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Abstract: The chemical properties, ecotoxicity, and microbiome of leachates from phytomanaged Cu-contaminated soils were analyzed. The phytomanagement was carried out using Cu-tolerant poplar *Populus trichocarpa* × *deltoides* cv. Beaupré and black bent *Agrostis gigantea* L., aided by soil amendments, i.e., dolomitic limestone (DL) and compost (OM), alone and in combination (OMDL). Plants plus either DL or OMDL amendments reduced in leachates the electrical conductivity, the Cu concentration, and the concentration of total organic C except for the OMDL treatment, and decreased leachate toxicity towards bacteria. Total N concentration increased in the OM leachates. The aided phytostabilization increased the culturable bacteria numbers and the proportion of Cu-resistant bacteria in the leachates, as compared to the leachate from the untreated soil. Phytomanagement also enriched the microbial communities of the leachates with plant beneficial bacteria. Overall, the Cu stabilization and phytomanagement induced positive changes in the microbial communities of the soil leachates.

Keywords: metal polluted soil; soil leachate; aided phytostabilization; soil toxicity; bacterial Cu-resistance; microbial diversity

1. Introduction

Soils host highly diverse microbial communities that may reach up to 10^6 of bacterial species per gram of soil [1–3]. Such extreme microbial richness is due to a large number of microhabitats within the soil structure [4,5] that offers, at the same time, conditions for the proliferation of the dominant microbial phylotypes and suitable protective niches for rare species, both playing a fundamental role in

microbially driven soil functions [6,7]. The composition and structure of the soil microbial communities are influenced by main soil properties such as pH value and nutrient availability [8], climate [9], vegetation cover due to the plant-induced microbial selection [10], and soil management [11,12]. Soil microbial diversity has been mainly assessed by analyzing the soil solid phases, and therefore the current knowledge refers to the bulk soil or soil aggregates [13]. However, soil is a heterogeneous environmental matrix consisting of solid, liquid, and gaseous phases, and the complexity of intact soils prevents a systematic study of the microbial communities adhering to the solid phases or present in the soil solution [14]. The soil solution is the liquid phase in which nutrients diffuse and become available for uptake by plant roots and microorganisms [15], in which the dissolved organic matter (DOM) is transported to the deeper soil horizons, and soil hydration influences the bacterial community composition and microbial physiological activity [16]. In addition, the soil solution is the medium in which bacterial and virus dispersal and transport occurs [17–19], and from the microbiological point of view, the soil solution can be considered as one of the most interactive soil phases. In metal(loid)-contaminated soils, generally only a fraction of metal(loid)s are present in the aqueous phase both as free ions after desorption from the surface of the solid phases or bound with soluble organic and inorganic ligands [20]. The DOM can increase the mobility and transport of several metal(loid)s such as Cu, Pb, and As [21], particularly for those having high affinity for the organic ligands in solution such as Cu [22].

