# Long-term phytomanagement with compost and a sunflower - Tobacco rotation influences the structural microbial diversity of a Cu-contaminated soil

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**Abstract**

At a former wood preservation site contaminated with Cu, various phytomanagement options have been assessed in the last decade through physicochemical, ecotoxicological and biological assays. In a field trial at this site, phytomanagement with a crop rotation based on tobacco and sunflower, combined with the incorporation of compost and dolomitic limestone, has proved to be efficient in Cu-associated risk mitigation, ecological soil functions recovery and net gain of economic and social benefits. To demonstrate the long-term effectiveness and sustainability of phytomanagement, we assessed here the influence of this remediation option on the diversity, composition and structure of microbial communities over time, through a metabarcoding approach. After 9 years of phytomanagement, no overall effect was identified on microbial diversity; the soil amendments, notably the repeated compost application, led to shifts in soil microbial populations. This phytomanagement option induced changes in the composition of soil microbial communities, promoting the growth of microbial groups belonging to *Alphaproteobacteria*, many being involved in N cycling. Populations of *Nitrososphaeria*, which are crucial in nitrification, as well as taxa from phyla *Planctomycetacia*, *Chloroflexi* and *Gemmatimonadetes*, which are tolerant to metal contamination and adapted to oligotrophic soil conditions, decreased in amended phytomanaged plots. Our study provides an insight into population dynamics within soil microbial communities under long-term phytomanagement, in line with the assessment of soil ecological functions and their recovery.

**Keywords**: phytoremediation; metal pollution; soil functions; organic amendments; metabarcoding.

## 1. Introduction

Long-term phytomanagement of metal(loid)-contaminated soils can (1) reduce soil phytotoxicity, promoting ecological soil functions while preserving the soil resource (Quintela-Sabaris et al., 2017; Mench et al., 2018), and (2) produce raw materials for local biomass processing technologies and incomes for the local communities (Mench et al., 2010; Evangelou et al., 2012; Witters et al., 2012; Van Slycken et al., 2013; Cundy et al., 2016). This was notably shown for Cu-contaminated soils due to mining and wood preservation activities (Kidd et al., 2015; Touceda-González et al., 2017a; Touceda-González et al., 2017b; Mench et al., 2018), which are often characterized by unfavourable soil properties, e.g. lack of structure with low OM content, low nutrient availability and acidic pH (Mench and Bes, 2009; Bes et al., 2010; Hattab-Hambli et al., 2016; Oustriere et al., 2016), .

The Biogeco site (a former wood preservation site, St-Médard d'Eyrans, France; Cu-contaminated soils) has received a lot of attention with the aim of demonstrating the benefits of long-term phytomanagement (Kolbas et al., 2011; Kumpiene et al., 2011; Marchand et al., 2011; Hattab-Hambli et al., 2016; Oustriere et al., 2016; Mench et al., 2018). Here, soil amendments, i.e. a single incorporation of compost combined with dolomitic limestone in year 1 (OMDL) and this OMDL treatment followed by compost application renewed in year 6 (OM2DL), and high yielding crops (sunflower, tobacco) improved soil organic matter (OM) and nutrient contents, soil pH and CEC and sustainably decreased Cu availability in year 9 (Mench et al., 2018). Both OMDL and OM2DL treatments led to higher shoot DW yields and Cu removals than the untreated treatment (UNT). Similarly, at the Touro site, NW Spain, compost incorporation into Cu-rich mine tailings, in combination with planting of *Salix* spp., *Populus nigra* L. and *Agrostis capillaris* L, improved soil properties, i.e. pH, CEC and fertility, and decreased

74 soil Cu availability, which notably promoted the growth of *Salix viminalis* L. and *A.*
75 *capillaris* (Touceda-González et al., 2017a).

76 Beside crop biomass and soil physico-chemical properties, there are increasing
77 evidences that phytomanagement, combined with soil amendments, can influence soil
78 microbial communities in the long term. Soil microorganisms are pivotal in the delivery
79 of numerous soil functions and underlying ecosystem services and the success of any
80 gentle remediation option (GRO), such as phytomanagement, should be evaluated in
81 terms of soil function recovery, through the assessment of soil microbial properties
82 (Epelde et al., 2009; Kumpiene et al., 2009; Burges et al., 2018). Phytomanagement
83 increased soil microbial biomass and activity at three out of six field trials with
84 metal(loid)-contaminated soils across Europe, obtaining the most pronounced effects at
85 the Biogeco site (Touceda-González et al., 2017b). At this one, enzyme activities
86 involved in the biogeochemical cycles of C, N, P, and S were 2 to 11-fold higher in
87 amended soils as compared to untreated soils. Furthermore, according to Touceda-
88 González et al. (2017b), changes in specific phylogenetic microbial groups could be
89 more responsive and informative about the effect of phytomanagement on microbial
90 communities than those observed in the community as a whole. In this respect,
91 phytomanagement also induced shifts in the microbial community structure and
92 increased the abundance of genes involved in the N cycle (*nirK*, *nirS*, *nosZ*, and *amoA*).
93 Similarly, at the Touro site, both compost-amendment and plant root activity induced
94 shifts in the bacterial community structure in year 3, along with enzyme activities
95 stimulation (Touceda-González et al., 2017a).

96 Other datasets were gained at the Biogeco site, over time and in field trials with
97 various plant covers. Biochemical activity and functional gene diversity were studied in
98 field plots revegetated with a mixed stand of willows, black poplar, and false indigo

bush, and amended or not with OMDL (Lagomarsino et al., 2011; Xue et al., 2018). In year 6, the OMDL treatment reduced Cu availability and soil toxicity, and increased microbial biomass and activity, as well as microbial functional diversity, including genes encoding for metal resistance, as compared to the unamended soil (UNT) (Xue et al., 2018).

Considering the key role of microbes in soil ecological processes, larger, biochemically more active and genetically more diverse microbial communities, as observed in above-mentioned studies, could suggest the recovery in soil functioning with phytomanagement. At the Biogeco site, one remaining question is the influence of the vegetation cover and soil amendments on soil microbial communities over time. Gathering long-term data based on evidence from the field is essential to demonstrate the sustainability and efficiency of phytotechnologies (Mench et al., 2010; Kidd et al., 2015; Cundy et al., 2016). Here, we decided to contribute to the monitoring of microbial communities in long-term phytomanaged sites with a metabarcoding approach. We assessed, at year 9, the effect of an application of compost and dolomitic limestone, with and without a renewed compost application , and annual rotation crop on the diversity, composition (the presence or the absence of microbial taxa) and structure (the relative abundance of the microbial taxa) of soil microbial communities.

## 2. Materials and methods

### *2.1 Site and experimental design*

The wood preservation site (about 10 ha, only 2 ha remaining in activity) is located at Saint-Médard d'Eyrans, Gironde, SW France (N 44°43.353, W 000°30.938) with a temperate Atlantic climate (variable mean rainfall and temperature; in 2017: 736 mm, 14.4 °C). Site history, soil characterization and zoning of soil ecotoxicity were

124 previously reported (Mench and Bes, 2009; Bes et al., 2010; Kolbas et al., 2011). Plant
125 communities were characterized in Bes et al. (2010). Copper is the major inorganic
126 contaminant in topsoil at the P1-3 sub-site with a high spatial variation (163-1170 mg
127 Cu $kg^{-1}$), while As, Zn, Cr and other metal(loid)s were at their background levels, and
128 some polycyclic aromatic hydrocarbons (PAH) reached high concentrations (in mg $kg^{-1}$
129 soil DW): fluoranthene (1.9), indeno[1,2,3-cd]pyrene (0.95), benzo[g,h,i]perylene (0.8),
130 and benzo[b]fluoranthene (0.8) (Mench and Bes, 2009; Kolbas et al., 2011). Soil texture
131 is sandy, i.e. 85.8% sand, 5.9% clay, and 8.3% silt, with 1.6% OM, C/N 17, soil pH 7,
132 and a low CEC (3.5 cmol $kg^{-1}$) (Kolbas et al., 2011).
133 The field trial, located at the P1-3 sub-site, consists of 4 blocks (2m x 10m): B1,
134 B2, B3 and B4 (Fig. 1). In March 2008 (year 1), plots in B1, B2 and B3 were amended
135 with compost (5% w/w, made from poultry manure and pine bark chips) and dolomitic
136 limestone (0.2% w/w). The amendment was mixed in the topsoil (0-0.25 m) with a
137 stainless steel spade after soil loosening (Marchand et al., 2011). In 2013, a second
138 compost dressing (5% w/w, made with green waste) was incorporated to half of the
139 plots in B1-B3 (hereafter referred to as OM2DL), while the remaining plots were not
140 additionally amended (OMDL), making 4 replicated plots per treatment and per block.
141 The composition of OMDL and OM2DL soil amendments are detailed in Mench et al.
142 (2018). The plot from B4 remained unamended throughout the whole study (UNT). All
143 plots were cultivated with an annual tobacco -sunflower rotation since 2008.
144
145 *2.2 Soil sampling and physicochemical characterization*
146 In March 2017 (year 9) four soil samples were collected from the topsoil (0–10 cm) of
147 each plot with a stainless sampling cylinder (ø 3.6 cm X 11.5 cm). Once fresh weight
148 was determined for soil bulk density, the four replicated samples were combined to

149 produce a composite soil sample (1 kg fresh weight, FW) for each plot of B1-B3, while
150 the four samples from the UNT plot in B4 were treated as separate samples, making a
151 total of 28 samples for analysis. Then, samples were air-dried, sieved at 2 mm and
152 manually homogenized. Subsamples (150 g FW) for soil microbial analysis were stored
153 fresh at -20°C until analysis. Element concentrations in soil samples, determined using
154 ICP-AES, and other physicochemical parameters were analysed following standard
155 methods and standard quality assurance employed by INRA LAS, Arras, France (2014)
156 (Mench et al., 2018).
157
158 *2.3 DNA extraction and sequencing, and bioinformatic analysis*
159 DNA was extracted from soil samples (0.25 g dry weight, DW) using Power Soil™ kits
160 (Mo Bio). Prior to DNA extraction, soil samples were washed twice in 120 mM
161 $K_2HPO_4$ (pH 8.0) to remove extracellular DNA (Kowalchuk et al., 1997).
162 Metabarcoding (amplicon) library preparations were carried out using a dual
163 indexing approach (Lanzen et al. (2016). Briefly, the V3-V4 hypervariable region of the
164 small subunit ribosomal RNA (SSU rRNA) was amplified from prokaryotes using the
165 primers 519F (CAGCMGCCGCGGTAA) adapted from Øvreås et al. (1997), and 806R
166 (GGACTACHVGGGTWTCTAAT) from Caporaso et al. (2012). Adapter-linked primer
167 pairs were used during a first PCR, followed by cleaning and a second PCR with
168 adapters linked to sample-specific barcodes. Pair-ended sequencing was carried out
169 using an Illumina MiSeq with the V2 kit at Tecnalia Corporation (Miñano, Spain).
170 Sequence read-pairs were quality-filtered and overlapped using vsearch (default
171 parameters; Rognes et al., 2016) and resulting sequences trimmed to remove N5 and
172 primer sequences, using cutadapt (Martin, 2011). Sequences were then truncated to 253
173 nt using vsearch, removing shorter sequences or those low quality based on expected

