiwari and Lata, 2018; Abdelkrim et al., 2020). PGPRs consist in a large group of microorganisms that can live and interact with plants and promote plant growth, including *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas,* and *Rhizobium*, etc (Ferreira et al., 2019). PGPRs can directly and/or indirectly promote plant growth under heavy metals stress, possibly by providing nutrients, increasing iron bioavailability, producing phytohormones, and reducing phytopathogens and harmful rhizobacteria (Hayat et al., 2010; Khan et al., 2020). In terms of providing nutrients, PGPRs promote the uptake of NO_3^--N by plants to meet their growth requirements (Calvo et al., 2019). PGPRs have been shown to promote nitrogen uptake by plants (Paungfoo-Lonhienne et al., 2019). Additionally, PGPRs enhance the uptake of metals by plants by regulating metal transporters expression (Pajuelo et al., 2011). Currently, only one study found that a bioremediation model using PGPRs, enhanced Cu phytoextraction and avoid Cu leaching (Ferrarini et al., 2021). Our previous study has found that *Paenibacillus* and *Rhizobium* strains inoculation increased the N and Cu contents in alfalfa tissues (Ju et al., 2019). Inoculation also affected rhizospheric soil enzyme activity and microbial community composition. Enzyme activity and microbial community composition, especially in the rhizosphere, play a key role in improving N transformation, promoting plant growth, and the fate of metal pollution (Duan et al., 2018; Kuzyakov and Razavi, 2019). Florio et al. (2019) reported that PGPRs inoculation could reduce the abundance and activity of denitrifiers in rhizospheric soil by increasing the competition for N. However, no studies have evaluated the effect of the exogenous application of PGPR on reducing the risk of metal and nitrate leaching, after EDDS-assisted phytoextraction process. To provide a better understanding on how the rizospheric environment is modified after such interventions, plant growth, phytoextraction capacity, and soil metal and available nitrogen content are worthy of be incorporated in the experimental design.

The present study aims to investigate the potential leaching risk of metal and nitrate residues from metal-contaminated soils of short-term EDDS-enhanced phytoextraction with addition of PGPRs. Hence, we used a pot EDDS phytoremediation model with alfalfa (*Medicago sativa*), to treat metal-contaminated soil. From this, we measure the Cu phytoextraction efficiency, nitrate concentration in soil, and N bio-cycling changes and compare them against the same model after

supplementation with two PGPRs strains. We hypothesize that the exogenous inoculation of PGPRs may help reduce the potential leaching risk of Cu and nitrate associated with EDDS use by improving plant growth and altering soil microbial communities. The results of this pioneer study will provide new insights in to reduce the potential risks in heavy metal contaminated soils of chelant-assisted phytoremediation.

2. Materials and methods

2.1. Pot model

The Cu-contaminated soil samples used in this experiment were collected from 0 to 20 cm layer of farmland surrounding a Cu smelter located in Huangshi City, Hubei Province, China (30°43′ N, 114°54′ E). The detailed physicochemical properties of soil samples can be consulted in our previous research (Ju et al., 2019). Two plant growth-promoting rhizobacteria (PGPR) strains were used in this study, Cu resistant strains *Paenibacillus mucilaginosus* (*P. mucilaginosus*, strain ACCC10013) and rhizobium *Sinorhizobium meliloti* (*S. meliloti*, strain CCNWSX0020). In addition, EDDS (Fluka Chemie GmbH; Buchs, Switzerland) and alfalfa seeds (*Medicago sativa*) were purchased from Sigma-Aldrich and Beijing Rytway Ecotechnology Co., Ltd., China, respectively. Approximately 20 pre-germinated alfalfa seeds were grown in each pot, followed by the addition of deionized water maintaining a soil water capacity of 80% to ensure optimal growth conditions. Twenty milliliters of bacterial suspensions were sprayed on to the plant roots once a week (three times in total). Afterwards, EDDS solution (5 mmol kg^{-1} soil) was applied to the soil when sprouts were grown for 54 days. More detailed information about the rhizobacteria strain source and culture, and experimental setup was reported in a previous study (Ju et al., 2020a). On this research five experimental treatments where compared: Control (soil + plant), ES (soil + plant + EDDS), ESP (soil + plant + EDDS + *P. mucilaginosus*), ESR (soil + plant + EDDS + rhizobium *S. meliloti*), and ESRP (soil + plant + EDDS + *S. meliloti* + *P. mucilaginosus*). All experiments were performed in triplicate. One week after adding EDDS, sub-samples of plant and rhizospheric soil were collected to determine their biochemical characteristics. It should be noted that the results of soil and plant biochemical characteristics in the control treatment (soil + plant) were reported previously (Ju et al., 2020a), but they serve to provide context to the present data.

2.2. Soil and plant characteristics

Soil organic carbon (SOC) and total nitrogen (TN) were determined using the K_2CrO_7–H_2SO_4 oxidation and Kjeldahl method, respectively. Ammonium (NH_4^+-N) and nitrate (NO_3^--N) considered as soil available nitrogen was determined using an auto-analyzer (SEAL, Auto-Analyzer, Germany). The total Cu concentration in soil solution was determined using flame atomic absorption spectrophotometry (PinAAcle 900F, PerkinElmer, Germany) after digestion with a modified USEPA "Method 3051 A". Methods for measuring soil enzymatic activity from saccharase, urease, and β-glucosidase activities were determined using a spectrophotometer (UV-2450, Shimadzu Corporation, Japan) at 508, 587, and 400 nm, respectively as described before by Guan et al. (1986). Catalase activity was determined by potassium permanganate titration. Plant dry biomass was determined after oven-drying at 70 °C for 3 d. The total Cu concentration of plant samples was determined using flame atomic absorption spectrophotometry after digestion with a 10-mL $HClO_4$ and HNO_3 (1:4, v/v) mixture. The bioconcentration factor (BCF) and translocation factor (TF) were used to assess the bioaccumulation and transfer of Cu in plants and calculated as described by Ju et al. (2020b). The uptake of Cu and total uptake (TU) of Cu were used to assess the ability of plants to extract Cu from the soil.