Copper is a micro-nutrient required as a cofactor in several oxo-reductase enzymes involved in basic cellular metabolic pathways, owing to its ability to alternate between Cu(I) and cupric Cu(II) oxidation states [23]. Copper, along with Cd, Cr, Pb, and Zn, may exceed its background levels in soils of anthropogenic areas [24], being released by many industrial and agricultural activities, and can reach particularly high concentrations in topsoils of industrial areas such as those where wood impregnation is practiced [25]. Soil Cu contamination results in a range of adverse effects, including phytotoxicity [26], reduced microbial activity, and diversity with deleterious effects on soil organic matter (SOM) decomposition and nutrient mineralization [27–30], leading to a general loss of soil fertility [31]. Excess Cu alters the microbial community structure [28,29] and can lead to the positive selection of Cu-tolerance within the microbial community [27]. The remediation of Cu-contaminated soils is relatively complicated due to strong Cu retention by the soil exchange complex and SOM. Soil washing with extractants and chelating agents is a common technology for Cu removal from soil [32–34], but although efficient, the engineering technologies are not sustainable for large-scale remediation interventions and can cause irreversible loss of soil ecological functions. Phytomanagement is a remediation approach for contaminated sites based on the use of plants, microorganisms, soil conditioners, and agro-ecological practices to reduce the environmental risks posed by soil contamination to an acceptable level, into a risk management based framework [35]. Unlike civil engineering technologies, the requirements of phytomanagement for chemicals and energy are low, as well as the total cost, making it use sustainable for the remediation of large contaminated areas, enhancing soil fertility resulting in high sustainability and social acceptance of such practice. Among the phytomanagement options for meta(loid)-contaminated soils, the cultivation of metal(loid)-tolerant woody plants aided by soil amendments can either mobilize or immobilize metal(loid)s, reduce the soil toxicity, and restore the soil microbial diversity and microbial functions in a relatively short time [36]. In contaminated soils, the metal(loid) pools in the aqueous phase are those having higher interactions with soil microorganisms [37]. While it is postulated that restoration of microbiological diversity and functions of metal(loid)-contaminated soils under aided phytostabilization is led by decreased metal(loid) solubility and bioavailability, such beneficial effects have been demonstrated for the microbial communities of the bulk soil. To our knowledge, the influence of phytomanagement on the microbial diversity and bacterial metal-resistance in the soil solution has been poorly studied.

We hypothesize that an aided phytostabilization approach can reduce the metal(loid) solubility in contaminated soils, as well as decrease their toxicity on soil microorganisms and increase the microbial diversity. We tested such a hypothesis by analyzing the water leached from a Cu-contaminated soil

of a wood preservation site, either untreated or amended with organic and inorganic amendments and cultivated with the Cu-tolerant poplar *Populus trichocarpa* × *deltoides* cv. Beaupré and black bent (*Agrostis gigantea* L). We analyzed the main chemical parameters, Cu concentration, and ecotoxicity of soil leachates from large outdoor lysimeters after three phytomanagement years and related these parameters to the microbial diversity and Cu-resistance of the endogenic bacteria.

Our results can improve the current knowledge of the chemical improvement and the composition of the microbial community in the solution of Cu-contaminated soils managed by aided phytostabilization.

2. Materials and Methods

2.1. Site Characteristics and Leachate Collection

The soil was collected at a wood preservation site (10 ha) located in Gironde (SW France, 44430N; 0300W) [25]. The soil is of alluvial origin, classified as Fluvisol-Eutric Gleysol (WRB, 2006), with a coarse sandy texture and neutral pH value.

In March 2007, vats of 75 dm^3 and 0.5 m diameter (Figure 1) were filled with three successive layers, including two undisturbed layers of sandy soil collected in a trench at the P3 subsite (Table A1) [25,38]: 5 cm of coarse gravels (1–3 cm, diameter), 22 cm of sub-soil (from the 30–60 cm soil layer), and 25 cm of topsoil (from the 0–30 cm soil layer). A geotextile separated gravels and the sub-soil. Total Cu concentrations (mg kg^{-1}) were 1110 in the topsoil and 111–153 in the subsoil (Table A1). Amendments were carefully mixed with the topsoil using a vat, alone and in combination (% air-dried soil DW, *w/w*), before filling the lysimeters, to consist the four soil treatments: bare untreated soil (Unt), 5% compost made of bark wood chips and poultry manure (OM, Orisol, Cestas, France), 0.2% dolomitic limestone (DL) [39], and OM with DL (OMDL). Lysimeters were prepared in triplicates for each treatment. Stem cuttings (roughly 20 cm long) of *P. trichocarpa* × *deltoides* cv. Beaupré, a commercial cultivar (INRA, Nancy, France), were sampled in January 2006 from trees established in a nursery (Gironde district, France) and were rooted in individual pots, placed in a greenhouse, on perlite imbibed with a quarter-strength Hoagland nutrient solution. *Agrostis gigantea* L. (2 patches, 5 cm in diameter of a population originated from the surrounding of a Cu/Ni smelter in Sudbury (Canada) and one Beaupré poplar (initial shoot length: 30 ± 5 cm) were transplanted in all lysimeters except for the Unt treatment. Lysimeters (n = 21) were placed in situ (March 2007). The soil pH value did not significantly vary across the treatments, ranging between 7.16 ± 0.12 for the Unt soils and 7.33 ± 0.12 for the OMDL ones (Table A1) [38]. The annual maintenance for the lysimeters was to harvest the senescent shoots of *A. gigantea* at the end of the winter (February). Lysimeter leachates were periodically collected in plastic bottles (1.5 dm^3) from 5 March 2007 after each major precipitation event (>30 mm, leachate volume >1.5 L). They were collected in March 2010, 3 years after the soil treatment, kept at 4 °C, and analyzed after 48 h from the collection.