174 incorrect read calls (fastq_maxee=0.5). All quality-filtered overlapped sequences were
175 then clustered into OTUs using Swarm v2 (Mahé et al., 2015). Singleton Swarm OTUs
176 were removed and the remaining subjected to both *de novo* and reference based chimera
177 filtering (with the rdp_gold reference database), using vsearch (UCHIME algorithm).
178 Remaining Swarm OTUs were further clustered into fixed similarity OTUs, taking into
179 account total read abundances, and using a maximum sequence divergence threshold of
180 3%, again using vsearch (Rognes et al., 2016). OTU abundances were obtained by
181 mapping reads back to the representative OTU sequences.
182 Taxonomic classification was carried out by aligning representative OTU
183 sequences to the SilvaMod database (v128) using blastn (v.2.2.25 + task megablast) and
184 the LCAClassifier of CREST (default parameters; Lanzén et al., 2012;
185 https://github.com/lanzen/CREST). Unclassified OTUs below the alignment threshold
186 and those classified as belonging to eukaryotic organellar rRNA were excluded from
187 further analysis.
188
189 *2.4 Bioinformatic and statistical analysis*
190 The effect of soil treatments on soil physicochemical properties, diversity indices, and
191 relative abundance of classified bacterial taxa (per phylogenetic level) was assessed by
192 means of one-way analysis of variance (ANOVA) using R. When significant differences
193 occurred between soil treatments, multiple comparisons of mean values were made
194 using post-hoc Tukey HSD test. A high variance in sequencing depth (number of reads)
195 occurred between samples, indicating that total OTU richness may not be an unbiased
196 measure of α-diversity in our study. Therefore, rarefied richness was evaluated, using
197 the read number of the smallest included dataset (7850 reads), along with Shannon (H')
198 and Simpson diversity indices. The influence of soil treatments on α-diversity indices

199 was further assessed accounting for the considerable variability in soil Cu across the
200 replicated plots (Fig. 1): (i) taking total soil Cu as co-variable, by means of analysis of
201 covariance (ANCOVA) and (ii) considering only plots with similarly higher values of
202 total soil Cu in the analysis, i.e. B3 and B4, by means of ANOVA. Even though total
203 soil Cu does not directly reflect the bioavailable fraction subject to interact with
204 organisms, values of 1 M $NH_4NO_3$-extractable Cu in the UNT plot were far beyond the
205 range of variability of values from OMDL and OM2DL plots (Fig. 1.b), making this
206 parameter unreliable, both as co-variable and categorizing factor, for the comparison of
207 α-diversity metrics among soil treatments.

208 Multivariate statistics, calculation of diversity indices and visualization of the
209 amplicon sequencing data was performed using the R package *vegan* (Oksanen et al.,
210 2019). Function *decostand* was used to transform OTU distributions into relative
211 abundances. Bray-Curtis dissimilarity matrices comparing community composition
212 between samples were calculated, as described by Lanzén et al. (2016), and were
213 subsequently used to perform non-metric multidimensional scaling (NMDS) with
214 function *metaMDS*. These matrices were also used to assess the significance of the
215 effect of soil treatments on the composition of microbial communities by means of
216 permutational multivariate analysis of variance (PERMANOVA).

217 We also explored the relationship between soil microbial communities and
218 changes in soil physicochemical properties induced by soil treatments. Firstly, a
219 selection of soil physicochemical parameters was fitted to the resulting NMDS space
220 using the function *envfit*. We used analysis of similarities (ANOSIM) based on Bray-
221 Curtis dissimilarities to evaluate the significance of the influence of soil
222 physicochemical properties on soil microbial composition. These correlation analyses
223 were subjected to Bonferroni correction and not reported unless $p<0.05$ after correction.

Only significantly correlated physicochemical properties were used in further analyses. In parallel, function *bioenv* was used to find the subset of physicochemical parameters that together showed maximum correlation with community dissimilarity. Secondly, multivariate analyses were performed by means of redundancy analysis (RDA) and variation partitioning analysis, using Canoco 5 (ter Braak and Šmilauer, 2012), with physicochemical properties and (i) α-diversity metrics, and (ii) abundance of the main bacterial taxa, at class level, that showed significant differences due to soil treatments.

## 3. Results

### *3.1 Analysis of soil microbial α-diversity*

In total, amplicon sequencing resulted in 1,042,643 16S rRNA reads, which clustered into 10,164 OTUs, after quality filtering and removal of singletons. No direct correlation between soil treatments and α-diversity estimates could be identified (Table 1). Accounting for the background influence of soil Cu variability among the plots showed that soil treatments did not significantly affect α-diversity metrics either (Fig. 2). An interaction between soil treatment and total soil Cu was found for Shannon diversity index, corroborating that its values increased in the OM2DL plots with increasing levels of total soil Cu, while they decreased in the OMDL and UNT plots. When considering only plots with similarly higher soil Cu levels, higher values of Shannon diversity were also found in the OM2DL plots (Supplementary Table 2), reflecting the influence of this soil treatment on microbial diversity.

### *3.2 Analysis of soil microbial composition*

PERMANOVA analysis of Bray-Curtis dissimilarities revealed a significant effect of the soil treatments on the composition of soil microbial communities (Table 2), the

post-hoc analysis corroborating significant differences in microbial composition between the soil treatments. This is illustrated by the NMDS ordination space (Fig. 3), which shows the clustering of soil microbial communities according to soil treatments. Consistently, the ANOSIM analysis also confirmed the composition discrimination driven by soil treatments ($R$ = 0.68; $P$ = 0.001). Based on the NMDS plot, the bacterial communities of OMDL and OM2DL plots clustered more closely, clearly separated from the UNT ones. This is corroborated by Bray-Curtis dissimilarities average values between soil treatments (mean ± SD; 0.62 ± 0.16 for OMDL-OM2DL, 0.71 ± 0.11 for OMDL-UNT, 0.70 ± 0.11 for OM2DL-UNT), indicating that the microbial composition of UNT soils is most different as compared to that from amended soils.

*3.3 Analysis of soil microbial structure*

Bacteria dominated the soil microbial community, representing 98.9-99.6% of the quality-filtered reads, whereas archaea accounted for only 0.4-1.0% (Supplementary Table 3). About 99, 98 and 83% of the 16S rRNA amplicons could be taxonomically classified to phylum, class and order level, respectively, resulting in 23 phyla, 61 classes and 76 orders. Fig. 4 illustrates the relative abundance of the most abundant taxa at class level. Among them, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*, which belong to phylum *Proteobacteria*, accounted for 39-47% of the total amplified amplicons dominating microbial communities in all plots, followed by classes belonging to phyla *Acidobacteria* (14-16%), *Bacteroidetes* (9-11%) and *Actinobacteria* (7-10%). Remaining predominant classes fell within phyla *Verrucomicrobia* (4-5%), *Planctomycetes* (4-5%), *Chloroflexi* (3-6%), *Gemmatimonadetes* (3-4%), *Firmicutes* (1-2%) and *Epsilonbacteraeota* (1%).

Out of the resulting classified microbial taxa, 13 phyla, 32 classes and 42 orders showed significant differences in their relative abundance due to soil treatments (Supplementary Table 3). Overall, the abundance of bacteria showed a trend to increase in amended pots, while archaeal population, represented mainly by the class *Nitrososphaeria*, significantly decreased to less than half in the OM2DL plots. Taking a closer look at class level (Fig. 5), soil amendments, particularly in the OM2DL soils, increased the population of *Aphaproteobacteria*, while *Thermoleophilia* (phylum *Actinobacteria*), *Blastocatellia* (*Acidobacteria*), *Spartobacteria* (*Verrucomicrobia*) and *Desulfurellia* (*Epsilonbacteraeota*) increased mainly in the OMDL soils. Soil amendments resulted in a reduction of the less predominant bacterial groups mostly belonging to phyla *Planctomycetes*, *Chloroflexi* and *Gemmatimonadetes*, as well as classes *Cytophagia* (phylum *Bacteroidetes*), *OPB35 soil group* (*Verrucomicrobia*) and *Acidobacteriia* (*Acidobacteria*).

*3.4 Relationship between soil physicochemical and microbial properties*

The influence of soil treatments on soil microbial communities was further explored through changes in physicochemical soil properties. Soil CEC, total and organic soil C, total N and total P influenced microbial diversity, being correlated to richness and Shannon diversity index (Supplementary Fig. 1; $F = 2.6$, $P < 0.05$;). Based on microbial composition, most physicochemical soil parameters strongly correlated to the NMDS ordination space (Table 3): soil CEC, WHC, total N and organic C, followed by total C, pH, Olsen's extractable and total P, correlated with microbial communities from amended soils, mainly the OM2DL ones; while total and extractable soil Cu correlated with those from the UNT soils (Fig. 3). BIOENV analysis indicated that microbial community dissimilarity is best explained by a combination of total and Olsen's

298 extractable P, total and extractable soil Cu, and soil WHC (R = 0.78). Regarding the
299 influence of soil physicochemical properties on soil microbial structure, most soil
300 physicochemical parameters positively correlated with *Alphaproteobacteria*, whereas
301 taxa belonging to phyla *Gemmatimonadetes*, *Planctomycetes* and *Chloroflexi*, and
302 archaea correlated with parameters related to soil Cu contamination, i.e., total and
303 $NH_4NO_3$-extractable soil Cu (Fig. 6).
304
305 **4. Discussion**
306 Phytomanagement of metal-contaminated soils combines phytoremediation options with
307 a sustainable site management, aiming at recovering soil functions and ecosystem
308 services, as well as achieving effective risk management (Kidd et al., 2015; Cundy et
309 al., 2016). After 9 years of phytomanagement in this field trial, Mench et al. (2018)
310 reported a long lasting positive effect of soil amendments and annual plant cultivation
311 on the improvement of soil physicochemical properties and the reduction of Cu
312 availability (Supplementary Table 1). In addition to the nutrient incorporation, this
313 allowed a higher production for sunflower and tobacco crops in amended plots, when
314 the annual climatic conditions (i.e. spring and summer drought, heat waves) were not
315 challenging it (Kolbas et al., 2011; Mench et al., 2018). Shoot Cu concentrations of
316 OMDL and OM2DL plants fitted into their common range and can be used by biomass
317 processing technologies and oilseeds as well. Several other studies performed at this
318 site, assessed through chemical and ecotoxicological assays, also corroborated the
319 effectiveness and sustainability of phytomanagement options for the remediation of Cu-
320 contaminated soils (Kolbas et al., 2011; Kumpiene et al., 2011; Marchand et al., 2011;
321 Hattab-Hambli et al., 2016; Oustriere et al., 2016; Mench et al., 2018). Likewise, other
322 studies at this site have evaluated soil microbial properties of activity, biomass and

diversity to provide further knowledge on the influence of these remediation options on the recovery of soil functions (Touceda-González et al., 2017b; Xue et al., 2018). Here, we contribute to assess the long-term effect of phytomanagement on soil microbial communities with a metabarcoding approach.

