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Abstract

Microbial communities dwelling in biogenic structures shaped by soil macroorganisms (e.g. rhizosphere of plants, drilosphere of earthworms) are often compared to communities in the bulk soil taken as a control. Two strategies are currently applied, by sampling either bulk soil surrounding the biogenic structures inside the same experimental unit ("surrounding bulk") or soil from a distinct control unit without macroorganism ("pristine bulk"). While surrounding bulk is commonly used, no studies explicitly compared these two bulk types. Moreover, the potential effect of plants and earthworms on microbial communities in the surrounding bulk could depend on soil properties. In controlled conditions, we exposed three soils with contrasting properties to either a plant, earthworms, both, or without macroorganisms (pristine bulk). Root-adhering soil, casts and their surrounding bulk were retrieved by meticulous sampling. We found that molecular abundances of bacteria, fungi and archaea were modified in surrounding compared to pristine bulk. In a non-trivial manner, bacterial community structure from surrounding bulk was significantly altered by plants in all soils, while the influence of earthworms was soil-dependent, in a way related to C and N contents rather than texture. When comparing macroorganism influenced versus non-influenced soils, the pristine bulk should thus be prefered, whereas the surrounding bulk is appropriate to characterize the sphere of influence of biogenic structures.

Keywords: Bacteria community structure; earthworm; experimental control; microbial abundance; plant; soil matrix

Soil physics, biogeochemistry and ecology studies investigating the effect of organisms on soil properties all require an experimental control. When studying the effects of these macroorganisms on soil microbial communities dwelling in biogenic structures, e.g. rhizosphere or drilosphere (Lavelle, 2002), two different methodological approaches relying on their own controls exist. The first assesses the impact of macroorganisms by comparing microbial communities of the biogenic structures with the surrounding and visually non-aggregated soil inside the same experimental unit (so-called "surrounding bulk soil") (Lundberg et al., 2012; Monard et al., 2011; Tkacz et al., 2015; Whalley et al., 2005). The second, less frequent, compares biogenic structures with the bulk soil of an independent control unit, without macroorganisms (so-called "pristine bulk soil") (Bomberg et al., 2011; Edwards et al., 2015; Hoeffner et al., 2018). Because controls are fundamentally used to calculate effect size, which enables generalization in meta-analyses (Borenstein et al., 2009), standardizing the reference situation is crucial. However, if beyond the biogenic structure, the surrounding bulk soil is also affected by the presence of a macroorganism (Lipiec et al., 2016), this could introduce a bias in effect size estimation. This potential long-range influence of macroorganisms on surrounding bulk microbial communities may depend on soil properties, which could modify microorganisms dispersal and growth. For instance, soil texture is expected to affect passive dispersal of microbes with water fluxes, while organic matter content may affect sorption and resource availability for microbes (Lindqvist and Bengtsson, 1991). Moreover, while plants gradually influence the soil from the rhizoplane towards the bulk soil (Tkacz et al., 2015), earthworms ingest and transform soil into casts (Medina-sauza et al., 2019). Thus, soil microbial dispersion patterns may significant differ depending on the macroorganism and its associated biogenic structure, with a potential interaction with the type of soil considered.

While effects of plants and earthworms on rhizosphere and drilosphere microbial communities are extensively studied, there is, to the best of our knowledge, no investigation aiming at comparing the different bulk soil controls (the surrounding and pristine bulks) to determine to which extent they might differ. Here, we hypothesized that (h1) microbial communities dwelling in the bulk soil

surrounding the rhizosphere and the drilosphere differ from those living in pristine bulk soil, and (h2) effects of plants and earthworms on surrounding bulk microbial communities depend on soil properties. We thus compared microbial communities from the bulk soil surrounding root-adhering soil and casts to the pristine bulk of a control treatment without macroorganisms in a controlled experiment using three contrasting soils (sand, loam, clay, properties described in Table S1) under the presence/absence of one plant (barley, *Hordeum vulgare* L. (1753)), three endogeic earthworms (*Aporrectodea caliginosa caliginosa,* Bouché (1972)), with a total average weight of 1 g) or both (design in Fig. S1). Microcosms were made of 1 kg of dry soil, maintained at 80% of the field capacity in a climatic chamber at 18/20°C night/day, 75% air humidity and 12h photoperiod for 28 days.