2.3. Bacterial community analysis

The composition of the bacterial community was determined by the sequencing of the 16S rRNA gene. DNA was isolated from 0.5 g soil and using the Fast DNA SPIN Kit (MP Biomedicals, Cleveland, USA) according to the manufacturer's instructions. Total DNA was processed by Novogene Bioinformatics Technology Co. Ltd (Beijing, China) for high-throughput sequencing on the Illumina MiSep platform. Briefly, the universal primer set 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) were used to amplify the V4–V5 region of the 16S rRNA genes. Then polymerase chain reaction (PCR) amplification was used according to a method previously described (Ju et al., 2019). The obtained high-quality and effective sequences were clustered by Quantitative Insights into Microbial Ecology (QIIME) software based on the UCLUST method and assigned to operational taxonomic units (OTUs) at similarities of 97% (Caporaso et al., 2010). For each representative sequence, the Silva reference database (http://www.arb-silva.de) with the RDP classifier was used to annotate the taxonomic information. Additionally, bacterial community diversity indicators were calculated by the Mothur software (http://www.mothur.org/).

2.4. Statistical analysis

A one-way analysis of variation (ANOVA) with Tukey's HSD test ($p < 0.05$) was used to assess differences amongst the treatments. Values are expressed as means ± standard deviation (n = 3). Linear discriminant analysis (LDA) effect size (LEfSe) was used to determine potential biomarkers within the soil bacterial community, based on an LDA threshold score (log 10) of 3.5 using the "MASS" package. The differences in bacterial genus level between treatments were further analyzed by the software of statistical analysis of taxonomic and functional profiles (STAMP, v.2.1.3) based on Welch's *t*-test. The beta (β)-diversity of bacterial community was assessed by nonmetric multidimensional scaling analysis (NMDS) based on the Bray-Curtis distance. Redundancy analysis (RDA) was used to ascertain the effects of environmental variables on bacterial community structure after the Hellinger transferred data of bacterial OTUs and the standardized data of environmental factors. Mantel test of the Bray-Curtis distance was used to identify the environmental variables correlating with the bacterial community composition. The NMDS, RDA, and Mantel test were performed in the "vegan" package. In addition, the stability of bacterial community in response to exogenous disturbances was estimated by the Resistance Index (Orwin and Wardle, 2004). Defined for this research as the comparison of the α-diversity (Shannon index) between the control and exogenous treatments (Liang et al., 2020). A correlation heat map was performed to determine the Pearson correlation between different variables using the "ggcorrplot" package. We determined the important variables explaining the variation of soil AN using the relative importance of regressors in linear models by the "relaimpo" package. We also used the variation partitioning analysis (VPA) in the "vegan" package to identify the contribution of the predictor variables to the variation of soil AN. All the above mentioned packages were used in R software v.3.6.3.

3. Results

3.1. Plant biomass and Cu concentration

The biomass of shoots and roots was measured to assess plant growth; the lowest found was in the ES treatment rather than the control (Table 1). The highest shoot and root biomass of plants treated with EDDS were observed in the ESRP treatment. The Cu concentration in shoots was slightly higher (15.8%) and in roots significantly ($p < 0.05$) lower (46.8%) in the ES treatment when compared to control. The Cu concentration in shoots was significantly higher by a factor of 1.53, 1.90,

Table 1
The biomass, Cu concentration, and Cu uptake in alfalfa.

Treatments	Dry biomass (g pot^{-1})		Cu concentration (mg kg^{-1})		Uptake of Cu (μg pot^{-1})		BCF_{Cu}		TF_{Cu}	TU_{Cu} (μg pot^{-1})
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root		
Control	3.04 ± 0.20 bc	2.22 ± 0.12 b	53.7 ± 5.76 a	58.6 ± 15.2 b	163 ± 10.8 a	129 ± 26.3 b	0.08 ± 0.01 a	0.09 ± 0.02 b	0.94 ± 0.17 a	292 ± 37.1 b
ES	1.96 ± 0.01 a	1.60 ± 0.04 a	73.2 ± 1.82 b	34.3 ± 7.63 a	143 ± 2.85 a	54.6 ± 10.6 a	0.09 ± 0.02 a	0.05 ± 0.02 a	2.06 ± 0.35 b	198 ± 7.80 a
ESP	2.55 ± 0.17 ab	1.96 ± 0.26 ab	95.4 ± 3.40 c	41.3 ± 7.84 ab	244 ± 24.4 b	82.2 ± 25.2 a	0.14 ± 0.01 b	0.06 ± 0.01 ab	2.37 ± 0.54 b	326 ± 4.81 b
ESR	3.21 ± 0.44 c	2.04 ± 0.11 b	119 ± 11.3 d	26.1 ± 3.17 a	378 ± 22.5 c	52.9 ± 3.59 a	0.18 ± 0.01 c	0.04 ± 0.00 a	4.56 ± 0.12 c	431 ± 20.3 c
ESRP	3.67 ± 0.15 c	2.18 ± 0.08 b	148 ± 4.48 e	29.5 ± 1.06 a	543 ± 16.9 d	64.4 ± 2.97 a	0.24 ± 0.00 d	0.05 ± 0.00 a	5.03 ± 0.33 c	607 ± 19.2 d

