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Functional activity and functional gene diversity of a Cu-contaminated soil remediated by aided phytostabilization using compost, dolomitic limestone and a mixed tree stand☆

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ABSTRACT

Trace elements (TEs) availability, biochemical activity and functional gene diversity was studied in a Cu-contaminated soil, revegetated for six years with a mixed stand of willow, black poplar, and false indigo-bush, and amended or not with compost plus dolomitic limestone (OMDL). The OMDL amendment significantly reduced Cu and As availability and soil toxicity, and increased the biochemical activity and microbial functional diversity assessed with the GEOCHIP technique, as compared to the unamended soil (Unt). The OMDL soil showed significantly higher abundance of 25 functional genes involved in decomposition organic compounds, and 11, 3 and 11 functional genes involved in the N, P and S biogeochemical cycles. Functional gene abundance was positively correlated with nutrient contents but negatively correlated with Cu availability and soil toxicity. The abundance of microbial functional genes encoding for resistance to various TEs also increased, possibly due to the microbial proliferation and lower Cu exposure in the presence of high total soil Cu concentration. Genes encoding for antibiotic resistance due to the co-occurrence of TEs and antibiotic resistant genes on genetic mobile elements. Overall, phytomanagement confirmed its potential to restore the biological fertility and diversity of a severely Cu-contaminated soil, but the increase of TEs and antibiotic resistant gene abundances deserve attention in future studies.

1. Introduction

Copper (Cu) is naturally present in all soils with a median concentration of 31.1 mg kg^{-1} (Heijerick et al., 2006; Tóth et al., 2016), but its concentrations in soils under conventional agriculture, vineyards, orchards, or surrounding mining areas, smelters and wood preservation sites can build up to very high concentrations due to anthropic activities, either producing or using Cu-based compounds and materials (Komárek et al., 2010; Mench and Bes, 2009; Ettler, 2016), and Cu sorption by clay minerals and the soil organic matter (SOM) (Quenea et al., 2009; Lagomarsino et al., 2011). Copper is a micronutrient with important physiological activities in all living microorganisms, but Cu excess in soil impacts the soil microbial communities (Sandaa et al., 1999; Lejon et al., 2008) and soil respiration, soil microbial biomass and enzyme activities (Kumpiene et al., 2009). Remediation of TE contaminated soils (TECS) can be carried out by civil engineering technologies such as “dig and dump” operations, thermal stabilization or soil washing, which rapidly reduce the pollutant linkages associated to TEs excess, but are expensive and cause the irreversible loss of soil properties and functions underlying beneficial ecosystem services (Khalid et al., 2017). Phytomanagement is a TECs remediation option carried out by using plants microorganisms and soil organic and inorganic amendments, capable of reducing the TEs bioavailability in soil and improving soil physico-chemical properties and nutrient status, and enhance the soil ecological functions (Raskin et al., 1997). Phytomanagement is effective in reducing soil

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phytotoxicity while preserving the soil resource (Quintela-Sabarís et al., 2017), and can produce income for the local communities (Mench et al., 2010; Ruttens et al., 2011; Van Slycken et al., 2013; Witters et al., 2012), especially when managed as short rotation coppice of biomass producing trees (Van Slycken et al., 2013).

Trace element contamination generally reduce soil microbial biomass and microbial diversity, due to negative selection of TE-sensitive microorganisms (Tyler et al., 1989; D'Ascoli et al., 2006; Azarbad et al., 2016). Moreover, TECS display reduced metabolic processes due to the dominance of less metabolically efficient TE-resistant microorganisms (Mergeay, 2000), or to the direct TE-induced inhibition of soil enzyme activity (Renella et al., 2006). Soils host highly diverse microbial communities involved in the biogeochemical cycles of nutrient in terrestrial ecosystems and for plant nutrition (Torsvik and Øvreås, 2002). Microbial diversity also confer stability and functional resilience to soil microbial activity and ecological functions (Girvan et al., 2005), and changes in the composition assessed by using DNA sequencing technologies of the soil microbial communities can indicate the impact of TEs bioavailability (Daniel, 2005; Singh et al., 2009). However, the assessment of changes in the microbial community composition, is not informative on the potential functional capacity of soils, nor directly related to the soil biochemical activity. The GeoChip technology allows the comparison of functional gene diversity and functional microbial groups across soils, and has been successfully used for studying the functional diversity of agricultural and forest soils under various management and vegetation cover, different climatic regimes and remediated TECS (Epelde et al., 2006; Xue et al., 2013, 2015). While the increase of microbial biomass and enzyme activity in TECS under phytoremediation has been reported (Ascher et al., 2009; Renella et al., 2008), the use of soil chemical and biochemical methods make impossible to understand whether the recovery of soil functionality is contributed by a higher functional gene diversity, preventing long term predictions of phytomanagement on soil quality.

We hypothesized that reduction of Cu solubility, along with the increased nutrient availability in TECS under aided phytostabilization, could increase not only microbial biomass and biochemical activity, but could also increase the functional gene diversity of soil microbial communities evaluated by the GEOCHIP (He et al., 2007). The increase of functional gene diversity could be used as an indication of the potentials of the aided phytostabilization technique to enhance microbial-driven soil ecosystem services. Because TE impacts on the soil microbial community and functionality are related to their availability, we also hypothesized that the long term effective Cu stabilization could reduce the proportion of metal resistant microorganisms within the soil microbial communities. We tested our hypotheses using the GeoChip technology to study the functional gene diversity in a Cu-contaminated soil after 6 years of aided-phytostabilization using soil amendments and a mixed tree stand. The functional gene diversity was compared with the mobile Cu concentrations evaluated by soil chemical extractions and soil toxicity.

