Chemosphere 138 (2015) 469–477

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

# Management with willow short rotation coppice increase the functional gene diversity and functional activity of a heavy metal polluted soil

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## HIGHLIGHTS

- We studied functional diversity of a heavy metal polluted soil under phytoremediation.
- Soils remediated with willow trees short rotations were compared to a grassland soil.
- Functional diversity and activities were higher in remediated than in grassland soil.

## ARTICLE INFO

*Article history:*
Received 24 March 2015
Received in revised form 1 June 2015
Accepted 15 June 2015

*Keywords:*
Phytoremediation
Short rotation coppice
GeoChip
Soil functionality
Soil microbial biomass
Soil enzyme activity

## ABSTRACT

We studied the microbial functional diversity, biochemical activity, heavy metals (HM) availability and soil toxicity of Cd, Pb and Zn contaminated soils, kept under grassland or short rotation coppice (SRC) to attenuate the risks associated with HM contamination and restore the soil ecological functions. Soil microbial functional diversity was analyzed by the GeoChip, a functional gene microarray containing probes for genes involved in nutrient cycling, metal resistance and stress response. Soil under SRC showed a higher abundance of microbial genes involved in C, N, P and S cycles and resistance to various HM, higher microbial biomass, respiration and enzyme activity rates, and lower HM availability than the grassland soil. The linkages between functional genes of soil microbial communities and soil chemical properties, HM availability and biochemical activity were also investigated. Soil toxicity and N, P and Pb availability were important factors in shaping the microbial functional diversity, as determined by CCA. We concluded that in HM contaminated soils the microbial functional diversity was positively influenced by SRC management through the reduction of HM availability and soil toxicity increase of nutrient cycling. The presented results can be important in predicting the long term environmental sustainability of plant-based soil remediation.

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## 1. Introduction

Heavy metal contaminated soils (HMCS) are one of the consequences of industrialization and represent a serious threat to human health and ecosystem stability. It has been estimated that of the $2.4 \times 10^6$ contaminated sites in the European Union, at least $3.4 \times 10^5$ require urgent remediation actions (Panagos et al., 2013). Among the available soil remediation techniques, conventional "dig and dump" operations and other civil engineering technologies such as thermal stabilization or soil washing, rapidly reduce the environmental risks associated with excessive heavy metals (HM) concentrations, but are expensive and lead to the irreversible loss of soil and its beneficial ecosystem services.

Phytoremediation is an alternative management option to attenuate environmental risks associated to HMCS, based on the use of plants and their associated microorganisms, in combination or not with organic and inorganic soil amendments. Phytoremediation is inexpensive, preserve the soil and restores its fertility and ecosystem functions, and may produce income

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http://dx.doi.org/10.1016/j.chemosphere.2015.06.062
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for local communities (Mench et al., 2010; Ruttens et al., 2011; van Slycken et al., 2013a).

In the last decade, the use of agro-forestry practices such as short rotation coppice (SRC) with high biomass producing tree clones has attracted great attention by HMCS owners, managers and policy makers because it can combines bioenergy production, risks attenuation, and restoration of soil fertility and ecosystem services (van Slycken et al., 2013b). Fast growing woody plants such as *Eucalyptus* sp., *Populus* spp. and *Salix* spp., are grown worldwide for bioenergy purposes under short rotation coppice regimes and it has been reported that high biomass yield can be obtained even on HMCS, with long term prospects for positive economic balance (Witters et al., 2012).

Phytoremediation experiments conducted at various scales have shown that the various options (i.e. phytoextraction, *in situ* immobilization, phytostabilization) can reduce risks associated with HM by reducing the bioavailable fractions, allowing plant growth, and increasing soil ecological functions (Ascher et al., 2009; Renella et al., 2008). Key soil ecological functions such as organic matter decomposition, nutrient mineralization, plant–microbe ecological interactions (e.g. growth promoting activities, host parasite relations), heavy metal resistance and decomposition of xenobiotics are mediated by microbial communities, and are of prime interest in the context of a sustainable agro-forestry management of contaminated sites. Microbial communities of HMCS are characterized by low diversity and are often dominated by selected resistant or tolerant/resistant species with reduced functional activity (Tyler et al., 1989; Mergeay, 2000; D'Ascoli et al., 2006).