Note: Control (soil + plant), ES (soil + plant + EDDS), ESP (soil + plant + EDDS + *P. mucilaginosus*), ESR (soil + plant + EDDS + rhizobium *S. meliloti*), ESRP (soil + plant + EDDS + *S. meliloti* + *P. mucilaginosus*). BCF, bioconcentration factor; TF, translocation factor; TU, total uptake. "BCF_{Cu} = Cu concentration in the plant/Cu concentration in the soil", and "TF_{Cu} = Cu concentration in the shoot/Cu concentration in the root". "Uptake of Cu = Cu concentration in plant tissues (shoots or roots) × biomass in plant tissues", and "TU_{Cu} = Uptake of Cu in shoots + Uptake of Cu in roots". Values are presented as means ± sd (n = 3). Different small letters stand for significant difference with the Tukey's HSD test ($p < 0.05$).

and 2.38 in the ESP, ESR, and ESRP treatments than in the ES treatment, respectively. The uptake of Cu in shoots and roots were reduced in the ES treatment, and the treatments inoculated with rhizobacteria strongly improved the extraction potential of Cu by plant shoots. The total uptake of Cu in plant (TU) was certainly reduced in the ES treatment compared to the control, moreover, rhizobacteria inoculation markedly increased the value of TU. This change was significant with values increased by a factor of 1.91, 2.52, and 3.55 in the ESP, ESR, and ESRP treatments respectively when compared to the ES treatment. EDDS treatment decreased the Cu bioconcentration factor (BCF) value in roots. The highest BCF in shoot was found in the ESRP treatment, which was significantly higher by 1.33–3.00 times compared to the other treatments. The translocation factor (TF) values for the ES, ESP, ESR, and ESRP treatments were 2.20, 2.50, 4.80, and 5.40 times higher than those for the control, respectively.

3.2. Soil nitrogen and Cu concentrations

The TN, NH_4^+-N, and NO_3^--N concentration of rhizospheric soil was significantly ($p < 0.05$) increased in the ES treatment, which was greater by a factor of 1.24, 10.5, and 755 compared to the control, respectively (Table 2). The individual inoculation or co-inoculation of the *S. meliloti* and *P. mucilaginosus* significantly increased the NH_4^+-N concentration in treatments applied with EDDS. The NH_4^+-N concentration was greater by 30.0% in the ESRP treatment compared to the ES treatment. Notably, the highest NO_3^--N concentration was observed in the ES treatment. The inoculation of rhizobium and *P. mucilaginosus* decreased rhizospheric NO_3^--N content. Compared to the ES treatment, the NO_3^--N concentrations were decreased by a factor of 1.98, 1.92, and 1.97 in the ESP, ESR, and ESRP treatments, respectively. The soil Cu concentration was the highest in the ES treatment and the lowest in the ESRP treatment. The Cu concentration was 7.98% and 10.2% lower in the ESRP treatment than that in the control and ES treatments, respectively.

Table 2
Rhizospheric soil nitrogen and Cu concentrations.

	SOC (g kg^{-1})	TN (g kg^{-1})	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	NH_4^+-N/ NO_3^--N	Cu (mg kg^{-1})
Control	15.9 ± 0.34 a	1.25 ± 0.02 a	1.24 ± 0.06 a	0.02 ± 0.00 a	65.5 ± 2.96 b	677 ± 5.27 ab
ES	17.0 ± 1.25 a	1.55 ± 0.03 bc	13.0 ± 0.43 b	15.1 ± 0.57 c	0.86 ± 0.02 a	694 ± 16.5 b
ESP	17.6 ± 0.34 a	1.57 ± 0.02 c	13.8 ± 0.23 b	7.64 ± 0.58 b	2.15 ± 0.10 a	686 ± 5.94 b
ESR	16.6 ± 0.85 a	1.43 ± 0.09 b	16.9 ± 0.96 c	7.86 ± 0.23 b	1.82 ± 0.16 a	649 ± 39.4 ab
ESRP	16.9 ± 0.30 a	1.44 ± 0.02 b	16.9 ± 1.39 c	7.66 ± 0.20 b	2.21 ± 0.12 a	623 ± 11.6 a

Note: Control (soil + plant), ES (soil + plant + EDDS), ESP (soil + plant + EDDS + *P. mucilaginosus*), ESR (soil + plant + EDDS + rhizobium *S. meliloti*), ESRP (soil + plant + EDDS + *S. meliloti* + *P. mucilaginosus*). Different small letters stand for significant difference with the Tukey's HSD test ($p < 0.05$).

3.3. Soil enzymatic activity

The changes of rhizospheric soil enzymes (i.e., saccharase, urease, β-glucosidase, and catalase) are shown in Fig. 1. The individual inoculation or co-inoculation of the *S. meliloti* and *P. mucilaginosus* increased soil saccharase, β-glucosidase, and catalase activities. The highest saccharase activity was observed in the ESRP treatment, which was significantly greater by a factor of 2.20 compared to the ES treatment. The ESP and ESRP treatments had higher soil β-glucosidase and catalase activities than the other treatments. The activity of soil urease was lower, but not significant in the inoculation treatment (ESR, ESP, and ESRP) than in the ES treatment. Altogether, short-term inoculation of rhizobacteria may not significantly affect the enzymatic activity of rhizospheric soil after EDDS treatment.

3.4. Soil bacterial community composition

Compared with the control, EDDS addition greatly decreased rhizospheric bacterial α-diversity and overall OTU numbers (Fig. S1). Exogenous inoculation with PGPRs did not significantly change the bacterial α-diversity index in the treatment with EDDS addition. Regarding all treatments, the dominant phyla of rhizospheric bacteria were Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria, and Bacteroidetes (Fig. 2a); the dominant genus were *Bacillus*, *Stenotrophomonas*, and *Sphingomonas* (Fig. 2b). The relative abundances of Proteobacteria and Bacteroidetes were higher in the ES treatment compared to the control, and the abundances of Firmicutes, Actinobacteria, and Acidobacteria were lower in the ES treatment. Conversely, in terms of the treatments added with EDDS, single inoculation and co-inoculation of *P. mucilaginosus* and *S. meliloti* markedly decreased the abundance of Proteobacteria but increased the abundance of Firmicutes. The LEfSe analysis identified the potential of Proteobacteria and Firmicutes taxa as biomarkers in the ES and ESRP treatments, respectively (Fig. S2). The abundance of *Stenotrophomonas* was higher in the ES treatment than that in the control, while the abundances of *Bacillus* and *Sphingomonas* were lower than that in the control. The inoculation of PGPRs increased the abundance of *Bacillus* in the treatments applied with EDDS, while decreased the abundance of *Stenotrophomonas*. Moreover, the largest abundance of *Bacillus* and the smallest abundance of *Stenotrophomonas* were observed in the ESRP treatment, respectively. STAMP analysis also

