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Rhizobiome diversity of field-collected hyperaccumulating *Noccaea* sp.

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Abstract

Hyperaccumulating plants are able to (hyper)accumulate high concentrations of metal(loid)s in their above-ground tissues without any signs of toxicity. Studies on the root-associated microbiome have been previously conducted in relation to hyperaccumulators, yet much remains unknown about the interactions between hyperaccumulating hosts and their microbiomes, as well as the dynamics within these microbial communities. Here, we assess the impact of the plant host on shaping microbial communities of three naturally occurring populations of *Noccaea* species in Slovenia: *Noccaea praecox* and co-occurring *N. caerulescens* from the non-metalliferous site and *N. praecox* from the metalliferous site. We investigated the effect of metal enrichment on microbial communities and explored the interactions within microbial groups and their environment. The abundance of bacterial phyla was more homogeneous than fungal classes across all three *Noccaea* populations and across the three root-associated compartments (roots, rhizosphere, and bulk soil). While most fungal and bacterial Operational Taxonomic Units (OTUs) were found at both sites, the metalliferous site comprised more unique OTUs in the root and rhizosphere compartments than the non-metalliferous site. In contrast to fungi, bacteria exhibited differentially significant abundance between the metalliferous and non-metalliferous sites as well as statistically significant correlations with most of the soil parameters. Results revealed *N. caerulescens* had the highest number of negative correlations between the bacterial phyla, whereas the population from the metalliferous site had the fewest. This decrease was accompanied by a big perturbation in the bacterial community at the metalliferous site, indicating increased selection between the bacterial taxa and the formation of potentially less stable rhizobiomes. These findings provide fundamentals for future research on the dynamics between hyperaccumulators and their associated microbiome.

Keywords Hyperaccumulating plants, *Noccaea praecox*, *Noccaea caerulescens*, Root-associated microbiome, Non-metalliferous soil, Metalliferous soil

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Background

High concentration of metal(loid)s in the substrate is a ubiquitous abiotic stressor that negatively affects plant growth and development [1]. Plants have evolved different mechanisms to tolerate the excess metal(loid) concentration in the soil, with hyperaccumulating species developing an extraordinary set of traits that enable them to (hyper)accumulate and tolerate high concentrations of one or more metal(loid)s in their aboveground biomass, without any visible signs of toxicity [2, 3]. To date, more than 700 plant species are recognized as hyperaccumulators [4], with several belonging to Brassicaceae [5]. Within this family, a group of hyperaccumulators has been identified in the genus *Noccaea*, including a model hyperaccumulating species *Noccaea caerulescens* [6], and a closely related *N. praecox* [7]. Both species are zinc (Zn) and cadmium (Cd) hyperaccumulators [8–10], which is a rare phenomenon; namely, 75% of hyperaccumulators (hyper)accumulate nickel (Ni) [11]. While Zn and Ni are essential elements for plants, Cd and Pb have no known function and are toxic at very low concentrations to the majority of plants [12–14].

The structure and the diversity of microbial communities are influenced by several factors, including the type of soil, soil compartments [15–17], and plant host [15]. The presence of metals in soil can negatively impact microbial community structure and reduce microbial abundance [18]. The effect of plant host on the root-associated microbiome is especially visible in hyperaccumulating plants, as they can select for metal-tolerant bacteria in the proximity of their roots, even if the plant grows in soil that is not enriched with metals [17]. By contrast, fungi seem to be more influenced by the soil [19, 20] and are more dominantly associated with non-hyperaccumulators than hyperaccumulators growing in the same location [17]. Nevertheless, a combination of plant host and environment significantly shapes the microbial communities [15, 21]. Although hyperaccumulators have a conserved core rhizosphere microbiota [22], the presence of metal(loid)s significantly impacts their microbial community structure in the soil [23, 24]. When growing in metalliferous soils, hyperaccumulators have been shown to harbour a higher number of bacterial and fungal taxa compared to non-metalliferous soils, including specific microbes that help the plant alleviate metal stress and facilitate its growth and survival [21, 25]. Plants actively recruit their microbiome [26]. In particular, hyperaccumulators growing on metalliferous soils have been shown to consistently attract soil microbes that may contribute to phytoextraction [15, 27, 28]. However, the root-associated microbiome of hyperaccumulating plants has been scarcely studied. Therefore, detailed investigations are required in different hyperaccumulating plants to pave the way for a better understanding of the hyperaccumulating traits to fill in the gaps of knowledge in rhizobiome diversity in different hyperaccumulators.

Recent advancements in soil microbial ecology, driven by high-throughput sequencing techniques, have highlighted a new challenge: understanding the intricate relationships between microorganisms. Network models compel us to view taxa not in isolation but as interconnected components within microbial communities and their environment [29]. Microorganisms can establish a range of relationships, such as mutualism, synergism, and antagonism, all of which can be considered positive (e.g., mutualism) and negative (e.g., antagonism). These ecological interactions represent critical evolutionary pressures for natural selection during microbial evolution. As such, network analysis offers profound insights into ecosystem organization, beyond its role as a visualization tool. Nevertheless, careful interpretation of results is crucial because significant spatial associations between species (or their absence) do not necessarily represent true ecological interactions [30, 31].

The multi-omics approaches have the potential to significantly expand and complement our understanding of unique physiological traits found in hyperaccumulators and of the ways they interact with the environment. Here, we report on the root-associated microbiomes of the hyperaccumulating *Noccaea* species in its natural environment. More specifically, we aim to: (i) estimate the impact of the plant host on the formation of the microbial communities unique to *N. praecox* and compare it to the microbiome of another hyperaccumulating species, *N. caerulescens*, growing in the same non-metalliferous location, (ii) identify changes in the microbial communities of *N. praecox* under metal enrichment compared to *N. praecox* from the non-metalliferous site, and (iii) estimate the connections within individual microbial groups and with their environment. We hypothesize that the plant host and soil metal status tailor the associated microbial community, thereby forming a specific core community. With this in mind, we: (i) analysed the bacterial and fungal rhizobiomes of the hyperaccumulating *N. caerulescens* in non-metalliferous soil and of hyperaccumulating *N. praecox* in metalliferous and non-metalliferous soils, (ii) assessed the adherence to the neutral model to eliminate stochastic processes, and (iii) evaluated the importance of the environment and microbial interactions on the formation of root-associated microbiomes of these two hyperaccumulating species.