2.2. Chemical Analysis of the Leachates and Microbial Toxicity Test

The leachates were split into aliquots. To measure the leachate Cu concentrations, samples were filtered at 0.22 µm, acidified with 0.2 mL HNO_3 prior to elemental quantification by inductively coupled plasma optical emission spectrometry (IRIS II XSP, Thermo Fisher Scientific, Courtaboeuf cedex, France).

Figure 1. Lysimeters filled with untreated and phytomanaged soils.

Microbial toxicity of leachate samples was assayed by using the BioTox™ Flash Test (Aboatox Oy, Turku, Finland), which is based on the inhibition of the luciferase activity of the bioluminescent *Alivibrio fisheri* bacteria, according to the standard method (ISO 21338:2010). The pH and conductivity values during the test were adjusted as recommended by the manufacturer. The *A. fisheri* bioluminescence was detected by Sirius luminometer (Berthold D.S., Pforzheim, Germany), allowing automatic correction of color and turbidity [40], and recorded by Sirius Software for Windows at time zero and after 15 min of sample–bacteria contact. The bioluminescence inhibition percentage (inh%) was determined by comparing the bioluminescence intensity of control bacteria suspensions with that of the tested materials, according to the following formula:

$$\text{inh\%} = 100 - [(IT_{15}/K_F \cdot IT_0) \times 100] \quad (1)$$

where IT_{15} and IT_0 are the luminescence values of samples at time 15 min and zero, respectively, and K_F is a correction factor given by the ratio between the luminescence after 15 min and that at zero time in control samples.

2.3. Cu Resistance of Culturable Bacteria

The Cu resistance of culturable bacteria was evaluated by plate counts of colony-forming units (CFU) of bacteria plated on Petri dishes. The bacteria resistance to Cu was evaluated, growing them on minimal salt medium containing 0.8 mM $CuSO_4$ [41]. The culturable oligotrophic bacteria were determined by the CFU formed onto the minimal salt medium without $CuSO_4$ addition, and the total culturable bacteria were counted after growth onto Luria Bertani broth (Sigma). For microbial colony development, Petri dishes were incubated at a constant 37 °C in the dark for 7 days [41]. The proportion of the Cu-resistant bacteria was expressed as a percentage of CFU on Cu-selective medium and the total culturable bacteria grown on LB broth.

2.4. High throughput Sequencing Analysis

Soil total DNA was extracted using the SPIN DNA kit (MP Biomedicals) according to the manufacturer instructions, except that a vortex at maximum speed for 10 minutes was used instead of the FastPrep disruptor and the DNA was eluted in 100 µL of sterile H_2O. DNA was purified by the DNA Clean Up Kit (MoBIO) according to the manufacturer's protocol with the following exceptions: clean up started at Step 11, and purified DNA was eluted using 50 µL of sterile water. The 16S

rRNA gene sequences were amplified by PCR using the specific forward (515F-SBS3) and reverse (806R:SBS12-1) primers. The PCR was performed with an initial denaturation temperature of 94 °C for 3 min, followed by 20 cycles of 94 °C for 45 s, 50 °C for 30 s, and 65 °C for 90 s. A final elongation step of 65 °C was run for 10 min. The amplicon sequencing was conducted on an Illumina IIx, with two 101 base pair, paired reads. The rRNA reads were trimmed to retain only reads longer than 100 bp and high-quality bases (Phred score > 30) using PRINSEQ [42]. Trimmed sequences were treated as previously described [43]. A taxonomic assignment was obtained using UCLUST on QIIME v1.9.1 [44]. The bacterial operational taxonomic units (OTUs) were summarized based on 97% sequence similarity, and taxonomic data were generated through the classification algorithm using the Silva database version 132 [45]. The total number of classified OTUs was used to calculate the following alpha diversity indices: diversity (Shannon), richness (Chao1), dominance, and evenness, followed by the one-way ANOVA statistical analysis using PAST 3.03 [46]. Differences among treatments were tested using Kruskal–Wallis, followed by Tukey posthoc test.