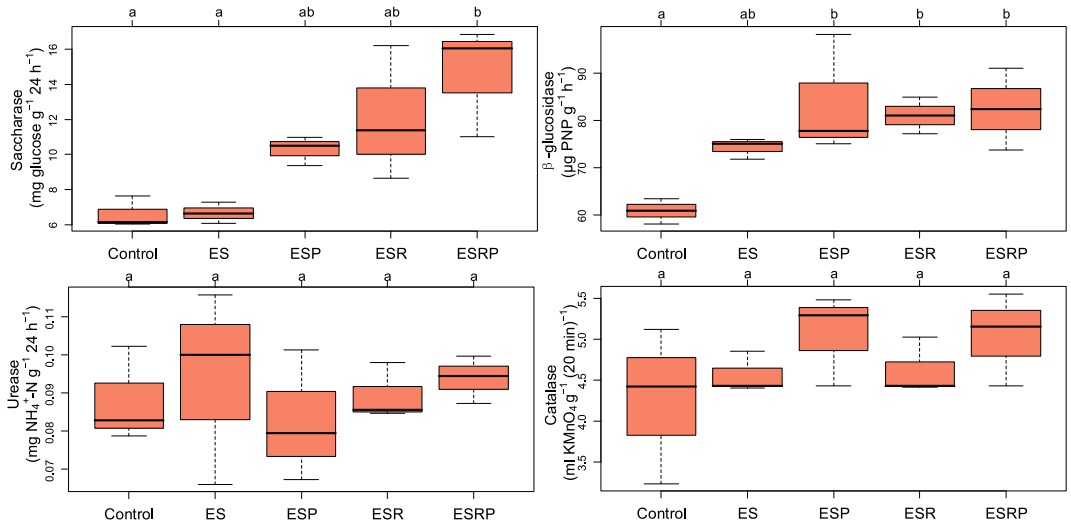

Fig. 1. Enzymatic activities in rhizospheric soil. Control (soil + plant), ES (soil + plant + EDDS), ESP (soil + plant + EDDS + *P. mucilaginosus*), ESR (soil + plant + EDDS + rhizobium *S. meliloti*), ESRP (soil + plant + EDDS + *S. meliloti* + *P. mucilaginosus*). Different small letters stand for significant difference after Tukey's HSD test ($p < 0.05$).

revealed that the difference in abundance of most dominant genera between the control and ES treatments was significant, and this difference was narrowed by rhizobium *S. meliloti* inoculation (Fig. S3).

The NMDS analysis revealed the structure of rhizospheric bacterial community for the treatments applied with EDDS distinctly separated from the control, and the distribution of bacterial community was largely overlapped among the ES, ESP, and ESRP treatments (Fig. 3a). The rhizospheric bacterial community structure did show slight separation between the ESR treatment and other treatments, as confirmed from the NMDS analysis and the Venn diagram based on OTU numbers (Fig. S4). The results of the bacterial resistance index showed that the presence or absence of exogenous beneficial bacteria had no significant effect on the stability of bacterial community (Fig. 3b). The results of RDA showed that soil TN, NH_4^+-N, NO_3^--N, and Cu contents as major factors significantly altered the rhizospheric bacterial community structure (Fig. 3c). The Mantel test also suggested that the major factors were significantly correlated with the overall compositions of the bacterial communities (Table S1).

3.5. Influencing factors and implications for available nitrogen

The Pearson correlation between different factors like soil nitrogen and soil enzymatic activity, microbial diversity, and the microbial community composition are shown in Fig. 4a–c. Soil available nitrogen concentration was positively correlated with enzyme activity, but only NH_4^+-N was significantly correlated with saccharase and β-glucosidase activity ($p < 0.05$). Soil NH_4^+-N/NO_3^--N value was positively correlated with bacterial OTU numbers and α-diversity index. Soil TN and NH_4^+-N concentrations were significantly correlated with OTU and Shannon index, NO_3^--N was only significantly correlated with OTU numbers. Additionally, soil NH_4^+-N and NO_3^--N were significantly positively correlated with the abundance of some dominant bacteria, such as Actinobacteria, Chloroflexi, and *Phenylobacterium*. The relative importance of regressors in the linear models showed that the selected soil enzymes, microbial diversity index, and dominant bacteria, as environmental variables, explained 96.8% and 96.7% of the variation in NH_4^+-N and NO_3^--N concentrations, respectively (Fig. 5a and b). The activity of β-glucosidase and the relative abundance of Bacteroidetes were identified as the most important variable affecting the available nitrogen content. Second, the relative influence of other dominant bacteria on the available nitrogen content was also relatively large. The VPA results further revealed the contribution of selected environmental variables to the variation in available nitrogen (Fig. 5c and d). The composition of soil dominant bacteria explained the most variation of available nitrogen, followed by bacterial diversity and soil enzymatic activity. Interactions among these three variables also accounted for 27.0% and 42.0% of the variation in NH_4^+-N and NO_3^--N, respectively. In terms of the correlation between soil nitrogen content and plant characteristics, soil NH_4^+-N concentration, NO_3^--N concentration, and their ratios had greater impacts on plant biomass, plant Cu content and Cu uptake (Fig. 4d).