2. Materials and methods

2.1. Site characteristics and soil sampling

Soil samples were collected at an industrial area (6 ha) located in Gironde (SW France, 44°43′N; 0°30′W) used to preserve and store timbers, posts, and utility poles for over a century (Mench and Bes, 2009; Bes et al., 2010). Soil Cu contamination resulted mainly from washing of treated wood. Total topsoil Cu varied from 65 to 2600 mg kg^{-1} on the whole site (Mench and Bes, 2009). Mean value for total topsoil Cu (0–25 cm) at the studied field trial was 1001 ± 279 mg Cu kg^{-1} and values did not significantly differ across the plots. The contaminated soil was of alluvial origin classified as Fluvisol - Eutric Gleysol (WRB, 2006), with a sandy texture and neutral pH value. The aided phytostabilization field trial started in May 2006 at site P1-3, consisting of 1 m × 3 m plots. The trial comprised the following four treatments: untreated (Unt), 0.2% w/w dolomitic limestone (DL), containing 30% CaO and 20% MgO combined with carbonates, fineness index < 80%, 0.16 mm, neutralizing power 58 (Prodical Carmeuse, Orthez, France), 5% w/w compost (OM) derived from poultry manure and pine bark chips (ORISOL, Cestas, France), and DL plus OM (OMDL) at the same rates as for the single treatments. Amendments were incorporated into the soil to a depth of 25 cm and the four treatments were randomly replicated in four blocks. the scheme of the treatments and a representative image of the site after 1 and 6 years of experiments are shown in supplementary materials (Figs. S-1, S2). The present study was conducted on soils under the Unt and OMDL treatments 6 years after the soil amendment because the latter proved to be the most successful in increasing the soil microbial biomass, biochemical activity and microbial diversity after two years of treatment (Šimek et al., 2017). The Unt and OMDL soils had a pH value of 7.16 ± 0.12 and 7.33 ± 0.12, respectively, and all plots were managed as short-rotation coppice (SRC) with a mixed stand of willows (*Salix viminalis* L.; *Salix caprea* L.), poplar (*Populus nigra* L.) and false indigo bush (*Amorpha fruticosa* L.). Soil samples made of three 1 kg subsamples per plot were collected from the 0–25 cm soil layer from all plots using a stainless steel spade and kept as independent replicates. Soil samples were immediately transported to the analytical laboratory, sieved (<2 mm) and pre-incubated at 25 °C for 1 week at 50% water holding capacity prior to biochemical determinations whereas soil samples for GEOCHIP analysis were stored at −80 °C prior to DNA extraction. Aliquots of each soil were air dried for chemical analyses.

2.2. Soil chemical and biochemical properties

The method of Walkley and Black (1934) was used to determine the total organic C (TOC) was used, whereas total N was determined using a Perkin Elmer 2400 Series II CHN Elemental Analyzer. Inorganic N (NH_4^+-N and NO_3^--N) was determined by extracting 5 g d.w. soil for 1 h with 1 M KCl (1:5 soil:solution ratio) according to Keeney and Nelson (1982), and analyzed by ion selective electrodes. The available P was measured according to Olsen and Sommers (1982) protocol.

The pseudototal concentrations of As, Cd, Cr, Cu, Mn, Ni, Pb, Zn were measured by microwave-assisted (Milestone 900) acid digestion in a 1:5 HF:HNO_3 solution at 600 W for 24 min using 0.25 g of dry soil. The residue was brought to a final volume of 25 mL with 0.15 M HCl prior to elemental quantification. The extractable TE fraction was determined by soil extractions with 0.05 M ethylenediaminetetraacetic (EDTA) according to Quevauviller (1998), using 10 g of air-dried sieved suspended in 100 mL of EDTA tetrammonium salt at pH 7.00, end-over-end shaken at 120 oscillations per min for 2 h at room temperature. The soil suspensions were filtered through Whatman 42 filter paper and immediately analysed. The exchangeable Cu fraction was determined by soil extractions with 1 M NH_4NO_3 (Pruess, 1998), with the modification that the 1 M NH_4NO_3 solution was buffered at pH 7.00 with concentrated NH_3 (Renella et al., 2004). Twenty grams of soil were suspended in 50 mL of 1 M NH_4NO_3 in polythene bottles end over end shaken at room temperature for 2 h at 20 oscillations per min. The soil suspensions were then filtered through Whatman 42 filter paper, and the extracts were acidified with 0.2 mL HNO_3 prior to elemental analysis. Elemental quantification was conducted by inductively coupled plasma optical

Table 1
Main physico-chemical properties of untreated and amended soils. Different superscripts indicate significant differences ($P < 0.05$) between mean values analysed the by two tailed *t*-test.

Soil	Sand	Silt	Clay	$pH_{(H2O)}$	TOC	N_{tot}	NH_4^+-N	NO_3^--N	P_{tot}	P_{avail}
	(%)	(%)	(%)		(g kg^{-1})					
Unt	85.8^a	8.3^a	5.9^a	7.16^a	0.23^a	0.08^a	1.31^a	0.28^a	46.8^a	8.99^a
OMDL	85.1^a	8.9^a	6.0^a	7.33^a	**3.01^b**	**0.32^b**	1.08^a	0.23^a	**379.9^b**	**28.5^b**

N_{tot} is total nitrogen; P_{tot} is total phosphorous; P_{avail} is the available phosphorous fraction (Olsen).
Values in bold indicate significant differences for values within columns

emission spectrometry (IRIS II XSP, Thermo Fisher Scientific).

Soil respiration rate was determined using 2 g of soil (dry weight equivalent) placed in 10 mL air tight vials (Exetainer, Labco, UK) and the headspace was sampled after 24 h using an air tight syringe. Soil respiration was estimated as CO_2-C concentration in the headspace by gas chromatography (HP 5890) according to Blackmer and Bremner (1977). Soil microbial biomass was estimated by determining the ATP content according to Ciardi and Nannipieri (1990) using the same sample previously used for measuring the soil respiration. The arylesterase activity was analyzed according to the method described by Zornoza et al. (2009). For the acid and alkaline phosphomonoesterase activities assay the method proposed by Tabatabai and Bremner (1969) was used, whereas for the phosphodiesterase activity was used the protocol as reported by Browman and Tabatabai (1978). The urease activity was measured according to Nannipieri et al. (1974), the protease activity assay was done according to Ladd and Butler (1972) and the β-glucosidase activity was assayed using the protocol proposed by Tabatabai (1982), and the arylsulfatase activity was determined with the method of Tabatabai and Bremner (1970). Soil toxicity was assessed by the BioTox test (Aboatox, Finland), according to Lappalainen et al. (2001), with bioluminescence inhibition values > 20% being the threshold for soil toxicity.

2.3. GeoChip analysis

The GeoChip analysis of the functional gene diversity was conducted on Soil DNA extracted by freeze-grinding mechanical lysis (Zhou et al., 1996), using the GeoChip 4.2. The chip contained 107.950 probes, covering 792 functional gene families from 11 major functional categories, including C, N, phosphorus and sulfur cycling categories (Tu et al., 2014). Full details on the DNA labeling, hybridization, image processing, and data processing were reported by Xue et al. (2015).

