Above and belowground biodiversity in adjacent and distinct serpentine soils

Manuscript Details

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Abstract

In this study we analysed the diversity of vascular plants, soil microorganisms and microarthropods on two serpentine outcrops in the Northern Apennines (Italy). The soils of the two sites are at different stages of evolution, as shown by soil depth and soil organic matter (SOM) content: the first site is a serpentine grassland clearing within sub-montane vegetation, while the second is a serpentinite scree rich in specialised flora only, including rare hyperaccumulator plant species such as Noccaea caerulescens and Alyssum bertolonii. The aboveground biodiversity was analysed via floristic relevés. The bacterial diversity was estimated through 16S rDNA profiling of the Ni hyperaccumulator N. caerulescens at the rhizosphere level, which thrives in both sites. Microarthropod communities were characterized by extracting and identifying organisms from the soil. The number of individuals per taxon, Acari/Collembola ratio, biodiversity indices and QBS-ar index were calculated. The two sites showed a clear difference as regards plant community: only three plant species were present in both sites, namely Euphorbia cyparissias, belonging to Euphorbiaceae, Galium spp., belonging to Rubiaceae, and N caerulescens, belonging to Brassicaeae. Belowground diversity was positively correlated with vegetation cover and SOM content. In particular, among the bacteria colonising the rhizosphere of N. caerulescens (present in both sites), Actinobacteria, which exhibit K-strategist attributes and are more successful in resource-limited, crowded environments, appeared to be more abundant in the site with lower SOM and higher content of Ni. Bacterioidetes, which are specialised in degradation of cellulose, chitin and plant detritus, were instead more abundant in the site with higher SOM. As for soil microarthropod communities, the site with higher concentration and bioavailability of heavy metals and lower SOM showed poorer and less structured community. In conclusion, the data observed with plant, microbial and microarhropod communities identify different micro-habitats, in spite of proximal geographical position and the common metal-rich substrate.

Keywords	Serpentinite vegetation; Noccaea caerulescens; Rhizosphere; Soil biodiversity; Soil fauna
Taxonomy	Biological Sciences, Environmental Science
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Suggested reviewers	Arthurita Venter, Sara Branco, Nishanta Rajakaruna

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Dr Andrey Zaitsev

Editor-in-Chief

Applied Soil Ecology

Parma 7 September, 2018

Dear Dr. Zaitsev,

Enclosed please find the revised manuscript "Above- and belowground biodiversity in adjacent and distinct serpentine soils", by Giovanna Visioli, Anna Maria Sanangelantoni, Federica D. Conti, Beatrice Bonati, Ciro Gardi and Cristina Menta for publication in *Applied Soil Ecology*. We have followed the Editor's and the Reviewers' suggestions carefully, and moved most of the Tables and Figures to the Supplementary Materials section. The MS has also been thoroughly proofread by an English language instructor at our University Language Centre, Dr. Anila R. Scott-Monkhouse. As for the issue of pseudireplication, we have added a sentence in the Conclusion, as suggested by Referee 2 too. Unfortunately, we found no other serpentine outcrop either nearby or elsewhere in the Apennines displaying the same conditions and plant species to perform comparative experiments. We have chosen to leave the Results and Discussion together since there are distinct subsets of data that were easily discussed independently.

We hope that this R1 version of the manuscript meets the standards required to be deemed suitable for publication in *Applied Soil Ecology*, as either a Research Article or a Short Communication.
Yours sincerely,

Giovanna Visioli

Corresponding author

The answers to the Editor's and the reviewers' comments are presented in bold:

Editor: Dear Authors, I received two reviews from the two independent Reviewers for your submission.

As you can see from their comments, they were very much satisfied with the editions you made. Please see below some further minor suggestions from the Reviewers.

However, after careful reading of your manuscript I still see that it needs more work before it could be considered for publication.

An important issue is language. Your text needs quite some editing. I tried to correct it but got discouraged after reading the abstract. I herewith kindly request you to let a native-speaker read and correct your manuscript.

I also suggest you to separate Results and Discussion. You have a full-size research paper and it is hard to follow the story with the current structure. Putting results and discussion together is more common in Short Communications. Alternatively, and this is my suggestion to convert this manuscript into a short communication as still the dataset is quite modest and the statistical analysis is very simple. Most of the results can be reported in supplementary materials.

Finally, there is a problem with pseudireplication of your study. It is understandable, that it is hard to find truly replicated sites on serpentine, but this problem should be treated.

ANSWER TO THE GENERAL EDITOR'S COMMENTS: please see the cover letter.

Editor: Additionally, I ask you to make further corrections as described below.

Highlight 1 is uninformative. Please bring it closer to the context of your study.
Add one more highlight (make it Highlight 1) with some sort of rationale of your study. Considering its novelty this should not be too difficult.
Current highlight 2: how is affected.
Current highlight 3: shorten to fit 85 symbols. Alternatively, you may split it into two.

ANSWER: We have rewritten all the highlights, and hope that this amended version will be suitable for the journal.

L15 ...we analysed vascular plant, soil microorganism and microarthropod diversity...**DONE**
L19 mention at least one or two species here for example here. **DONE**
L21 ...bacterial and microarthropod diversity... This is easy to remember. We say e.g. "tree diversity", similarly we should say "microarthropod diversity". Please correct elsewhere. **DONE**
L23 ...thriving in both sites...**DONE**
L24 "Abundances" sounds strange. What kind of abundances? Microarthropod taxa separately, total, per species? **DONE - We have specified in the MS**
L27 ...and <italics/>N. caerulescens</italics> belonging...**DONE**
L28 ...Belowground diversity...**DONE**
L33 ...abundant in the site with higher OC content in soil...**DONE**
L37 ...highest concentration and bioavailability of heavy metals...**DONE**
L38 Some sort of conclusion is needed (1 sentence). **DONE**
L45 ...of a stressful environment...**DONE**

NOTE! From now on I will not make any further corrections of language and will focus only on the scientific side of your work. A thorough check of the text by a native-speaker is required.

L46 correct Reference format. See https://www.elsevier.com/journals/applied-soil-ecology/0929-1393/guide-for-authors **DONE**
L80-84 The sentence is too long and hard to follow. Consider revision and splitting. **DONE**
L81 ...and nutrient elements cycling... sounds weird. **DONE**
L90 Single plant species? If yes, I would call it here "a model plant species" **DONE**
L97-98 "mountain range ridge" sounds strange**- DONE we eliminated the phrase**
L101-102 do these two sites have some proper names? If MP1 and MP2 have already been used in some earlier published work, it would be nice to have a reference here. **DONE**
L104 Specify horizons.:**DONE**
L106 Specify metals analyzed. **DONE**
L127 Format reference as required by teh Guide for Authors. **DONE**
L131 Please explain, what is a "uclust".

ANSWER: A uclust algorithm divides a set of sequences into clusters.

L132 Explain, what is "QIIME".

ANSWER: Quantitative Insight Into Microbial Ecology is the bioinformatic pipeline used to analyse the microbial communities sampled through marker gene (e.g. 16S rRNA gene) amplicon sequencing.

L135 Please briefly explain, what is a "UniFrac".

ANSWER: Unifrac is a distance metric used to compare biological communities. It differs from dissimilarity measures such as Bray-Curtis dissimilarity in that it incorporates information on the relative relatedness of community members by incorporating phylogenetic distances between observed organisms in the computation. Both weighted (quantitative) and unweighted (qualitative) variants of UniFrac are widely used in microbial ecology.

L139 Did you split your monolith into horizontal layers during extraction? If not there is a fair chance that your extraction was not efficient. Please provide more details on that.

ANSWER: We collected a 10x10x10 cm soil core and distributed the sample in a 22 cm sieve of splitting the monolith into two parts only when the soil remained compact.