4. Discussion

The biodegradable chelating agent EDDS assisted phytoextraction can improve the uptake of Cu by plants and is used as an effective remediation technique for heavy metal pollution in the field. In this study, short-term (7 d) application of EDDS significantly increased Cu concentration in shoots and Cu transfer coefficient without changing the uptake of Cu by alfalfa shoots (Table 1). This phenomenon caused by EDDS application could be driven by the decrease of shoot biomass in alfalfa despite the increase in Cu bioaccumulation. More metallic Cu in the soil solution is mobilized after the EDDS application, however these mobilized Cu instantly negatively affects the sustainability of the plant-soil system (Muehlbachova, 2011; Vamerali et al., 2015; Ju et al., 2020b). EDDS has a half-life of 4.18–5.60 days, but it is degraded in the soil after a lag phase of 7–11 days (Tandy et al., 2006). One week after the application of EDDS, the existing Cu-EDDS complex is a less bioavailable to plants and that could cause some physiological damage to the root (Wei et al., 2007; Cestone et al., 2010; Borker et al., 2020) and lead to limited normal plant growth and reduced biomass. In addition, EDDS improves metal transport from roots to shoots by forming Cu-EDDS complex to improve the fluidity of Cu in plant tissues and reduce the storage of Cu by roots (Cestone et al., 2010; Zhao et al., 2018). Here we confirm this finding, since the Cu concentration in the alfalfa shoot was higher than that in the root for the ES treatment in this pot experiment. Meanwhile, inoculation of PGPRs facilitated the process of Cu ions transfer from the root to the shoot. On the contrary, the total uptake of Cu by alfalfa was greatly increased in the inoculation treatment applied with EDDS. The improved phytoextraction performance of the inoculated plants can be attributed to the properties of the PGPR strains that promote plant growth and reduce metal stress (Hayat et al., 2010; Khan et al., 2020; Abbaszadeh-Dahaji et al., 2021; Rathi and Yogalakshmi, 2021). Moreover, the phytoextraction efficiency of Cu was

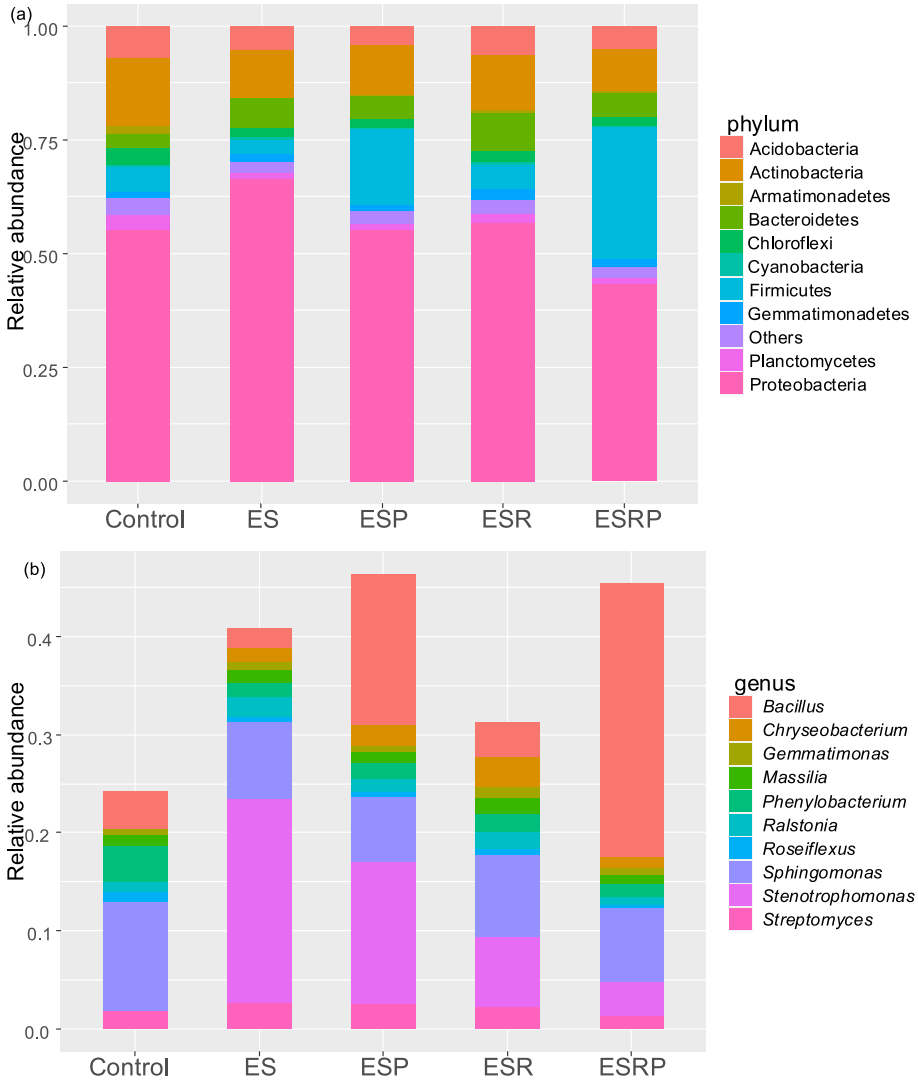

Fig. 2. Composition and abundance of rhizospheric bacterial community. (a), bacterial community composition at top 10 phylum level and (b), at top 10 genus level. Control (soil + plant), ES (soil + plant + EDDS), ESP (soil + plant + EDDS + *P. mucilaginosus*), ESR (soil + plant + EDDS + rhizobium *S. meliloti*), ESRP (soil + plant + EDDS + *S. meliloti* + *P. mucilaginosus*).