2.4. Data analysis

All soils were analyzed in four replicates originating by composite sampling of the original four field replicates. Treatment comparisons for the soil chemical and biochemical data were performed by two tailed *t*-test, while permutation *t*-test was performed for the gene diversity indices. To analyze the GeoChip data, we used detrended correspondence analysis (DCA), non-parametric similarity tests such as permutational multivariate analysis of variance (Adonis), multiple response permutation procedure (MRPP) and analysis of similarity (ANOSIM), based on Horn, Bray-Curtis, and Euclidean dissimilarity indices, respectively. Canonical correlation analysis (CCA) and Mantel test were used to assess the relationship between soil microbial functional gene structure and environmental variables. The BioTox value, the variance inflation factors (VIF), contents of organic C, total N, total P, and extractable Cu were selected for the CCA model. Statistics were performed using the R 3.0.2 software (The R Foundation for Statistical Computing, Vienna, Austria), and the significant differences were defined as $P < 0.05$, or with listed *P* values.

3. Results

3.1. Soil physico-chemical properties and biochemical activity

The OMDL soils had significantly higher concentrations of TOC, total N, total and available P than the Unt soils, whereas texture, pH values and inorganic N concentrations (NH_4^+-N, NO_3^--N) were similar for both treatments (Table 1). Soils were mainly contaminated by Cu, other total soil TE being at the background levels for French sandy soils (Table 2). EDTA-extractable soil Cu was

Table 2
Pseudo-total, EDTA-extractable and NH_4NO_3-exchangeable TE concentrations of untreated and amended soils. Different superscripts indicate significant differences ($P < 0.05$) between mean values analysed the by two tailed *t*-test. ND indicates TE concentrations not determined.

Soil	As	Cd	Cr	Cu	Mn	Ni	Pb	Zn
Pseudo-total concentrations (mg kg^{-1} DW)								
Unt	8.92^a	0.14^a	8.26^a	1172^a	152^a	5.01^a	21.8^a	50.8^a
OMDL	7.29^a	0.10^a	7.54^a	1081^a	160^a	4.73^a	20.7^a	55.7^a
EDTA-extractable concentrations (mg kg^{-1} DW)								
Unt	0.45^a	0.05^a	ND	883^a	32.1^a	0.09^a	17.2^a	5.07^a
OMDL	0.50^a	0.05^a	ND	**724^b**	27.9^a	0.09^a	13.4^a	7.00^a
NH_4NO_3 exchangeable concentrations (mg kg^{-1} DW)								
Unt	0.32^a	0.002^a	ND	22.9^a	57.7^b	0.09^a	0.83^a	0.10^a
OMDL	**0.04^b**	0.002^a	ND	**4.69^b**	**72.2^a**	0.09^a	0.92^a	0.07^a
Background values for French sandy soils [a]	1–25 [b]	0.03–0.24	14.1–40.2	3.2–8.4	72–376	4.2–14.5	16.4–58.7	17–48
Screening values for French soils [c]		0.7	100	35		70	60	150

Values in bold indicate significant differences for values within columns.
[a] Median and upper whisker values except for As (Baize, 1997).
[b] Frequent As mean values for all French soil types (Baize, 2016).
[c] Baize (2009).

Table 3
Soil toxicity, microbial biomass, soil respiration and enzyme activities of Unt and OMDL soils. Different superscripts indicate significant differences ($P < 0.05$) between mean values analysed the by two tailed *t*-test.

Soil	Soil toxicity	Microbial biomass	Soil respiration	Arylest	Arylsulf	Ac ph	Alk ph	Phosphod	β-gluc	β-gal	Urease	Protease
	(bioluminescence inhibition %)	(ng ATP kg^{-1})	(μg CO_2-C g^{-1} soil*d^{-1})	(mg p-np kg^{-1}·h^{-1})							(mg NH_4^+-N kg^{-1}·h^{-1})	
Unt	36.2^a	698^a	7.43^a	82.9^a	22.4^a	391.8^a	215.2^a	90.7^a	113.9^a	84.8^a	2.66^a	1.04^a
OMDL	**12.2^b**	**2335^b**	**23.7^b**	**168.5^b**	**258.0^b**	**1161^b**	**621.9^b**	**142.5^b**	**414.8^b**	**186.5^b**	**23.3^b**	**3.41^b**

Arylest = Arylesterase activity; Arylsulf = Arylsulfatase; Ac. ph. = acid phosphomonoesterase activity; Alk. ph. = alkaline phosphomonoesterase activity; Phosphod. = phosphodiesterase activity; β-gluc. = β-glucosidase activity; β-gal. = β-galactosidase activity. Different superscripts indicate significant differences ($P < 0.05$) between mean values (letters in bold for significant differences).

decreased by 18% in the OMDL soil. The 1 M NH_4NO_3-exchangeable soil Cu and As were significantly reduced by 79% and 91%, respectively, and exchangeable soil Mn increased by 25% in the OMDL soil as compared to the Unt soil (Table 2).

3.2. Soil respiration, soil microbial biomass and soil toxicity

The OMDL soils had significantly higher microbial biomass and respiration rates, and all measured enzyme activities were enhanced (Table 3). The Unt soil was slightly toxic, as the bioluminescence inhibition for this treatment was higher than 20%, and its bioluminescence inhibition value significantly higher than that of OMDL soil (Table 3).

3.3. Functional gene diversity of soil microbial communities

GeoChip detected a total of 42580 probes across all samples, of which 31450 and 37910 were in the Unt and OMDL soils, respectively. Among the detected probes, 11.0% and 26.1% were unique to the Unt and OMDL soils, respectively, while 62.9% were detected in both (Table 4). The detected probe number (richness), Simpson Reciprocal (1/D) and Shannon–Weaver (H) indices showed a significantly higher ($P < 0.05$) functional gene diversity of microbial communities in the OMDL than in the Unt soils (Table 4). The DCA profile showed that the Unt soils clustered separately from the OMDL soils (Fig. 1), indicating that the community composition of both soil treatments was different. Consistently, the MRPP, ADONIS and ANOSIM dissimilarity tests also indicated significant ($P < 0.05$) difference in the composition of the functional genes between the Unt and OMDL soils (Table 5). Moreover, the Unt soils clustered more closely in the DCA profile than in the OMDL soils. Based on the Euclidean dissimilarity index, the dissimilarity of replicated OMDL samples (100.92 ± 0.95) was significantly higher ($P = 0.003$) than that for the Unt samples (91.1 ± 1.40).