Results

Microbial communities associated with *Noccaea* hyperaccumulators

Microbiome sequencing of *N. caerulescens* and *N. praecox* roots with accompanying rhizosphere and bulk soil revealed 2996 and 39 unique OTUs for bacteria and

fungi, respectively. PerMANOVA confirmed that soil compartments (i.e., roots, rhizosphere, and bulk soil) harbor statistically different bacterial and fungal communities (Table S2). The α-diversity within the bacterial community showed nearly identical values for rhizosphere and bulk soil. The number of observed OTUs was statistically lower in roots than in rhizosphere and bulk soil, whereas Chao1 richness was statistically higher. In contrast, Shannon index did not show a statistically significant difference between the soil compartments (Table S3). In the fungal community, the number of observed OTUs was the highest in roots and decreased from the root to the rhizosphere and bulk soil. Chao1 richness and Shannon index showed no statistically significant differences between the soil compartments (Table S3). Overall, the diversity and richness of fungi were significantly lower compared to those of bacteria (Table S3). Actinobacteria and Proteobacteria (44% and 35% average relative abundance, respectively) were dominant across all three *Noccaea* populations, and root-associated compartments (Fig. 1a). Among the fungal community, Ascomycota was the most dominant group, with Dothiomycetes and Leotiomycetes representing on average over 73% of all fungal sequences (Fig. 1b). Notably, arbuscular mycorrhizal fungi (Glomeromycetes) from Archaeosporales and Diversisporales comprised 9% of all fungal sequences. Additionally, Agaricomycetes displayed a significant level of abundance in the rhizosphere of *N. praecox* from the metalliferous site.

The majority of bacterial phyla indicated no clear preferences for specific soil compartments (Fig. 1c, Suppl. Mat. Fig.S1), and a large majority of bacterial OTUs were found in all three compartments (Fig. 1e, Suppl. Mat. Fig. S1). For individual species/location combination, 1884–2089 bacterial OTUs (69–77% of all bacterial OTUs) were shared between all three soil compartments (Suppl. Mat. Fig.S1). Bulk soil showed the lowest number of unique bacterial OTUs with 43–61 OTUs (2% of all bacterial OTUs). The number of unique OTUs in roots and rhizosphere was similar for both *Noccaea* species at Lokovec but lower compared to the rhizosphere at the metalliferous site Žerjav (Suppl. Mat. Fig. S1). Similar to the bacterial community, a large portion of fungal OTUs revealed no preference for a particular soil compartment (Fig. 1f, Suppl. Mat. Fig. S2), although the distribution among individual compartments indicated that fungal OTUs preferred the root-rhizosphere environment over the bulk soil (Fig. 1d, Suppl. Mat. Fig. S2). The majority of unique OTUs were observed for the root compartment with 7–13 OTUs (26–52% of all fungal OTUs).

Effect of metal-enrichment on microbial communities of *N. praecox*

Next, we compared the microbial communities in *N. praecox* only, i.e., from metalliferous and non-metalliferous sites. The comparison showed that not only the compartment but also the location is a significant factor that shapes the root-associated microbiome in *Noccaea* hyperaccumulatos (Table S2 The α-diversity of the bacterial community revealed that the non-metalliferous site in Lokovec has higher diversity and species richness compared to the metalliferous site in Žerjav (Table S4), although differences were not statistically significant. In contrast, the fungal community was more diverse with more species richness in the metalliferous site (Table S4). Again, the sites did not differ significantly. In root and rhizosphere compartments of *N. praecox*, the majority of fungal and bacterial OTUs identified were found at both sampling sites (Fig. 2). At the metalliferous site in Žerjav, eight unique fungal OTUs (24% of all fungal OTUs) and 262 unique bacterial OTUs (9% of all bacterial OTUs) were found (Fig. 2a), while at the non-metalliferous site in Lokovec six unique fungal OTUs (18% of all OTUs) and 211 unique bacterial OTUs (7% of all OTUs) were found (Fig. 2b).

The follow-up analysis of keystone bacterial OTUs in the root and rhizosphere compartments revealed no shared keystone bacterial OTUs between the two populations of *N. praecox* (Table 1). In the non-metalliferous site in Lokovec, one keystone OTU (Unclassified Devosiaceae) belonging to the class Alphaproteobacteria was detected, whereas at the metalliferous site in Žerjav, Fimbriiglobus and Phycicoccus, belonging to the classes Planctomycetia and Actinomycetes, respectively, were identified. Notably, the fungal community exhibited a higher number of keystone OTUs compared to bacteria, as outlined in Table 1. Although no common keystone OTUs were detected, Acephala and Golovinomycetes from the control site belong to the class Leotiomycetes, which was found as a keystone OTU in the metalliferous site. Additionally, Thelephora (Agaricomycetes) was found as a keystone OTU in Lokovec, whereas in Žerjav, a keystone belonging to the Dothideomycetes was detected (Unclassified Pleosporales).