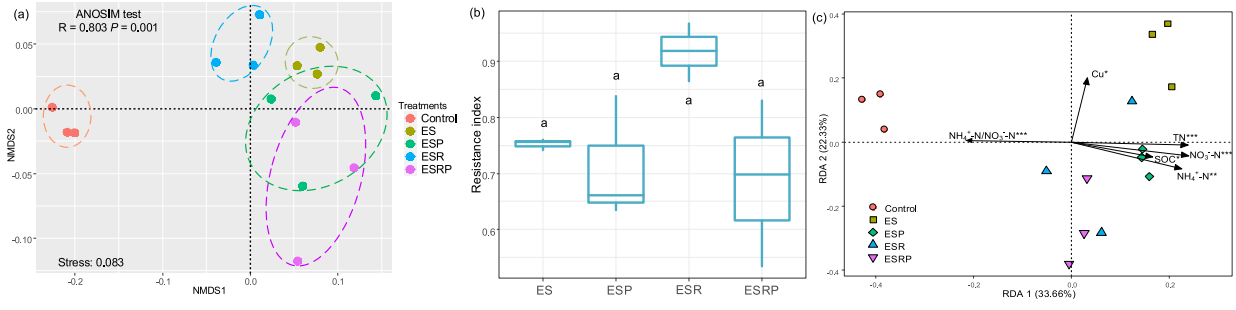

Fig. 3. Bacterial β-diversity, resistance index, and the relationship between soil properties and bacterial community composition. (a), the β-diversity of bacterial community was assessed by nonmetric multidimensional scaling analysis (NMDS) based on the Bray-Curtis distance; (b), resistance index was used to estimate the stability of bacterial community, different small letters stand for significant difference after Tukey's HSD test ($p < 0.05$); (c), redundancy analysis (RDA) revealed the relationship between soil properties and bacterial community composition. Rhizospheric bacterial community composition based on the operational taxonomic unit (OTU) table.

maximized after the co-inoculation of two PGPRs, which can be confirmed by the highest total uptake of Cu in the ESRP treatment. Our data showed an increase in plant growth and activated Cu when plants were grown in presence of PGPRs, this metal was extracted then by plants as much as possible and could avoid Cu leaching into the surrounding environment. On the other hand, the phytoextraction of Cu was weakly correlated with soil enzymatic activity, microbial diversity index, and microbial dominant species abundance (Fig. S5). In contrast,

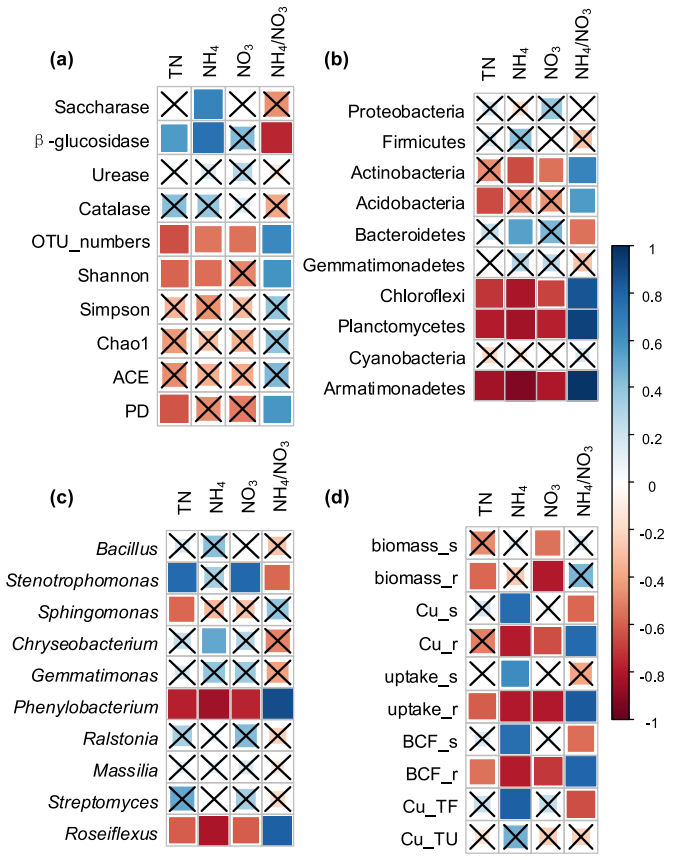

Fig. 4. Correlations between rhizospheric nitrogen content and enzymatic activities, bacterial diversity, bacterial community composition, plant growth, and Cu phytoextraction. (a), enzymatic activities and bacterial community diversity index; (b), bacterial community composition at top 10 phylum level; (c), bacterial community composition at top 10 genus level; (d), plant biomass, s-shoot, r-root, Cu concentration, Cu uptake, Cu bioconcentration factor, Cu translocation factor, and Cu total uptake. TN, NH_4 (NH_4^+-N), NO_3 (NO_3^--N), NH_4/NO_3 (NH_4^+-N/NO_3^--N). The correlation was evaluated by Pearson correlation. " × " denotes insignificance at a $p > 0.05$.

the biomass, Cu bioaccumulation, and Cu extraction of alfalfa in this study were significantly related to soil NH_4^+-N, NO_3^--N, and their proportion (Fig. 4d). Therefore, the variation of soil NH_4^+-N and NO_3^--N in the system after EDDS treatment and together with PGPRs inoculation will be critical in influencing the system to assist in phytoextraction of Cu-contaminated soil.