2.5. Statistical Analysis

The chemical parameters and ecotoxicity values of the leachates from all treatments were the mean values of three independent lysimeters, and the significance of differences was assessed by one-way ANOVA followed by the Fisher HSD test.

3. Results

3.1. Chemical Parameters and Toxicity of the Soil Leachates

The soil leachates had pH values ranging between 5.63 and 5.91 with no significant differences among the treatments, whereas the EC value was significantly higher for the leachate of the bare untreated soil (Unt) than that of the amended soils under phytomanagement (Table 1). The TOC concentrations showed higher and similar values in the Unt, OM, and OMDL leachates as compared to the DL ones, whereas the total N concentration was significantly higher in the OM leachates than in other treatments (Table 1). The Cu concentrations were highest in the Unt and OM leachates and lowest in the DL and OMDL ones. The highest value of bioluminescence inhibition of *A. fischeri* determined by the BioTox test was observed for the Unt leachates (28.9%); an inhibition value of 22.5% was observed for the OM leachates, whereas the bioluminescence inhibition was below the toxicity threshold for the DL and OMDL leachates (Table 1).

Table 1. Chemical properties and ecotoxicity of the leachates from untreated and phytomanaged soils.

Treatment	pH	EC (µS)	Ca (mg L^{-1})	Mg (mg L^{-1})	Cu Concentration (µM)	Total Organic C (mg L^{-1})	Total N (mg kg^{-1})	Microbial Toxicity (Inhibition %)
Unt	5.91 (±0.32) a	255 (±75) a	4.70 (±1.33) a	1.21 (±0.17) a	17.0 (±0.37) a	12.9 (±5.8) a	1.15 (±0.16) b	28.9 (±5.4) a
DL	5.83 (±0.32) a	121 (±19) b	8.78 (±4.39) a	2.66 (±0.66) bc	7.40 (±0.12) b	7.58 (±1.5) b	1.05 (±0.18) b	11.4 (±3.9) c
OM	5.63 (±0.09) a	124 (±37) b	5.15 (±0.57) a	1.85 (±0.20) ab	16.2 (±0.13) a	13.7 (±1.1) a	1.56 (±0.13) a	22.5 (±5.0) b
OMDL	5.81 (±0.75) a	155 (±19) b	10.25 (±2.21) a	3.14 (±0.21) c	6.77 (±0.22) b	14.8 (±3.36) a	0.86 (±0.73) b	9.6 (±5.0) c

Values are the average of three replicates, and values in brackets are the standard deviation of the means. Values followed by the same letter(s) are not significantly different at p value ≤ 0.05 by ANOVA and the Fisher HSD test.

3.2. Microbial Diversity, Cultural Bacteria, and Cu Resistant Bacteria

The total culturable bacteria showed a significant increase in CFU values for the OM and OMDL leachates, whereas the culturable oligotrophic bacteria showed the lowers CFU values in the leachates from the Unt soils (Table 2). The culturable Cu-resistant bacteria showed significantly higher CFU values in the OM and OMDL than in the Unt and DL leachates (Table 2); however, by comparison with

the culturable oligotrophic, the highest proportion of Cu-resistant bacteria, defined as the percentage of the culturable oligotrophic bacteria, were detected in the leachates from the Unt soil and the lowest proportions in the DL and OMDL leachates (Table 2).

Table 2. Total culturable bacteria, Cu-resistant bacteria, and the proportion of Cu-resistant bacteria in the leachates of the untreated and phytomanaged soils.