L142-143 It is very uncommon to place Myriapoda, all Insecta, all Chelicerata and Crustacea among microarthropods. For example, all myriapods and crustaceans (isopods actually?) are macroarthropods. Please clarify this issue and make changes accordingly throughout the text.

ANSWER: We agree with the Editor's comment and have understood the reason for this comment. However, we use the term "microarthropod" because we extracted and analysed only arthropods having 2 mm body size, in relation to the sieve mesh (we specified this aspect in the reviewed version). If the Editor believes that this use is wrong, we will replace the term with "arthropods".

Collembolans are often put separately from insects. You should be careful with your macrosystem. Please provide references.

ANSWER: We agree with the Editor's comment, and we know that Collembola are included in superclass Hexapoda, class Entognata (like Protura and Diplura). Considering the Editor's suggestion, we have replaced Insect with Hexapoda in the text.

L144-147 It is quite not correct to calculate diversity indices basing on different taxonomic resolution levels for different soil animal taxa. How did you deal with this?

ANSWER: We have understood the Editor's comment. We adopted this approach several times in previously published papers and the Reviewers accepted it. If the Editor considers this approach as being not entirely correct, we can calculate the indices using the orders only.

L148 Please explain, what is a "soil adaptation level"?

ANSWER: This concept is in relation to the QBS-ar index approach and the EMI (EcoMorfological score) assignment used for the QBS-ar computation. We did not explain this concept in the text because it was explained in several previous scientific papers, two of which are reported in the references.

L151 Obviously those are packages of R. Please explicitly state that and include the version of the software and a proper reference into the Reference list. **DONE**
L154 observed number of taxa? **ANSWER: J was calculated dividing H' by the logarithm of the number of taxa. We have clarified this in the text.**
L159 Results are always reported in the past tense. **DONE**
L162-163 Move to site description**. DONE**
L180-181 Are those SDs or SEs? Explain in Methods **DONE.**
L185 You mean "weakly?" **DONE**
L192-194 This belongs to Discussion.
ANSWER: We have considered the Editor's suggestion of shortening the manuscript and left the Results and discussion section together.
L199 ...*N. caerulescens*... At first mentioning of a species in the text you write it in full, then you abbreviate the genus name. Same is applicable for the Abstract as a standalone text. **DONE**
L210 ...Venter et al. (2018). **DONE.**
L215-220 The sentence is too long and unintelligible. Should be split into several sentences. The second part should go to Discussion. **ANSWER: We have modified the sentence. As explained above, we have considered the Editor's suggestion of shortening the manuscript and left the Results and discussion section together.**

L231 You refer to Fig. 2a, but not to 2b. Later you refer to Fig. 2 as a whole. Do you really need ä"and "b"then? **DONE**
L238 in the rhyzosphere? **DONE**
L242-243 to Discussion and L248-250 to Discussion. **ANSWER:**
As explained above, we have considered the Editor's suggestion of shortening the manuscript and left the Results and discussion section together.

.
L252-255, L257-262 to Discussion. Further, please make very clear separation between Results and Discussion. I will not make further comments on that but anticipate, that this is fixed. **ANSWER:**
We have considered the Editor's suggestion of shortening the manuscript and left the Results and discussion section together.

L263 "degree of biodiversity" is not a common term. **DONE**
L276 From what I read here, there is no way to call all taxa collected as microarthropods. You have a mixture of macro- and mesofauna. Please adjust elsewhere that you were studying soil arthropods.
ANSWER: Please refer to the explanation above (L 142-143).

L305 The story ends nowhere and makes a paper rather confirmatory. A deeper discussion on the correlation of plant, microbial and arthropod diversity is needed.
Conclusions are also rather confirmatory and can be or replaced by a take-home message. The only interesting statement on the importance of OC content and Ni content is actually not based on any kind of statistical analysis or extensive literature search.
ANSWER: A final sentence was added in the Conclusion, following the suggestions of Referee 2 too.

Table 1 to Supplementary Material with most important data to methods.
ANSWER: DONE

Table 2 Are those means and SEs? In fact those are not replicates but pseudoreplicates. How have you treated this in your design? A star marking significance should be not after the mean value, but after the SE. **ANSWER: DONE**
L492 Organic matter or Organic carbon? If the former, how have you recalculated this? **ANSWER: This was indicated in the Materials and Methods section.**
L499 Space after "x"**DONE**
Table 3 can go to Supplementary Material.**DONE**

Figures.
First, it is hard to understand which Figure is which one. They are not signed.
Further I will call them in accordance to their appearance.
Figure 1 - to Supplementary material **DONE**
Figure 2 - What do percent refer to? Frequencies? Y-axis should be signed. Also in the text to refer to Figure 2a. Here there are no any "a's" and b's"...**DONE. This figure is now Figure 1. Percentagae of number of sequences of the phyla respect to the total number of sequences**
Figure 3 is not readable. Increase fonts. Overall it can be replaced with a Table and go to Supplementary Material. You have "a" and "b" in the Figure, but nothing in the figure caption about it. Also no separate references for Fig. 3a and Fig. 3b in the text. ANSWER: **We put Figure 3 in supplementary materials increasing font.**
.
Figure 4 - How can you prove significance of differences? This figure does not bring too much, particularly if you had multiple taxonomic resolution levels in your dataset. Consider skipping.

ANSWER: After carefully considering the Reviewer's suggestion, we have chosen to maintain the figure in the MS because the statistical analysis was made by comparing the couple of data (MP1 vs MP2) for each taxon. In our opinion this figure adds important explanation and would be best kept it in the Results discussion section.
References.
Please see attached file with the results of the references check. The color coding should be pretty much self-explanatory: Red - reference missing in text or vice-versa. Purple - problems with formatting.
ANSWER: All the references were checked according to the indications. Where there are open assess articles we put the doi

REVIEWER 1 The revisions have greatly improved the paper. Look forward to seeing the paper in print.

ANSWER: We thank Reviewer1 for their positive evaluation of the manuscript in its present form.

REVIEWER 2
The study characterizes the diversity of two different serpentine sites, having different environmental features, particularly regarding microclimate and soil structure. The manuscript is interesting, really well written, pleasant to read and rich in details and analyses. The only concern I have is about the only two sites where the authors made the experiment. Although they temporary and spatially replicate the sampling, only one site per factor level risks being not sufficient to make general conclusions about the aim of the study. However, if the sampling area provides spatial constraints as declared by the authors, this problem cannot be actually solved, so I think they have done a really good job anyway. Nonetheless, I suggest the authors to add a sentence in the

conclusion section where they declare that the study may suffer from this problem, and that it should be repeated in different contexts to ensure a general reliability of results. Adding a figure showing the outcrop and the relative position of the two sites, may help to better understand this issue.

However, I think that the manuscript is suitable for publication. I have only a few minor comments listed below.

Lines 54-68: I suggest the authors to consider these two studies:

> Mengoni et al (2004). Genetic diversity of bacterial communities of serpentine soil and of rhizosphere of the nickel-hyperaccumulator plant Alyssum bertolonii. Microbial ecology, 48(2), 209-217.
>
> Mengoni et al. (2001). Characterization of nickel-resistant bacteria isolated from serpentine soil. Environmental Microbiology, 3(11), 691-698.

ANSWER: We thank Reviewer 2 for their positive evaluation of the manuscript. We have followed their suggestions by adding a comment in the Conclusion section concerning the problem of the small number of samples. We have also added the studies suggested. The figure showing the outcrop and the relative position of the two sites is reported as supplementary figure 1.