Measurement of Zn, Cd and Pb root concentrations with ICP-MS showed that root Zn and Cd concentrations were higher in *N. praecox* than in *N. caerulescens* regardless of the location, with concentration at metalliferous site reaching 816 ± 87.5 mg kg^{-1} and 699 ± 158 mg kg^{-1}, respectively (Table 2). Root Pb concentrations in *N. praecox* from Žerjav reached 5161 ± 1242 mg kg^{-1}, which is over 500 times the concentration in the roots of *Noccaea* plants at the non-metalliferous site. In contrast, no difference in root Pb concentrations was observed in *Noccaea* species at the non-metalliferous site in Lokovec.
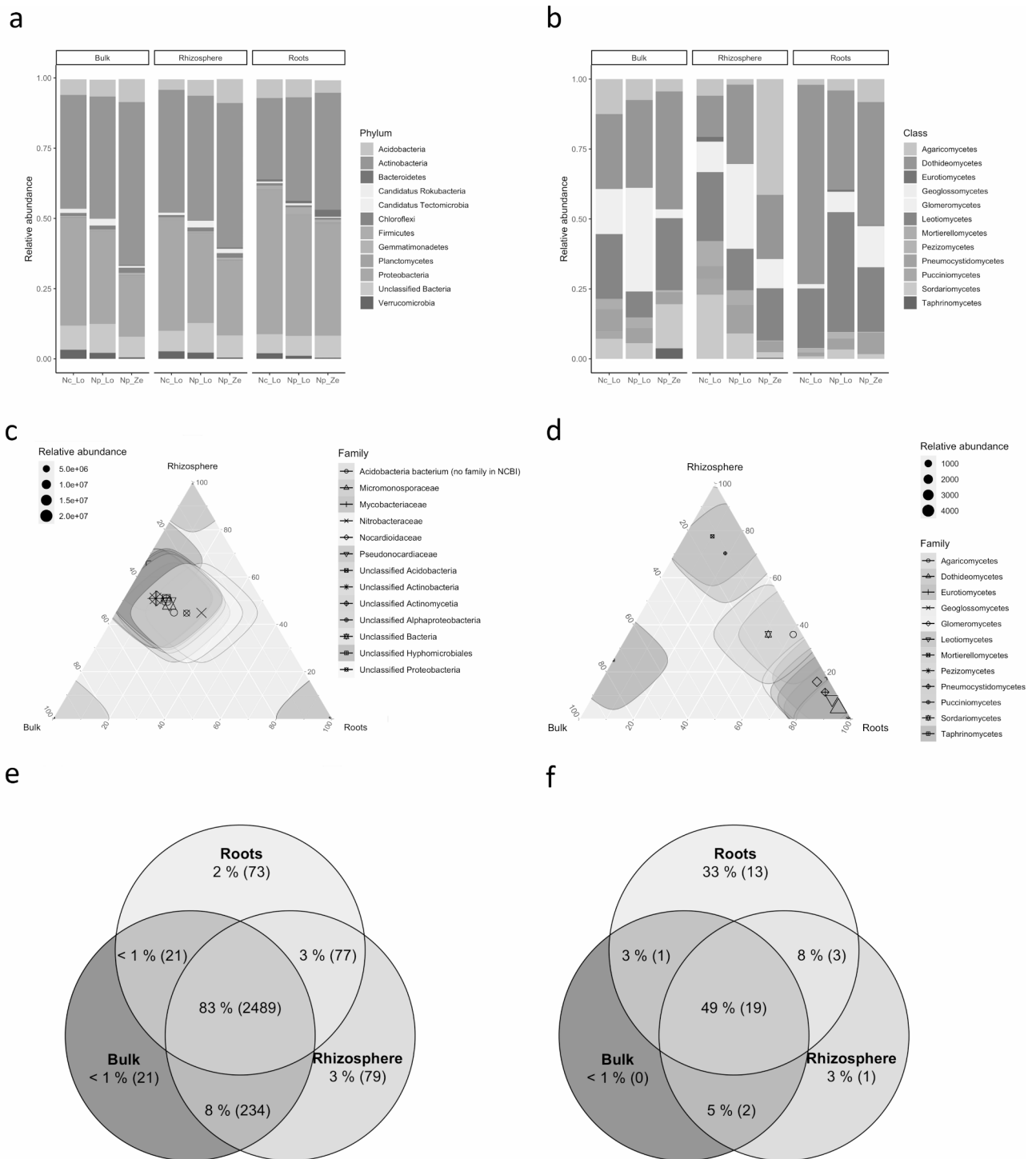
Fig. 1 Top ten abundances of **(a)** bacterial phyla and **(b)** fungal classes of root and rhizosphere compartments of *Noccaea praecox* (Np) and *N. caerulescens* (Nc) and bulk soil from the non-metalliferous (Lo) and metalliferous (Ze) sites; ternary plots indicate the fraction of **(c)** bacterial families and **(d)** fungal classes with the top ten abundances in each soil compartment (for all sites and plant species). The symbol size (black circle) represents the average abundance; the Venn diagram shows the number of common and specific Operational Taxonomic Units (OTUs) in **(e)** bacterial and **(f)** fungal communities and the proportion of the total number of sequences in each compartment (for all sites and plant species)
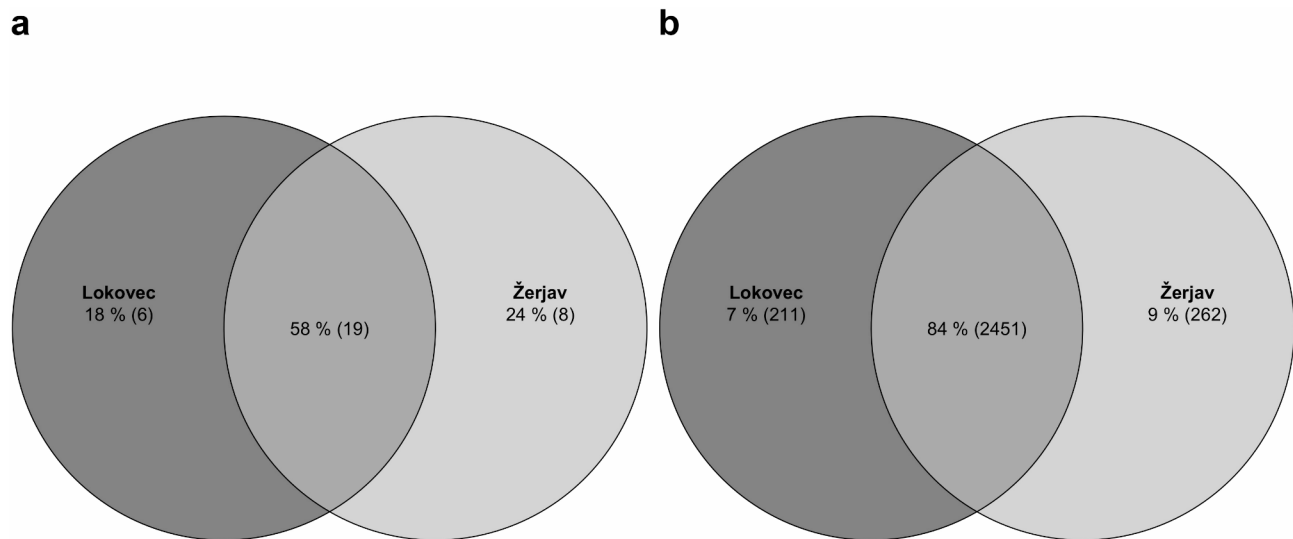
Fig. 2 Venn diagrams of common and unique Operational Taxonomic Units of rhizosphere and root compartments for **(a)** fungal and **(b)** bacterial communities of *Noccaea praecox* at the non-metalliferous (Lokovec) and metalliferous (Žerjav) sites. Numbers represent percentages of all taxa with the absolute numbers in parentheses

Table 1 Presence (indicated by "x") of keystone bacterial operational taxonomic units (OTUs) in the rhizosphere and root compartments of *Noccaea praecox* from the non-metalliferous site (Lokovec) and metalliferous site (Žerjav)

Bacterial OTUs \ population	Lokovec	Žerjav
Unclassified Devosiaceae	x	
Fimbriiglobus		x
Phycicoccus		x
Fungal OTUs		
Acephala	x	
Golovinomyces	x	
Thelephora	x	
Unclassified Leotiomycetes		x
Unclassified Pleosporales		x

Table 2 Concentration of zinc (zn), cadmium (cd), and lead (pb) in roots of *Noccaea caerulescens* and *N. praecox* naturally occurring on non-metalliferous (Lokovec) or metalliferous (Žerjav) sites. Shown are means ± standard errors (*n* = 5). Different letters in bold depict statistically significant differences between populations (Dunn's test at *p* < 0.05)

Population	Zn [mg kg⁻¹]	Cd [mg kg⁻¹]	Pb [mg kg⁻¹]
N. caerulescens (Lokovec)	82 ± 13.8**a**	9.15 ± 2.02**a**	11.8 ± 1.32**a**
N. praecox (Lokovec)	231 ± 43.9**a**	77.9 ± 14.9**a**	8.25 ± 3.18**a**
N. praecox (Žerjav)	816 ± 87.5**b**	699 ± 158**b**	5161 ± 1242**b**

To evaluate the impacts of the two locations and the plant host on the microbial community, we employed DESeq analysis alongside the Sloan neutral model evaluation. No fungal genera that deviated from the neutral model exhibited differentially significant abundance between both sampling sites. In contrast, according to the Sloan neutral model, the bacterial community included 394 bacterial genera that were above the prediction and 282 genera that were below the prediction. Among these, 92 and 37 bacterial genera were enriched and under-enriched, respectively, and statistically significant according to the DESeq analysis. The highest number of genera not following the neutral model and displaying statistically significant enrichment was observed for Actinobacteria (6 taxa), Bacterioidetes (6 taxa), and Proteobacteria (6 taxa). Proteobacteria also exhibited the highest number of genera that were below prediction (23 taxa), followed by Verrucomicrobia (7 taxa) and Cyanobacteria (6 taxa).