Treatment	Total Culturable Bacteria (CFU mL^{-1})	Oligotrophic Bacteria (CFU mL^{-1})	Cu Resistant Bacteria (CFU mL^{-1})	Cu Resistant Bacteria (%)
Unt	2.4 (±2.1) × 10^3 b	2.7 (±2.1) × 10^3 b	5.8 (±1.9) × 10^2 b	21.4 (±2.1) a
DL	9.4 (±3.7) × 10^3 b	1.5 (±3.7) × 10^4 a	5.6 (±1.0) × 10^2 b	3.8 (±1.3) d
OM	2.8 (±2.6) × 10^4 a	8.3 (±2.6) × 10^4 a	1.3 (±2.8) × 10^3 a	15.6 (±2.4) b
OMDL	1.0 (±0.1) × 10^4 a	1.4 (±0.1) × 10^4 a	1.3 (±4.7) × 10^3 a	9.8 (±2.1) c

Values are the average of three replicates, and values in brackets are the standard deviation of the means. Values followed by the same letter(s) are not significantly different at p value 0.05 by ANOVA and the Fisher HSD test.

The relative abundance of the prokaryotic phyla accounting for ≥1% of the total of sequences detected in at least one replicate per treatment, showed that Proteobacteria was the most abundant phylogenetic group ranging from 39.3% to 47.2% of the total sequences, followed by Bacteroidetes, Chlamydiae, and Planctomycetes in variable relative abundances depending on the treatment (Figure 2). The largest changes in the relative abundance of bacterial phylotypes were observed in the DL treatment, in which the Spirochaetes group appeared, and the Planctomycetes, Proteobacteria, Planctomycetes, and Bacteroidetes groups were reduced (Figure 2).

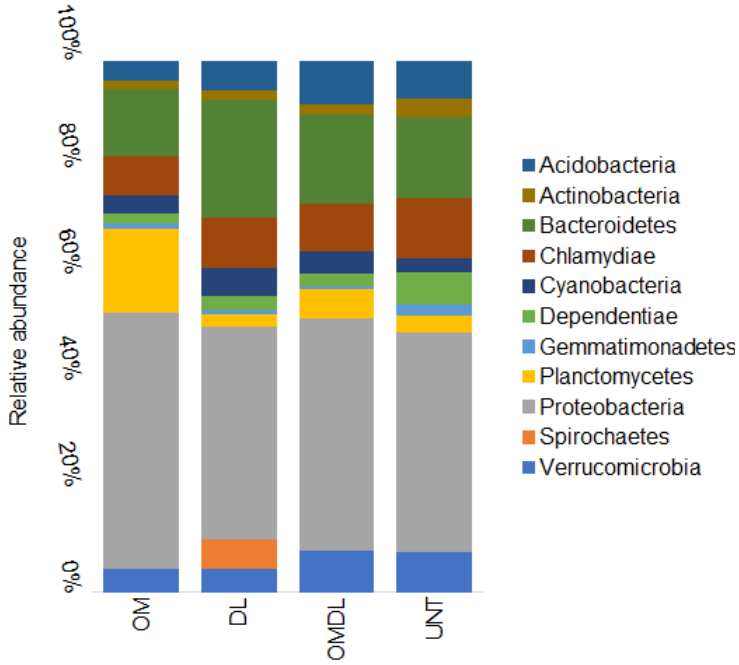

Figure 2. Prokaryotic phyla with relative abundance ≥1% detected in at least one treatment replicate of the total sequences revealed by the analysis of the 16S rRNA gene using high throughput sequencing.