Soil at 70 % w/w humidity was meticulously dismantled by manual sorting of aggregates, i.e. root-adhering soil for the rhizosphere and belowground casts for the drilosphere (no burrow sampling). As soil was sieved before the experiment, it was easy to visually identified casts as round-shaped aggregates (Velasquez et al., 2007), wheareas the surrounding bulk consisted in non aggregated soil. We focused on molecular microbial abundances (i.e. bacteria, archaea and fungi) based on quantitative PCR estimates, followed by bacterial community structure *via* 16S rRNA gene amplicon sequencing due to their importance in plant-earthworm interactions (Medina-sauza et al., 2019). A detailed description of the experimental protocol, sampling and molecular analyses is available in a previous study (Jacquiod et al., 2020).

First, we focussed on the effects of plant and earthworms on the abundance of bacteria, fungi and archaea in the surrounding bulk relative to the pristine bulk (Fig. 1, with z-score corresponding to molecular copy counts standardized against average and standard deviation values of the pristine bulk from the same soil). Plants were responsible for an increased abundance of bacteria in the soil surrounding the rhizospheres, whereas earthworms reduced it in the soil surrounding the casts, with a neutralization of effects when both macroorganisms were present (Fig. 1a). Plants had no effect on archaea but earthworms had a strong negative effect that disappeared under the presence of plant (Fig. 1b). Finally, plants stimulated the fungal abundance, but no significant effect was observed for earthworms and the combination of both macroorganisms (Fig. 1c). Bacteria, archaea and fungal

88 abundances were thus all influenced in the bulk surrounding biogenic structures (h1), being often
89 stimulated by the plant while inhibited by earthworms.

90 Bacteria community structure clearly differed inside and outside biogenic structures, ruling out the
91 possibility that we inadvertently mixed root-adhering soil or casts with the surrounding bulk during
92 sampling. Indeed, rhizospheres (Fig. 2a-c) separated from surrounding and pristine bulks for all soils
93 (CAP1 = 34-55% of the explained variance). Casts separated from surrounding and pristine bulks in
94 the sand soil (Fig. 2f, CAP1 = 20%), and to a lesser extent in the clay soil (Fig. 2d, CAP2 = 8%).

95 Regarding our two hypotheses, we found that the bulk soil surrounding rhizospheres was always
96 separated from the pristine bulk in all soils, although weakly (CAP2 = 4-8%, Fig. 2a-c). The pattern
97 was variable for earthworms (Fig. 2d-f): in the clay soil, the main source of variance was the
98 difference between the surrounding and pristine bulk (CAP1 = 23%), being less important for the sand
99 soil (CAP2 = 6%) and not significant for the loam soil (p = 0.07). Plants and earthworms were thus
100 influencing bacterial community structure beyond the physical boundaries of their own biogenic
101 structures (h1), whatever the soil for plants but only in some soils for earthworms (h2). A confirmation
102 of the soil-independent plant effect and soil-dependent earthworm effect was observed when both
103 macroorganisms were present (Fig. 2g-i).

104 When restricting the analysis to the bulk samples of the four treatments (pristine bulk in the control
105 without macroorganisms and surrounding bulk in the others) (Fig. S2), we found that bacterial
106 communities differed significantly between the different bulks (h1), except in the loam soil when
107 considering only the presence/absence of Operational Taxonomic Units (OTUs) (Fig. S2b). In the clay
108 soil, the presence of the plant, earthworms and their interaction had a significant effect on the bacterial
109 community structure in the different bulks, either based on presence/absence (Fig. S2a) or abundance
110 (Fig. S2d). For the sand soil, only the plant effect was significant (Fig. S2c and f). For the loam soil,
111 plant and earthworm effects were found only based on abundances (Fig. S2e). These results concur
112 with Fig. 2, stressing the soil-dependent effect of earthworms and their interaction with plant (h2).

113 Analysis of OTUs distribution from these four bulks in Venn diagrams (systematic presence amongst
114 the four biological replicates in the three soils simultaneously) showed diversity fractions specificaly
115 found in each bulk soil and not shared with the others (Fig. S3). There were 15 OTUs specific of
116 pristine bulks, 19 from cast-surrounding bulks, 16 from rhizosphere-surrounding bulks and 18 from
117 cast and rhizosphere-surrounding bulks (Fig. S3a). In the four bulks, about 80% of these specific
118 OTUs were members of the Actinobacteria, Proteobacteria and Acidobacteria (Fig. S3b), which are
119 taxa typically reported in casts (Medina-sauza et al., 2019) or rhizosphere (Philippot et al., 2013).