While previous studies have shown that phytoremediation can restore specific soil functions (e.g., nutrient mineralization) since the early stages of implementation, information on the functional diversity of the microbial communities in soils under phytoremediation is still scarce.

Among the currently available molecular or metagenomic technologies, a comprehensive characterization of the functional diversity of the soil microbial communities can be obtained with the microarray-based GeoChip technology (He et al., 2007). The GeoChip 4.2 can be used for the detection of $1.5 \times 104$ genes from more than 400 gene categories of microbial groups involved in various functions such as nutrient cycling, metal resistance, and degradation of organic contaminants and ecological interactions (Tu et al., 2014).

We hypothesized that the SRC management of a heavy metal polluted soils using willow trees could increase the functional gene diversity of the soil microbial communities and the soil functional activity as compared to the same soil kept under mixed volunteer vegetation representing a 'no intervention' scenario. We tested our working hypothesis by studying the functional diversity, biochemical activity, and properties HM solubility in soils under long term phytoextraction management. Overall, our work aimed to clarify the potential of SRC remediation to restore soil ecosystem services in a sustainable way over the long term.

## 2. Materials and methods

### 2.1. Site characteristics, management and sampling

Soils were sampled from a HM-contaminated site (51°12′41″N; 5°14′32″E) located in Lommel (Campine region, Belgium) that has been diffusely contaminated with Pb, Cd and Zn by historic smelter activities from 1889. The experimental site is located 500 m NE from the Balen smelter and was used for maize cultivation until 2001. The willow plantation is part of a more complex field trial for research on phytoremediation (~10 ha) initiated by Hasselt University and extended in 2006 together with Ghent University and the Research Institute for Nature and Forest (INBO). A 2100 $m^2$ area was used to grow eight commercially available willow clones in a double row design (alternating inter-row distances of 0.75 and 1.5 m, spacing of 0.6 m between cuttings within the rows). The present study was conducted on the soil from the plots where the willow clone 'Tora' (*Salix schwerinii* × *Salix viminalis*) was planted at a density equivalent to 15,000 cuttings per ha. The initial soil acidic pH value 4.8 was increased by lime application (6 ton $ha^{-1}$) in the upper 25 cm soil layer one month before willow planting in 2006 by a rotary tiller and mechanical weeding was carried out in the inter-row in the first year to optimize plant growth. Soil sampling was done in 2012 from plots planted with willow and adjacent area uniformly covered by a mixed grassland dominated by *Agrostis capillaris*, *Holcus lanatus*, *Epilobium angustifolium*, *Juncus effusus*, *Poa pratensis*, and *Rumex acetosa*. The sampling date was before the willow harvest after two 3-years growth cycles. A total of six soil samples of 2 kg each were collected from three SRC plots and three points under mixed grassland, from the 0 to 30 cm depth soil layer, placed into separate plastic bags as independent samples, and immediately shipped to the analytical laboratories in refrigerated boxes. In the laboratory, soils were sieved with a stainless steel mesh (2 mm), and portions for chemical analyses were air dried, portions for the analysis of soil microbial biomass, soil respiration, soil enzyme activity and functional diversity were moistened to 50% water holding capacity and pre-incubated at 25 °C for 7 d prior to analysis, to stabilize the erratic soil microbial communities and biochemical activity after sieving and adjustment of soil moisture level, whereas soils for GeoChip analysis were immediately frozen.