No significant differences among treatments were observed at the phylum level. Differences were observed among the leachates from different treatments in the prokaryotic OTUs at the genus level with an abundance of ≥1% of the total sequences and detected in at least one replicate per treatment (Figure 3). Among the genera detected in single treatments, Verrucomicrobia Pedosphaeraceae ADurb.Bin063-1 were characteristic of the Unt treatment, Bacteroidetes *Chitinofaga* sp., Proteobacteria Paracaedibacteraceae, Proteobacteria *Oligoflexales* 0319-6G20, Spirochaetes *Leptospira*

sp. were characteristic of the DL treatment, Planctomycetes Planctomycetales, Proteobacteria Desulfobulbaceae, Proteobacteria Gammaproteobacteria sp. Verrucomicrobia *Prosthecobacter* sp. were characteristic of the OMDL treatment (Figure 3). Among the genera common to two treatments, the Bacteroidetes *Chitinophagaceae_2*, Planctomycetes *Gemmata*, Bacteroidetes *Chitinophagaceae*_2, Proteobacteria Gammaproteobacteria EC3 characteristic of both DL and OMDL treatments, Proteobacteria Reyranellaceae (*Reyranella* sp.), Proteobacteria Sphingomonadaceae (*Sphingomonas* sp.) characteristic of both OM and OMDL treatments (Figure 3).

Figure 3. Heatmap prokaryotic operational taxonomic units (OTUs) at genus level with relative abundance >1% of the total sequences detected in at least one treatment replicate revealed by the analysis of the 16S rRNA gene using high throughput sequencing.

No significant differences were observed in the alpha diversity indexes among treatments ($p > 0.05$), although the diversity was slightly lower in the leachates of from all amended soils, with lowest values for the OM and DL ones (Figure 4). Differently, the Unt soil was the soil with lower dominance, whereas the highest values were found for the DL and OM soils (Figure 4).

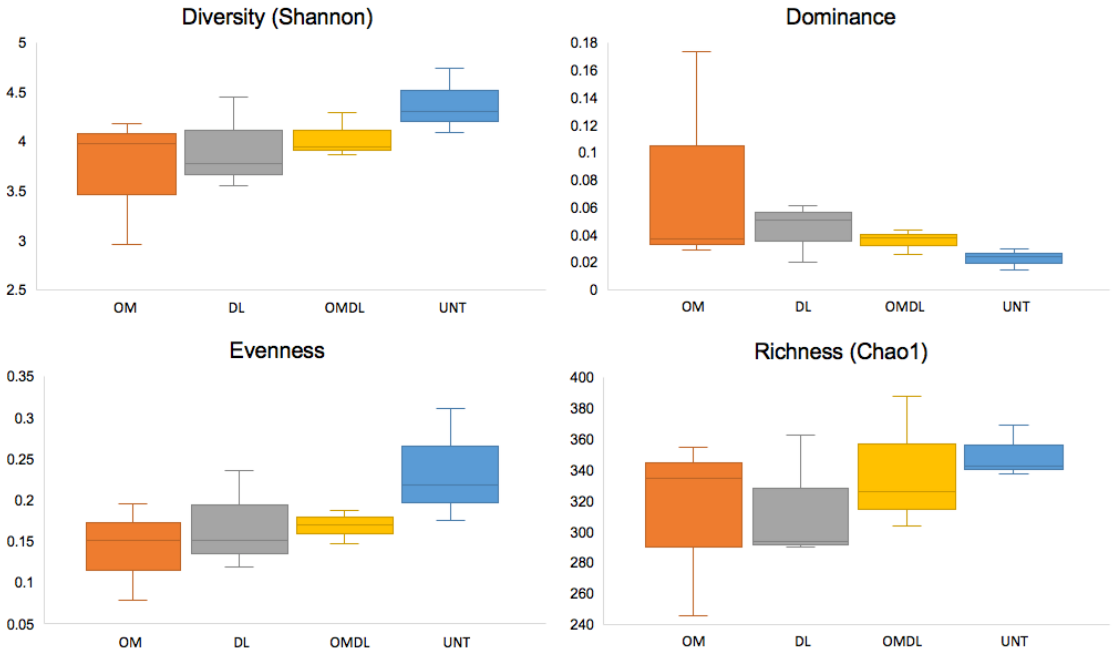

Figure 4. Values of alpha-diversity of the bacterial community of leachates from untreated and phytomanaged soils. Black bar corresponds to the median. No significant differences were observed ($p > 0.05$).