Table 4
Functional gene overlap (italicized) and uniqueness (bold) between untreated and DLOM soils, and diversity indices (mean ± standard error). Expressed gene probes were considered as "species" and their abundances were represented by the normalized signal intensities. Shannon–Weaver index is defined as $H = -\sum p_i \times ln(p_i)$, where *pi* is the proportional abundance of species *i*. The Simpson's index was based on $D = \sum p_i^2$ and invsimpson returns *1/D*. The richness was considered as detected probe numbers. Different superscripts indicate significant differences ($P < 0.05$) between untreated and DLOM treatments by permutation two tailed *t*-test.

Treatment	Untreated	DLOM
Untreated	**4670** (11.0%)	*26780* (62.9%)
DLOM		**11130** (26.1%)
Gene no. in treatments	31450	37910
Richness	23102 (±807)^a	**27625 (±888)^b**
Shannon-Weaver (H)	10.04 (±0.04)^a	**10.22 (±0.03)^b**
Invsimpson (1/D)	23004.49 (±807)^a	**27532.27 (±884)^b**

Values in bold indicate significant differences for values within columns.

3.4. Functional genes involved in biogeochemical cycles

Among the detected genes encoding enzymes for degrading various C substrates, 24 out of 33 had significantly higher abundances ($P < 0.05$) in the OMDL soils than in the Unt soils. The significantly higher abundant genes in the OMDL soils included those encoding α-amylase (*amyA*), cyclomaltodextrinase (*cda*), isopullulanase and pullulanase (*pulA*) for starch degradation, arabinofuranosidase (*ara*) either from bacterial and fungal origin, xylanase (*xylA*), mannanase and xylanase for hemicellulose degradation, cellobiose dehydrogenase (*CDH*), cellobiase, endoglucanase and exoglucanase for cellulose degradation, acetylglucosaminidase and endochitinase for chitin degradation, and pectinase for pectin degradation (Fig. 2). Genes encoding for isocitrate lyase (*AceA*), malate synthase (*AceB*), limonene-1,2-epoxide hydrolase (*limEH*), vanillate O-demethylase oxygenase (*vanA*), and vanillin dehydrogenase (*Vdh*) for aromatic compound degradation, and genes encoding the lignin peroxidase (*lip*), manganese peroxidase (*mnp*) and phenol-oxidase for lignin degradation were significantly higher in the OMDL soils than in the Unt ones (Fig. 2). Differently, the abundance of *cam*DCAB operon involved in the degradation of D-camphor in the for aromatic compound degradation pathway was significantly lower ($P = 0.04$) in the OMDL soils than the Unt soils (Fig. 2).

Eighteen functional genes, involved in N cycling, were detected in the analyzed soils. Among them, genes encoding the glutamate dehydrogenase (*gdh*) and urease (α-subunit of *ureC*) for

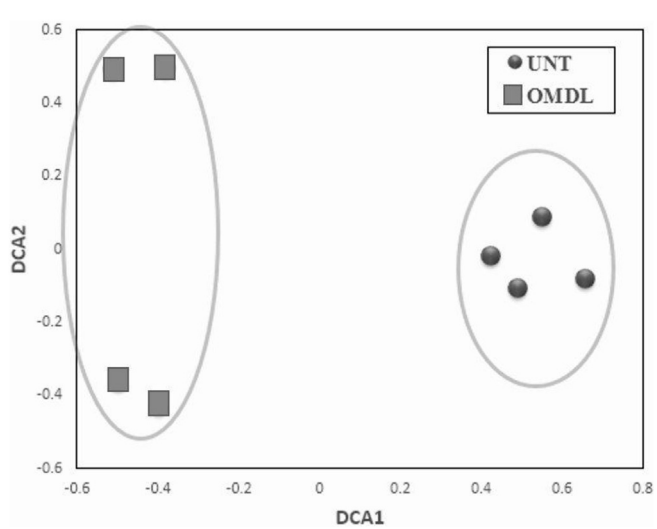

Fig. 1. Detrended correspondence analysis of the functional gene diversity in the Unt and OMDL soils.

Table 5
Non-parametric analyses to test dissimilarity of communities between untreated and DLOM treatments. All three tests are multivariate analyses based on *Bray-Curtis*, *Horn* and *Euclidean* dissimilarity indexes.

SRC *vs.* grassland	ADONIS[a]		ANOSIM[b]		MRPP[c]	
	F	P^d	R	*P*	δ	*P*
Bray-Curtis	6.871	0.030	1.000	0.029	0.196	0.032
Horn	7.198	0.032	1.000	0.032	0.182	0.022
Euclidean	3.350	0.032	1.000	0.035	96.034	0.023

[a] Permutational multivariate analysis of variance using distance matrices. Significance tests were performed by *F*-tests based on sequential sums of squares from permutations of the raw data.
[b] Analysis of similarities. Statistic *R* is based on the difference of mean ranks between groups and within groups. The significance of observed *R* is assessed by permuting the grouping vector to obtain the empirical distribution of *R* under null-model.
[c] Multi-response permutation procedure. Statistic δ is the overall weighted mean of within-group means of the pairwise dissimilarities among sampling units. The significance test is the fraction of permuted deltas that are less than the observed delta.
[d] *P*-value of corresponding significance test.

ammonification, the nitrate reductase (*nasA*) for assimilatory N reduction, the nitrate reductase (α-subunit *narG*), the nitrite reductase (*nirS*), the nitric oxide reductase (subunit B *norB*) and the nitrous-oxide reductase (*nosZ*) for denitrification, periplasmic nitrate reductase catalytic subunit (*napA*) and c-type cytochrome nitrite reductase (*nrfA*) for dissimilatory N reduction, ammonia monooxygenase (*amoA*) either by Bacteria or Archaea for nitrification, and nitrogenase (*nifH*) for nitrogen fixation, were significantly higher ($P < 0.05$) in the OMDL soils than in the Unt soils (Fig. 3). The abundance of *hzo*, encoding hydrazine oxidoreductase for Anammox, was lower in the OMDL soils than in the Unt soils (Fig. 3) although the difference was not significant ($P = 0.96$). All of the 11 detected functional genes involved in S cycling, encoding sulfite reductase (*CysI, CysJ*, *dsrB*, *dsrA* and *sir*), adenylylsulfate reductase (*AprA, APS_AprA, APS_AprB*), flavocytochrome c (*fccAB*) with sulfide dehydrogenase activity and sulfide-quinone reductase (*sqr*) for sulfide oxidation, and sulphur oxidation protein (*sox*) were significantly higher ($P < 0.05$) in the OMDL soils than in the Unt soils (Fig. 4). Two of the three detected functional genes involved in P cycling were significantly higher ($P < 0.05$) in the