### 2.2. Soil chemical, biochemical and toxicological analyses

The total organic C (TOC) was determined by the method of Walkley and Black (1934). Total soil 121 C and N were determined by dry combustion using a Multi N/C analyzer (Analytik Jena, Germany). Inorganic ($NH_4^+$-N and $NO_3^-$-N) N was analyzed according to Keeney and Nelson (1982). The available P was determined according to Olsen and Sommers (1982). Total element extraction was performed by microwave pressurized digestion (CEM Mars Xpress) using 0.5 g of dry soils suspended in 10 ml of aqua regia 170 °C for 25 min. Analysis of blank samples and reference materials (2711a Montana II Soil from the National Institute of Standards and Technology, USA) were also performed to assess the HM extraction efficiency, which was in the range 80–120%. The elemental analysis was performed by ICP-MS (Agilent 7500ce). The TE availability was determined by extractions with ethylenediaminetetraacetic acid disodium-dihydrate (EDTA), according to the Austrian standard method (Önorm, 2005). The 1 M $NH_4NO_3$ exchangeable concentrations were determined by the standard method (DIN ISO 131 19730:2008-E).

Soil toxicity was assessed by the BioTox test (Aboatox, Finland), based on the inhibition of the bioluminescence of *Vibrio fischeri*. Samples of 2 g of both soils under SRC or grassland were suspended with 8 ml of 2% NaCl in 20 ml polyethylene test tubes, shaken for 5 min by hand and settled for 30 min and the pH value of the slurry was adjusted 7.0 with 0.1 M NaOH added dropwise. Then, 1 ml of each soil suspension was transferred into new plastic tubes (Sarstedt 68.752). Freeze-dried *V. fischeri* cells were reconstituted with a sterile 2% NaCl solution at 4 °C for 30 min, following 15 min at 15 °C on a dry cooling block (Torrey Pines, USA). For toxicity tests, 300 μl of the soil slurry was pipetted into measurement cuvettes (Sarstedt 55.476), then 300 μl of the *V. fischeri* suspension was injected into the soil slurry, and bioluminescence was measured with a high performance Sirius Luminometer. The bioluminescence output was automatically recorded by FB12 Software

(Berthold Detection Systems, Pforzheim, Germany) after 30 s and 15 min and the soil toxicity was calculated by the formula:

$$inh\% = 100 - [100 \cdot (IT_{15}/KF \cdot IT_0)]$$

where KF is the ratio between the bioluminescence at time 0 and 15 min ($IC_{15}/IC_0$) of *V. fischeri* not in contact with soil, and IT0 and IT15 the bioluminescence of *V. fischeri* after contact with soil for at time 0 and 15 min, respectively. With this test, soil is toxic for bioluminescence inhibition values >20%.

Soil microbial biomass was determined by the ATP content according to Ciardi and Nannipieri (1990) and the soil respiration was measured by determining the $CO_2$-C evolution rate by gas chromatography according to Blackmer and Bremner (1977). Arylesterase activity was determined as described by Zornoza et al. (2009). Acid and alkaline phosphomonoesterase activities were assayed according to Tabatabai and Bremner (1969), and phosphodiesterase activity as reported by Browman and Tabatabai (1978). The β-glucosidase activity was assayed according to Tabatabai (1982). All soil hydrolase activities were determined at 37 °C for 1 h; after centrifugation at 6000 g at 4 °C, the concentration of p-nitrophenol (p-NP) was determined at 400 nm (Lambda 2, Perkin Elmer). Protease activity was determined according to Ladd and Butler (1972), and the urease activity was determined according to Nannipieri et al. (1974), and the released $NH_4^+$-N was extracted with 1 M KCl and quantified at 660 nm after reaction with the Nessler reagent. Recovery efficiency of $NH_4^+$-N released by evaluated by urease and protease activities was evaluated by standard additions of $NH_4^+$-N to soil slurries at concentrations in the range of those released by the enzyme activities and was higher than 95% for all soils.