4. Discussion

The adopted phytomanagement was effective in Cu immobilization for both DL and OMDL soils, based on Cu concentration in the soil leachates (Table 1) likely by precipitation reactions driven by the addition of dolomitic limestone, containing Ca and Mg oxides and carbonates, and sorption with the OM from the compost incorporation. The limestone addition in soil can increase the availability of macronutrients such as Ca and Mg, facilitating uptake by plants and reducing the effect of Cu toxicity, as reported by Juang et al. [47] and Ambrosini et al. [48]. Franceschi and Nakata [49] showed that higher Ca concentration in shoots may decrease the phytotoxic effect of Cu, facilitating the formation of calcium oxalate crystals, which incorporate metals, such as Cu, in their structure, whereas Yruela [50] reported that higher Mg concentration in plant tissue may compete with Cu ions and prevent the replacement of the central Mg ion of the chlorophyll molecule. Our results were in line with those by Fan et al. [51], reporting effective Cu immobilization in contaminated soils amended with Ca-rich sludge from water treatment residues. Moreover, our results confirmed those reported by Trentin et al. [52] about the effectiveness of the use of the limestone to reduce the Cu availability and phytotoxicity. The Cu stabilization significantly reduced the leachate toxicity to microorganisms, which could, in turn, explain both the reduced proportion of Cu-resistant bacteria in the phytomanaged soils, also in absolute values for the DL treatment (Table 2). In fact, tolerable environmental Cu concentrations for non-resistant bacteria are lower than 10 μM [53], and such low concentrations were only detected in the DL and OMDL leachates (Table 1). Concerning the higher bacterial Cu-resistance in the OM treatment as compared to DL and OMDL, this could be explained by the bacterial proliferation triggered by the larger C availability as compared to the latter treatments, indicated by the increase of the total and oligotrophic bacteria (Table 2) which contained a high proportion of Cu-resistant bacteria (i.e. Unt bacterial community in Table 2) in the presence of relatively high Cu concentrations in solution (Table 1). This explanation was also supported by slight toxicity of the OM leachates indicated by the BioTox test and by the increase of species dominance in the bacterial community of the OM leachates (Figure 3) coupled to the lack of significant differences in soil bacterial communities among treatments at the phylum level (Figure 2).

The microbial community of the soil leachates showed the typical composition of the soil microbiome with the Acidobacteria, Actinobacteria, Proteobacteria, Bacteriodetes, and Firmicutes being the dominating phyla [54]. Proteobacteria, Chloroflexi, Acidobacteria, Actinobacteria, Gemmatimonadetes, Verrucomicrobia, Thaumarchaeota, Firmicutes, and Nitrospirae were reported as the dominant phyla in a long-term Cd contaminated soil [55]. Nevertheless, the phytomanagement influenced the microbial community composition, with major changes observed for Verrucomicrobia and Actinobacteria, Gemmatimonadetes, and Dependentiae, and in the increase of phyla of Proteobacteria, Planctomyces, and Cyanobacteria. Such changes could be attributed to the decreased Cu concentration in the soil leachates, and this relationship was observed for several most abundant and rare phylogenetic groups. The Actinobacteria, Gemmatimonadetes, and Dependentiae phyla comprise several metal-tolerant species; their relative abundances in the leachates from phytomanaged soil paralleled that of the Cu resistance in culturable bacteria and could be attributed to the reduced Cu concentration in the soil leachates. The same reduction of Cu-induced selective pressure could also be hypothesized for the slight reduction of Chlamydiae and Patescibacteria, which tolerate soil metal(loid) pollution [56] and are generally detected in contaminated environments [57,58]. Species belonging to Gemmatimonadetes have also been detected in metal-contaminated soils [59]. Acidobacteria and Thaumarchaeota comprise species with variable tolerance to metal(loid)s and display divergent lifestyles [60,61]. This may explain the lack of definite trends observed for these phylogenetic groups. The observed reduction of relative abundance of Verrucomicrobia was in line with their negative correlation with pollution levels in Cu-contaminated soils [62], and support the hypothesis that this phylum may be used as a molecular biomarker of soil contamination [63].