Further, it was explored how fungal and bacterial communities that do not follow the Sloan neutral model correlated with soil properties. Therefore, we employed the Mantel test for rhizosphere and root compartments. Surprisingly, the fungal community exhibited no statistically significant correlation with any of the soil parameters in either compartment. In contrast, the majority of soil parameters were statistically significantly correlated with the bacterial community in both compartments (Fig. 3). Among all soil parameters, only base saturation did not reveal a statistically significant correlation with the bacterial community in either compartment. In addition, root metal concentrations also showed a statistically significant Mantel correlation with the bacterial communities in the roots and rhizosphere compartments.
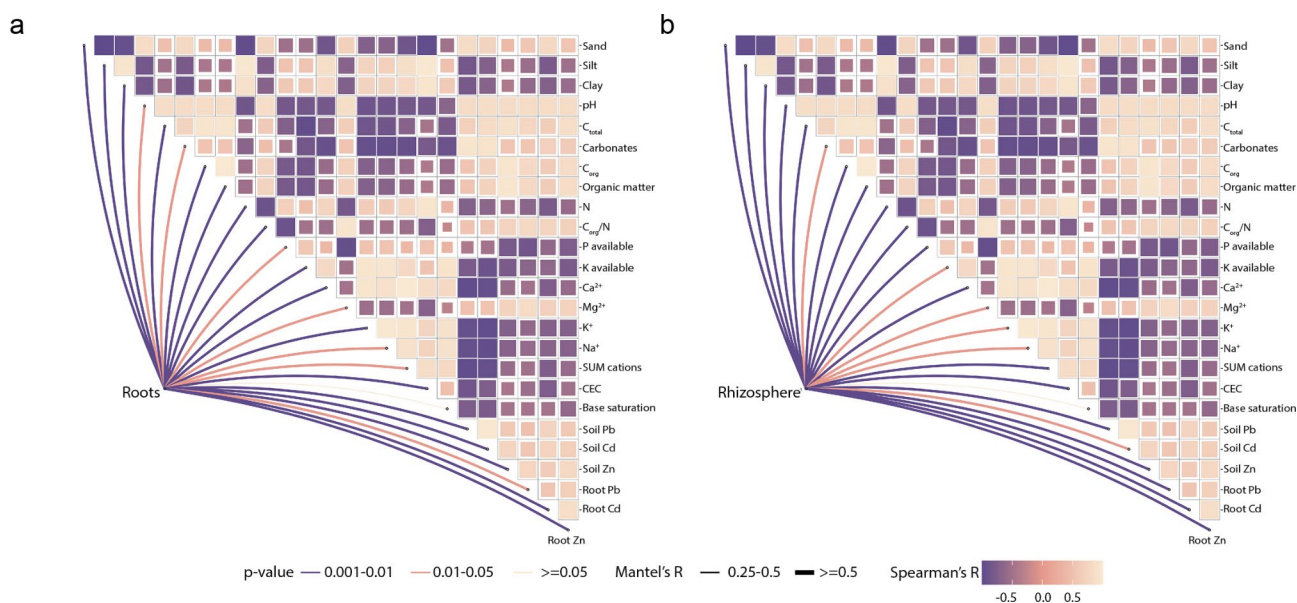
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Fig. 3 Mantel test for **(a)** root and **(b)** rhizosphere compartments of bacterial community that did not follow the Sloan neutral model evaluation. Color gradient in squares represents Spearman's correlation coefficient, line color statistical significance, and line width Mantel's correlation coefficient ($n = 9$)

Bacterial networks of *Noccaea* rhizobiome

The co-occurrence network analysis revealed that the majority of bacterial phyla in all three compartments form positive interactions within the bacterial community (Fig. 4a, b), with the exception of Firmicutes, Gemmatimonades, and Bacteroidetes.

Bacteroides and Firmicutes exhibited several negative interactions with other bacterial phyla in the roots and rhizosphere of both plant species and at both locations. *N. praecox* population from Lokovec has a higher number of statistically significant correlations within the root and rhizosphere compartments compared to the *N. praecox* from the metalliferous site. The number of negative interactions between the bacterial phyla was the highest for *N. caerulescens* (Fig. 4b). In contrast, the overall number of negative edges was the highest for *N. praecox* at the non-metaliferous site (849 negative edges) and the lowest for *N. praecox* at the metaliferous site (259 negative edges) (Fig. 5). This change was a result of a severe decrease in negative edges of the dominant bacterial phyla Proteobacteria (from 401 down to 165 negative edges) and Actinobacteria (from 295 down to 38 negative edges) and a slight increase in negative edges in Bacteroides (from 9 up to 27 negative edges) and Firmicutes (from 5 up to 19 negative edges).

The *Noccaea* population from metalliferous soil had the highest number of bacterial phyla with only positive correlations: Acidobacteria, Candidatus Rokubacteria, Candidatus Tectomicrobia, and Planctomycetes. The other two populations from Lokovec had one phylum each that did not form negative correlations

– Acidobacteria and Chloroflexi in *N. praecox* and *N. caerulescens*, respectively.