Lines 84-91: In my opinion, the aim as described by the authors is a little bit weak if compared to the in-depth study they performed. Given that the study provides a comparative approach between two different sites, I think that the aim has to be rephrased also focusing this aspect, not only the 'simple' characterization of the two site. The authors should also list their expectations on the basis of the available literature, and answer to each expectation in the discussion section. **DONE**

Lines 173-174: Please add the type of test used and the value of the statistical parameter. **DONE**

Bullet points

- Above and belowground biodiversity in serpentine soils are still little known.
- Vascular plant, soil microorganism and microarthropod diversity were studied.
- Different micro-habitats on serpentinite showed high vascular plant diversity.
- *Noccaea caerulescens* rhizosphere is modulated by soil characteristics.
- Microarthropods are richer in lower Ni and higher soil organic matter contents.

Above and belowground biodiversity in adjacent and distinct serpentine soils

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Abstract

In this study we analysed the diversity of vascular plants, soil microorganisms and microarthropods on two serpentine outcrops in the Northern Apennines (Italy). The soils of the two sites are at different stages of evolution, as shown by soil depth and soil organic matter (SOM) content: the first site is a serpentine grassland clearing within sub-montane vegetation, while the second is a serpentinite scree rich in specialised flora only, including rare hyperaccumulator plant species such as *Noccaea caerulescens* and *Alyssum bertolonii*. The aboveground biodiversity was analysed via floristic relevés. The bacterial diversity was estimated through 16S rDNA profiling of the Ni hyperaccumulator *N. caerulescens* at the rhizosphere level, which thrives in both sites. Microarthropod communities were characterized by extracting and identifying organisms from the soil. The number of individuals per taxon, Acari/Collembola ratio, biodiversity indices and QBS-ar index were calculated. The two sites showed a clear difference as regards plant community: only three plant species were present in both sites, namely *Euphorbia cyparissias*, belonging to Euphorbiaceae, *Galium* spp., belonging to Rubiaceae, and *N caerulescens*, belonging to Brassicaeae. Belowground diversity was positively correlated with vegetation cover and SOM content. In particular, among the bacteria colonising the rhizosphere of *N. caerulescens* (present in both sites), Actinobacteria, which exhibit *K-strategist* attributes and are more successful in resource-limited, crowded environments, appeared to be more abundant in the site with lower SOM and higher content of Ni. Bacterioidetes, which are specialised in degradation of cellulose, chitin and plant detritus, were instead more abundant in the site with higher SOM. As for soil microarthropod communities, the site with higher concentration and bioavailability of heavy metals and lower SOM showed poorer and less structured community. In conclusion, the data observed with plant, microbial and microarhropod communities identify different micro-habitats, in spite of proximal geographical position and the common metal-rich substrate.

Keywords: Serpentinite vegetation; *Noccaea caerulescens*; Rhizosphere; Soil biodiversity; Soil fauna

1. Introduction

Serpentinites are widespread and represent a well-known example of a stressful environment (Harrison and Rajakaruna, 2011), being constituted by tectonic blocks and intrusion of ultramafic rock and by sedimentary and metamorphosed derivatives (Coleman and Jove, 1992). These soils are generally characterised by low levels of macronutrients such as N, K, P, Fe. In addition, the high Mg causes a low Ca/Mg molar ratio, inhibiting Ca uptake, and the high concentration of heavy metals, particularly Ni, exerts toxic effects on plants and soil biota (Chiarucci et al., 2003; Lombini et al., 1998). Dwarf plant communities often inhabit serpentine areas and are remarkably different from the ones growing in adjacent soils (Lombini et al., 1998). Even microbial communities are an important, highly specialised component in belowground serpentine environments (Branco, 2010; Mengoni et al., 2004; 2001; Venter et al., 2018; 2015). Several studies based on culture-dependent techniques have revealed that serpentine microorganisms show a very high degree of multi-metal resistance. Moreover, bacteria present in serpentine soils surrounding plant roots (rhizobiome) are known to enhance plant nutrient and water uptake and protect plants against toxic conditions, thus greatly contributing to plant growth and fitness under harsh edaphic conditions (Seneviratne et al., 2016). The culturable bacteria isolated from serpentine soils benefit plants by producing siderophores and carboxylic acids, and help solubilisation of phosphates, which increase the mobility of macro- and micronutrients in the rhizosphere (Rajkumar and Freitas, 2008) and produce phytohormones (Visioli et al., 2014). Isolation-based microbiological techniques are limited because culturable microorganisms represent only a tiny percentage of the soil's microbial community. Metagenomics and metaproteomics are ideal approaches when investigating the

63 diversity and ecology of microbiome in serpentine soils, though very little information is currently
64 available (Mattarozzi et al., 2017).

65 Even the soil fauna community is affected by high metal concentrations, but to date
66 knowledge about soil animal communities in serpentine soils is scanty. There are examples of high-
67 Ni herbivorous insects which are well adapted to these severe habitats (Boyd, 2009). Frizzi et al.
68 (2017) reported an interesting pattern of mortality in the *Crematogaster scutellaris* ant in long-term
69 metal exposure in serpentine soils, while ants from intermediate contaminated sites are more
70 tolerant to acute exposure to nickel. Moreover, the authors found no difference in genetic diversity
71 between ant colonies from different sites, and hypothesised that this was due to queen mediated
72 gene flow during nuptial flights across contaminated and uncontaminated areas of limited
73 geographical extent. Visioli et al. (2013) reported significant differences in terms of microarthropod
74 communities between four serpentine soils characterized by different metal content. The site
75 characterized by lower metal content showed the richer community. Soil fauna is involved in
76 important processes such as organic matter decomposition, humus formation, nutrient cycles
77 (nitrogen, sulphur, and carbon), and bioturbation (Bird et al., 2004). For these reasons, soil animals
78 play an important role in the distribution and bioavailability of the trace elements generally present
79 in high concentration in serpentine soils.

80 The aim of this study was to compare the above- and belowground biodiversity in two micro-
81 habitats on the serpentine outcrop of Mt. Prinzera, an ophiolite mountain in the Italian Northern
82 Apennines, characterised by sharp changes in geomorphology and significant edaphic and
83 microclimatic variations due to exposure (Lombini et al., 1998). The study adopts a
84 multidisciplinary approach, from aboveground plant cover to soil microbial and fauna community.
85 In both sites we compared: i) vegetation cover; ii) bacterial diversity via 16S rDNA profiling within
86 the rhizosphere of a model plant species present in both sites*;* iii) composition and abundance of
87 microarthropod community.

88

2. Materials and Methods

2.1 Study area, soil and aboveground classification

The area studied is located on Mt. Prinzera (MP) (736 m a.s.l.), one of the most extreme ophiolitic outcrops of the Italian Northern Apennines (Lombini et al., 1998). The landscape and ecological features of Mt. Prinzera and its surroundings (237 ha) are protected as a nature reserve of the Emilia–Romagna region. For this reason, the collection of few samples only was allowed, and only two previously identified different micro-habitats were analysed in this study: MP1 (GPS 44.65138°N – 10.08330°E) and MP2 **(**GPS 44.64282°N – 10.07951°E) (Visioli et al., 2013, 2012). The soil profile description and sampling were carried out in the two sites by collecting three soil samples per horizon (horizon A and horizon A/C). The soils were classified according to the World Reference Base for soil resources 2014 (IUSS Working Group, 2015) as Skeletic Mollic Leptosol and Orthoskeletic Leptosol for site MP1 and site MP2 respectively (Figure SI1, Table SI1). The water content, pH value and total Ca, K, Mg, Fe, Ni, Zn, Co and DTPA extractable Ni, Zn, Co contents in sites MP1 and MP2 were analysed according to Visioli et al. (2012). SOM was determined by "weight loss on ignition method. Two-gram aliquots of each soil were placed in ceramic crucibles at 450°C for 3h. The SOM was then calculated with the equation: SOM % = (initial weight−final weight) × 100.