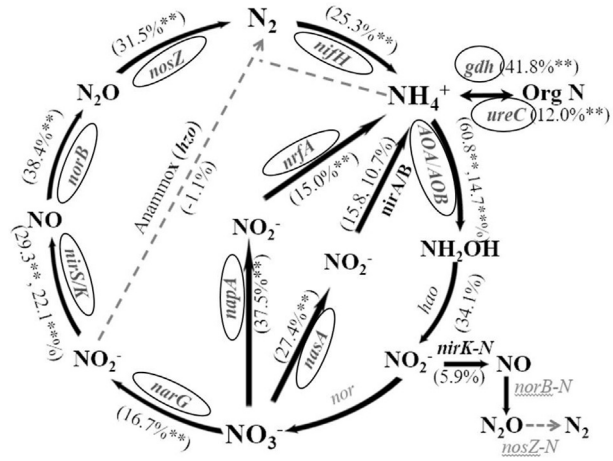

Fig. 3. Percentage change of normalized signal intensity from detected functional genes involved in nitrogen (N) cycling in the OMDL and Unt soils. Symbols ** and * indicate significant differences at $P < 0.05$ and < 0.10, respectively. The circled * symbols indicate the case when Unt > OMDL. Genes of *nor, norB* and *nosZ* for nitrification were not detected.

OMDL than in the Unt soils, including those encoding exopolyphosphatase (*ppx*) and polyphosphate kinase (*ppk*) for phosphorus utilization (Fig. 4).

3.5. Functional genes encoding TE and antibiotic resistance mechanisms

Among the genes encoding for TEs resistance, 1 out of 5 Cu-resistance genes (*copA* encoding Cu-transporting ATPase) was significantly higher ($P = 0.008$) in the OMDL soils than in the Unt soils, whereas other Cu-resistance genes did not differ between the treatments (Fig. 5). Four out of five As-resistance genes (*aoxB, arsB, ArsC* and *arsM*) were significantly higher ($P < 0.05$) in the OMDL as compared to the Unt soil, whereas the *ArsA* was higher but not significantly ($P = 0.09$) in the OMDL soils than in the Unt soil (Fig. 5). For other TE resistance genes, significantly higher abundances ($P < 0.05$) in the OMDL soils were found for *CadA*

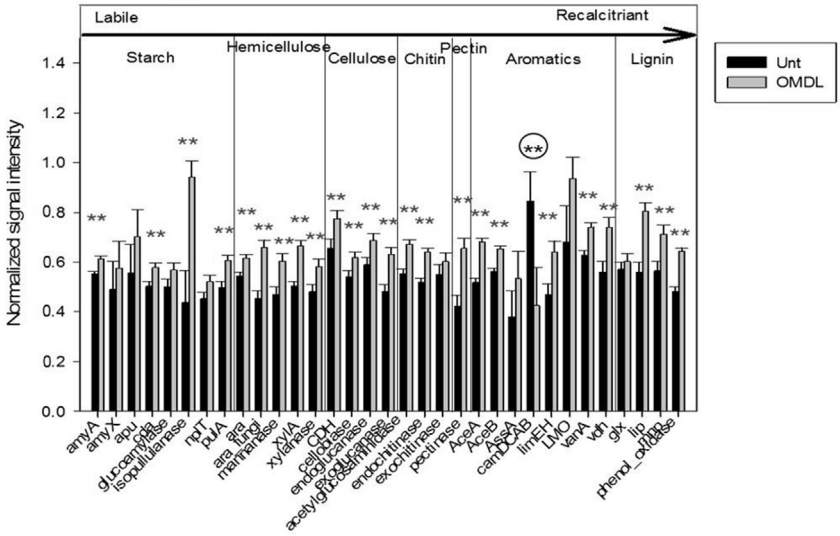

Fig. 2. Normalized signal intensity of detected functional genes encoding enzymes involved in carbon substrate degradation in the Unt and OMDL soils. The complexity of carbon is presented in order from labile to recalcitrant from left to right. Error bars represent standard error and symbols ** significant differences at $P < 0.05$. The circled * symbols indicate the case when Unt > OMDL.

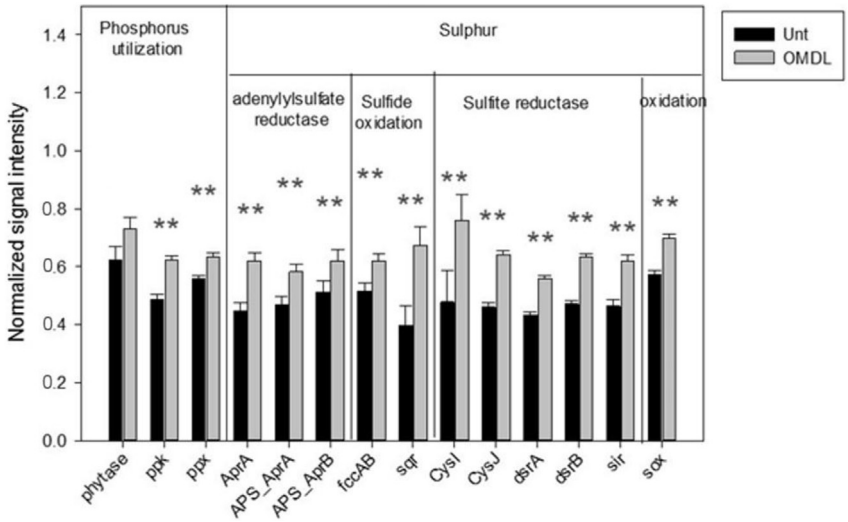

Fig. 4. Normalized signal intensity of detected functional genes encoding enzymes involved in S and P cycles in the OMDL and Unt soils. Error bars represent standard error and symbols ** significant differences at $P < 0.05$. The * symbols in red indicate that OMDL > Unt.

encoding Cd-translocating P-type ATPase for Cd resistance (1 of 2), *czcA* and *czcD* encoding cation efflux system proteins for Cd, Co and Zn resistance (2 of 3), the *ChrA* encoding chromate ion transporter protein for Cr resistance (1 of 1), the *CorC* encoding Mg and Co efflux protein for Mg and Co resistance (1 of 1), the *pbrT* encoding Pb uptake protein for Pb resistance (1 of 3), the *mer*, *merT* and *merC* encoding mercuric transport proteins for Hg resistance (3 of 7), the *nreB* encoding a Ni-induced transporter for Ni resistance (1 of 1), the *silA* and *silC* encoding outer membrane cation efflux protein for Ag resistance (2 of 4), *TehB*, *TerC*, *TerD*, *TerZ* encoding for TE resistance proteins (4 of 4), the *ZntA* encoding cation transport ATPase for Zn resistance (1 of 2), and the *SmtA* encoding metallothionein resistance to various TEs (Fig. 5).