*4.1 Effect of phytomanagement on microbial α-diversity*

As previously reported for this field trial (Kolbas et al., 2011; Hattab-Hambli et al., 2016; Mench et al., 2018), both total and extractable soil Cu displayed an increasing gradient across the plots, from B1 to B4. This spatial variability in soil Cu contamination, mainly attributed to variability in cumulative wood washings resulting from long-term storage of preserved wood (Oustriere et al., 2016; Mench et al., 2018), may account for a large part of the variance in α-diversity metrics among soil treatments, leading to no significant differences. Nevertheless, when we took into account the background influence of total soil Cu variability across the plots (Fig. 2), no significant effect of the soil treatments on microbial diversity was observed either.

Focusing on plots with higher soil Cu contamination, the OM2DL treatment had a stimulating effect on microbial diversity, only reflected in higher Shannon values. In fact, Shannon and Simpson indices are diversity metrics that account for both richness and evenness (Bent and Forney, 2008), being more sensitive to changes in microbial populations than only richness-based metrics. Interestingly, the response of microbial diversity to the renewed compost application in the most contaminated plots followed the same pattern as crop yield: plant biomass in the OM2DL plots remained steady as levels of total soil Cu increased, whereas it decreased in the OMDL plots (Mench et al., 2018). Plant biomass can strongly affect and promote soil microbial communities (Epelde et al., 2010) and enhance microbial diversity (Burges et al., 2017). Root

exudates are an excellent source of energy and nutrients for soil microorganisms, and differences in their amount and quality, due to changes in plant biomass, induce changes in soil microbial diversity (Lucisine et al., 2014; Lopez et al., 2019). Accordingly, the renewed compost application may have counterbalanced the detrimental effects of the increasing levels of total soil Cu on microbial diversity, partly by promoting plant growth. This also demonstrates the benefits to renew compost supply not just to produce higher yield of annual crops at high total soil Cu but also to stimulate microbial diversity through the improvement of soil revegetation.

In any case, no overall effect of soil treatments on microbial diversity across all plots could be identified. However, considering the evidence of season variability in soil microbial communities (Bouskill et al., 2010; Pereira et al., 2014), microbial diversity at the sampling time (right before the growing season) may not be corresponding to its full potential. Importantly, we must bear in mind that diversity is relative and constrained by method of measurement, and without sufficient context it can turn out to be uninformative or liable to misinterpretation (Shade, 2016). In addition, α-diversity metrics are simplified estimations of microbial diversity calculated from the vast amount of data provided by next generation sequencing techniques. They are still useful tools that facilitate interpretation of metabarcoding results and provide valuable information that could serve as a first step to provide key insights into underlying ecological processes that drive microbial community patterns (Shade, 2016). Therefore, it is fundamental to assess soil microbial composition and structure, along with microbial diversity, for determining the effect of long-term phytomanagement on soil microbial communities.

372 *4.2 Effect of phytomanagement on the composition and structure of soil microbial*
373 *communities*

374 As opposed to α-diversity, compositional analysis showed higher differences among
375 soil treatments (Fig. 3, Table 2), revealing information on soil microbial communities
376 not implied by diversity metrics alone. These changes in microbial composition could
377 reflect the influence of the soil amendments on microbe-mediated soil ecological
378 processes. For instance, in the same field trial, 6 years after the application of the
379 OMDL amendment, Touceda-Gonzalez et al. (2017b) observed an increase in microbial
380 biomass and activity, as well as in the abundance of genes involved in N cycling.
381 Furthermore, organic amendments, like the ones used here, can increase genes encoding
382 for metal and antibiotic resistance (Caban et al., 2018; Garbisu et al., 2018). At this site,
383 in a field trial phytomanaged with the OMDL treatment combined with a mixed stand of
384 willows, poplar and false indigo bush, Xue et al. (2018) reported higher abundance of
385 functional genes encoding for resistance to metals and antibiotics. They suggested that
386 this could be due to proliferation of certain microbial groups caused by OM
387 mineralization, as well as introduction of exogenous metal-resistant microbes with the
388 soil amendment.

389 Compositional analysis also allowed a more comprehensive interpretation of the
390 effect of soil treatments based on differences in the chemical composition and
391 application rate of soil amendments. The compost incorporated in both OMDL and
392 OM2DL plots in year 1 was made with poultry manure and pine bark chips, the latter
393 known to contain, along with cellulose and xylans, a high lignin content (Vane et al.,
394 2006; Xue et al., 2018). The second compost dressing, incorporated in the OM2DL
395 plots in year 6, was made from green waste and sandy soils (Jones et al., 2016),
396 containing more easily biodegradable OM. Since the biochemical nature of the plant

residue can determine the mineralization rate of green manures (Tejada et al., 2008), this would certainly shape soil microbial communities. For instance, 6 years after the incorporation of the OMDL soil amendment in the field trial phytomanaged with trees, Xue et al. (2018) reported a stimulation of microbial functional genes involved in both labile (e.g. cellulose) and recalcitrant (e.g. lignin and aromatic components) C decomposition. Here, we hypothesize that by year 9 much of the relatively labile C in the OMDL soils may be mineralized, with the bulk of soil OM accounting for the more recalcitrant forms of C; while the second compost dressing, in contrast, contributed with a more recent input of nutrients and easily degradable OM (Oustriere et al., 2016) in the OM2DL soils. Based on this, we could suggest that the active fraction of the microbes in the OMDL soils may be mainly represented by decomposers of lignin-rich OM; whereas microbial populations in the OM2DL soils may have shifted to more easily biodegradable OM-decomposers.

After 9 years of phytomanagement, plots under different soil treatments sheltered genetically diverse microbial communities, reflected in changes in microbial composition (Fig. 3), i.e. the presence or the absence of microbial taxa. Likewise, organic amendments will inexorably promote the growth of certain microbial groups in detriment of others, resulting in changes in microbial structure, i.e. the proportion of the different microbial taxa.

The incorporation of soil amendments, particularly the second compost dressing, stimulated the growth of several microbial groups of the class *Alphaproteobacteria*. Among them, the order *Rhizobiales*, typically $N_2$-fixing bacteria living in root nodules of legumes (Hartwig, 1998), was notably abundant in OM2DL plots where the green waste compost promoted the development of clover vegetation in winter time (Mench et al., 2018). The OM2DL soil treatment also increased the populations of

*Rhodospirillales*, another $N_2$-fixer that can live both free in soil or associated with the rhizosphere of host plants, and *Sphingomonadales*, whose several members can play various roles in microbe-assisted metal phytoremediation (Waigi et al., 2017). Another taxon from *Alphaproteobacteria* whose abundance was higher in the OM2DL plots was *Caulobacterales*, which can be positively influenced by root exudates, along with *Rhizobiales* and *Sphingomonadales* (Shi et al., 2011). The fact that these taxa are generally associated with plants may justify their predominance in plots that received the second compost dressing, where plant biomass was the highest and contributed to their growth, corroborating that above and belowground communities are tightly interlinked in many soil processes. In addition, *Alphaproteobacteria* belong to *Proteobacteria*, a phylum with great importance to global C, N and S cycling (Spain et al., 2009; Li et al., 2019), which explains that they are the main microbial group profiting from the amendment-derived benefits on soil properties (Fig. 6).

Archaeal populations, on the contrary, decreased with the incorporation of soil amendments, reflected in the lower abundance of *Nitrososphaeria*, a class that encompasses several ammonia oxidizing archaea (AOA). At year 6, Touceda-González et al. (2017b) reported an increase in denitrification genes and urease activity with the OMDL treatment, whereas no differences in AOA nitrification genes were found. However, in a phytoextraction experiment with *Sedum alfredii* growing on a Cd/Zn contaminated soil, Luo et al. (2019) observed a decrease in AOA groups belonging to *Nitrososphaeria* with successive crops, resulting in a reduction in the potential nitrification rate and nitrogen loss. They also indicated a negative correlation between the nitrification rate and root exudates. Accordingly, the lower abundance of *Nitrososphaeria* observed here in year 9 in amended plots, along with the higher plant biomass, could indicate a decrease in the nitrification rate with time. This, paired with

447 the increase in $N_2$-fixing bacteria, suggests a beneficial effect of the incorporation of
448 soil amendments on the ecological processes involved in N cycling partly through the
449 physiological improvement of the annual rotation crop.

450 The abundance of bacterial taxa belonging to *Planctomycetes*, *Chloroflexi* and
451 *Gemmatimonadetes*, was also negatively affected by soil amendments. These phyla are
452 generally abundant in metal-contaminated soils owing to their tolerance to metal excess,
453 including Cu (DeBruyn et al., 2011; Chodak et al., 2013; Singh et al., 2014; Azarbad et
454 al., 2015; Jiang et al., 2019), and contain slow-growing bacteria adapted to oligotrophic
455 habitats, like *Chloroflexi* (Davis et al., 2011; Barton et al., 2014), which enables them to
456 successfully occupy extreme environments (Durand et al., 2018). This may explain that
457 the most predominant groups of these phyla were favoured by the rather oligotrophic
458 and highly contaminated conditions of the unamended plots (Fig. 6). The ecological
459 traits that make these phyla highly adaptive and efficient in oligotrophic, contaminated
460 soils may put them, however, in disadvantage against fast-growing bacterial groups,
461 more efficient in competing under the more favourable conditions of the amended plots.

462 These results indicate there was a consistency in the direction and magnitude of
463 the response to soil treatments, both at class and phylum level. However, the phyla
464 *Acidobacteria*, *Actinobacteria* and *Verrucomicrobia* include several taxa, e.g.
465 *Blastocatellia*, *Thermoleophilia* and *Spartobacteria*, whose abundance increased mainly
466 in the OMDL plots, demonstrating the long lasting effect of the first soil amendment;
467 whereas they also include groups, e.g. *OP35 soil group* and *Acidobacteriia*, that
468 decreased. These microbial groups present a wide range of lifestyle, metal-resistance
469 and metabolic properties. For instance, *Acidobacteria* has a diversity of members with
470 varying tolerance to Cu contamination (Pereira et al., 2014; Singh et al., 2014) and
471 metabolic activity, with many groups reported to be degraders of plant-derived-OM,

while others, like *Blastocatellia*, prefer oligotrophic environments (Navarrete et al., 2015; Li et al., 2019). *Actinobacteria* are also a heterogeneous group among the metabolically active bacteria in metal(loid)-contaminated soils (Gremion et al., 2004). *Verrucomicrobia* are relatively abundant in subsurface horizons due to their preference for rather oligotrophic soils (Bergmann et al., 2011). The ecological diversity of these microbial groups may account for the divergent response among their members to soil treatments, which results in no overall changes at phylum level. As suggested by Spain et al. (2009), this highlights the detailed resolution and the importance of subphylum phylogenetic analysis of metabarcoding datasets.