Plant diversity was analysed by recording vascular flora in the sites being studied. Vascular plant species were recorded as present during field surveys from May to October 2015. The aboveground biomass was collected within three plots (0.25 x 0.25 m) for each sampling site, oven dried at 105° C, and weighted, and standard deviations were calculated. The nomenclature of taxa follows Pignatti (1982).

2.2 Rhizosphere biodiversity

2.2.1 Plant samples and extraction of total DNA from rhizosphere soil

N. caerulescens plants (which accumulate up to 2,000 mg kg^{-1} DW) were collected in May 2015 from the previously studied sites, MP1 and MP2 (Visioli et al., 2012). A total of 6 *N. caerulescens* plants per site, at the same phenological stage were uprooted manually (i.e. with steles full of immature inflorescences). *N. caerulescens* rhizospheric soils were separated from the roots and collected. The DNA was extracted from 500 mg of soil with the FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) and visualised by electrophoresis on 0.8% (w/v) agarose gels to test the DNA integrity.

2.2.2 16S rRNA gene-based community analysis

Partial 16S rDNA sequences were amplified from the DNA extracted by using primer pair Probio_Uni and /Probio_Rev, which target region V3 of the 16S rRNA gene sequence, and 16S rRNA gene amplification and amplicon checks were carried out as described (Mancabelli et al., 2016). The 16S rDNA sequencing was performed via MiSeq (Illumina) at the facility of GenProbio srl (www.genprobio.com) following the protocol previously reported (Mancabelli et al., 2016). In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥ 97 % sequence identity by using uclust algorithm (Edgar, 2010). All reads were classified at the lowest possible taxonomic rank by using QIIME (Quantitative Insights into Microbial Ecology) and a reference dataset from the SILVA database (Quast et al., 2013). The biodiversity of the samples (alpha-diversity) was calculated with Chao1 and Shannon indices. Similarities between samples (beta-diversity) were calculated by unweighted UniFrac distance metric (Lozupone and Knight, 2005). The range of similarities was calculated between the values 0 and 1.

2.3 Microarthropod community

Three soil cores (100 cm^2 and 10 cm in depth) per site were collected in May and October 2015, and put on the Berlese-Tüllgren funnel (2 mm sieve mesh) for microarthropods extraction. The specimens extracted were collected in a 2 alcohol/1 glycerine (v/v) solution and identified via a stereomicroscope. We considered class taxonomic level for Myriapoda, and order level for Hexapoda, Chelicerata and Crustacea (http://taxonomicon.taxonomy.nl/), counted the number of specimens present per taxon and calculated their abundance (ind/m^2). Based on this classification level, we assessed taxa richness and diversity using Margalef index (D; Margalef, 1958), Shannon diversity index (H'; Shannon and Weaver, 1963), Pielou's evenness index (J; Pielou, 1975) and Simpson's index 1-D (Simpson, 1949). We applied QBS-ar index in order to describe the soil microarthropods community in terms of soil adaptation level (Menta et al., 2018).

2.4 Statistical analyses

Richness and biodiversity indices (Shannon diversity - H'-, Pielou's evenness -J- and Simpon's dominance -1-D-) were calculated by using the *vegan* package (Oksanen et al., 2017), in particular applying the *diversity* function to the absolute abundances observed. While H' and 1-D were directly calculated through the function, J was calculated by dividing H' by the logarithm of the number of taxa observed. These estimates were performed via R software (R Core Team, 2016, version 3.0.2). The Mann–Whitney U-test (U) was used to compare MP1/MP2 chemical and physical parameters and MP1/MP2 number of microarthropods for different taxa, and the comparison of MP1/MP2 richness, biodiversity and soil biological indices.

3. Results and discussion

3.1 Soil characteristics

The soil of site MP2 was at early stages of differentiation, and was formed essentially by the material resulting from the physical degradation of serpentine rocks. Skeleton was dominant and the fine earth fraction was only 22% and 9% in horizon A and A/C respectively. The soil organic matter (SOM) in the horizon A was 2.95%. Stones cover more than 90% of soil surface as a result of selective erosion (Fig. SI1 and Table SI1). The soil from site MP1 showed evidence of evolution in terms of physical and chemical weathering and SOM accumulation; the fine earth fraction was 40% and 15% respectively for horizon A and A/C and WW, while the SOM in horizon A was 4.84% (Fig. SI1 and Table SI1).

Soil chemical-physical analyses highlighted the differences between the two sites reported in Table 1. The amount of total Ni in the two sites was very high, but MP2 showed a significant higher content in total Ni ($P<0.05$). The amount of Fe and K was also significantly higher in MP2 than in MP1 ($P<0.05$), while Co was significantly higher in MP1 ($P<0.05$). The lowest Ni, Zn and Co DTPA extractable fractions were observed in MP1. Ca, Mg and Zn did not reveal any significant variation between the two sites. SOM and water content were significantly higher in MP1, while pH was similar in the two sites (around 7.00).

3.2 Plant diversity

The two sites were different in terms of aboveground biomass, with about 158±32 g m^{-2} d.w. in MP1 and about 114±6 g m^{-2} d.w. in MP2. Site MP2 is characterized by the typical vegetation of scree slopes, with species highly adapted to extreme conditions (extremely coarse and stony, weakly developed soil, low in organic matter and nutrient content, higher Ni concentration) (Tables 1, SI1); the vegetation cover is discontinuous, and the aboveground biomass low. Site MP1 is a weakly developed soil too (Leptosol according to WRB), but the percentage of fine earth and SOM is higher compared to MP2, and this is reflected in plant species composition and aboveground biomass (Tables 1, SI1); the vegetation cover is continuous and the aboveground biomass

187 significantly higher than in MP2. A survey of vascular plant diversity present in sites MP1 and MP2
188 is reported in Table S2. Poaceae, Carofillaceae and Fabaceae were present in MP1 only, while
189 Asteraceae and Brassicaceae were predominant in MP2. Interestingly, most of the genera and
190 species present in MP2 (e.g. *Echinops, Thymus*, *Globularia bisnagarica, Helichrysum italicum,*
191 *Artemisia annua,* and *Sedum montanum*) are known to be adapted to dry rocky soils and to produce
192 secondary metabolites with anti-inflammatory and antimicrobial proprieties (Pignatti, 1982). The
193 species identified in site MP1 are typical of forest margins and humid basic soils (i.e. *Cornus*
194 *sanguinea, Clematis vitalba, Hypericum perfoliatum*) (Pignatti, 1982). Moreover, only three plant
195 species/genera were present in both sites*, Euphorbia cyparissias*, belonging to Euphorbiaceae,
196 *Galium*, belonging to the Rubiaceae, and *N. caerulescens*, belonging to Brassicaeae (Table SI2).
197 The former two are annual and perennial herbaceous plants, quite commonly found in a wide region
198 of Europe, while *Noccaea caerulescens* is a well-known Ni hyperaccumulator, endemic to Mt.
199 Prinzera and present with different populations which colonise various micro-habitats and display
200 distinguishing phenotypic traits (Visioli et al., 2012). *Alyssum bertolonii,* the other Ni
201 hyperaccumulator which colonises serpentinite in the Italian Apennines (Galardi et al., 2007;
202 Lombini et al., 1998), is present in site MP2 only. These analyses confirmed previous studies which
203 indicated that specialised flora is found on serpentine rocks and screes only (Ferrari et al., 1992) and
204 is of great interest for its adaptation to such stress-inducing habitats.
205