Short-term application of EDDS obviously increased the content of total nitrogen and available nitrogen in rhizospheric soil (Table 2). The biodegradation products of EDDS in a short period can provide available nutrients to microorganisms and/or plants, such as NH_4^+-N (Beiyuan et al., 2021). Our previous study also suggested that EDDS application could affect the nitrification and denitrification of the soil, thereby accelerating the release and transformation of nitrogen (Fang et al., 2017). The obvious decrease in NH_4^+-N/NO_3^--N values in the ES treatment also suggested that EDDS promoted microbial nitrification to allow more conversion of ammonium-N to nitrate-N. In addition, NO_3^--N, this form can be easily taken up by plants, yet it was negatively correlated with plant biomass in this study. This result may suggest that EDDS application will increase the risk of groundwater contamination by nitrates because the reduction of NO_3^--N uptake by alfalfa could lead to increase NO_3^--N leaching from subsurface in the short term (Hashimoto et al., 2007; Kettering et al., 2013). The soil NH_4^+-N content was higher in the inoculated treatment with EDDS than that in the uninoculated treatment. In the same way, the inoculation of rhizobium was more favorable for the accumulation of NH_4^+-N in the soil. Some possible reasons for the increase of NH_4^+-N content in the system in the presence of EDDS-PGPRs: 1) PGPRs, especially rhizobium inoculation, can enhance rhizospheric N fixation and promote the N cycle (Hayat et al., 2010; Ju et al., 2019); 2) it is speculated that the addition of exogenous rhizobacteria alters indigenous microbial activity (Kalam et al., 2017) may have accelerated the biodegradation of EDDS leading an increase in N release. In contrast, the inoculation of PGPRs reduced the accumulation of nitrate in the EDDS-treated soil. We speculate that the possible reasons include 1) exogenous rhizobacteria inoculation directly reduce NO_3^--N concentrations dissolved in soil (Mercl et al., 2018); 2) nitrate is highly mobile (Prendergast-Miller et al., 2011) and easily captured by plants and microorganisms, and PGPRs promote NO_3^--N uptake by plants and microorganisms to meet growth requirements (Calvo et al., 2019). These two reasons are inseparable from the biochemical processes and nutrient cycling in rhizospheric soil, such as soil enzyme activity, microbial diversity, and microbial community composition (Yang et al., 2013; Duan et al., 2018; Kuzyakov and Razavi, 2019). These results are consistent with our hypothesis that the exogenous application of beneficial rhizobacteria may minimize the possible risk of nitrate leaching associated with short-term applications of EDDS.

In the present study, the short-term application of EDDS alone slightly increased the activities of saccharase, urease, β-glucosidase, and catalase (Fig. 1). Consistently, urease and β-glucosidase activities in EDDS-added soils increased to a certain extent within a week (Yang et al., 2013; Beiyuan et al., 2017). Yang et al. (2013) also reported that a significant decrease in soil saccharase and catalase activities on the seventh day after EDDS application. And the effect of EDDS application on soil enzyme activity gradually weakened or even disappeared with the degradation of EDDS. Kaurin et al. (2020) found that no significant difference in enzyme activity in EDDS-washed soils compared to original soils. The exogenous application of *P. mucilaginosus* and *S. meliloti* slightly increased soil saccharase and β-glucosidase activities but did not significantly affect the activities of urease and catalase in EDDS-treated soils. The inoculation of PGPRs could promote organic matter decomposition and C-cycling in metal-contaminated soils due to the increase of saccharase and β-glucosidase, which can provide more carbohydrates for plants and microorganisms (Abdelkrim et al., 2020; Ju et al., 2020a). The insignificant changes in urease indicated that the EDDS-PGPRs system had less effect on soil nitrogen form by regulating nitrogen cycle-related enzyme activities. The relative importance of regressors in the linear models and VPA analysis also confirms this statement (Fig. 5).

Moreover, this study found that rhizospheric bacterial diversity and community composition contributed more to the variation in available nitrogen forms in EDDS-treated and PGPRs-inoculated soils relative to soil enzyme activity. Short-term treatment with EDDS reduced the bacterial community diversity in the rhizosphere (Fig. S1). EDDS is known to effectively increase the available Cu content in the soil (Luo et al., 2015; Vamerali et al., 2015), and higher levels of Cu will increase the dispersion of the microbial community and reduce the alpha diversity of the microbiome (Rocca et al., 2019). The application of *P. mucilaginosus* did not eliminate the negative impact of EDDS application on bacterial alpha diversity, while *S. meliloti* enhanced bacterial diversity to a certain extent. In addition to Cu content, N content was also a major factor affecting the structure of the rhizospheric bacterial community (Fig. 3c and Table S1). The nitrogen-fixing *Rhizobium* can increase the uptake of Cu by plants, reduce Cu residues in soil, and provide more nitrogen sources for the rhizospheric microorganisms through N fixation (Kong et al., 2015; Ju et al., 2020a). Sufficient nutrients stimulate the growth of microorganisms and have a positive effect on microbial diversity. In this study, dominant bacterial species composition was a key factor in the variation of NH_4^+-N and NO_3^--N in rhizospheric soils (Fig. 5c and d). Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria, and Bacteroidetes were dominant taxa for all treatment, and the abundances of Proteobacteria and Bacteroidetes were increased in the ES treatment (Fig. 2a). Proteobacteria was also a biomarker for the ES treatment (Fig. S2). This was in agreement with the