Finally, the relationship between patterns in physicochemical and microbial properties demonstrated the influence of soil treatments on soil microbial communities through changes in soil physicochemical properties. Soil organic amendments can incorporate OM and available nutrients, reduce metal bioavailability, and modify pH and other physicochemical properties (Alvarenga et al., 2009a, 2009b; Epelde et al., 2009), that strongly affect microbial communities. pH has often been highlighted as a most important factor in metal-contaminated soils as it highly affects metal mobility, as well as availability of nutrients, soil OC and OM, N, etc. (Kumpiene et al., 2011; Jiang et al., 2016; Mench et al., 2018; Jiang et al., 2019). However, the pH effect here was rather limited and nutrients level, OM, water holding capacity or CEC would influence soil microbial communities in a greater way (Fig. 3; Table 2; Fig. 6; Supplementary Fig. 1). Indeed, Xue et al. (2018) reported the amelioration of soil properties such as nutrient content and aggregate formation as major drivers of soil biochemical activity.

In any case, the reported influence of the soil treatments was mainly reflected in microbial composition and structure, in the same way that other studies have reported that soil metal contamination had more impact on compositional and structural diversity

497 than on diversity metrics *per se* (Pereira et al., 2014; Azarbad et al., 2015; Epelde et al.,
498 2015; Jiang et al., 2016). This sensitive response of microbial composition and structure
499 may be reflecting an eventual positive influence on soil ecological processes, such as N
500 cycling, demonstrating the potential benefits of phytomanagement on Cu-contaminated
501 soils even in the long-term. Based on this, the monitoring of soil microbial functional
502 genes, along with compositional and structural diversity, should be considered for
503 evaluating the effectiveness of long-term phytomanagement in Cu-contaminated soils,
504 like this site. Lastly, and considering that Cu is a well-known fungicide (Singh et al.,
505 2014), the composition and structure of fungal communities should also be assessed.
506

507 **5. Conclusions**

508 The long-term phytomanagement of Cu-contaminated soils, based on a crop rotation
509 with tobacco and sunflower and the incorporation of compost with dolomitic limestone
510 (compost dressing being renewed or not in year 6), contributed to shift the composition
511 and structure of soil microbial communities in year 9, even though it had no effect on
512 microbial diversity This phytomanagement option induced changes in soil
513 physicochemical properties in the long term that led to genetically diverse microbial
514 communities even 9 years after the compost application. The input of organic matter
515 and nutrients, the reduction in Cu availability and the improvement of soil properties,
516 particularly with the renewed compost application in year 6, enhanced the growth of
517 specific soil microbial groups, which are involved in different soil ecological processes.
518 The stimulation of plant biomass in the amended plots, induced by the direct and
519 indirect effects of amendments on soil quality, also contributed to shape soil microbial
520 communities. Further research is needed to determine the long-term sustainability and
521 effectiveness of phytomanagement in Cu-contaminated soils by (i) monitoring microbial

functional diversity, (ii) assessing also the genetic structure of soil fungal communities, (iii) evaluating the influence of temporal variability through the sampling of plots during and after the growth season, and (iv) exploring the relationship between above and belowground communities through the assessment of plant physiological status.

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736
737

**Figure captions**

**Fig. 1**: Spatial variability of (a) total soil Cu (mg Cu $kg^{-1}$ DW), and (b) 1M $NH_4NO_3$-extractable Cu (µg Cu $kg^{-1}$ DW) within the field trial.

**Fig. 2**. Influence of soil treatments on α-diversity indices, assessed using ANCOVA, considering total soil Cu concentration as co-variable. Probability values from ANCOVA (ns: non-significant; * represents significance of $P<0.05$) for rarefied richness: amendment, ns; soil Cu, ns; amendment x soil Cu, ns; Shannon diversity index: amendment, ns; soil Cu, ns; amendment x soil Cu, *; and Simpson diversity index: amendment, ns; soil Cu, ns; amendment x soil Cu, ns.

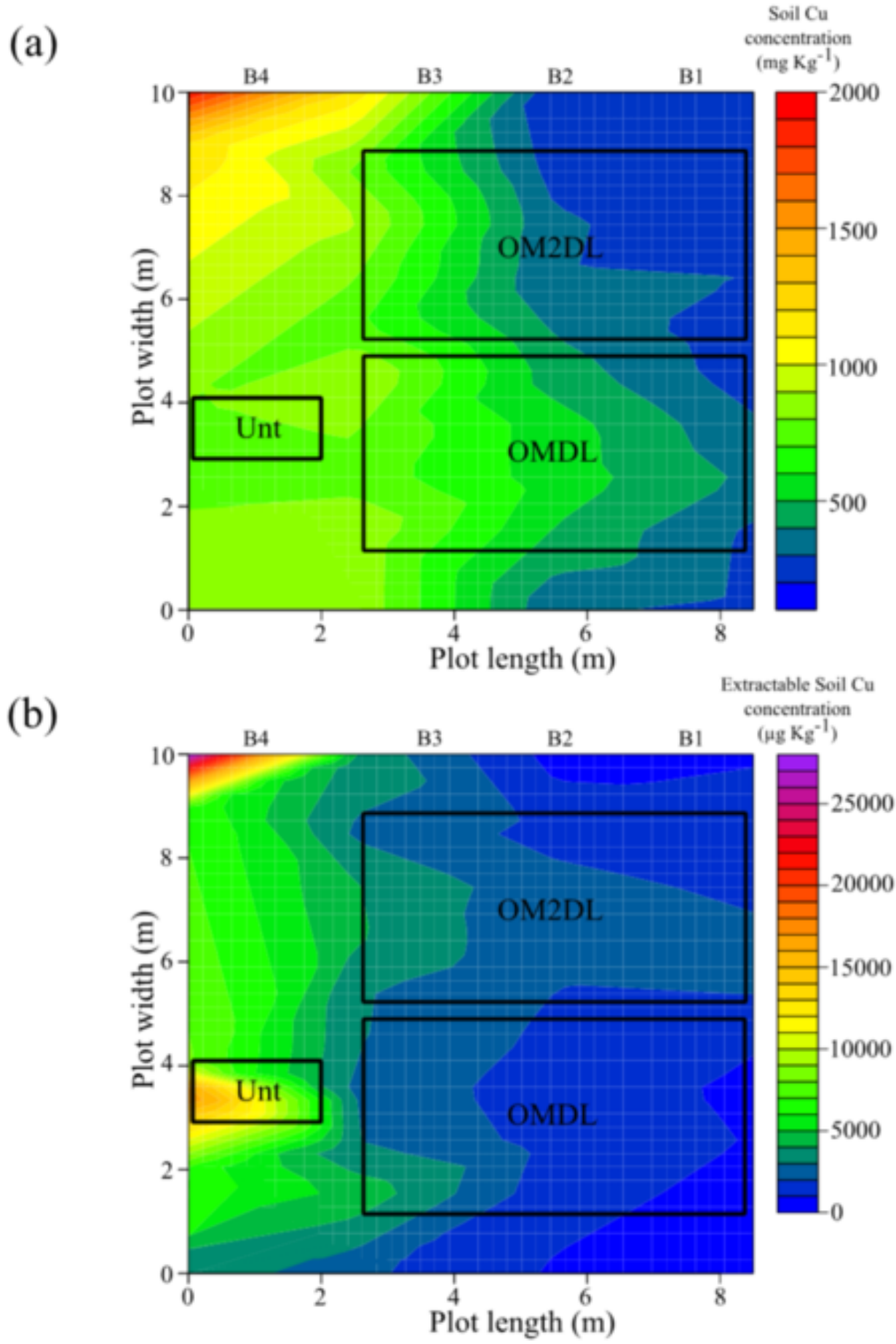
**Fig. 3**. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of the composition of soil microbial communities. Fitted physicochemical soil parameters with significant correlation to the multidimensional NMDS space are included. Tot_N: total N; Tot_P: total P; P_Olsen: Olsen's extractable P; Tot_Cu: total Cu; Ext_Cu: 1 M $NH_4NO_3$-extractable Cu; Tot_C: total C; OC: organic C; WHC: water holding capacity; CEC: cationic exchange capacity.

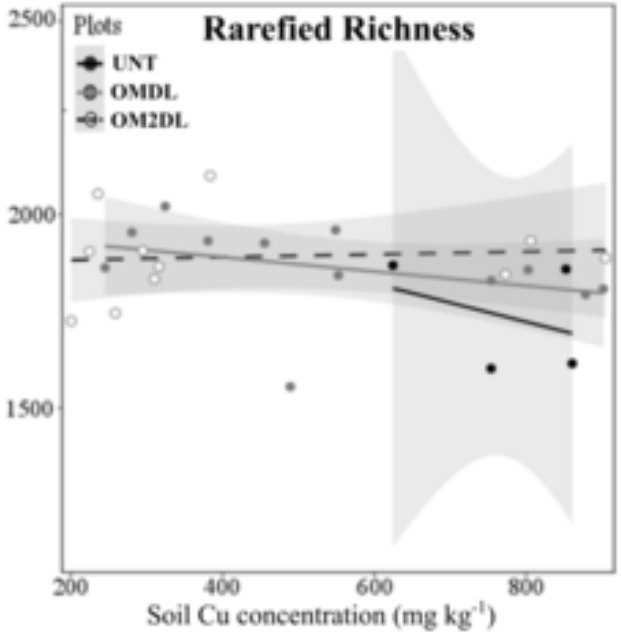
**Fig. 4**. Average relative abundance of the main microbial taxa, at class level, per soil treatment.

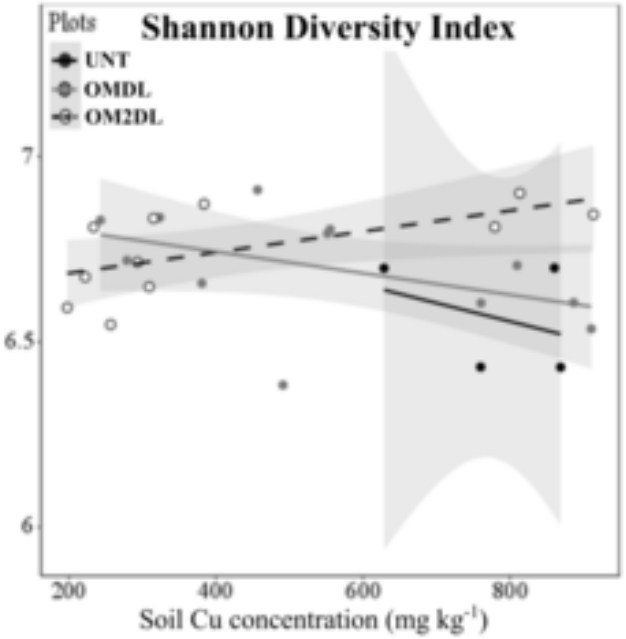
**Fig. 5.** Heat map based on relative abundance of the top 15 microbial taxa, at class level, that showed significance differences among soil treatments.