206 *3.3 Rhizosphere bacterial biodiversity*

207 Previous studies have shown that variations in the structure of bacterial communities may be
208 due to the presence of different plants in non-serpentine and serpentine sites (Pessoa-Filho et al.,
209 2015). By comparing 16 soils belonging to both serpentine and non-serpentine geology, Venter et
210 al. (2018) demonstrated that the local heterogeneity in variables such as soil characteristics or
211 microclimate gradients may impact cyanobacteria and algal biodiversity. In addition, when

212 considering the same serpentine site, the structure and functions of the rhizosphere bacterial
213 community varied between hyperaccumulator and tolerant plants (Mattarozzi et al., 2017).
214 Considering all these aspects, despite the substantial differences in plant biodiversity between sites
215 MP1 and MP2, the presence of *N. caerulescens* in both sites enabled us to investigate possible
216 changes in bacterial diversity associated with the rhizosphere of the same plants in the two micro-
217 habitats. *N. caerulescens,* endemic to Mt. Prinzera, is a Ni hyperaccumulator accession and different
218 populations were previously analysed for morphological and molecular traits highlighting the high
219 phenotypic plasticity of this accession (Visioli et al., 2012). In particular, *N. caerulescens*
220 individuals in MP1 and MP2 were diversified for both growth parameters and Ni accumulation
221 capacity (Visioli et al., 2012). The 16S rDNA based community profiling highlighted the bacterial
222 diversity in the rhizosphere of *N. caerulescens* sampled in MP1 and MP2. A total 153,482 raw
223 pyrosequencing reads was obtained from the two rhizospheres. After quality trimming 138,756
224 sequences were obtained: 44,307 reads for MP1 and 94,449 reads for MP2 (Table SI3). The
225 phylogenetic analysis revealed high bacterial complexity phylum, family and genus levels, with 11
226 phyla representing >1% in both samples (Fig. 1; supplementary file SI4).

227 Bacterial diversity in sites MP1 and MP2 measured on the basis of OTUs, and calculated
228 bacterial diversity indices, i.e. Shannon diversity index and Chao1 estimator of richness, showed
229 that there are no significant differences between samples. The rarefaction curves using the Chao and
230 Shannon indices reached a plateau, thus indicating that further sequencing would not have resulted
231 in the retrieval of more OTUs (Fig. SI2).

232 Actinobacteria (MP1 37%, MP2 48%), Proteobacteria (MP1 32%, MP2 31%) and
233 Acidobacteria (MP1 7.94%, MP2 6.51%) were the predominant phyla in the rhizosphere. The
234 significant differences observed in the abundance of Actinobacteria sequences between the two sites
235 were not surprising as the soil in site MP2 was expected to be more selective because of the lower
236 SOM (2.95% versus 4.84% in MP1) and the higher content of Ni (2032 μg g^{-1} of soil dry weight
237 versus 1700 μg g^{-1} in MP1) (Table 1), and Actinobacteria exhibit *K-strategist* attributes, i.e. are

more successful in resource-limited environments. The higher richness in Actinobacteria of site MP2 can be ascribed to the higher percentage of Mycobacteriaceae, Geodermatophilaceae, Micromonosporaceae, Pseudonocardiaceae, Rubrobacteriaceae families and U. m. of Frankiales order (Fig. 2). Other phyla with > 2% relative abundance were Bacterioidetes, more present in MP1 (6.3%) with Cytophagaceae, Chitinophagaceae and Saprospiraceae families, than in MP2 (1.94%). This can probably be attributed to the more abundant aboveground biomass (either plant or animal) in MP1, giving a greater concentration of carbon sources suitable for these bacteria specialised in degradation of cellulose and chitin (Beier and Bertison, 2013).

Despite the selective conditions, the bacterial diversity was high in both sites, with forty-four families representing >1% (26% of them belonging to unculturable bacteria) (Fig. SI3). The high bacterial biodiversity found on serpentine soils is in accordance with previous studies on ECM fungal communities, demonstrating that serpentine soils are not depauperate and not characteristically composed of specialised species, compared to non-serpentine ones (Branco, 2010).

The greater richness in Acidobacteria in site MP1 was mainly due to U. m. of subgroup 6 order (Fig. SI3). Because of the difficulty in cultivating most of its members, this phylum changed from being virtually unknown to being recognized as one of the most abundant and diverse on the planet only after cultivation-independent analysis methods were fully developed (Kielak et al., 2016). In particular, subgroup 6 has been reported as a subgroup of Acidobacteria with a marked preference for soil environments with increased availability of nutrients and higher organic carbon content (Navarrete et al., 2015).

By analysing these data, we can conclude that there is no difference in the degree of biodiversity between the rhizosphere of *N. caerulescens* in the two environments, probably because rhizosphere is a "protected" environment for bacteria. Authors who analysed ECM fungal biodiversity on serpentine soils previously speculated that the high diversity may be maintained by a combination of environmental effects and competitive relationships among species (Branco,

2010). However, in our situation, environmental factors such as differences in SOM, humidity and Ni concentration shape the bacterial communities in the rhizosphere of the same plant species. This is not surprising as several studies concerning the diversity and community composition of microorganisms (either bacteria or fungi) in the rhizosphere of plants have identified the environment as the most important factor shaping the community and influencing richness and diversity, rather than the root itself (Turrini et al., 2017; Zhang et al., 2018).

3.4 Microarthropods biodiversity

We found an average abundance of 13,248 ind/m^2 belonging to 16 taxa and 6,065 ind/m^2 belonging to 9 taxa in MP1, in May and October respectively. In MP2 we observed an average abundance of 4,628 ind/m^2 belonging to 12 taxa in May, and a mean of 524 ind/m^2 belonging to 8 taxa in October. In both periods the number of taxa was higher in MP1 compared to MP2, but the difference was greater in May (Fig.2). This datum is similar to the trend reported in Visioli et al. (2013) and confirms the different arthropod community supported by the two soils. Acari and Collembola showed the highest abundance in both sites, except for MP2 in October (Fig. 2). Comparing MP1 and MP2, the former showed the highest abundance for both taxa and for both periods. This trend is followed by many other taxa. All taxa extracted in May were more abundant in MP1 than in MP2, except for Psocoptera. Statistical analysis showed significant differences for Symphyla and Coleoptera only (both larvae and adult) (Fig. 2a). Moreover, Pseudoscorpiones, Diplopoda and Pauropoda were absent in MP2.

Pseudoscorpiones, Diplopoda, Pauropoda, Chilopoda, Diplura and Tysanoptera were found in neither sites in October. The other taxa showed a trend similar to the one observed in May, except for Araneae (Fig. 2b). Symphyla and Protura were found in MP1 only. Statistical computation highlighted significant differences for Symphyla and Coleoptera (adult and larvae) as in May (Fig. 2a, b).

As previously established by other studies (Menta et al., 2018; 2013), the more adapted microarthropod taxa were associated with stable soil conditions with a constant supply of organic matter. Moreover, vegetal cover enhanced soil protection, mitigating thermal and hydric stresses (Bird et al., 2004; Eaton et al., 2004). We can hypothesise that the richer and diversified cover vegetation, and the higher organic matter content in MP1 improve soil conditions for the soil fauna community. Indeed, the trend observed for the taxa was confirmed by the index computation (Table 2): Margalef richness index, Shannon diversity index, Simpson index and QBS-ar revealed higher values in MP1 than MP2 in both periods. Only Pielou's Evenness was similar in the two soils (Table 2), thereby highlighting the relevant presence of Acari and Collembola as major communities, and thus not an equal distribution between the groups.