### 2.3. GeoChip analysis

Soil DNA was extracted by freeze-grinding mechanical lysis as described previously (Zhou et al., 1996) from 5 g samples and was purified using a low melting agarose gel followed by a butanol–phenol extraction. GeoChip 4.2 was used in this study to investigate microbial functional structures. GeoChip 4.2 is a comprehensive gene array containing 107,950 probes designed for covering 792 functional gene families from 11 major functional categories, including C, N, phosphorus and sulfur cycling categories (Tu et al., 2014). GeoChip analysis was performed with the following steps:

#### 2.3.1. Template labeling

For each sample, 1 μg DNA was diluted to a volume of 25 μL and was mixed with 20 μL random primers (Invitrogen by Life Technologies, Grand Island, NY, USA), denatured at 99.9 °C for 5 min and immediately chilled on ice. Then, 2.5 μL dNTP (5 mM dAGC-TP, 2.5 mM dTTP), 1 μL fluorescent dye Cy-3 dUTP (GE Healthcare UK Limited, Buckinghamshire, UK) and 80 U of the large Klenow fragment (Imer Inc., CA, USA) were added into the denatured DNA. The 50 μL mixture was incubated at 37 °C for 6 h, followed by heating at 95 °C for 3 min. Labeled DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Cy-3 dye concentration was measured on a NanoDrop ND-1000 spectrophotometer (NanoDrop Products). The labeled DNA was then dried in a Savant SPD1010 SpeedVac concentrator system (Thermo Fisher Scientific) at 45 °C for 45 min.

#### 2.3.2. Hybridization and image processing

The labeled DNA was resuspended in 10 μL hybridization solution containing 2.68 μL sample tracking control (Roche NimbleGen, Inc., Madison, WI, USA), 40% formamide, 25% SSC, 1% SDS, 1.6% Cy3-labeled alignment oligo, 1.6% Cy5-labeled alignment oligo and 2% Cy5-labeled common oligonucleotide reference standard target (NimbleGen). After denaturing at 95 °C for 5 min, the mixtures were spun down and kept at 42 °C to be deposited onto the glass microarray.

GeoChip 4.2 slides were synthesized by NimbleGen in a 12-plex format (i.e., 12 arrays per glass slide). An HX12 mixer (NimbleGen) was affixed onto the array using a Precision Mixer Alignment Tool (NimbleGen). The array is preheated at 42 °C in the 12-Bay MAUI Hybridization System (BioMicro Systems, Inc., Salt Lake City, UT, USA) for at least 5 min before the samples were loaded onto the array. Hybridizations were performed on the MAUI Hybridization System at 42 °C for about 16 h with mixing power. Then the mixers were removed from the slides while soaking in buffer I. The slides were manually washed in buffer I for 120 s, in buffer II for 60 s and in buffer III for 15 s and spun to dry up in a NimbleGen slide spinner. All washing buffers were provided by NimbleGen.

The dried microarray was scanned with a laser power of 100% and a photomultiplier (PMT) gain of 100% by a MS 200 Microarray Scanner (NimbleGen) at the wavelengths of 532 nm and 635 nm. NimbleScan software version 2.5 (NimbleGen) was used to grid the images according to GeoChip 4.2 design file. After gridding, every spot (containing one unique probe) on the GeoChip array was fixed into a 7 × 7 pixel square and was adjacent to four blank equal-sized squares (void). The probe signal was then calculated as the average intensity of the centered 5 × 5 pixels for each spot, and the background noise signal was the average void intensity for each spot. The signal and background intensity report was generated also in NimbleScan software.