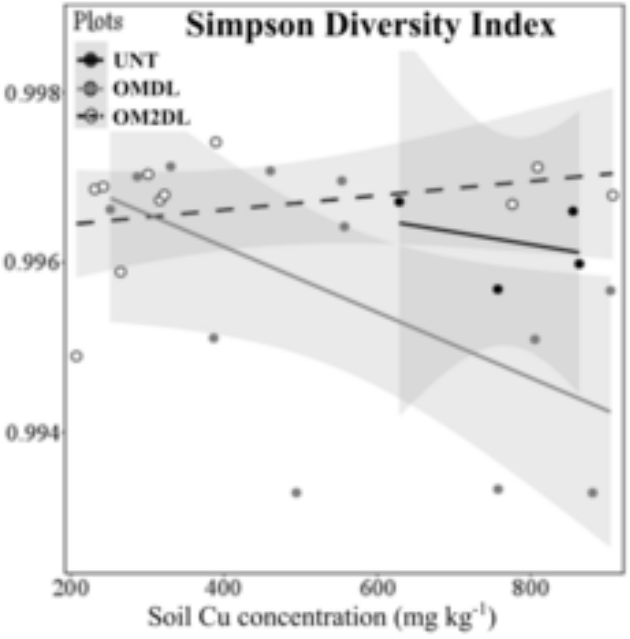
**Fig. 6**. Biplot of redundancy analysis (RDA) performed between soil physicochemical properties and relative abundance of the main microbial taxa, al class level, considering only taxa showing significant differences in their abundance due to soil treatment ($F$ = 3.0, $P$ < 0.01). RDA1 and RDA2 account for 29 and 15% of the total variance, respectively. Tot_N: total N; Tot_P: total P; P_Olsen: Olsen's extractable P; Tot_Cu: total Cu; Ext_Cu: $NH_4NO_3$-extractable Cu; Tot_C: total C; OC: total organic C; WHC: water holding capacity; CEC: cationic exchange capacity

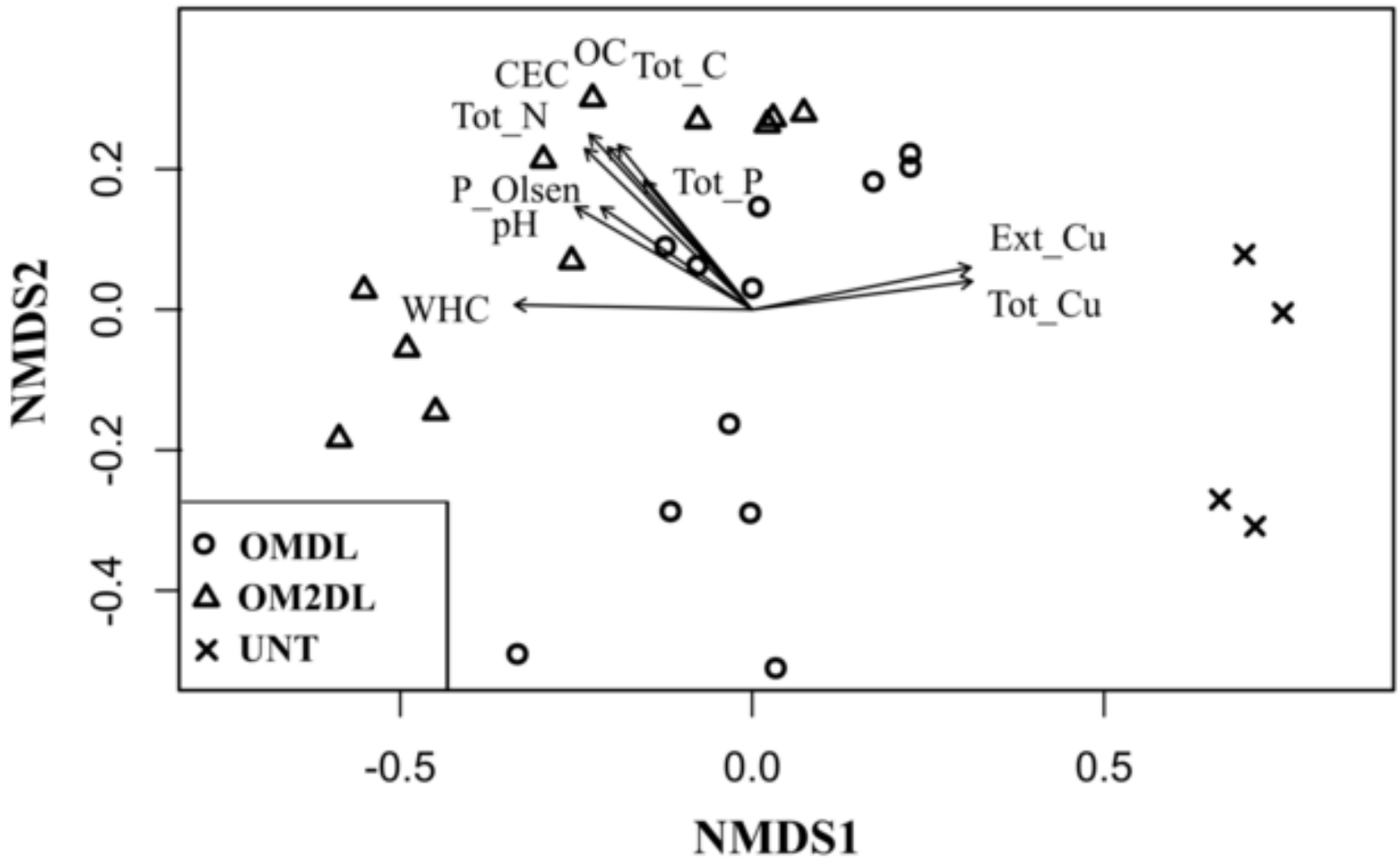
**Supplementary Fig. 1**. Biplot of the redundancy analysis (RDA) from α-diversity metrics explained by physicochemical topsoil parameters ($F$ = 2.6, $P$ < 0.05); RDA1 and RDA2 account for 61 and 1% of the variance, respectively. Richness: rarefied total richness; Shannon: Shannon diversity index; Simpson: Simpson diversity index; Tot_N: total N; P_Olsen: Olsen's extractable P; Tot_P: total P; Tot_Cu: total Cu; Ext_Cu: extractable Cu; Org_C: organic C; WHC: water holding capacity; CEC: cationic exchange capacity.


(a)
Soil Cu concentration (mg Kg-1)
B4
B3
B2
B1
OM2DL
OMDL
Unt
Plot width (m)
Plot length (m)
2000
1500
1000
500
(b)
Extractable Soil Cu concentration (µg Kg-1)
25000
20000
15000
10000
5000
0


Plots
Rarefied Richness
UNT
OMDL
OM2DL
2500
2000
1500
200
400
600
800
Soil Cu concentration (mg kg-1)


Plots
Shannon Diversity Index
UNT
OMDL
OM2DL
7
6.5
6
200
400
600
800
Soil Cu concentration (mg kg-1)


Plots
Simpson Diversity Index
UNT
OMDL
OM2DL
0.998
0.996
0.994
200
400
600
800
Soil Cu concentration (mg kg-1)


CEC
OC
Tot_C
Tot_N
Tot_P
P_Olsen
pH
Ext_Cu
Tot_Cu
WHC
OMDL
OM2DL
UNT
NMDS1
NMDS2
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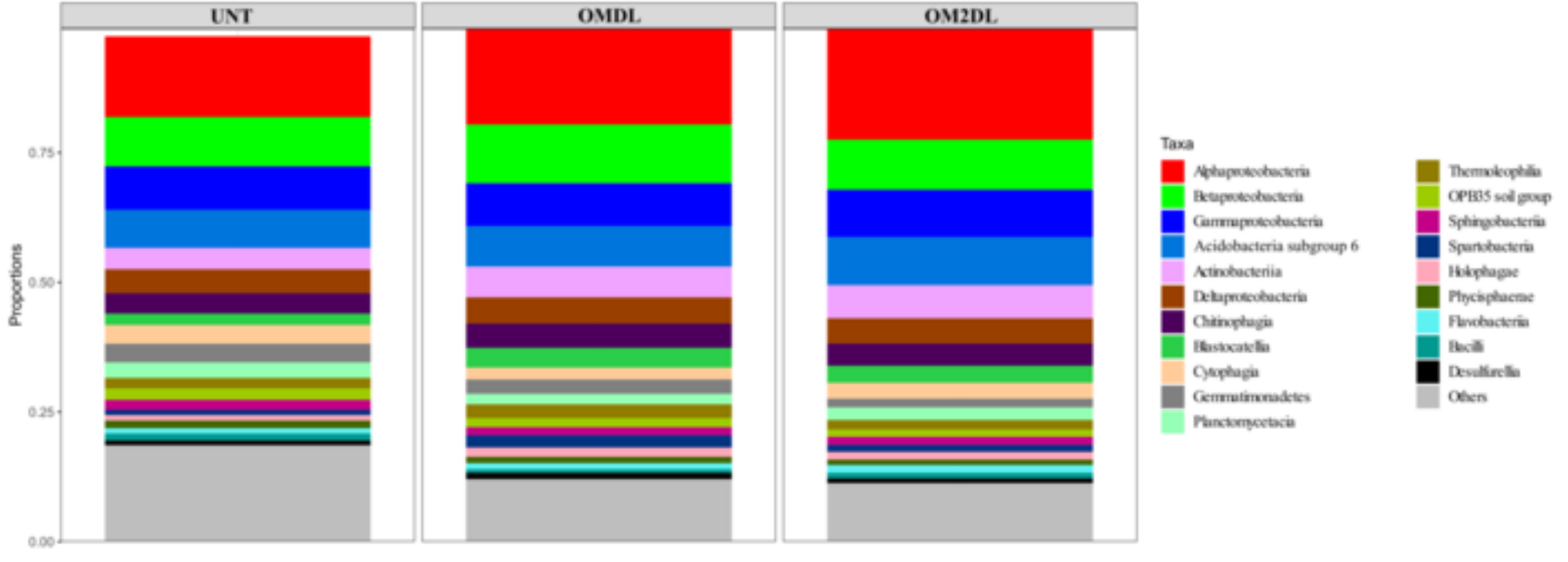
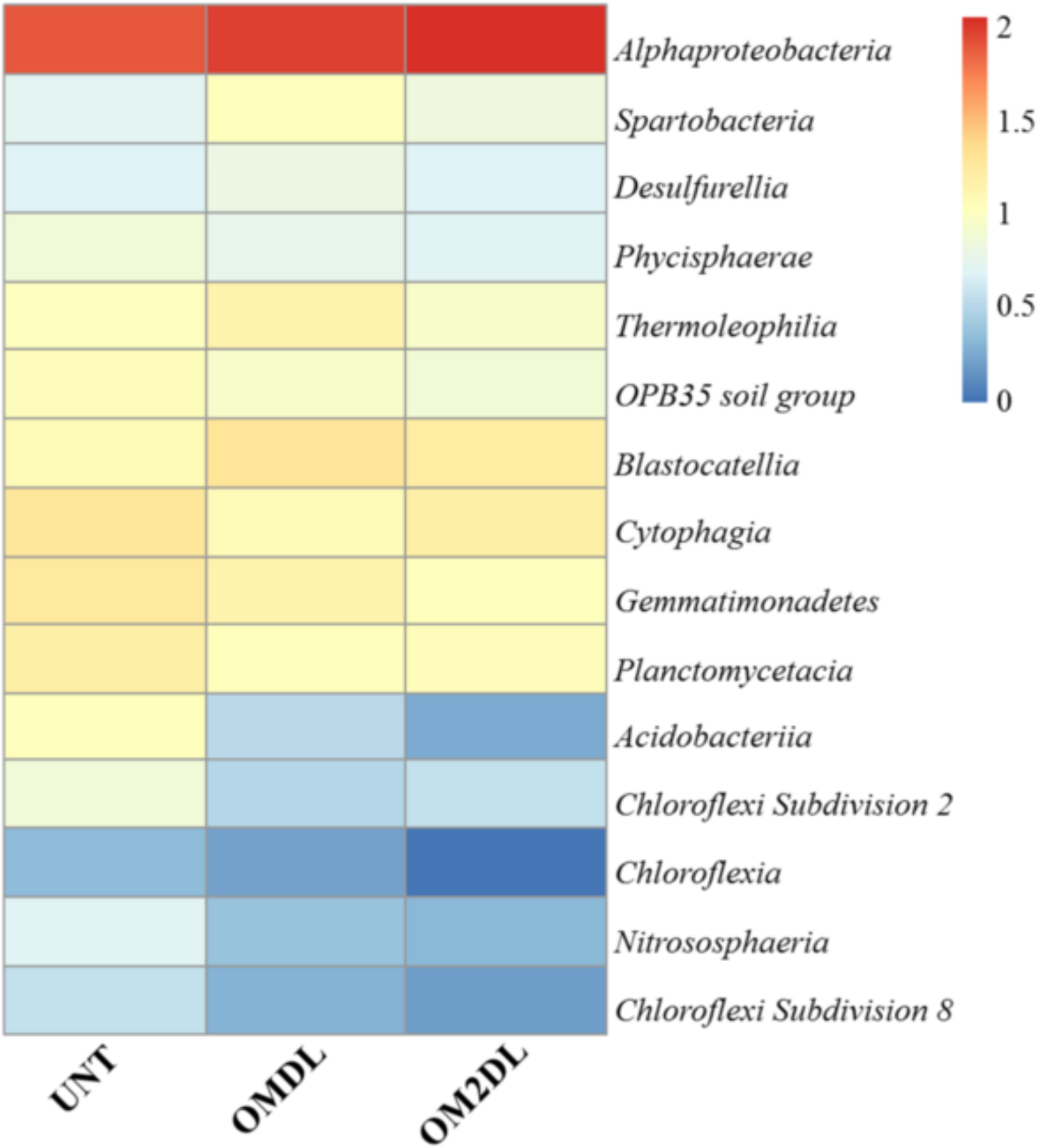
0.0
0.5
0.2
0.0
-0.2
-0.4