Discussion

Microbial communities associated with *Noccaea* hyperaccumulators

In our study, root-associated compartments of the hyperaccumulating *Noccaea* species – bulk soil, rhizosphere, and roots – showed statistically significant differences in abundance and diversity of bacteria and fungi. The α-diversity of the bacterial and fungal communities differed between compartments, indicating that they represent important niches for different microorganisms. In the bacterial community, the diversity was similar between bulk soil and rhizosphere, with a significant decrease in the root compartment, whereas fungal community diversity declined from the root compartment to bulk soil. Other authors report similar observations for bacterial [15] and fungal communities [32]. Species richness, determined by the Chao1 index, was the highest for the root compartment for both bacterial and fungal communities, indicating that the host's roots harbor a higher number of different genera compared to the other two (soil) compartments studied. While fungi indicated a preference for the root-rhizosphere compartments, bacteria did not exhibit a clear preference for any particular soil compartment. This aligns with the results observed by Xiao et al. (2020) [32] in their study on hyperaccumulating fern *Pteris vittata*. Nevertheless, in roots, Glomeromycetes were significantly more abundant in *N. praecox* from the metalliferous site in Žerjav compared to both *Noccaea* populations from the non-metalliferous
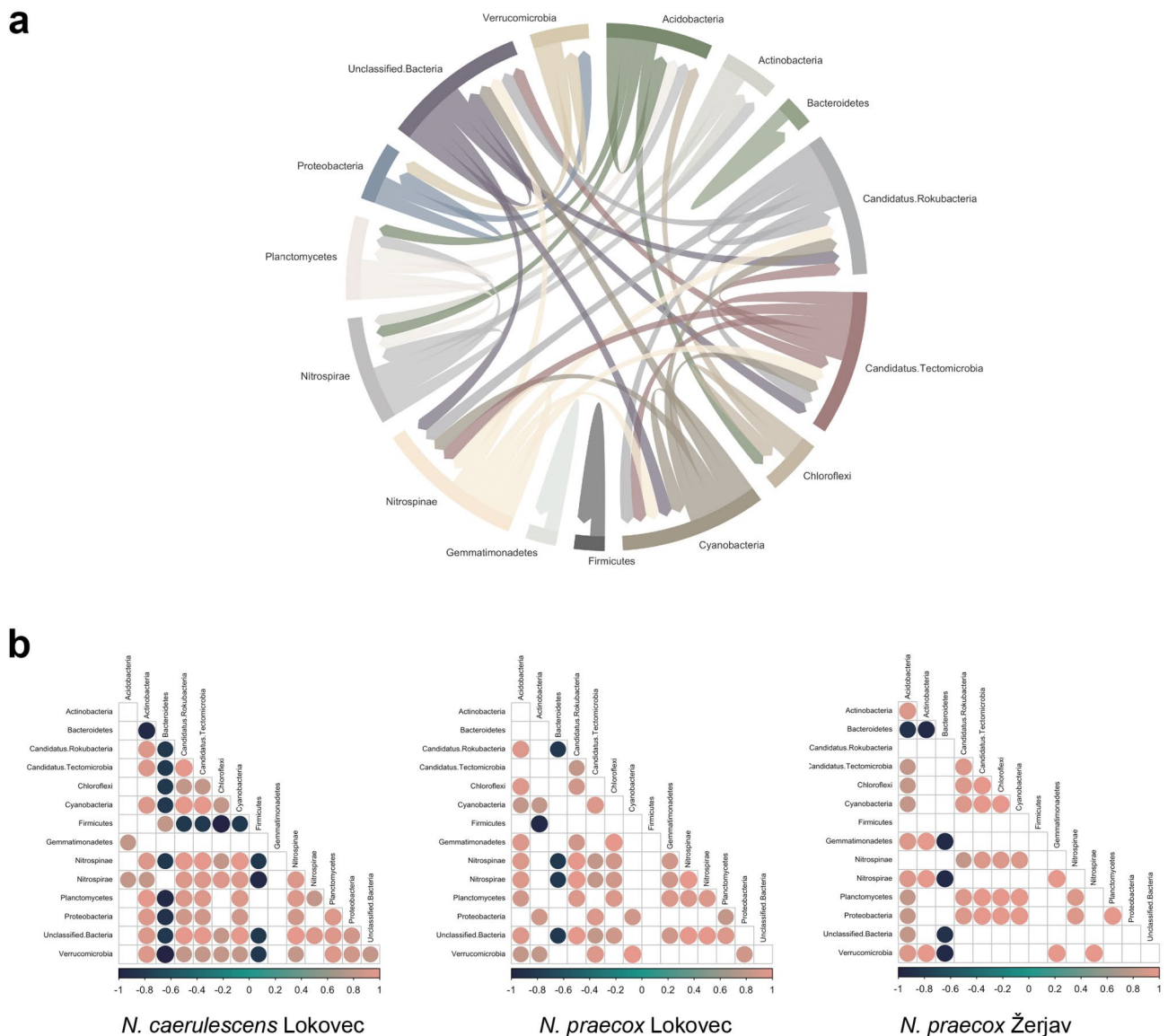
Fig. 4 Co-occurrence networks for **(a)** top 15 bacterial phyla of all three compartments (bulk soil, rhizosphere, and roots) and all three *Noccaea* populations with rho > 0.8 and **(b)** Spearman correlation plots for bacterial phyla in the rhizosphere and root compartments from the individual *Noccaea* population with (only correlations with $p < 0.05$ are shown, $n = 6$). Lokovec is a non-metalliferous and Žerjav is metalliferous site

site in Lokovec. Glomeromycetes are known to form beneficial associations with plants through arbuscular mycorrhiza [33], playing an important role in the diversification and stability of soil microbiota [34]. They are also found in environments rich in metals [35, 36], explaining their presence in Žerjav. However, there is a common belief that Brassicaceae, which includes the genus *Noccaea*, do not form mycorrhizal associations [37]. Despite this, several studies using both morphological observations as well as molecular have confirmed their presence in the roots of representatives from this family, including *N. praecox* [8, 38–40].

Actinobacteria and Proteobacteria dominance over other bacterial phyla, independent of the soil metal status

seems to be a common pattern. Both phyla were also prevalent in other hyperaccumulating species [15, 17] and are positively correlated with Cd/Zn hyperaccumulation in plants [28]. Actinobacteria are known to promote metal absorption and plant growth [41, 42] and are well-represented soil microorganisms [43, 44]. They were also shown to contribute to the increased plant tolerance to heavy metals [45]. Proteobacteria, on the other hand, are also known for their plant growth-promoting effects and resistance to some metals, commonly found on both metalliferous and non-metalliferous sites [46, 47]. Thus, our findings suggest that the studied *Noccaea* hyperaccumulators are associated with potentially beneficial organisms that could positively influence plant growth and
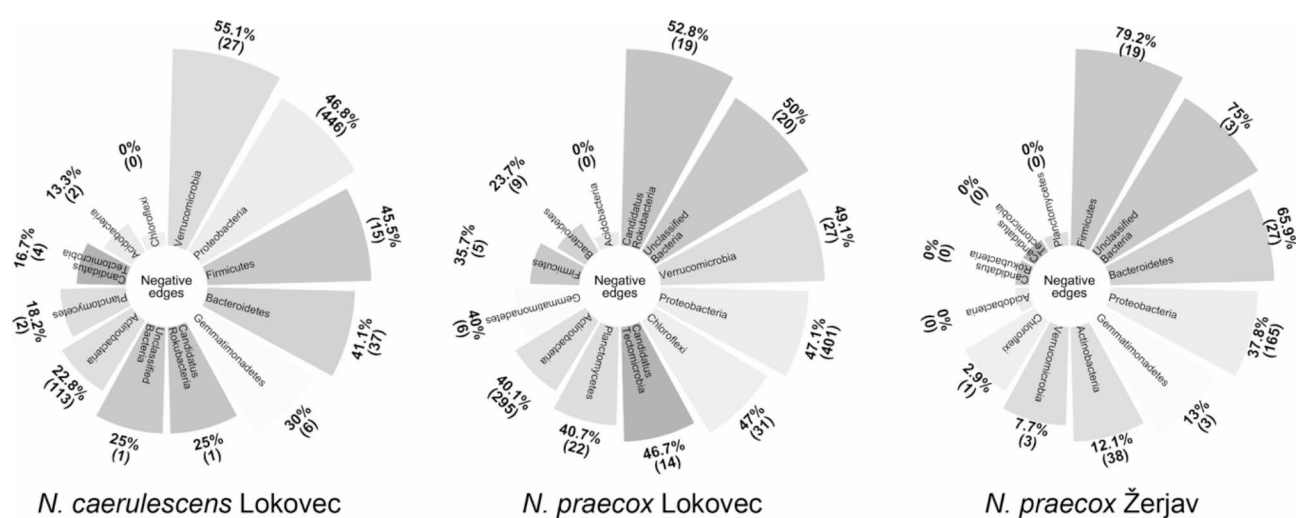
Fig. 5 Number of negative edges in bacterial co-occurrence networks for root and rhizosphere compartments of *Noccaea* populations from the non-metalliferous (Lokovec) and metalliferous (Žerjav) sites. Only correlations with *p* < 0.05 are presented on the pie charts. Percentages represent the proportion of negative correlations out of the total number of correlations for each phylum, whereas numbers in parentheses present the cumulative sum of the number of negative correlations for each phylum