#### 2.3.3. Data pre-processing

Raw data from NimbleScan were submitted to the Microarray Data Manager on the IEG website (http://ieg.ou.edu/microarray/) and analyzed using the data analysis pipeline with the following steps: Raw signal intensities (Cy3 channel) on each array are background subtracted and then multiplied by a normalization weight I, which is the ratio of the maximum average universal standard intensity (Cy5 channel) among all the samples divided by the average universal standard intensity of each array. Then, the signal intensities on each array were further multiplied by a normalization weight II, which is the ratio of the maximum total raw intensity (Cy3 channel) among all the samples divided by the total raw intensity of each array. In each sample, the signal to noise ratio (SNR) of spots were used to maintain the hybridized thermophile probes at less than 5% of the total positive probes. Because very small changes in hybridization conditions such as temperature and ozone concentration can cause variations in signal intensity and background noise distribution between hybridizations, spots with signal intensities lower than 1000 were considered to be noise and removed. Results were also screened for possible false positive signals and if a probe appeared in only 33% or less of the samples in one treatment group (one out of three samples) it was treated as a false positive and removed from further analyses. Intensity values plus 1 were transformed to natural logarithm and the mean ratio of each sample was calculated by dividing the signal intensity of each probe by the average signal intensity of all detected probes in each sample.

### 2.4. Data analysis

All analyzed soils were independent samples from three SRC and grassland field replicate plots. The soil chemical and biochemical data were analyzed by ANOVA followed by the Fischer LSD test for sample comparisons. The GeoChip data were analyzed by detrended correspondence analysis (DCA), non-parametric similarity tests such as multiple response permutation procedure (MRPP), permutational multivariate analysis of variance (Adonis), analysis

of similarity (ANOSIM), based on Bray-Curtis, Horn and Euclidean dissimilarity indices, respectively. The canonical correlation analysis (CCA) and Mantel test and analysis of variance (ANOVA) were performed by R version 3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria). To remove redundant information in the CCA model and control the variance inflation factors (VIF), the BioTox values, $NH_4^+$, total P and Pb contents were selected for the CCA model whereas soil chemical parameters with similar impact on the functional gene composition (e.g. Zn and Cd contents) were not used. Individual probes were included as a factor in the employed ANOVA model for partitioning the variance from various probes within each gene catalog. Differences between treatments were compared by *Post hoc* Fisher's least significant difference (LSD) test with Holm–Bonferroni adjustment. The significant differences were defined as $P < 0.05$, or with listed exact $P$ values.

## 3. Results

### 3.1. Soil chemical, biochemical properties and toxicology

Soil under SRC presented similar values for texture, pH, TOC, TN and total P as the soil under the grassland regime, whereas the SRC soil had significantly lower $NH_4^+$-N and available P and higher $NO_3^-$-N concentrations than the soil under grassland (Table 1). Soils under SRC presented values for microbial biomass, soil respiration, Arylesterase, Acid and alkaline phosphomonoesterase, phosphodiesterase and β-glucosidase activities, similar to those of the soil under grassland, whereas Arylsulfatase, Protease and urease activities were significantly higher (Table 1). The bioluminescence inhibition % detected by the BioTox test was significantly higher for the soil under grassland than for the soil under SRC (Table 1), although the soil under grassland was only slightly above the toxicity threshold for this test (20% of bioluminescence inhibition).

The studied soils were mainly contaminated with Cd, Pb and Zn, whereas the other measured HM presented typical background concentrations for the soils of the area (Table 2). Among the extractable TE fractions, the EDTA fraction contained higher amounts of TE than $NH_4NO_3$ (Table 2). The EDTA extractable Pb and Zn concentrations were significantly lower in the SRC soil than in the soil from the grassland soil, whereas the lower Cd concentration in the SRC soil than in the grassland soil was not significant (Table 2). The $NH_4NO_3$-extractable fractions of Cd, Pb and Zn were lower in the SRC soil than in the grassland soil but the differences were not significant (Table 2).