UNT
OMDL
OM2DL
Proportions
0.00
0.25
0.50
0.75
Taxa
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Acidobacteria subgroup 6
Actinobacteriia
Deltaproteobacteria
Chitinophagia
Blastocatellia
Cytophagia
Gemmatimonadetes
Planctomycetacia
Thermoleophilia
OPB35 soil group
Sphingobacteriia
Spartobacteria
Holophagae
Phycisphaerae
Flavobacteriia
Bacilli
Desulfurellia
Others


Alphaproteobacteria
Spartobacteria
Desulfurellia
Phycisphaerae
Thermoleophilia
OPB35 soil group
Blastocatellia
Cytophagia
Gemmatimonadetes
Planctomycetacia
Acidobacteriia
Chloroflexi Subdivision 2
Chloroflexia
Nitrososphaeria
Chloroflexi Subdivision 8
2
1.5
1
0.5
0
UNT
OMDL
OM2DL

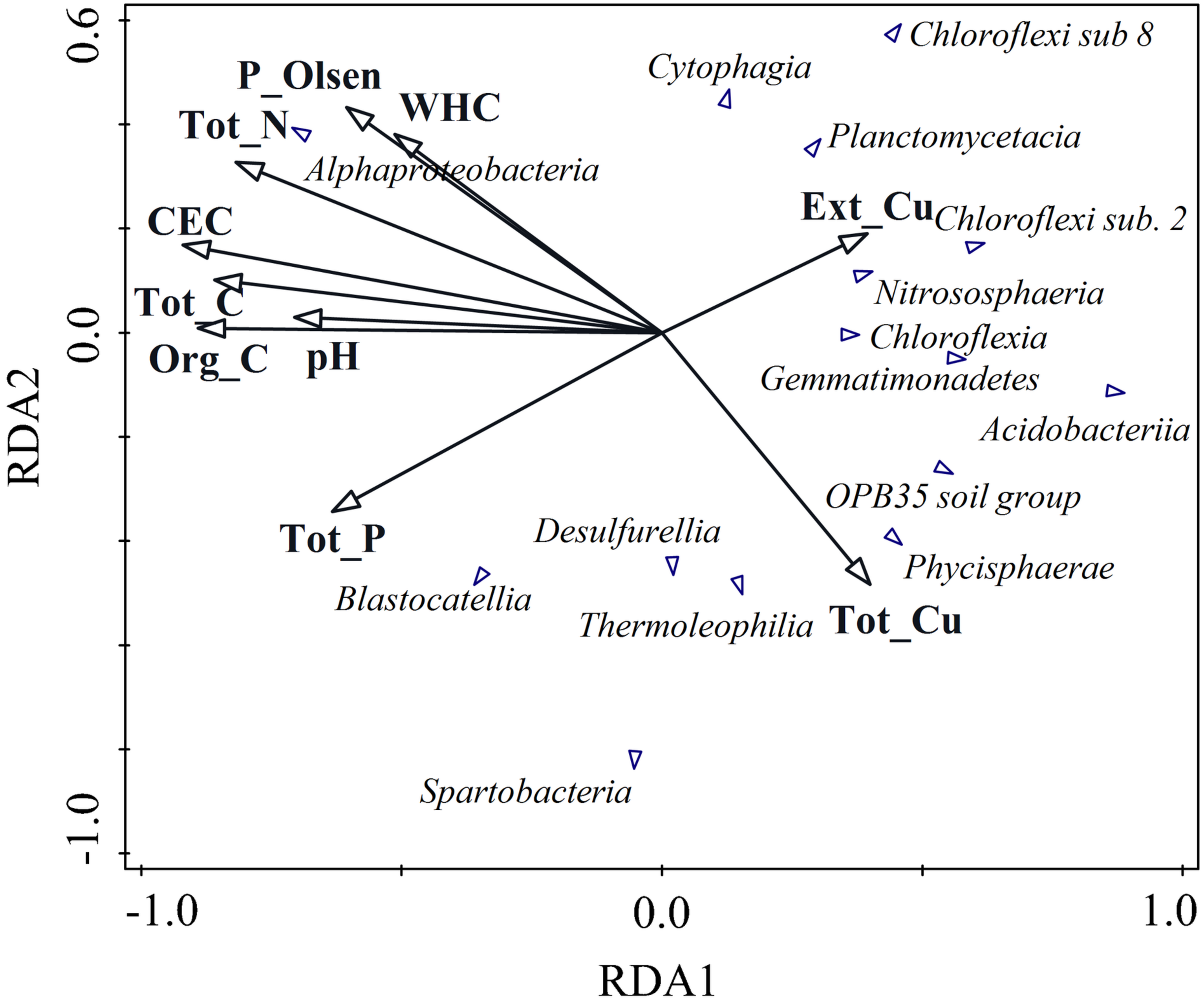

P_Olsen
WHC
Tot_N
Alphaproteobacteria
CEC
Tot_C
Org_C
pH
Tot_P
Blastocatellia
Cytophagia
Chloroflexi sub 8
Planctomycetacia
Ext_Cu
Chloroflexi sub. 2
Nitrososphaeria
Chloroflexia
Gemmatimonadetes
Acidobacteriia
OPB35 soil group
Desulfurellia
Phycisphaerae
Thermoleophilia
Tot_Cu
Spartobacteria
0.6
0.0
-1.0
RDA2
-1.0
0.0
1.0
RDA1

**Table 1**. Values of amplified reads and α-diversity metrics per soil treatment (Mean values ± standard deviation). Values followed by different letters are significantly different ($P<0.05$) according to Tukey's test.

| | UNT | OMDL | OM2DL |
|---|---|---|---|
| **Reads** | 10956 ± 1092[a] | 46058 ± 32597[a] | 40557 ± 33994[a] |
| **Rarefied richness** | 1736 ± 148[a] | 1862 ± 87[a] | 1891 ± 113[a] |
| **Shannon index (H')** | 6.57 ± 0.15[a] | 6.70 ± 0.11[a] | 6.75 ± 0.12[a] |
| **Simpson index** | 0.996 ± 0.001[a] | 0.996 ± 0.000[a] | 0.997± 0.001[a] |

**Table 2.** (a) Effect of soil treatments, total soil Cu, and their interaction, and (b) pair-wise comparisons between soil treatments, on the composition of soil microbial communities, assessed using PERMANOVA (asterisks represent strength of significance).

| | | Df | F | $R^2$ | Pr(>F) | |
|---|---|---|---|---|---|---|
| a | Amendment | 2 | 7.0002 | 0.3327 | 0.001 | ** |
| | Soil Cu | 1 | 4.7321 | 0.1124 | 0.001 | ** |
| | Amendment x Soil Cu | 2 | 1.1723 | 0.0557 | 0.252 | |
| | Residuals | 21 | | | | |
| b | OMDL - OM2DL | 22 | 7.886 | 0.273 | 0.006 | ** |
| | OMDL - UNT | 15 | 18.0655 | 0.5633 | 0.006 | ** |
| | OM2DL - UNT | 14 | 27.5414 | 0.6793 | 0.006 | ** |

Df: degree of freedom; F: Fisher value; $R^2$: coefficient of determination;

Pr: probability value.

**Table 3**. Correlation of physicochemical soil properties to NMDS coordinates (asterisks represent strength of significance)

| | $R^2$ | Pr(>r) | |
|---|---|---|---|
| CEC | 0.630 | 0.001 | *** |
| Water Holding Capacity | 0.620 | 0.001 | *** |
| Total N | 0.593 | 0.001 | *** |
| 1M $NH_4NO_3$-extractable Cu | 0.544 | 0.001 | *** |
| Total Cu | 0.541 | 0.001 | *** |
| Organic C | 0.519 | 0.001 | *** |
| Total C | 0.496 | 0.001 | *** |
| pH | 0.459 | 0.002 | ** |
| Olsen's extractable P | 0.367 | 0.003 | ** |
| Total P | 0.312 | 0.013 | * |

$R^2$: coefficient of determination; Pr: probability value.