120 Our results demonstrated that the influence of macroorganisms on microbial abundance (Fig 1) and
121 community structure (Fig. 2) goes beyond what is condidered as rhizosphere and drilosphere on the
122 basis of physical aggregation. Some studies have investigated the gradual influence of a
123 macroorganism on microbial community structures by comparing the endosphere, rhizoplane,
124 rhizosphere and bulk soil (Edwards et al., 2015), or on enzymatic activities and functional diversity
125 between the burrow wall (0-3 mm from the burrow), the "transitional zone" (3-7 mm), the bulk soil (at
126 least 20 mm) and the casts (Lipiec et al., 2016). Our study shows that when the research objectives are
127 to quantify the effect of a macroorganism on microbial communities by comparing two *a priori*
128 "influenced" and "non-influenced" soils or to characterize the influence gradient of a macroorganism,
129 pristine bulk soil without macroorganism should be preffered as a control when the range of influence
130 is not known. Otherwise, the effect size of a macroorganism influence could be biased by not taking
131 into account its impact on the bulk soil surrounding biogenic structures. While this can be easily
132 achieved with microcosms, including such controls can be challenging for field experiments (Furlong
133 et al., 2002; Smalla et al., 2001). Meta-analyses aiming at establishing quantitative assessments of
134 plants, earthworms (and likely other soil invertebrates) on microbial communities should consider the
135 type of control bulk, either for laboratory or field experiments, through "sub-group analyses"
136 (Borenstein et al., 2009).

137 Our study also shows that soil properties interacted with macroorganism's influence in a non-trivial
138 way: they were modifying the influence of earthworms but not plants (Fig. 2 & S2). The aim of this
139 study was not to identify a specific soil property associated with the soil-dependant effect of

earthworms. Noteworthy, we observed that the progressive decrease in the effect size from the clay, to sand and loam soils was not correlating with soil texture, which was predicted to alterwater fluxes and microbes' dispersal. Instead, it was correlating with carbon and nitrogen contents (Table S1), which could reflect the nutrient availability status for microorganisms. The soil-independent effect of plant may be due to volatile signals, known to trigger specific microbial responses (Lebeis et al., 2015), or a stronger priming of microbial activities by plants compared to earthworms. The specific OTU fractions identified in the pristine, cast-surrounding, rhizosphere-surrounding and cast/rhizosphere-surrounding bulks respectively, showed that bacterial species can segregate their niches according to the influence of macroorganisms and their interactions, even in the non-aggregated soil. A precise sampling with relevant control will become mandatory to understand the complex interaction network between soil macroorganism's and microorganism's communities.