Eleven functional genes involved in antibiotic resistance were detected in the analyzed soils. Genes encoding the class A and C of β-lactamases that utilize serine for β-lactam hydrolysis were significantly ($P < 0.05$) increased by the OMDL as compared to the Unt treatment, whereas the class B of β-lactamases were significantly ($P = 0.005$) decreased by the OMDL treatment as compared to the Unt treatment (Fig. 5).

3.6. Relationship between soil microbial functional gene diversity, biochemical activity and soil chemical properties

The CCA permitted to investigate the relationships between selected soil parameters and the structure of all detected functional genes in soil microbial communities. Soil toxicity assessed by BioTox and contents of organic C, total N, total P and extractable Cu were selected for the CCA model ($F = 1.43$, $P = 0.02$). In the CCA profile (Fig. 6), along the first canonical axis (CCA 1) the OMDL soils clustered separately from the Unt soils, explaining 36.8% of the total change in functional gene composition. We observed that the projections of soil chemical parameters, analyzed by CCA, revealed that OMDL samples were positively related with contents of organic C, total N and total P, but negatively related to the extractable Cu content and BioTox responses. The Mantel test results confirmed the link between soil chemical and biochemical properties and all detected functional gene composition, showing that the functional gene composition was significantly correlated with BioTox (Mantel statistic $r = 0.547$, $P = 0.026$), EDTA-extractable Cu (Mantel statistic $r = 0.547$, $P = 0.011$), organic C (Mantel statistic $r = 0.932$, $P = 0.011$), total P (Mantel statistic $r = 0.746$, $P = 0.024$) and total N (Mantel statistic $r = 0.786$, $P = 0.016$). Correspondingly, BioTox response was from 36% to 12% of bioluminescence inhibition (58%, $P = 0.05$) in the OMDL soils as compared to the Unt soils (Table 2). Total, NH_4NO_3-exchangeable and EDTA-extractable Cu concentrations decreased in the OMDL soils by 8% ($P = 0.18$), 79% ($P < 0.05$) and 18% ($P < 0.05$), respectively (Table 2). Compared to Unt soils, the OMDL treatment significantly increased soil contents of organic C ($P < 0.001$), total N ($P < 0.01$) and total P ($P < 0.01$) (Table 1).

All of the measured soil enzyme activities, ATP activity and soil respiration increased significantly ($P < 0.05$) by 57.7%–1052.1% in the OMDL soils compared to the Unt soils, except protease that was increased by 22.1% but not significantly ($P = 0.12$). By Mantel tests, they were all significantly correlated ($P < 0.05$) with the whole composition of all detected functional genes. Specifically, arylesterase and arylsulfatase were significantly correlated ($P < 0.05$) with recalcitrant C degradation genes (i.e. *glx*, *lip*, *mnp* and the gene encoding phenol oxidase). Activities of β-glucosidase and β-galactosidase were significantly correlated ($P < 0.05$) with most starch degradation genes (i.e. *amy*A, *cda*, *npl*T, *pul*A, genes encoding glucoamylase and isopullulanase), both enzymes activities were not significantly correlated with the *apu* gene ($P > 0.05$) and the β-galactosidase activity was not significantly correlated with the *amy*X gene ($P = 0.092$). Urease activity was significantly correlated ($P < 0.05$) with all detected nitrification genes (i.e. *amo*A, *hao*, and *nir*K). Activities of acid phosphatase, alkaline phosphatase and phosphodiesterase were significantly correlated ($P < 0.05$) with all functional genes involved in P cycles (i.e. *ppk*, *ppx* and the gene encoding phytase).

By Mantel tests, all of the antibiotic resistance genes were significantly correlated with either exchangeable or extractable Cu contents in soils ($P < 0.05$), as well as with exchangeable Pb ($P = 0.005$) and total Mg contents ($P = 0.009$). Specifically, the exchangeable Cu content was only significantly correlated with the composition of antibiotic transporter genes ($P = 0.033$), but not with the composition of β-lactamases or other antibiotic resistance genes ($P > 0.05$). In contrast, extractable Cu, exchangeable Pb and total Mg contents were significantly correlated with all of the antibiotic transporter, β-lactamases or other antibiotic resistance genes.