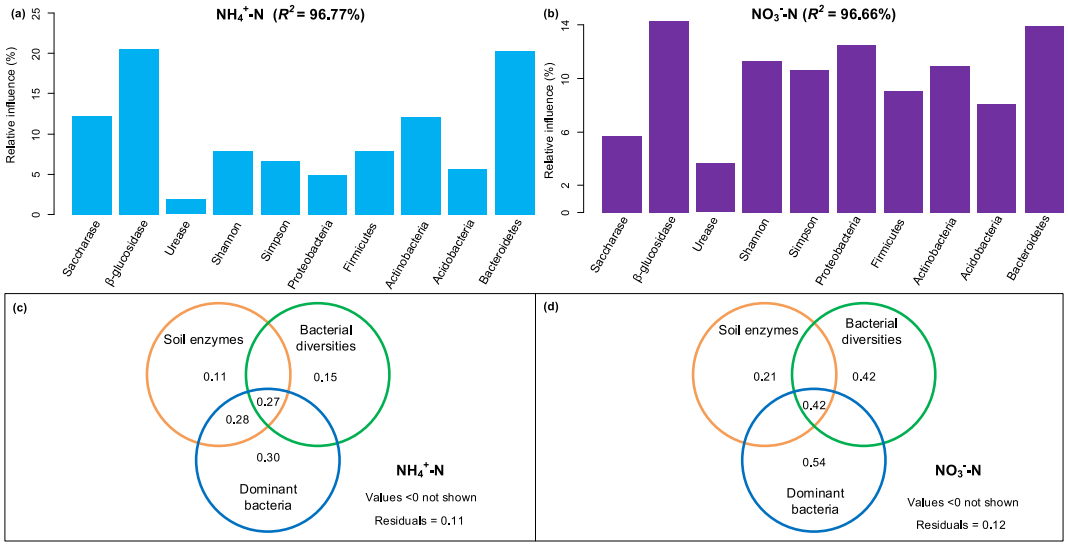

Fig. 5. Variation partitioning analysis (VPA) and the relative importance of regressors in linear models reveal the contribution of environmental factors to the variation of soil NH_4^+-N and NO_3^--N. (a–b), R^2 represents the proportion of variance explained by linear models; (a), relative importance of regressors in the linear model in NH_4^+-N; (b), relative importance of regressors in the linear model in NO_3^--N; (c), VPA in NH_4^+-N; (d), VPA in NO_3^--N. Environmental factors include soil enzymatic activities, bacterial diversity indices, and bacterial community dominant taxa. Soil enzymes include Saccharase, β-glucosidase, and urease; bacterial diversity indices include Shannon index and Simpson index; bacterial community dominant taxa include Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria, and Bacteroidetes.

fact that EDDS releases N in a short period of time, and the copiotrophic taxa Proteobacteria and Bacteroidetes usually increase with high N availability (Fierer et al., 2012). Seems like Bacteroides is devoted to N denitrification (Diamond et al., 2019) since is known that EDDS treatment can promote N cycling, which is consistent with our previous findings on the positive effect of EDDS treatment in nitrite reductase genes, especially *nirS* (Fang et al., 2017). Altogether, this also confirmed that Bacteroides contributes more to the variation of soil available nitrogen forms than other dominant taxa (Fig. 5a and b). However, the inoculation of PGPRs decreased the abundance of Proteobacteria and Bacteroidetes and increased the abundance of Firmicutes. Similar trends for Proteobacteria and Firmicutes were found in the Cu-contaminated soil after 50 days inoculation with PGPRs (Ju et al., 2020a). This result indicates that, in the short term, the inoculation of exogenous PGPRs may limit the indigenous copiotrophic bacteria in EDDS-treated soil. When Firmicutes is used as a biomarker for ESRP treatment showed a significant increase in the abundance of *Bacillus* at the genus level (Fig. S3). *Bacillus* is associated with promoting plant growth and protecting plants from pathogens (Molina-Santiago et al., 2019). Moreover, the inoculation of PGPRs inhibited the denitrification of N, disrupted the N cycle, and indirectly reduced the production of nitrate. This was also evidenced by the decrease in *Stenotrophomonas* abundance after inoculation of PGPRs (Fig. 2b and Fig. S3), this may be due to *Stenotrophomonas* exhibiting a moderate denitrification ability (Cyplik et al., 2013). If denitrifying bacteria are limited by nitrogen rather than carbon, PGPRs inoculation will reduce the abundance and activity of denitrifying bacteria by increasing competition for nitrogen (Florio et al., 2019). In addition, the increased plant growth mentioned in the above discussion can facilitate the uptake and utilization of NO_3^--N by plants. This result may imply that PGPRs inoculation can reduce residual nitrate pollution caused by short-term application of EDDS by regulating the abundance of bacteria associated plant growth and nitrogen cycling.

In conclusion, the potential for plant growth and Cu phytoextraction in EDDS-treated groups were further stimulated after inoculation with PGPRs, and these effects are linked to the available nitrogen in rhizospheric soil. In addition to activating metal ions, short-term treatment with EDDS greatly increased the levels of NH_4^+-N and NO_3^--N in soil, which altered the composition and structure (Fig. 3) of the same soil bacterial community. The composition of rhizospheric microbial community contributed more to the variation in soil available nitrogen than microbial diversity and enzymatic activity (Fig. 5). The exogenous application of PGPRs reduced the soil NO_3^--N content mainly by altering the abundance of bacterial dominant taxa without significantly affecting microbial community stability. This probably indicate that the PGPRs/EDDS system can indirectly and directly reduce the risk of Cu and nitrate leaching in the short term based on the promoted plant growth, improved phytoextraction efficiency, and regulated microbial communities.

5. Conclusions

The short-term metal activation and N release performance of the biodegradable EDDS enhanced the transfer and bioaccumulation of Cu from soil/roots to shoots, yet it also inhibited plant growth and increased the possible risk of Cu and nitrate residues. The application of PGPRs *P. mucilaginosus* and *S. meliloti* alleviated the inhibition of EDDS on plant growth and further greatly increased the efficiency of Cu phytoextraction. Plant growth and phytoextraction potential were related to rhizospheric available nitrogen, and bacterial dominant taxa was a key factor in regulating the content of soil NH_4^+-N and NO_3^--N. The inoculation of PGPRs may reduce the level of soil nitrate by modulating the abundance of bacteria associated with plant growth and denitrification, respectively. Our study suggests that inoculation of exogenous beneficial bacteria can provide an effective measure to minimize the potential risk associated with the use of biodegradable chelants, such as the risk of metal and/or nitrate leaching. Moreover, additional efforts are required to better investigate the presence/migration status of metal, metal-chelant complexes, and nitrate in plant-rhizospheric-bulk soil-groundwater in the chelant-PGPRs system.

Credit author statement

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administration, Funding acquisition, Writing - Reviewing & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.132288.

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