Conclusion

In conclusion, huge differences were observed in plant genera in the two serpentine micro-habitats considered in this study, located in Mt Prinzera Natural Reserve in the Northern Apennines, with the serpentinite scree rich in peculiar flora such as rare hyperaccumulator plant species. SOM and Ni seem to be the main factors influencing the differences in abundance between bacterial taxa. The results obtained for soil microarthropod communities confirmed this trend, showing that the habitat with lower SOM and higher Ni concentration is less suitable for soil fauna. This may be the result of concerted effects driven by both the higher Ni concentration and the lower SOM content, as a consequence of scanty cover vegetation. Indeed, cover plants play a decisive role in determining soil biodiversity, and due to the scarce vegetation in this area the soil is more affected by chemical-physical variations in terms of temperature, moisture, SOM content, erosion, etc. Investigating which taxa are more sensitive to high Ni concentrations can be a step towards clarifying how serpentine soil affects both above- and belowground living communities. In conclusion, the data observed with plant, microbial and microarhropod communities identify different micro-habitats, in spite of proximal geographical position and the common metal-rich

substrate. This study may have been affected by the limitations imposed by the Natural Reserve of Mt. Prinzera. However, the same approach could be repeated in different contexts (e.g. other serpentine outcrops in Italy) to ensure general reliability of these results.

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434

435 **Figure captions**

436 **Fig. 1**: Taxonomic distribution at phylum levels based on 16S rDNA reads across the two soil
437 samples (MP1 and MP2) according to the percentage of sequences considered >1% in abundance
438 respect to the total number of sequences at least in one sample (x-axis).

439 **Fig. 2**: Mean (± Std Dev) of microarthropod density (ind/m^2) observed in MP1 and MP2 in May (a)
440 and October (b) (A: adult; L: larvae). * $p<0.05$

441

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445

446 **Table 1**: Soil parameters in MP1 and MP2 sites: total Ca, K, Mg, Ni, Fe, Zn, Co contents (µg g^{-1} of soil d.w), DTPA extractable fractions of metals
447 indicated in branckets, pH values, water content (WC) %(, Soil organic matter (SOM)%. Five replicates were taken from the 2 different sites MP1
448 and MP2 in around an area of 10 m^2 each. Data are means of measures of five samples (± Std Dev). Significant different values between samples
449 are indicated with an Asterisk * $p<0.05$

Sites	Ca	K	Mg	Ni	Fe	Zn	Co	pH	WC (%)	SOM (%)
MP1	84±1.2	146±3.5	395±5.1	1700±24	45500±341	75±0.2	112±1.3*	7.10	25.38±0.2*	4.84±0.21*
				(34.8±3.4)		(1.4±0.05)	(7.4±0.04)			
MP2	82±2.1	160±2.7*	322±3.6	2032±34*	62120±236*	71±0.5	97±1.7	7.20	19.01±0.4	2.95±0.32
				(50±2.5*)		(1.7±0.06)	(8.5±0.1)			

450

451

453 **Table 2** – Mean (± Std Dev) of Margalef's Index (D), Shannon diversity index (H'), Evenness (J), Simpson dominance index (1-D), Soil Biological Quality

454 index (QBS-ar) calculated for the microarthropod communities. * $p<0.05$

	May			October		
Indices	**MP1**	**MP2**	**p value**	**MP1**	**MP2**	**p value**
D	1.86 ± 0.52	0.92 ± 0.46	-	1.27 ± 0.12	0.93 ± 0.41	*
H'	1.32 ± 0.35	0.93 ± 0.28	*	1.37 ± 0.20	0.79 ± 0.21	-
J	0.52 ± 0.12	0.55 ± 0.05	-	0.66 ± 0.10	0.66 ± 0.18	-
1-D	2.89 ± 0.66	2.16 ± 0.43	*	3.11 ± 0.99	1.83 ± 0.33	-
QBS-ar	182 ± 31	89 ± 42	*	102 ± 8	43 ± 11	*

455

458

Figure 1

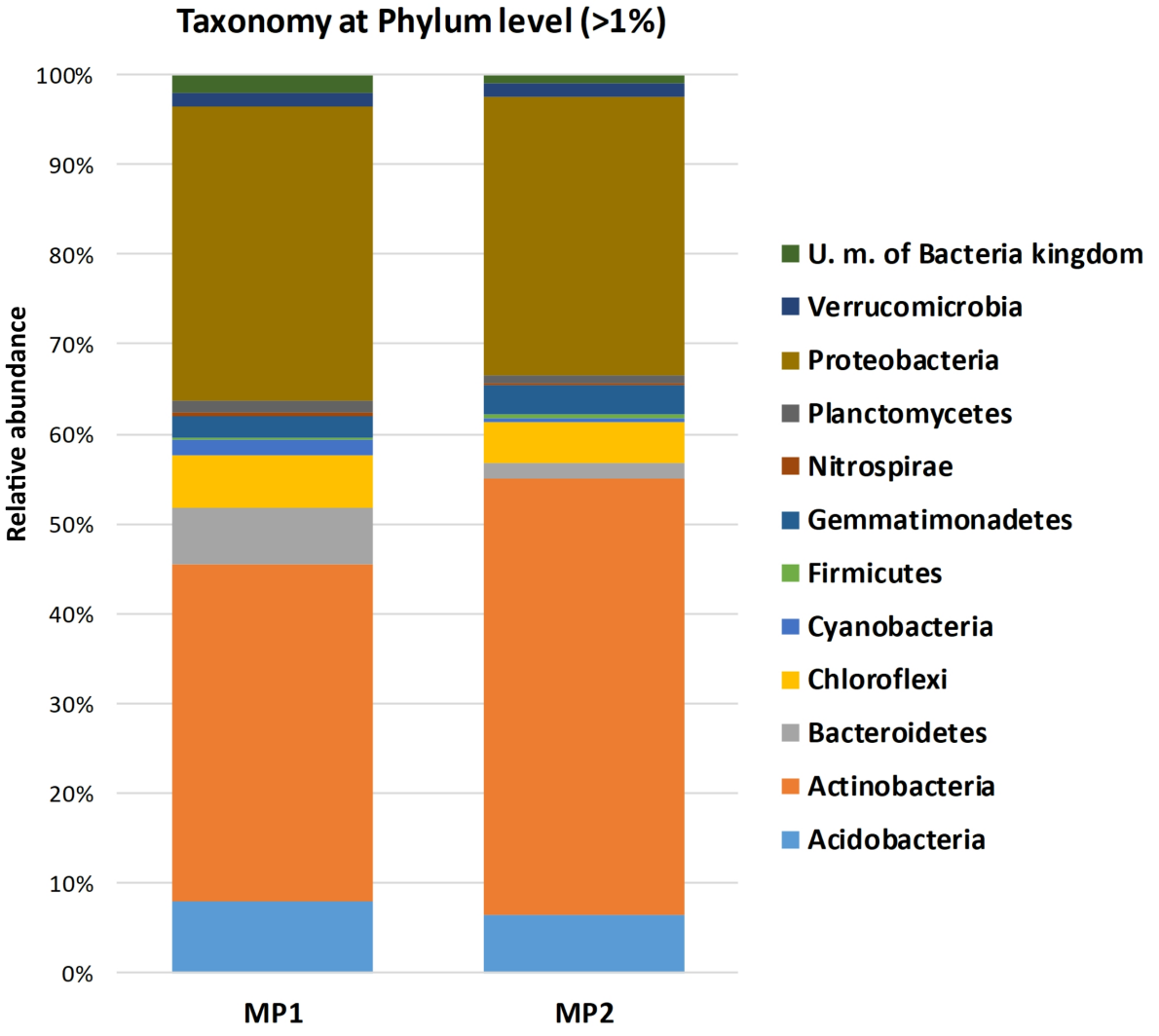
Taxonomy at Phylum level (>1%)
Relative abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
MP1
MP2
U. m. of Bacteria kingdom
Verrucomicrobia
Proteobacteria
Planctomycetes
Nitrospirae
Gemmatimonadetes
Firmicutes
Cyanobacteria
Chloroflexi
Bacteroidetes
Actinobacteria
Acidobacteria