fitness. Interestingly, no differences in the abundances of bacterial genera were observed between the populations from Lokovec, suggesting that the plant host plays a less important role in shaping the microbiota than the location itself.

Effect of metal-enrichment on microbial communities of *N. praecox*

In addition to compartments, soil metal enrichment was found to be an important factor in shaping the root-associated microbiome. The concentration of Zn, Cd, and Pb were the highest in the roots of *N. praecox* growing at the metalliferous site, forming an additional selective pressure on the microbial community. In line with this, we observed more unique bacterial OTUs than the non-metalliferous site and a lower α-diversity and species richness in Žerjav compared to Lokovec. Additionally, the uniqueness of fungal genera was slightly higher in Žerjav than in Lokovec, but the difference was not as pronounced as for bacteria, possibly due to the lower number of fungal to bacterial genera detected. Nevertheless, most bacterial and fungal genera were present on both sampling sites. This could be explained by similar soil conditions, i.e., pH, organic matter content, and cation exchange capacity. These soil parameters also influence the bioavailability of metals. A neutral pH combined with a high organic matter content and a high cation exchange capacity increases the adsorption of metals to soil particles and decreases the bioavailability of metals. Conversely to the bacterial community, the α-diversity and species richness of the fungal community were higher in metalliferous compared to non-metalliferous sites. These results partially align with other studies on fungi [21], indicating

that polluted sites have significantly higher microbial diversity than non-metalliferous sites [48, 49].

No bacterial keystone taxa of *N. praecox* were characteristic for the two locations. Phycicoccus and Fimbriiglobus were found as keystone OTUs in the population from Žerjav, whereas unclassified Devosiaceae were found only in *N. praecox* from Lokovec. Both Phycioccocus and Fimbriiglobus are known to be resistant to environmental contaminants such as heavy metals [50, 51]. Devosiaceae, on the other hand, prefer non-metalliferous environments and are mostly associated with the root compartment [52, 53]. These results suggest the environment is a major factor in determining bacteria that present the core of the local ecosystem. Conversely, the fungal community was more similar between locations, with Leotiomycetes being keystone taxa in both Lokovec and Žerjav, although due to a lack of lower taxonomic level information, it remains unknown whether any keystone species from both locations are the same biological species. Not negligibly, Leotiomycetes were one of the most abundant fungal classes in our study. Pleosporales, which are known for being resistant to metals [54], were found as keystone taxa in metalliferous sites. At Lokovec, Acephala and Golovinomyces (both Leotiomycetes) were detected along with Thelephora basidiomycete. Interestingly, Acephala and Thelephora are fungal genera with well-documented tolerance to metal pollution [55–57]. Acephala is classified as a dark septate endophyte (DSE) [58], whereas Thelephora is an ectomycorrhizal fungus [59]. Although ectomycorrhiza is not formed by *Noccaea*, the presence of these fungi in the rhizosphere could alter the soil conditions in favour of the plant. Indeed, the abundance of Thelephora was even higher in Žerjav than

in Lokovec. Nevertheless, we cannot rule out opportunistic commensalism due to root exudate attraction. In addition, a low number of unique fungal genera was observed in our study, hindering straightforward conclusions. Nevertheless, the low diversity of fungal taxa could well be the state of the norm for Brassicaceae, which contain many anti-fungal compounds [60] and are believed to have lost the ability to form typical symbiotic associations with beneficial fungi [33]. A study of endophytes of the related *Microthlaspi* (Brassicaceae) yielded a wide range of OTUs per population (109–272 OTUs per population) [61], with a lower range just above the numbers observed in our study.

The identified bacterial keystone taxa followed Sloan neutral model, which is in accordance with Wang et al. (2021) [62], who examined microbial communities in a freshwater river continuum in subtropical China. This would suggest that bacterial keystones as module hubs of the communities were mostly neutrally distributed generalists with high abundances and were beneficial to many related OTUs. This was not the case with fungal keystone taxa that mainly deviated from the neutral model. It seems that fungi are more intimately connected to the plant host, resulting in different responses to the environment when compared to bacteria.

To evaluate the stochastics within microbial communities, we applied the Sloan neutral model to assess the distribution of microorganisms associated with *N. praecox*. The bacterial community revealed the statistical significance of genera that were above and below the prediction according to the Sloan neutral model. This suggests certain bacterial genera are either favored by the plant host and/or environmental conditions or associated with active migration and negative interactions within the community. The fungal community also contained the genera that did not follow the Sloan neutral model, but there was no statistical significance in their abundances between both sampling sites. It indicates that these genera follow the dynamics of passive dispersal [63], possibly by animals, precipitation, or other abiotic and/or biotic vectors. Moreover, host-associated microbial community variation does not come only from plant hosts or associated microorganisms. Neutral processes like drift and dispersal are powerful enough to create a large amount of diversity within and among hosts [64] or environmental conditions [65], explaining a significant part of the microbial community structure (64).

Eliminating the randomness of the bacterial community allowed us to evaluate the influence of individual abiotic soil parameters and biotic interactions on the composition of the bacterial OTUs that did not follow the Sloan neutral model. As fungi were less connected to the environment, only bacterial OTUs showed statistically significant correlations with environmental parameters,

with the majority affecting the composition of the bacterial community. The root compartment displayed a pattern similar to that of the rhizosphere, suggesting that the environment impacts microbial taxa that colonize the root endosphere or at least the rhizoplane. However, there is still no consensus on how much environmental conditions affect the fungal communities, as different studies report different results [19, 20, 66, 67] In contrast, it is well established that soil bacteria are much more susceptible to biotic and abiotic factors [68, 69] than fungi, which aligns with our results.