**Table 1**
Main physico-chemical properties, soil microbial biomass, soil respiration and enzyme activities of soils under grassland or SRC management. Values are means ($n = 3$) ± standard error. Different superscripts of within columns indicate significant differences ($P < 0.05$) between mean values.

| Soil parameter | Soil management | |
|---|---|---|
| | Grassland | SRC |
| Sand | 88 (±2.2)[a] | 88 (±2.1)[a] |
| Silt | 8.4 (±0.9)[a] | 8.5 (±1.5)[a] |
| Clay | 4.2 (±0.7)[a] | 4.0 (±0.4)[a] |
| $pH_{H20}$ | 6.3 (±0.2)[a] | 6.1 (±0.3)[a] |
| TOC (g $kg^{-1}$) | 4.0 (±0.4)[a] | 4.2 (±0.5)[a] |
| total N (g $kg^{-1}$) | 0.58 (±0.4)[a] | 0.45 (±0.03)[a] |
| $NH_4^+$-N (g $kg^{-1}$) | 1.5 (±0.1)[a] | 0.43 (±0.02)[b] |
| $NO_3^-$-N (g $kg^{-1}$) | 0.08 (±0.02)[b] | 0.67 (±0.3)[a] |
| total P (g $kg^{-1}$) | 111 (±9.3)[a] | 135 (±7.6)[a] |
| Organic P (g $kg^{-1}$) | 75 (±6.1)[a] | 56 (±5.1)[b] |
| Available P (mg $kg^{-1}$) | 81 (±3.8)[a] | 53 (±1.2)[b] |
| BioTox (inhibition %) | 22 (±3.3)[b] | 13 (±2.0)[a] |
| ATP (ng ATP $kg^{-1}$) | 524 (±12)[a] | 591 (±35)[a] |
| $CO_2$-C (mg $CO_2$-C $kg^{-1}$ $d^{-1}$) | 17 (±1.4)[a] | 19 (±0.4)[a] |
| Arylesterase (mg p-np $kg^{-1}$ $h^{-1}$) | 58 (±2.4)[a] | 64 (±1.4)[a] |
| Arylsulfatase (mg p-np $kg^{-1}$ $h^{-1}$) | 277 (±25)[b] | 372 (±11)[a] |
| Acid phosphatase (mg p-np $kg^{-1}$ $h^{-1}$) | 1579 (±51)[a] | 1814 (±93)[a] |
| Alkaline phosphatase (mg p-np $kg^{-1}$ $h^{-1}$) | 523 (±6.1)[a] | 589 (±25)[a] |
| Phosphodiesterase (mg p-np $kg^{-1}$ $h^{-1}$) | 90 (±3.8)[a] | 107 (±1.5)[a] |
| β-glucosidase (mg p-np $kg^{-1}$ $h^{-1}$) | 617 (±31)[a] | 626 (±32)[a] |
| Urease (mg $NH_4^+$-N $kg^{-1}$ $h^{-1}$) | 14 (±1.9)[b] | 19 (±1.4)[a] |
| Protease (mg $NH_4^+$-N $kg^{-1}$ $h^{-1}$) | 8.0 (±0.8)[b] | 11 (±0.7)[a] |

**Table 2**
Total and available HM concentrations in soils under grassland or SRC management. Values in bold indicate total HM concentrations exceeding the current EU thresholds for agricultural soils. Values of HM total concentrations in bold indicate the soil contaminants. Different superscripts indicate significant differences ($P < 0.05$) between mean values.

| Soil management | As | Cd | Cr | Cu | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|
| *Pseudototal concentrations (mg $kg^{-1}$)* | | | | | | | |
| Grassland | 7.4[a] | **10.9**[a] | 4.8[a] | 26.7[a] | 1.6[a] | **238.3**[a] | **682.1**[a] |
| SRC | 7.0[a] | **8.9**[a] | 4.0[a] | 21.1[a] | 1.4[a] | **182.4**[a] | **579.8**[a] |
| *EDTA extractable concentrations (mg $kg^{-1}$)* | | | | | | | |
| Grassland | nd | 6.2[a] | nd | nd | nd | 203.5[a] | 308.8[a] |
| SRC | nd | 5.0[a] | nd | nd | nd | 151.2[b] | 248.2[b] |
| *$NH_4NO_3$ exchangeable concentrations (mg $kg^{-1}$)* | | | | | | | |
| Grassland | nd | 0.39[a] | nd | nd | nd | 0.37[a] | 35.3[a] |
| SRC | nd | 0.31[a] | nd | nd | nd | 0.33[a] | 24.8[a] |

Symbols 'nd' indicate elemental concentrations not determined.