Figure 2

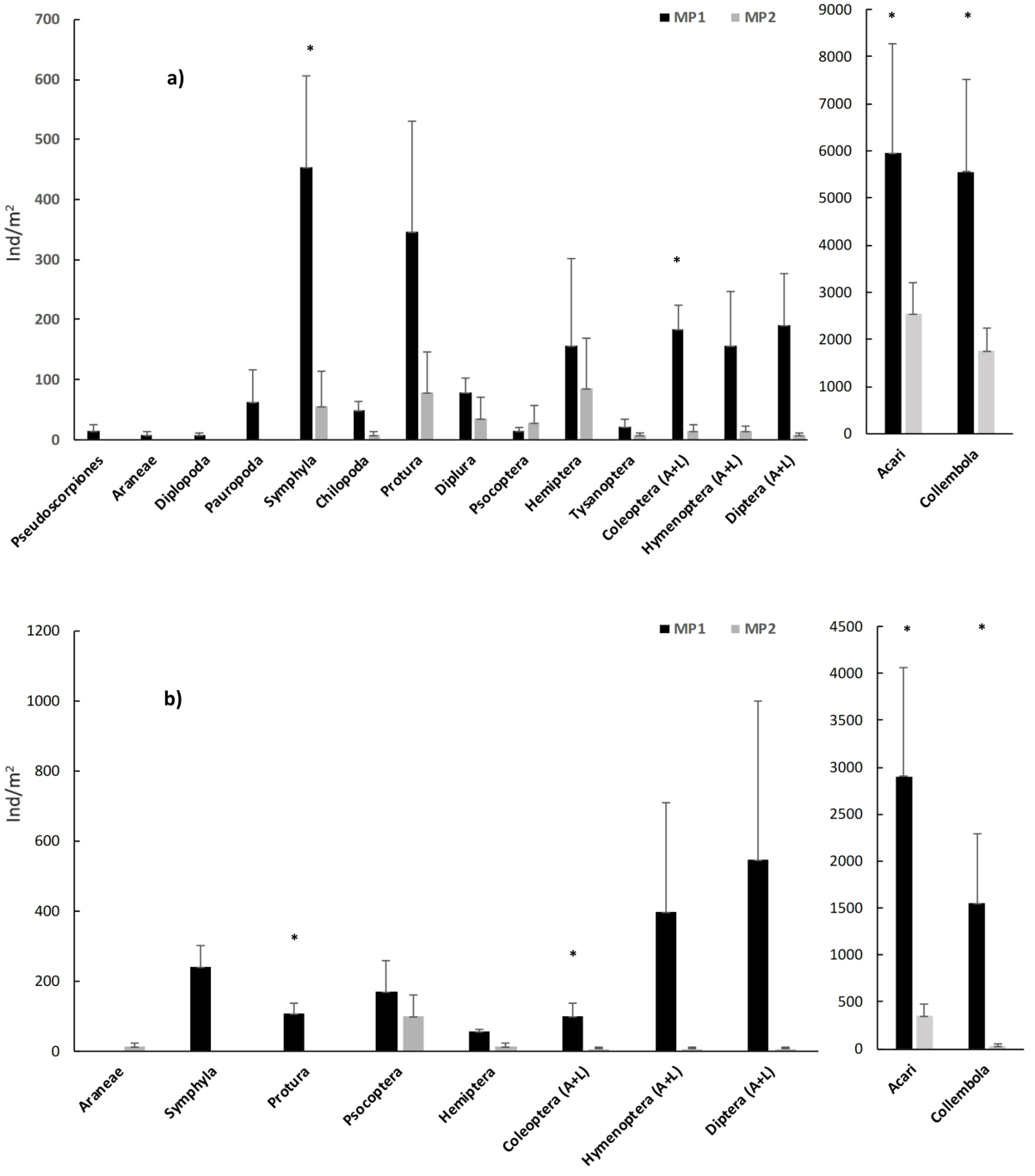
a)
MP1
MP2
Ind/m²
Pseudoscorpiones
Araneae
Diplopoda
Pauropoda
Symphyla
Chilopoda
Protura
Diplura
Psocoptera
Hemiptera
Tysanoptera
Coleoptera (A+L)
Hymenoptera (A+L)
Diptera (A+L)
Acari
Collembola
b)
MP1
MP2
Ind/m²
Araneae
Symphyla
Protura
Psocoptera
Hemiptera
Coleoptera (A+L)
Hymenoptera (A+L)
Diptera (A+L)
Acari
Collembola

Figure SI1: Pictures of MP1 and MP2 sites with GPS coordinates, and the corresponding *Noccaea caerulescens* plants growing at each site.

Figure SI2: Rarefaction curves generated for 16S rRNA gene sequences obtained from MP1 and MP2 samples. Panel a) displays the rarefaction curves using the Chao1 index. Panel b) displays rarefaction curves using the Shannon index

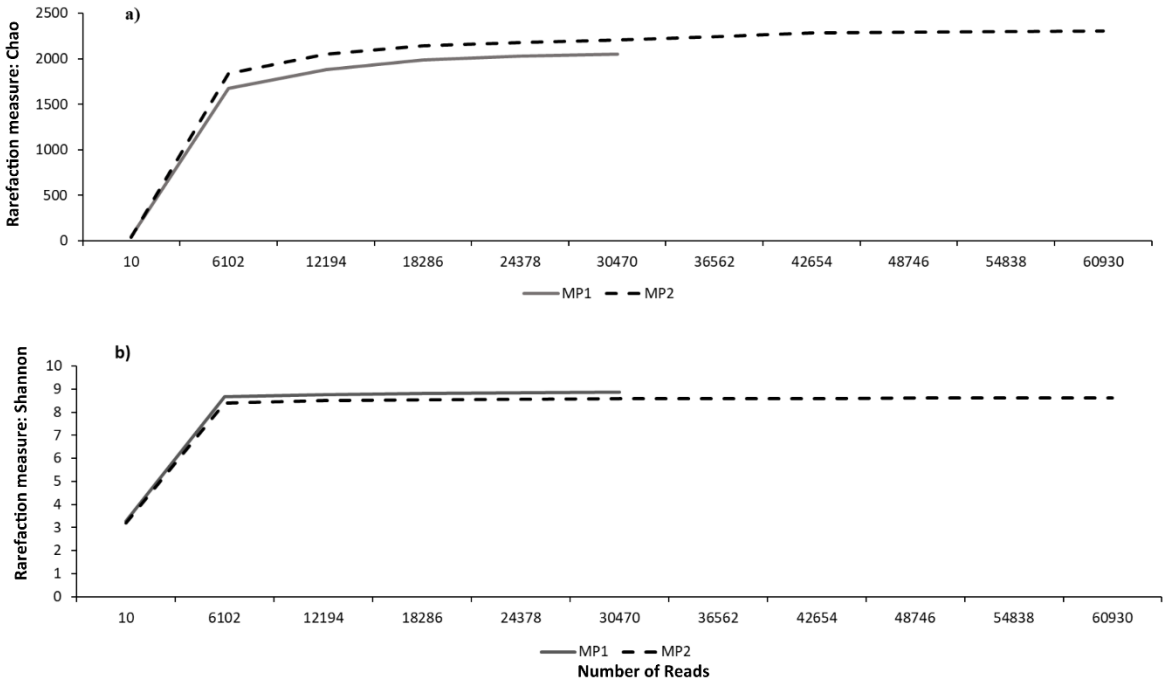

Figure SI3: Taxonomic distribution at family levels based on 16S rDNA reads across the two soil samples (MP1 and MP2) according to the percentage of sequences considered >1% at least in one sample (x-axis)