Bacterial networks of *Noccaea* rhizobiome

Along with abiotic environmental factors, microbial interactions also shape the structure and function of the microbiome [70, 71]. The predominance of positive interactions over negative edges indicates a prevalence of cooperative or syntrophic interactions with the potential for extensive mutualistic interactions [28, 72] and is probably a result of heterogeneous microenvironments that reduce direct competition [73].

However, we observed a few negative interactions associated with Gemmatimonadetes, Firmicutes, and Bacteroidetes, indicating potential antagonistic behaviors, possibly arising from competition for the same ecological niche among these microbial entities. Most negative correlations in all three *Noccaea* populations were associated with Firmicutes, which are a common bacterial phylum involved in various processes, including the decomposition of organic matter and nutrient cycling, and thus important for the maintenance of the soil ecosystem [74, 75]. Firmicutes are highly resistant to toxic metals, and the high percentage of negative correlations could reflect their high competitiveness, as they can displace other phyla in metalliferous soil [76]. Similarly, Bacteroidetes are another metal-resistant bacterial phylum known for their rapid growth and ability to adapt to various environments, including metal-polluted soils [77–79]. They are successful competitors [80] and are known to produce antimicrobial compounds that regulate population-level interactions [81, 82], which might explain negative correlations towards other bacteria within the community. Interestingly, no negative edges were associated with Acidobacteria that were observed to carry a substantial proportion of negative edges in several environments, including soils [73].

We further compared the prevalence of negative edges among bacterial phyla in the root and rhizosphere compartments between both studied locations and *Noccaea* species. Proteobacteria and Actinobacteria exhibited the highest number of negative correlations. Notably, they were also the most abundant phyla within the bacterial community, allowing them to compete for their root-associated niche with other bacterial phyla efficiently

[28]. Nevertheless, the number of negative edges in Proteobacteria and Actinobacteria decreased dramatically at the metalliferous site and increased in Firmicutes and Bacterioidetes at the metalliferous site. The overall presence of negative edges inside the community can be an important factor, as they are hypothesized to promote community stability due to negative feedback [83]. In contrast, positive interactions may destabilize the community through the generation of co-dependencies [83]. *N. praecox* growing at the metalliferous site exhibited Zn, Cd, and Pb root concentrations that were up to 10-times, 76-times, and 737-times those at the non-metalliferous site, respectively, thus increasing the environmental pressure and selection of bacterial groups. In this context, the results of network analyses suggest that conditions at the metalliferous site may increase competition in some bacterial phyla, as indicated by an increased number of negative edges within Firmicutes and Bacterioidetes. However, the overall frequency of these interactions is significantly reduced due to the negative selection of dominant bacterial groups, which could potentially lead to a more unstable community.

Nevertheless, the network analysis included three samples of each soil compartment for the individual soil/habitat combinations, which could introduce some bias into the results. To strengthen the generalizability of these findings, further research with a larger sample size is recommended.

Conclusions
Our study revealed differences in bacterial and fungal communities from *Noccaea* plants growing at polluted and unpolluted sites, as well as in root-associated compartments. While fungal OTUs varied among populations and compartments, bacterial OTUs displayed more consistent abundance and significant correlations with soil parameters, particularly in the metalliferous site. *N. caerulescens* exhibited the highest number of negative correlations between the bacterial phyla, while *N. praecox* population from the metalliferous site the fewest, suggesting microbial specialization or reduced competition. However, the evaluation showed a big perturbation in the bacterial community at the metalliferous site, suggesting increased selection between the bacterial taxa and the formation of potentially less stable rhizobiomes. These results offer valuable insights for future research on hyperaccumulator-microbiome dynamics.

Methods
Site description
Sampling took place at two sites in Slovenia where *Noccaea* species occur naturally: in Lokovec (N 46° 2′ 39.2706″, E 13° 46′ 8.9934″), a non-metalliferous site where both species co-occur, and in Žerjav (N 46° 28′ 26.1258″, E 14° 51′ 56.0118″), a metal-polluted site where only *N. praecox* grows. The soils at both sites belong to the reference group of Phaeozems with calcareous rocks, namely limestone in Lokovec and dolomite in Žerjav, as the parent material. Both soils are eutric, with a pH of 6.8 and 6.9 for Lokovec and Žerjav, respectively, and a high base saturation. Among the exchangeable basic cations, calcium cations predominate $30.2–52.1$ mmol$_c$ 100 g^{-1}, followed by magnesium $2.9–9.3$ mmol$_c$ 100 g^{-1} and potassium $0.39–0.61$ mmol$_c$ 100 g^{-1}. Exchangeable sodium is negligible in both soils. Both soils have a high cation exchange capacity, which is due to the high organic matter content, which is 17.1 and 20.2% for Lokovec and Žerjav, respectively (Table S1). The soils differ in soil texture and carbonates content. The high proportion of sand and carbonates in Žerjav is the result of the typical physical weathering of dolomite. On the other hand, the chemical weathering of limestone leads to a more clayey texture and the dissolution of carbonates (Table S1).

Sample collection
Three replicates of flowering plants, including their rhizosphere and the surrounding soil, were dug out in spring 2022: both species in Lokovec and *N. praecox* in Žerjav. The plant material was formally identified by Matevž Likar. In addition, at each sampling site, we took a composite soil sample prepared from ten subsamples of the topsoil (0–10 cm). Subsamples were collected across the entire sampling area, tightly packed in a plastic bag to prevent potential cross-contamination, and taken to the laboratory. Before combining and thoroughly mixing the soil subsamples from each location into a single composite sample, we removed the top organic layer containing dry plant material.

During the transfer, plants were kept in plastic pots and were processed the next day, when the roots, the rhizosphere soil, and the bulk soil were separated. Rhizosphere soil was collected by gently shaking the plant to gather the soil adhering to the roots [84], while the roots were thoroughly washed with tap water, followed by distilled water, and finally dried with paper towels. Water and equipment were sterilized by autoclaving for 30 min at 121 °C. All procedures followed appropriate sterile conditions required to prevent contamination of the material. Subsequently, all samples were stored at -80 °C until further use. With this approach we separated the collected microbial communities into three compartments: (i) roots that include endophytes, epiphytes, and the microbes inhabiting the nearest soil particles; (ii) rhizosphere that includes only microbes not in contact with plant roots and therefore less influenced by the root exudates; (iii) bulk soil that was well removed from the plant roots.