### 3.2. Diversity and composition of functional genes in soil microbial community

GeoChip detected a total of 27,004 probes across all samples, of which 23,234 and 24,680 were in soils under grassland and SRC, respectively. Among all detected probes, 8.6% and 14.0% were unique to grassland and SRC soils, respectively, while 77.4% were detected in both (Table 3). The functional gene diversity of the microbial communities in grassland and SRC soils, assessed by richness (detected probe number), Shannon–Weaver (H) and Simpson Reciprocal ($1/D$) indices, was not significantly different ($P \geqslant 0.48$) (Table 3). However, the DCA profile showed that SRC soils clustered separately from grassland soils along DCA 1 (Fig. 1), revealing differences in the community composition of the two soils, whereas the MRPP, ADONIS and ANOSIM dissimilarity tests indicated that the differences in the composition of the functional genes between soils under SRC or grassland were not significant ($P > 0.05$).

### 3.3. Functional genes involved in biogeochemical cycles

Among the 33 detected genes encoding enzymes for degrading plant-produced polymers, 6 including *amyA* (encoding α-amylase),

**Table 3**
Functional gene overlap (italicized) and uniqueness (bold) between soils under grassland or SRC treatments, 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.

| Treatment | Grassland | SRC |
|---|---|---|
| Grassland | **2324** (8.6%) | *20,910* (77.4%) |
| SRC | | **3770** (14.0%) |
| Gene no. in treatments | 23,234 | 24,680 |
| Richness[a] | 20,833 (±1421) | 22,266 (±1072) |
| Shannon–Weaver (H)[2] | 9.94 (±0.07) | 10.0 (±0.05) |
| Invsimpson (1/D)[3] | 20,771 (±1420) | 22,201 (±1074) |

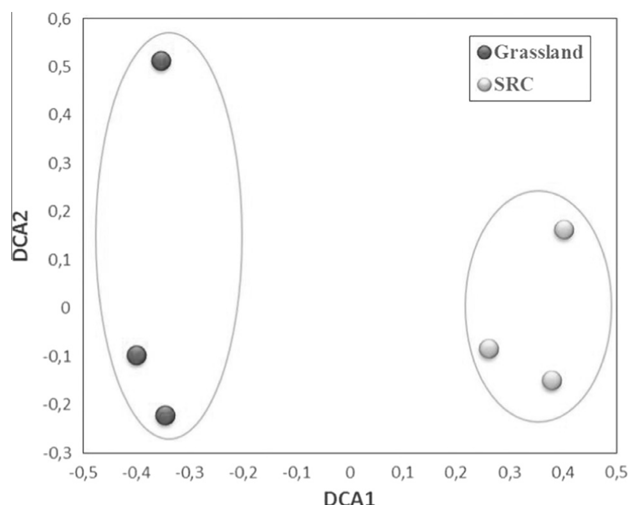


**Fig. 1.** Detrended correspondence analysis of the functional gene diversity in the soils under grassland or SRC regimes.

*cda* (encoding cyclomaltodextrinase), *AceB* (encoding malate synthase), the genes encoding for glucoamylase, exoglucanase, cellulase, phenol oxidase and for other aromatics had significantly higher abundance ($P \leqslant 0.05$) in SRC than grassland soils (Fig. 2A). Differently, the abundance of *amyX* encoding pullulanase for starch decomposition was significantly lower ($P = 0.006$) in SRC than grassland soils (Fig. 2A).