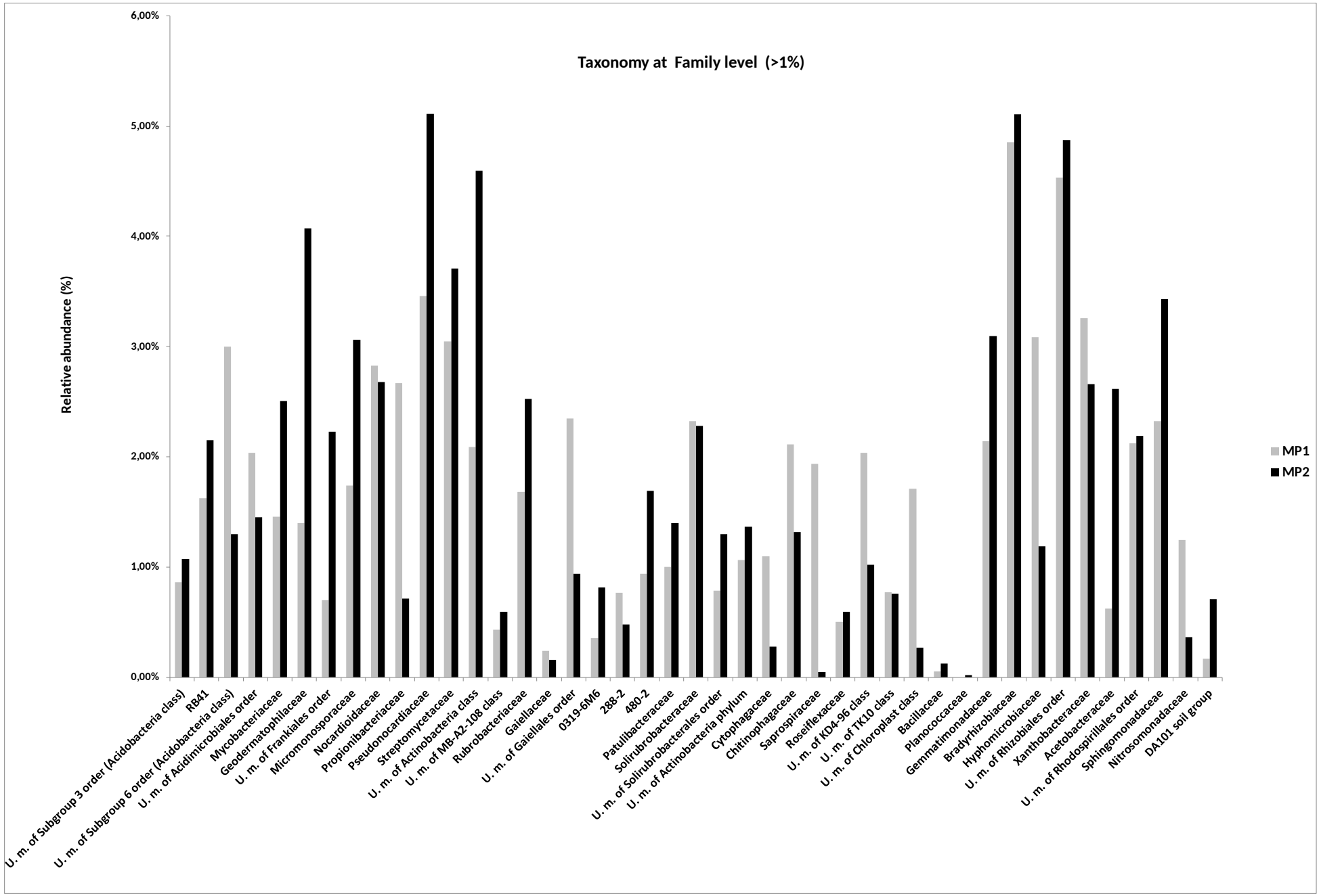
Taxonomy at Family level (>1%)
Relative abundance (%)
6,00%
5,00%
4,00%
3,00%
2,00%
1,00%
0,00%
MP1
MP2
U. m. of Subgroup 3 order (Acidobacteria class)
RB41
U. m. of Subgroup 6 order (Acidobacteria class)
U. m. of Acidimicrobiales order
Mycobacteriaceae
Geodermatophilaceae
U. m. of Frankiales order
Micromonosporaceae
Nocardioidaceae
Propionibacteriaceae
Pseudonocardiaceae
Streptomycetaceae
U. m. of Actinobacteria class
U. m. of MB-A2-108 class
Rubrobacteriaceae
Gaiellaceae
U. m. of Gaiellales order
0319-6M6
288-2
480-2
Patulibacteraceae
Solirubrobacteraceae
U. m. of Solirubrobacterales order
U. m. of Actinobacteria phylum
Cytophagaceae
Chitinophagaceae
Saprospiraceae
Roseiflexaceae
U. m. of KD4-96 class
U. m. of TK10 class
U. m. of Chloroplast class
Bacillaceae
Planococcaceae
Gemmatimonadaceae
Bradyrhizobiaceae
Hyphomicrobiaceae
U. m. of Rhizobiales order
Xanthobacteraceae
Acetobacteraceae
U. m. of Rhodospirillales order
Sphingomonadaceae
Nitrosomonadaceae
DA101 soil group

Table SI1: Soil mini-pit description

MP1 –Skeletic Mollic Leptosol

Horizon	Depth (cm)	Description
A	0-30	10 YR 3/2, sandy-loam, moderate fine granular structure, common rock fragments, abundant fine roots.
A/C	>30	10 YR 3/3, sandy, massive, abundant rock fragments

MP2 – Orthoskeletic Leptosol

Horizon	Depth (cm)	Description
A	0-25	10 YR 4/3, sandy, moderate fine granular structure, abundant rock fragments, common fine and medium roots.
A/C	>25	10 YR 4/4, sandy, massive, very abundant rock fragments

Table SI2: Plants (Genus and species and the corresponding Families) found in MP1 and MP2 site in Spring 2015 (x represents presence)

Family	Genus/species	MP1 site	MP2 site
Asteraceae	*Sonchus*	x	
	Artemisia annua		x
	Echinops		x
	Helichrysum italicum		x
	Hieracium pilosella		x
Boraginaceae	*Myosotis*	x	
Brassicaceae	*Biscutella coronipilolia*		x
	Noccaea caerulescens	x	x
	Alyssum bertolonii		x
Caryophyllaceae	*Silene*	x	
Ciperaceae	*Carex*	x	
Cornaceae	*Cornus sanguinea*	x	
Crassulaceae	*Sedum montanum*		x
Euphorbiaceae	*Euphorbia cyparissias*	x	x
Fabaceae	*Lotus cornicatus*	x	
	Robinia	x	
Hypericaceae	*Hypericum perfoliatun*	x	
Lamiaceae	*Thymus*		x
Orobanchaceae			x
Plantaginaceae	*Globularia bisnagarica*		x
Poaceae	*Sesleria pichiana*		x
	Bromus	x	
	Poa	x	
Ranunculaceae	*Clematis vitalba*	x	
Rosaceae	*Rubus fruticosus*	x	
Rubiaceae	*Galium*	x	x
Umbelliferae			x

Table SI3: Data filtering report of single sample runs. MP1=rhizosphere of N. caerulescens plants at MP1 site MP2= rhizosphere of N. caerulescens plants at MP2 site

Sample	Number of sequenced pe reads	Number of pe reads with mean quality > 20	Number of merged pe reads	Number of reads removed because of:						Final Read Number
				Human sequences	Outside bounds (100-400)	Ambiguous bases	Homopolymers > 7	Mismatch in primers >1	Reverse primer not found	
MP1	48739	48168	46668	65	0	0	23	2254	19	44307
MP2	104743	103865	100055	36	0	0	8	5532	29	94449