Soil analysis

The soil samples were air-dried, crushed, and sieved through a 2 mm sieve (SIST ISO 11464, 2006). For the metal analyses, the soil samples were additionally crushed and sieved through a 160 μm sieve. The soil analysis procedures were carried out as follows: soil texture was determined using a sedimentation pipette method (SIST ISO 11277, 2011). For soil pH measurement, a 1:5 (v/v) ratio of soil and 0.01 M $CaCl_2$ suspension was employed (SIST ISO 10390, 2006). Total carbon (C_{total}) and nitrogen (N) were determined through dry combustion (ISO 10694, 1995) using an elemental analyzer (Elementar Vario MAX Instrument, Germany). Carbonates content (Carbonates) was determined volumetrically using Scheibler calcimeter following soil reaction with hydrochloric acid (SIST ISO 10693, 1995). The inorganic carbon was calculated from the content of carbonates to further assess organic carbon (C_{org}), which is approximated as the difference between the total carbon (C_{total}) and the inorganic carbon [85]. Soil organic matter content (Organic matter) was calculated as the product of organic carbon × 1.724 [85], and the ratio between organic carbon and total nitrogen was also determined (C_{org}/N). Plant-available phosphorus (P available) and potassium (K available) were measured post-extraction with ammonium lactate solution (ÖNORM L 1087, 1993) – phosphorus spectrophotometrically with the development of a blue color and potassium using the FAAS method. The cation exchange capacity (CEC) was determined by a two-step extraction procedure: first by extraction with ammonium acetate (pH=7), whereby the concentrations of the basic cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+) were measured, and then by extraction with potassium chloride (KCl). The sum of base cations (SUM cations) was measured by FAAS after the first extraction. Base saturation (Base saturation) was then calculated as the sum of base cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+) divided by the total CEC. Pseudototal concentrations of Pb, Cd, and Zn were determined by FAAS after digestion with aqua regia in a microwave oven system (SIST ISO 11047:1999).

Each soil parameter was measured three times. Mean values and standard deviations were calculated and are presented in Table S1.

Inductively coupled plasma mass spectrometry (ICP-MS)

The concentrations of Zn, Cd, and Pb in dried roots ($n=5-6$) were determined following acid digestion by inductively coupled plasma mass spectrometry (ICP-MS). Dried samples were milled to a fine powder using a mortar and a pestle and accurately weighed powdered material was digested in closed vessels using a microwave digester (MARS Xpress, CEM Microwave Technology, Italy). Samples were digested with 6 mL of concentrated HNO_3 and 2 mL of 30% H_2O2 at 180 °C (holding for 60 min). Digested samples were diluted to 30 mL with MilliQ water before element analyses. Total Zn, Cd, and Pb concentrations of digested samples were determined by ICP-MS (Nexion 1000, PerkinElmer, Waltham, MA, USA). Blank digestions were performed to determine background concentrations of elements, and a tomato leaf standard (Reference 1573a; National Institute of Standards and Technology, NIST, Gaithersburg, MD, USA) was used as an analytical control [86].

Metagenomics

Whole-community DNA from rhizosphere and bulk soil was extracted using the DNeasy® PowerSoil® Pro Kit (Qiagen) and total genomic DNA from roots using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma Aldrich) following the manufacturer's instructions. All DNA samples were stored at -80 °C until sequencing. The DNA quality control and shotgun metagenomic sequencing were conducted by Macrogen company using the Illumina HiSeqX platform (2×150 pair-ends) in accordance with the manufacturer's guidelines, employing TruSeq DNA kit (Illumina). Altogether, 24 samples were sequenced: three samples from rhizosphere and plant roots and two bulk soil samples for each population, yielding on average 29,776,694±6,228,348 reads/sample. For the taxonomic assignment of metagenomic reads, SqueezeMeta [86] pipeline that implements a fast-last common ancestor (LCA) algorithm was used to analyze each query gene hit results as the Diamond [87] search query against the GenBank nr database. Operational taxonomic units (OTUs) were defined at the level of genus.

Bioinformatics and statistical analysis

All statistical analyses were performed in R (v4.3.2). Metal concentrations in plant roots and microbial diversity measures were compared using the Kruskal-Wallis test and Dunn's test for multiple comparisons. Microbial communities were analyzed using the phyloseq library (v1.46.0) [88], while the results were visualized using the ggplot2 (v3.5.0) or additional R libraries specified below. Before any analyses, abundances were normalized to median sequencing depth to eliminate any bias due to differences in the sampling sequencing depth. Differences between the grouping parameters (compartment, location, and *Noccaea* population) were tested for all bacterial and fungal OTUs with permutational analysis of variance [89] perMANOVA), using *adonis2* function in the vegan (v2.6-6.1) library with 1000 permutations. Differentially abundant OTUs were identified using DESeq analysis, DESeq library (v1.44.0). Only OTUs with padj<0.05 and $-1<log2FC>1$ were flagged as differentially abundant between comparisons. The Sloan neutral model for fungal and bacterial communities was

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assessed according to the protocol outlined by Likar et al. (2023) [65]. Calculations of 95% confidence intervals around for the model-calculated frequencies were done by bootstrapping with 1000 bootstrap replicates. OTUs were sorted into three partitions depending on whether they occurred more frequently than ('above' partition), less frequently than ('below' partition), or within ('neutral' partition) the 95% confidence interval of the neutral model predictions. Only microbial genera that did not adhere to the neutral model were used for evaluation of the effects of plant host and abiotic environment on the microbial community.

Libraries ggcor (v0.9.4) and vegan (v2.6-4) were used to conduct the Mantel test for bacterial communities on root and rhizosphere compartments ($n=9$). To evaluate the correlation between compartments and soil parameters, Spearman and Mantel correlation coefficients were applied, assessing the statistical significance between parameters. We ensured that the data followed all the assumptions for Spearman's correlation, e.i. ordinality, paired observations, and monotonic relationship.

The co-occurrence network was inferred based on Spearman's Rho between the pairwise OTUs matrix constructed by the R psych (v2.1.9) library. The *p*-values for multiple testing were calculated using the false discovery rate (FDR) controlling procedure. A valid co-occurrence event was considered robust if the correlation coefficient $r > |0.8|$ and if it was statistically significant at $p < 0.05$. To reduce network complexity, only the top 15 bacterial phyla were included (Fig. 4a). For the triangular correlation plots of the rhizosphere and root compartments of each *Noccaea* population ($n=6$) only data with a $p < 0.05$ was considered. R libraries chorddiag (v0.1.3) and corrplot (0.92) were used for visualization.

Keystone OTUs were calculated using phylosmith (v1.0.7), igraph (v2.0.3), and microbiome libraries (v1.24.). For the fungal community, all OTUs were included in the keystone analysis, whereas for the bacterial community, only OTUs with more than 500 reads with a statistical significance $p < 0.05$ were retained. We identified keystone OTUs separately for the generated networks and defined them as those nodes within the top 1% of node degree values for each network [90, 91].

The analysis of negative edges for bacterial phyla was conducted using igraph (v2.0.3), tidygraph (v. 1.3.1), and microbiome (v. 1.24) libraries. Negative edges were analysed for the top 100 most abundant bacterial genera in the rhizosphere and root compartments, and genera with statistical significance of $p < 0.05$ were included in the analysis.

Abbreviations
OTUs   Operational Taxonomic Units
Zn   Zinc
Cd   Cadmium
Pb   Lead

Declarations

Ethics approval and consent to participate
All necessary permissions and licences were obtained before the collection. A voucher specimen was deposited in the Herbarium of the University in Ljubljana under identification LJU10147538.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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