The reduced Cu solubility and toxicity could also have interactive effects with the TOC concentration in the soil leachates. In particular, the significantly lower TOC in the DL leachates could be due to the stabilization of the SOM by reaction with Ca- and Mg carbonates [64]. This lower TOC could have influenced the bacterial community, in particular, the balance between copiotrophic and oligotrophic microorganisms. For example, the higher TOC in the OM and OMDL leachates as compared to the Unt leachates (Table 1) could explain the increase of the Proteobacteria [65] and of Planctomycetes [66]. Lejon et al. [67] suggested that adaptation of soil microorganisms to Cu was related to the SOM quality. Moreover, the lower TOC concentration in the DL leachates could also have reduced the microbial dispersal in the soil leachates. In fact, higher DOM content can increase the groundwater transport of bacteria, mainly coating and saturating the sorption sites of the soil solid phases [18]. Owing to their high metal sensitivity [68], the observed increase of Cyanobacteria, particularly in the DL leachates, could be attributed to the strong reduction of Cu concentrations. The leachate pH value was not significantly changed by the phytomanagement (Table 1). This allowed us to better discriminate the phytomanagement effects on the microorganisms, as the pH value is an important factor shaping the structure of soil microbial community [69].

Long-term field experiments indicated a rapid influence and sustainable efficiency of the OMDL soil treatment over a 10-year period [30,36]. The cost is low as compared to dig-and-dump and other ex-situ physico-chemical solutions as this in situ option only included costs for soil loosening, dolomite and compost and their application, annual cultural practices (including inorganic N–P–K fertilization in case of the cultivation of annual high yielding plants), and harvest.

5. Conclusions

Our results provided insights on the changes in the chemical properties and ecotoxicity and on the microbial diversity and metal-resistance of the leachates from a Cu-contaminated soil, either untreated or under aided phytomanagement. The adopted phytomanagement significantly reduced the Cu in the soil leachates, the soil toxicity and the incidence of Cu-resistant bacteria, and also led to an increase of Cyanobacteria, Proteobacteria, and Planctomyces phylogenetic groups, which are generally plant beneficial and metal sensitive that tend to decrease in metal(loid) contaminated soils. Overall, the results confirmed the potential of phytomanagement as an ecological remediation

strategy. To our knowledge, this was the first study on the effects of aided phytostabilization on the microbial communities of the soil leachate, a potential proxy for the soil solution, and we suggest that further investigations should be conducted to assess the effects of phytomanagement on arbuscular mycorrhizal as well as non-symbiotic fungal communities.

Author Contributions: L.G., A.d.O.S. and M.M. carried out the experiments. L.G. and G.R. supervised the experimental plan. L.G.d.A.B. and A.G. performed the amplicon sequencing analysis. E.W.T. and A.N.A. generated the amplicon sequencing and prepared the raw data. L.G. and G.R. wrote the manuscript with the support of M.M. All authors discussed the results and contributed to the final manuscript. G.R. and M.M. conceived the original idea and G.R. supervised the project. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this article.

Appendix A

Table A1. Main properties of the soil layers used to fill the lysimeters and main soil characteristics [38].

Soil Properties	0–30 cm Soil Layer	30–60 Soil Layer
Sand %	85.8	85.5
Silt%	8.3	11.2
Clay %	5.9	3.3
Organic matter (g/Kg)	16	5.42
C/N	17.2	8.63
CEC (cmol/Kg)	3.5	1.17
pH	7	4.04
As (mg/Kg)	9.8	4.7
Cd (mg/Kg)	0.12	
Co (mg/Kg)	<2	2.3
Cu (mg/Kg)	1110-1460	111-153
Cr (mg/Kg)	23	18.4
Fe (mg/Kg)	6090	7900
Mn (mg/Kg)	181	185
Ni (mg/Kg)	5	8
Pb (mg/Kg)	27	
Tl (mg/Kg)	0.24	
Zn (mg/Kg)	46	28.7
pCu^{2+}	7.66	5.12

$pCu^{2+} = 3.20 + 1.47\ pH - 1.84\ \log_{10}$ (total soil Cu).

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