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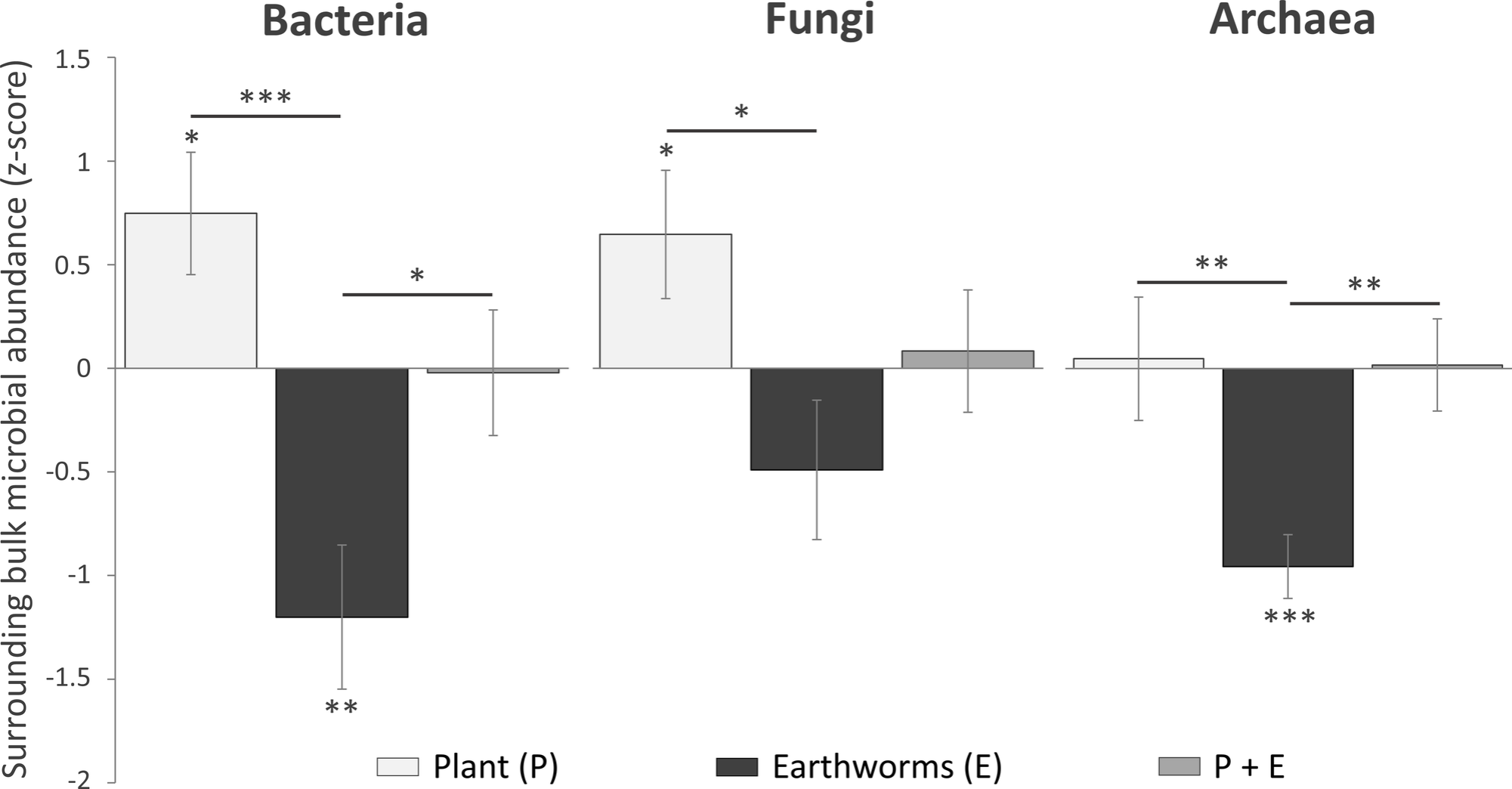
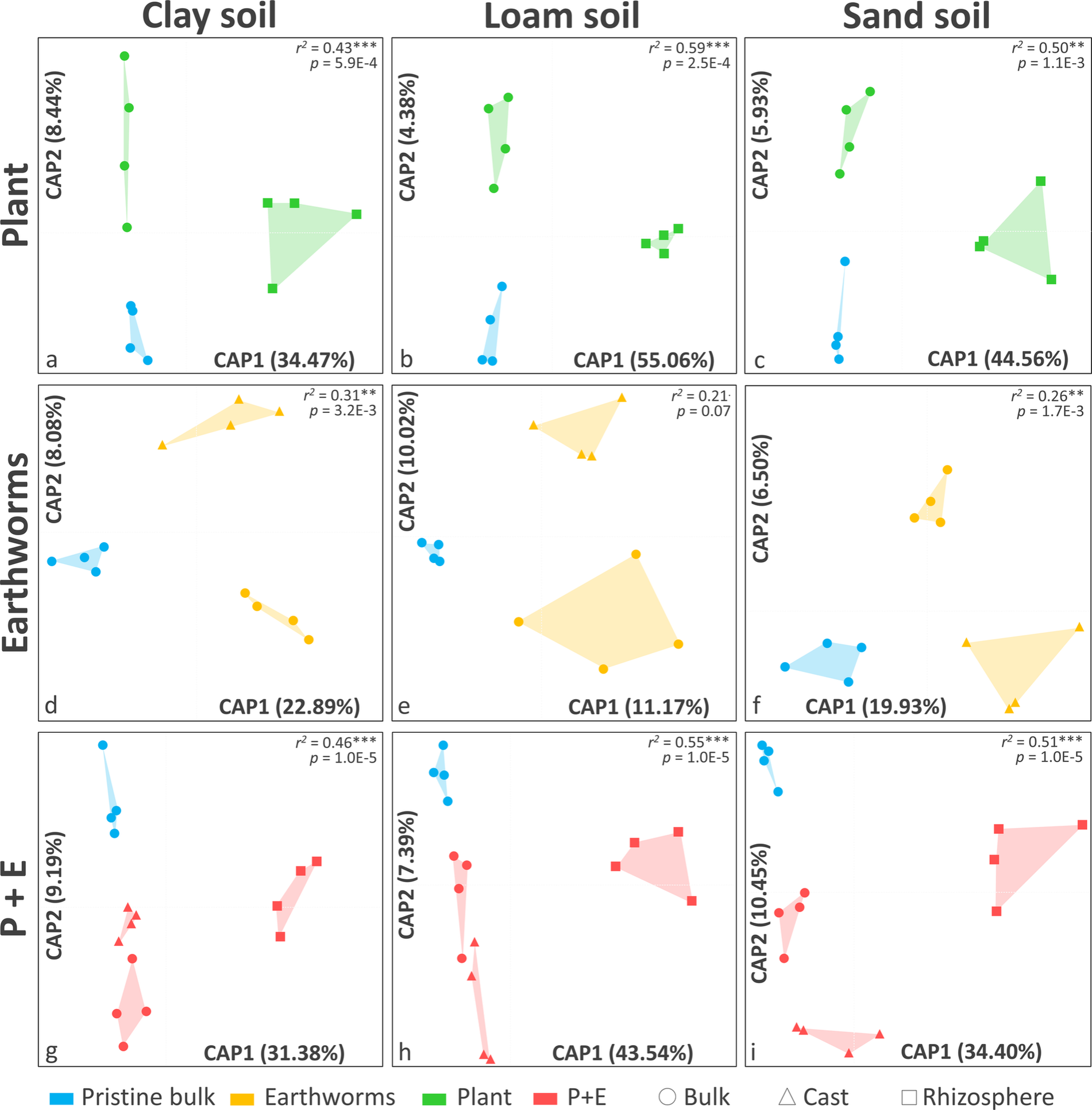
Figure legends