**Supplementary Table. 1.** Soil physicochemical parameters per block and per soil treatments (Mean values ± standard deviation). Values followed by different letters are significantly different (P<0.05) according to Tukey's test. No letter in a row indicates no significant difference.

| | BLOCK 4 | BLOCK 3 | | BLOCK 2 | | BLOCK 1 | |
|---|---|---|---|---|---|---|---|
| | UNT | OMDL | OM2DL | OMDL | OM2DL | OMDL | OM2DL |
| pH water | 6.8 ± 0.3$^{b}$ | 7.0 ± 0.2$^{ab}$ | 7.0 ± 0.2$^{ab}$ | 7.0 ± 0.1$^{ab}$ | 7.1 ± 0.1$^{ab}$ | 7.0 ± 0$^{ab}$ | 7.2 ± 0.1$^{a}$ |
| CEC (cmol kg$^{-1}$) | 3.3 ± 0.2$^{d}$ | 7.0 ± 3.3$^{bcd}$ | 10.4 ± 2.6$^{abc}$ | 6.4 ± 0.2$^{cd}$ | 10.8 ± 0.5$^{a}$ | 6.0 ± 1.1$^{d}$ | 10.4 ± 1.1$^{ab}$ |
| Organic C (g kg$^{-1}$) | 8.3 ± 2.1$^{c}$ | 19.0 ± 8.2$^{ab}$ | 23.7 ± 8.5$^{ab}$ | 14.5 ± 0.6$^{abc}$ | 24.3 ± 1.7$^{a}$ | 14.0 ± 1.4$^{bc}$ | 22.8 ± 1.0$^{ab}$ |
| OM (g kg$^{-1}$) | 17 ± 1$^{c}$ | 33 ± 14$^{abc}$ | 41 ± 15$^{ab}$ | 25 ± 1$^{abc}$ | 42 ± 3$^{a}$ | 24 ± 3$^{bc}$ | 40 ± 2$^{ab}$ |
| Total C (g kg$^{-1}$) | 9.8 ± 0.5$^{c}$ | 19.0 ± 8.2$^{abc}$ | 23.7 ± 8.5$^{ab}$ | 14.8 ± 0.5$^{abc}$ | 24.3 ± 1.7$^{a}$ | 14.0 ± 1.4$^{bc}$ | 23.3 ± 1.3$^{ab}$ |
| Total N (g kg$^{-1}$) | 0.7 ± 0.0$^{c}$ | 1.2 ± 0.5$^{bc}$ | 1.7 ± 0.6$^{ab}$ | 1.0 ± 0.0$^{bc}$ | 2.0 ± 0.0$^{a}$ | 1.0 ± 0.0$^{c}$ | 2.0 ± 0.0$^{a}$ |
| C/N | 13.8 ± 1.3 | 14.0 ± 0.0 | 13.3 ± 0.6 | 13.8 ± 0.5 | 13.8 ± 0.5 | 13.5 ± 0.6 | 14.0 ± 0.0 |
| Olsen extractable P (mg kg$^{-1}$) | 72 ± 2$^{c}$ | 78 ± 9$^{bc}$ | 84 ± 5$^{abc}$ | 72 ± 6$^{c}$ | 96 ± 10$^{a}$ | 75 ± 4$^{c}$ | 92 ± 7$^{ab}$ |
| Total $CaCO_3$ (g kg$^{-1}$)[1] | <1 | 1.0 ± 0.7$^{b}$ | 1.7 ± 0.6$^{ab}$ | 1.0 ± 0.0$^{b}$ | 1.5 ± 0.6$^{ab}$ | 1.0 ± 0.0$^{b}$ | 2.5 ± 0.6$^{a}$ |
| Water holding capacity (%) | 6.8 ± 0.6$^{d}$ | 7.8 ± 1.7$^{cd}$ | 8.0 ± 0.0$^{cd}$ | 10.0 ± 0.3$^{bc}$ | 12.8 ± 0.9$^{a}$ | 9.3 ± 1.2$^{cd}$ | 11.9 ± 1.4$^{ab}$ |
| **Elements (mg kg$^{-1}$)** | | | | | | | |
| Total Cu | 780 ± 112$^{a}$ | 842 ± 69$^{a}$ | 836 ± 70$^{a}$ | 514 ± 48$^{b}$ | 316 ± 52$^{c}$ | 307 ± 60$^{c}$ | 237 ± 41$^{c}$ |
| Extractable Cu (µg kg$^{-1}$)[2] | 6274 ± 2639$^{a}$ | 3398 ± 879$^{b}$ | 3667 ± 770$^{ab}$ | 1543 ± 215$^{b}$ | 2025 ± 270$^{b}$ | 889 ± 161$^{b}$ | 1903 ± 320$^{b}$ |
| **Nutrients (mg kg$^{-1}$)** | | | | | | | |
| Ca | 1391 ± 50$^{ab}$ | 2292 ± 1728$^{ab}$ | 3072 ± 1563$^{ab}$ | 1611 ± 92$^{ab}$ | 3284 ± 235$^{a}$ | 1285 ± 250$^{b}$ | 3304 ± 388$^{a}$ |
| Fe | 6199 ± 316 | 6397 ± 301 | 6209 ± 238 | 6516 ± 253 | 6399 ± 116 | 6271 ± 19 | 6403 ± 322 |
| K[1] | 7893 | 7645 ± 269$^{abc}$ | 7322 ± 219$^{c}$ | 7760 ± 118$^{ab}$ | 7501 ± 158$^{bc}$ | 7992 ± 28$^{a}$ | 7372 ± 101$^{c}$ |
| Mg | 771 ± 26$^{b}$ | 919 ± 83$^{a}$ | 909 ± 36$^{a}$ | 887 ± 26$^{a}$ | 885 ± 34$^{a}$ | 828 ± 17$^{ab}$ | 840 ± 51$^{ab}$ |
| Na[1] | 2015 | 1925 ± 50$^{b}$ | 1942 ± 42$^{b}$ | 2098 ± 31$^{a}$ | 1907 ± 93$^{b}$ | 2115 ± 31$^{a}$ | 2005 ± 47$^{ab}$ |
| P | 259 ± 14$^{b}$ | 940 ± 128$^{a}$ | 910 ± 68$^{a}$ | 956 ± 63$^{a}$ | 739 ± 435$^{a}$ | 878 ± 37$^{a}$ | 874 ± 139$^{a}$ |
| **Texture (g kg$^{-1}$)** | | | | | | | |
| Clay[1] | 54 | 60 ± 6 | 65 ± 9 | 63 ± 1 | 70 ± 3 | 64 ± 4 | 70 ± 2 |
| Silt[1] | 109 | 96 ± 8 | 97 ± 9 | 99 ± 3 | 97 ± 3 | 101 ± 3 | 101 ± 5 |
| Sand[1] | 837 | 844 ± 13 | 839 ± 17 | 838 ± 3 | 833 ± 5 | 836 ± 5 | 829 ± 5 |

[1]n = 1 for UNT; [2]1M $NH_4NO_3$-extractable soil Cu.

**Supplementary Table 2.** Values of α-diversity metrics per soil treatment in B3 and B4 (Mean values ± standard deviation). Values followed by different letters are significantly different (P<0.05) according to Tukey's test. No letter in a row indicates no significant difference.

| | UNT | OMDL | OM2DL |
|---|---|---|---|
| **Rarefied richness** | 1877 ± 159 | 1980 ± 65 | 2052 ± 48 |
| **Shannon's index (H')** | 6.56 ± 0.15[b] | 6.60 ± 0.16[b] | 6.84 ± 0.05[a] |
| **Simpson index** | 0.996 ± 0.000[a] | 0.994 ± 0.001[a] | 0.997± 0.000[a] |

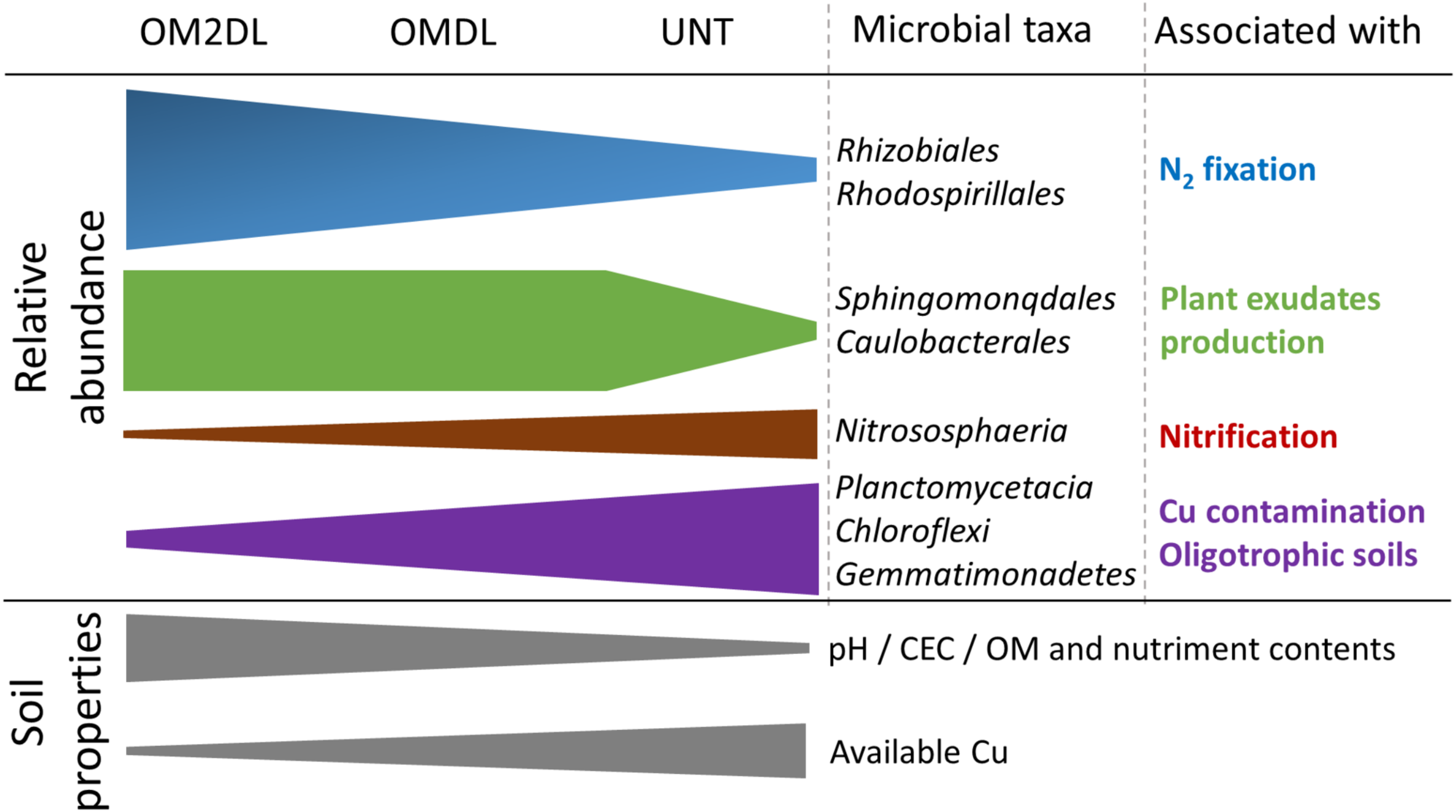
**Supplementary Table 3**. Effect of the soil treatments on the relative abundance of classified soil microbial taxa.