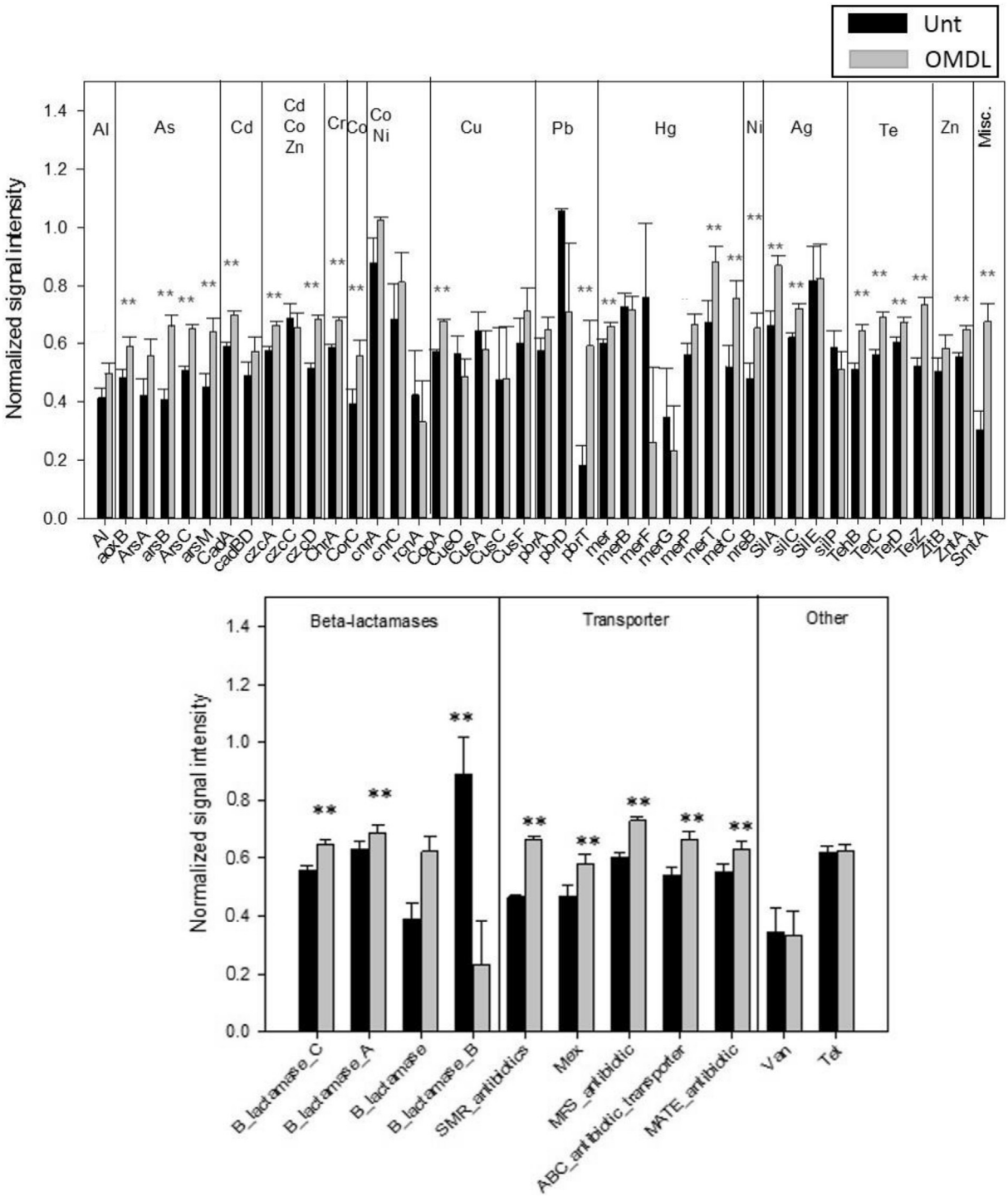

Fig. 5. Normalized signal intensity of detected genes encoding for TEs antibiotic and resistance in the OMDL and Unt soils. Error bars represent standard error and symbols ** significant differences at $P < 0.05$.

4. Discussion

Analysis of Cu-contaminated soils in year 6 of phytomanagement indicated a significant decrease in the Cu availability, estimated by the NH_4NO_3-exchangeable fraction, in the OMDL soils (Table 2). The EDTA-extractable and NH_4NO_3-exchangeable Cu fractions accounted on average for 67% and 1% of total soil Cu, respectively, the same order of magnitude of previous results (Tsang et al., 2007), and confirmed the high Cu affinity for the SOM (Di Palma et al., 2007). The high EDTA-extractable Cu fraction in both OMDL and Unt soils was likely due to the soft acid and complexing capacities of EDTA towards metals associated with potential acido-soluble bearing phases such as carbonates and Fe/Mn oxyhydroxides and organic matter (Di Palma et al., 2007; Tsang et al., 2007). Our results also agreed with those of Rinklebe and Shaheen (2015) who reported that the DTPA extractable Cu in a polluted soil accounted for 53% of the total Cu, and that soil amendment with various alkaline materials, including limestone,

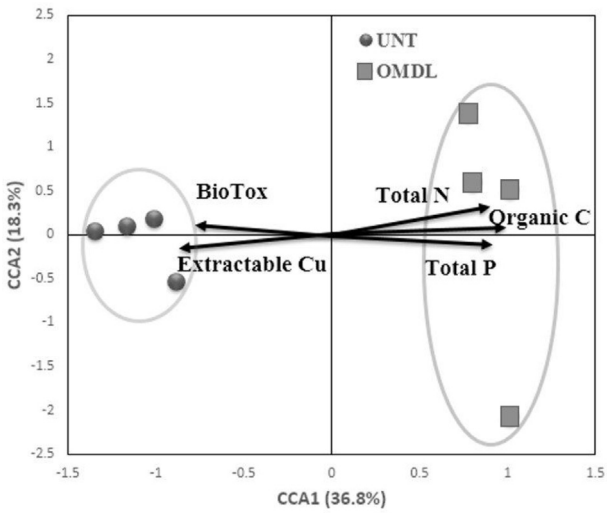

Fig. 6. Canonical correspondence analysis (CCA) profile between selected soil parameters and the structure of detected functional genes in the microbial communities of the OMDL and Unt soils.

significantly reduced the Cu solubility. Both the EDTA-extractable and NH_4NO_3-exchangeable TE fractions generally correlate to plant uptake (Ure and Davidson, 2002; Polettini et al., 2006), although the EDTA-extractable fraction has higher variability than the NH_4NO_3-exchangeable one (Kumpiene et al., 2017). This could explain why in the CCA analysis the functional gene structure of OMDL samples positively responded to the reduced extractable Cu fraction and reduced toxicity (Fig. 5). By comparison with the same soils analyzed after 2 years (Šimek et al., 2017), the exchangeable Cu fraction was decreased in the OMDL soils from 5.08 mg kg^{-1} (year 2) to 4.69 mg kg^{-1} (year 6), whereas the exchangeable Cu was reduced from 22.8 mg kg^{-1} (year 2) to 17.8 mg kg^{-1} (year 6).