Fig. 1. qPCR estimation of bacterial (16S rRNA), fungal (ITS) and archaeal (16S rRNA) genetic markers in the surronding bulk, reported to the pristine bulk (0 value). Molecular copy counts were standardized against average and standard deviation values of pristine bulk from the same soil (z-score). Bar charts are representing z-score averages ± standard error (n = 3–5). Significance between treatments were assessed by two-sample, two-sided Student tests (top horizontal lines between treatments). Significance relative to the reference pristine bulk (0 value) were assessed by one-sample, two-sided Student tests (indications above bars). Significance codes: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; • $p < 0.1$.

Fig. 2. Distance-based redundancy analysis showing the principal constrained coordinates of bacterial communities in each soil (weighted unifrac distances, 10.000 permutations). The four treatments are indicated by different colors ; microhabitats (bulk soil, rhizosphere, casts) are indicated by different marker shapes.


Bacteria
Fungi
Archaea
Surrounding bulk microbial abundance (z-score)
1.5
1
0.5
0
-0.5
-1
-1.5
-2
***
*
*
*
*
**
**
**
***
Plant (P)
Earthworms (E)
P + E


Clay soil
Loam soil
Sand soil
Plant
Earthworms
P + E
r^2 = 0.43*** p = 5.9E-4
CAP2 (8.44%)
CAP1 (34.47%)
a
r^2 = 0.59*** p = 2.5E-4
CAP2 (4.38%)
CAP1 (55.06%)
b
r^2 = 0.50** p = 1.1E-3
CAP2 (5.93%)
CAP1 (44.56%)
c
r^2 = 0.31** p = 3.2E-3
CAP2 (8.08%)
CAP1 (22.89%)
d
r^2 = 0.21· p = 0.07
CAP2 (10.02%)
CAP1 (11.17%)
e
r^2 = 0.26** p = 1.7E-3
CAP2 (6.50%)
CAP1 (19.93%)
f
r^2 = 0.46*** p = 1.0E-5
CAP2 (9.19%)
CAP1 (31.38%)
g
r^2 = 0.55*** p = 1.0E-5
CAP2 (7.39%)
CAP1 (43.54%)
h
r^2 = 0.51*** p = 1.0E-5
CAP2 (10.45%)
CAP1 (34.40%)
i
Pristine bulk
Earthworms
Plant
P+E
Bulk
Cast
Rhizosphere