| TAXA | UNT | | OMDL | | OM2DL | | P val |
|---|---|---|---|---|---|---|---|
| ***Domain*** | | | | | | | |
| Bacteria | 9.89E-01 | b | 9.95E-01 | a | 9.96E-01 | a | 0.000 |
| Archaea | 1.06E-02 | a | 4.65E-03 | b | 4.25E-03 | b | 0.000 |
| ***Phylum*** | | | | | | | |
| Proteobacteria | 3.83E-01 | b | 4.31E-01 | ab | 4.70E-01 | a | 0.003 |
| Verrucomicrobia | 4.57E-02 | ab | 4.97E-02 | a | 4.11E-02 | b | 0.002 |
| Planctomycetes | 5.49E-02 | a | 3.74E-02 | b | 3.63E-02 | b | 0.000 |
| Gemmatimonadetes | 4.42E-02 | a | 3.48E-02 | ab | 2.57E-02 | b | 0.012 |
| Chloroflexi | 5.97E-02 | a | 3.15E-02 | b | 2.81E-02 | b | 0.000 |
| Epsilonbacteraeota | 9.21E-03 | b | 1.23E-02 | a | 9.27E-03 | b | 0.004 |
| Armatimonadetes | 4.12E-03 | b | 7.21E-03 | a | 5.47E-03 | b | 0.004 |
| Thaumarchaeota | 9.92E-03 | a | 4.51E-03 | b | 3.91E-03 | b | 0.001 |
| Ca. Latescibacteria WS3 | 9.39E-03 | a | 2.77E-03 | b | 2.57E-03 | b | 0.000 |
| Chlorobi | 1.31E-03 | b | 2.39E-03 | a | 1.97E-03 | ab | 0.032 |
| Ca. Tectomicrobia | 2.39E-03 | a | 1.14E-03 | b | 8.33E-04 | b | 0.000 |
| Ca. Dependentiae TM6 | 1.25E-03 | a | 7.25E-04 | b | 6.81E-04 | b | 0.002 |
| Ca. Parcubacteria | 1.56E-03 | a | 5.83E-04 | b | 7.82E-04 | b | 0.000 |
| ***Class*** | | | | | | | |
| Alphaproteobacteria | 1.57E-01 | b | 1.85E-01 | b | 2.21E-01 | a | 0.000 |
| Blastocatellia | 2.27E-02 | b | 3.86E-02 | a | 3.23E-02 | ab | 0.006 |
| Thermoleophilia | 1.94E-02 | ab | 2.77E-02 | a | 1.76E-02 | b | 0.008 |
| Gemmatimonadetes | 3.60E-02 | a | 2.76E-02 | ab | 2.07E-02 | b | 0.009 |
| Cytophagia | 3.67E-02 | a | 2.32E-02 | b | 3.09E-02 | a | 0.001 |
| Spartobacteria | 1.02E-02 | b | 2.10E-02 | a | 1.38E-02 | b | 0.004 |
| Planctomycetacia | 3.00E-02 | a | 2.05E-02 | b | 2.17E-02 | ab | 0.003 |
| OPB35 soil group | 2.24E-02 | a | 1.75E-02 | b | 1.52E-02 | c | 0.000 |
| Desulfurellia | 9.21E-03 | b | 1.23E-02 | a | 9.27E-03 | b | 0.004 |
| Phycisphaerae | 1.46E-02 | a | 1.15E-02 | ab | 9.96E-03 | b | 0.009 |
| Acidobacteriia | 2.12E-02 | a | 6.45E-03 | b | 3.33E-03 | c | 0.000 |
| Chloroflexi Subdivision 2 | 1.40E-02 | a | 5.95E-03 | b | 6.81E-03 | b | 0.000 |
| Nitrososphaeria | 9.92E-03 | a | 4.51E-03 | b | 3.91E-03 | b | 0.001 |
| Chloroflexi Subdivision 8 | 7.12E-03 | a | 3.75E-03 | b | 2.90E-03 | b | 0.001 |
| Chloroflexia | 4.06E-03 | a | 3.16E-03 | ab | 1.88E-03 | b | 0.027 |
| OM190 | 6.93E-03 | a | 2.76E-03 | b | 2.89E-03 | b | 0.000 |
| Armatimonadetes Group 4 | 1.61E-03 | b | 2.47E-03 | a | 1.64E-03 | b | 0.023 |
| Chlorobia | 1.31E-03 | b | 2.39E-03 | a | 1.97E-03 | ab | 0.032 |
| Clostridia | 2.20E-03 | ab | 2.35E-03 | b | 3.10E-03 | a | 0.023 |
| S085 | 4.06E-03 | a | 2.23E-03 | b | 2.57E-03 | b | 0.002 |
| Anaerolineae | 4.59E-03 | a | 1.93E-03 | b | 2.21E-03 | b | 0.000 |
| Chthonomonadetes | 7.50E-04 | b | 1.85E-03 | a | 9.68E-04 | b | 0.000 |
| Verrucomicrobiae | 1.94E-03 | ab | 1.72E-03 | b | 2.33E-03 | a | 0.040 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| JG30 KF CM66 | 3.72E-03 | a | 1.69E-03 | b | 1.26E-03 | b | 0.000 |
| Saprospiria | 2.34E-03 | ab | 1.50E-03 | b | 2.85E-03 | a | 0.018 |
| Thermomicrobia | 2.69E-03 | a | 1.22E-03 | b | 6.08E-04 | b | 0.003 |
| vadinHA49 | 1.21E-03 | a | 1.15E-03 | a | 5.93E-04 | b | 0.002 |
| Acidobacteria clade RB25 | 2.42E-03 | a | 1.04E-03 | b | 1.01E-03 | b | 0.000 |
| Ktedonobacteria | 5.95E-03 | a | 9.86E-04 | b | 5.30E-04 | b | 0.000 |
| BD2 11 terrestrial group | 1.30E-03 | a | 4.85E-04 | b | 9.83E-04 | a | 0.000 |
| Acidobacteria group 2 | 3.33E-03 | a | 4.27E-04 | b | 3.21E-04 | b | 0.000 |
| Caldilineae | 7.68E-04 | a | 4.15E-04 | b | 6.37E-04 | a | 0.001 |
| ***Order*** | | | | | | | |
| Rhizobiales | 6.80E-02 | b | 8.43E-02 | ab | 9.38E-02 | a | 0.032 |
| Sphingomonadales | 3.96E-02 | b | 6.06E-02 | a | 6.85E-02 | a | 0.013 |
| Xanthomonadales | 6.71E-02 | a | 5.46E-02 | b | 6.67E-02 | a | 0.001 |
| Blastocatellales | 2.26E-02 | b | 3.86E-02 | a | 3.22E-02 | ab | 0.006 |
| Gemmatimonadales | 3.60E-02 | a | 2.76E-02 | ab | 2.07E-02 | b | 0.009 |
| Rhodospirillales | 2.93E-02 | ab | 2.43E-02 | b | 3.57E-02 | a | 0.002 |
| Cytophagales | 3.66E-02 | a | 2.31E-02 | b | 3.08E-02 | a | 0.001 |
| Chthoniobacterales | 9.91E-03 | b | 2.08E-02 | a | 1.31E-02 | b | 0.003 |
| Planctomycetales | 3.00E-02 | a | 2.05E-02 | b | 2.17E-02 | ab | 0.003 |
| Solirubrobacterales | 8.73E-03 | b | 1.66E-02 | a | 1.18E-02 | b | 0.010 |
| Desulfurellales | 9.21E-03 | b | 1.23E-02 | a | 9.27E-03 | b | 0.004 |
| Caulobacterales | 1.29E-02 | ab | 1.11E-02 | b | 1.78E-02 | a | 0.002 |
| Frankiales | 6.43E-03 | ab | 8.80E-03 | a | 5.52E-03 | b | 0.009 |
| TRA3 20 | 8.17E-03 | ab | 7.41E-03 | b | 9.66E-03 | a | 0.002 |
| Propionibacteriales | 5.64E-03 | b | 7.35E-03 | b | 1.06E-02 | a | 0.000 |
| Gaiellales | 9.63E-03 | a | 7.24E-03 | a | 4.19E-03 | b | 0.000 |
| Acidobacteriales | 2.12E-02 | a | 6.45E-03 | b | 3.33E-03 | c | 0.000 |
| Nitrososphaerales | 8.02E-03 | a | 4.46E-03 | b | 3.88E-03 | b | 0.013 |
| SC I 84 | 4.11E-03 | a | 3.57E-03 | a | 2.19E-03 | b | 0.000 |
| Kineosporiales | 1.15E-03 | b | 3.31E-03 | a | 1.34E-03 | b | 0.000 |
| Chloroflexales | 2.74E-03 | a | 2.67E-03 | a | 1.42E-03 | b | 0.037 |
| Chlorobiales | 1.31E-03 | b | 2.39E-03 | a | 1.97E-03 | ab | 0.032 |
| Clostridiales | 2.09E-03 | ab | 2.12E-03 | b | 2.86E-03 | a | 0.021 |
| Anaerolineales | 4.59E-03 | a | 1.93E-03 | b | 2.21E-03 | b | 0.000 |
| Chthonomonadales | 7.50E-04 | b | 1.85E-03 | a | 9.68E-04 | b | 0.000 |
| Verrucomicrobiales | 1.89E-03 | ab | 1.69E-03 | b | 2.27E-03 | a | 0.033 |
| Saprospirales | 2.34E-03 | ab | 1.50E-03 | b | 2.85E-03 | a | 0.018 |
| Streptosporangiales | 9.94E-04 | b | 1.44E-03 | b | 2.79E-03 | a | 0.012 |
| Phycisphaerales | 3.20E-03 | a | 1.25E-03 | b | 1.62E-03 | b | 0.000 |
| Rhodobacterales | 2.85E-03 | a | 1.11E-03 | b | 9.63E-04 | b | 0.000 |
| Acidobacteria group 22 | 2.42E-03 | a | 1.04E-03 | b | 1.01E-03 | b | 0.000 |
| Acidobacteria group 7a | 6.09E-04 | ab | 9.19E-04 | a | 3.71E-04 | b | 0.006 |
| JG30 KF CM45 | 2.17E-03 | a | 8.72E-04 | b | 4.45E-04 | b | 0.003 |
| Lineage IIa | 3.49E-04 | a | 7.50E-04 | a | 5.07E-04 | a | 0.025 |
| C0119 | 1.55E-03 | a | 7.36E-04 | b | 3.86E-04 | c | 0.000 |
| NB1 j | 1.86E-03 | a | 7.05E-04 | b | 7.56E-04 | b | 0.000 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Methylophilales | 2.30E-03 | a | 6.77E-04 | b | 4.09E-04 | b | 0.000 |
| Gitt GS 136 | 1.21E-03 | a | 6.41E-04 | ab | 9.26E-04 | a | 0.024 |
| Vicinamibacter order incertae sedis | 2.39E-04 | b | 6.14E-04 | b | 1.29E-03 | a | 0.000 |
| HTA4 | 1.65E-03 | a | 6.12E-04 | b | 3.64E-04 | b | 0.000 |
| Oceanospirillales | 6.28E-04 | b | 5.68E-04 | b | 1.35E-03 | a | 0.002 |
| Caldilineales | 7.68E-04 | a | 4.15E-04 | b | 6.37E-04 | a | 0.001 |

Ca., candidate


OM2DL
OMDL
UNT
Microbial taxa
Associated with
Relative abundance
Rhizobiales
Rhodospirillales
N2 fixation
Sphingomonqdales
Caulobacterales
Plant exudates production
Nitrososphaeria
Nitrification
Planctomycetacia
Chloroflexi
Gemmatimonadetes
Cu contamination
Oligotrophic soils
Soil properties
pH / CEC / OM and nutriment contents
Available Cu