The phytomanagement, combining the OMDL soil amendment and the mixed tree stand, significantly increased the soil microbial biomass, soil respiration and all the measured enzyme activities (Table 3), resembling results from previous field experiments of TECS phytomanagement (Ascher et al., 2009; Mench et al., 2006; Renella et al., 2008). Increase of biochemical activity is due to beneficial effects of organic matter inputs and reduced Cu availability and soil toxicity, and amelioration of soil properties such as nutrient contents (e.g. available P, TOC, and total N, Table 1) and aggregate formation (Kivlin and Treseder, 2014). The Cu availability, soil toxicity, soil organic C, total N and total P contents, also significantly shaped the composition of soil microbial functional genes, as revealed by CCA analysis. Although the decreased Cu exchangeable fraction from year 2 to year 6 was not significant, it indicated a long-term stabilization effect of the adopted aided phytomanagement, and explained why the Unt soil was still toxic with 54 and 32% of bioluminescence inhibition in soils from years 2 and 6, respectively, determined with the Biotox test (cfr Šimek et al., 2017). The effective long term Cu stabilization along with the fertilization effect of the OMDL amendment also allowed a significant increase ($P < 0.05$) of soil ATP content from 1167 ng kg^{-1} (year 2) to 2335 ng kg^{-1} (year 6), whereas for the Unt treatment the soil ATP content increased, but not significantly, from 576 ng kg^{-1} (year 2) to 698 ng kg^{-1} (year 6) (cfr Šimek et al., 2017).

The increase of soil biochemical activity in the OMDL as compared to the Unt soil (Table 3) was sustained by enriched microbial functional genes (Figs. 2–5). In year 6, the soil amendment with compost and dolomitic limestone still increased all potential C, N, P and S biogeochemical cycles, with a balanced growth for C present in both labile and recalcitrant forms (Fig. 2), and functional genes involved in N mineralization and N oxidation, as reported by (Šimek et al., 2017) on C and N dynamics in the same soils after 2 years of phytomanagement. Mertens et al. (2010) reported high impact of Cu contamination on soil nitrification potential. These results, along with the stimulated genes involved in P and S biogeochemical cycles, can be considered key factors contributing to the efficiency of the OMDL treatment, notably through potential nutrient supply for plant uptake.

Usually, SOM decomposition is reduced in Cu-polluted soils (Sauvé, 2006). This may also explain why microbial functional genes involved in C degradation were lower in the Unt soils. Moreover, the compost used in the OMDL treatment was made of bark pine chips and poultry manure. Cellulose and xylans are main structural components of bark pine chips with high lignin content (Vane et al., 2006) and other aromatic components like tannins. The addition of compost with remained polysaccharides, lignin and other aromatic components from bark pine chips may contribute to the stimulated microbial functional genes involved in relatively labile carbon (e.g. cellulose) and recalcitrant C (e.g. lignin and aromatic component) decomposition. Overall, the combined results of soil enzyme activities and abundance of functional genes support the hypothesis that in the OMDL soil the observed recovery of soil functions is sustained by a more active and genetically diverse microbial community as compared to the Unt soil.

The increase of genes encoding for TE-resistance in soil microorganisms in the OMDL soils was not expected. High TE concentrations are considered the main factors inducing the selection of resistance mechanisms within the microbial communities (Nies, 1999), and Cu impact on soil microorganisms in soils polluted by wood preservation activities has been reported (Turpeinen et al., 2004). In fact, all the measured microbiological endpoints showed that the OMDL incorporation into the Cu polluted soil significantly increased the microbial biomass and activity, as compared to the sole plants (Unt). We therefore hypothesize that the stimulating effect of OMDL towards soil microorganisms has led to the proliferation of the previously selected microorganisms carrying TE resistance genes due to their greater fitness under the high Cu concentrations. Increased abundance of genes encoding for resistance to various TEs in phytomanaged TECS, in spite of the reduction of both total and available TE fractions, was reported by Epelde et al. (2006) and Xue et al. (2015). Another potential factor favoring the TE resistant microorganisms. Higher abundance of functional genes encoding resistance to various TEs in the OMDL soils could be due to the higher microbial proliferation induced by the organic matter mineralization in the presence of high total soil Cu. Another possible explanation of higher metal resistance genes in the OMDL soils as compared to the Unt soils could be the introduction of exogenous TE-resistant microorganisms. The antibiotic resistance was also higher in the OMDL than in the Unt soil, with all five detected genes encoding the transporter that confer antibiotic resistance to microorganisms were significantly ($P < 0.05$) increased by the OMDL amendment, except the Zn-dependent class B of β-lactamases utilize divalent Zn ions for β-lactam hydrolysis (Fig. 5). The β-lactamases provide resistance through breaking the structure of β-lactam antibiotic (Bush and Jacoby, 2010). The parallel increase of metal and antibiotic resistance in the OMDL soil can be ascribed to the fact that various TE and antibiotic resistance genes are often located on the same mobile genetic elements (Mergeay, 2000), and the genetic linkage of TE and antibiotic resistance genes in bacteria has been reported in various environments due to the horizontal gene transfer within metabolically active microbial communities (Hobman and Crossman, 2014). The different trend of genes encoding for class B β-lactamases could be explained by the fact that these genes are

found as resident chromosomally-encoded enzymes in some microbial environmental strains (Rossolini et al., 2017). Overall, results of the metal and antibiotic resistance genes highlighted that phytomanagement of TECS while leading to microbial proliferation, also passes through phase that favors the affirmation of TE and antibiotic resistance encoding genes, that can persist for relatively long time (i.e. 6 y) after the effective TEs stabilization and reduction of soil toxicity. This has implications for the surrounding environment, as discussed in previous literature (Baker-Austin et al., 2006; Singer et al., 2006; Stepanauskas et al., 2006; Alekshun and Levy, 2007; Aminov and Mackie, 2007; Allen et al., 2010). In fact, spread of antibiotic resistance within soil microbial communities due to due to horizontal gene transfer may have not only negative repercussions on the soil ecological functions, but may potentially threats humans and wildlife once resistance is transferred to human or animal pathogens from environmental bacteria (Bengtsson-Palme and Larsson, 2015).

5. Conclusions

A single incorporation of compost and dolomitic limestone into a Cu-contaminated soil, coupled with revegetation with mixed stand of willows, black poplar and false indigo-bush, significantly reduced Cu availability and soil toxicity, and significantly increase microbial biomass, soil enzyme activity and abundance of functional genes involved in SOM decomposition and C, N, P and S mineralization, indicating a potential long term sustainability of this phytomanagement option. Unexpectedly, microbial proliferation also resulted in higher abundance of functional genes encoding resistance to various TEs and antibiotics in the OMDL soils, even after 6 y of effective reduction of Cu availability and soil toxicity. Future research is needed to understand whether this phenomenon indicates a relatively long transition phase during soil restoration, what are the potential impact of and the potential countermeasures to prevent the spreading of genetic mobile elements encoding for TEs and antibiotic resistance during TECS phytomanagement.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.06.057.

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Further reading

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