Nineteen functional genes involved in N cycling were detected in the analyzed soils. Ten out of 19 genes, including *gdh* encoding glutamate dehydrogenase for ammonification, *nasA* encoding nitrate reductase, *nirA* encoding nitrite reductase for assimilatory N reduction, *narG* encoding nitrate reductase subunit $\alpha$, *nirS* encoding nitrite reductase for denitrification, *napA* encoding periplasmic nitrate reductase catalytic subunit and *nrfA* encoding c-type cytochrome nitrite reductase for dissimilatory N reduction, *amoA* encoding ammonia monooxygenase either from bacterial or archaea for nitrification, and *nifH* encoding nitrogenase for N fixation, were significantly higher ($P \leqslant 0.05$) in SRC than in grassland soils (Fig. 3). The abundance of *nirB*, encoding nitrite reductase for assimilatory N reduction, was lower in SRC than in grassland soils although the difference was not significant ($P = 0.069$).

Functional genes involved in S and P cycles were significantly higher ($P \leqslant 0.05$) in SRC than in grassland soils, in particular genes encoding sulfite reductase (4 of 5: *CysJ*, *dsrB*, *dsrA* and *sir*) and *ppx* genes encoding exopolyphosphatase for P utilization and *fccAB* encoding flavocytochrome c sulfide dehydrogenase for sulfide oxidation (Fig. 2B).

Several genes encoding for metal resistance were significantly higher ($P \leqslant 0.05$) in SRC than in grassland soils (Fig. 2C). In particular, the *ArsC* encoding arsenate reductase for As resistance, *cadA* encoding cadmium-translocating P-type ATPase for Cd resistance, *ChrA* encoding chromate ion transporter protein for Cr resistance, *CopA* encoding Cu-transporting ATPase and *CusC* encoding cation efflux system protein for Cu resistance, *pbrA* encoding P-type Pb-efflux ATPase and pbrT encoding Pb uptake protein for lead resistance, *merB* encoding alkylmercury lyase protein for mercury resistance, *silC* encoding outer membrane cation efflux protein and *silP* encoding cation-transporting P-type ATPase for Ag resistance, *TehB*, *TerD* and *TerZ* encoding Te resistance proteins, *zntA* encoding cation transport ATPase for Zn resistance, *czcA* and *czcD* encoding a cation efflux system protein for Cd, Co and Zn resistance.

### 3.4. Link between soil chemical properties, biochemical activity and functional gene structure

The CCA was performed to investigate the relationship between selected soil parameters and the structure of functional genes in soil microbial communities. The BioTox values, $NH_4^+$, total P and Pb concentrations were selected for the CCA model ($F = 2.24$, $P = 0.06$), whereas soil parameters that provided redundant information in the CCA model based on the VIF scores were removed. Therefore, factors having similar impacts on the functional gene composition like soil total Zn and Cd concentrations, which were similar to the soil total Pb content were not selected. The final VIF ranged from 3.3 to 7.0. In the CCA profile (Fig. 4), SRC soils clustered separately from grassland soils along the first canonical axis (CCA 1), which explained 45.5% of the total variation in functional gene composition. Projections of soil chemical parameters by CCA revealed that SRC samples were positively correlated with soil $NH_4^+$ content, total Pb content and BioTox, but negatively correlated with total P content. Afterward, Variation Portioning Analysis (VPA) was performed based on partial CCA results, and results indicated that the BioTox explained 15.3%, soil Pb content explained 16.2%, both soil $NH_4^+$ and Pb contents explained 34.7% of the total variance in functional gene structure, whereas their combined interactions explained 24.2%, leaving 10.0% unexplained (Fig. 4).

The link between soil chemical and biochemical properties and functional gene composition was confirmed by the Mantel test results showing that the functional gene composition was significantly correlated with BioTox ($P = 0.01$), $NH_4^+$-N ($P = 0.02$), $NO_3^-$-N ($P = 0.01$), organic P ($P \leqslant 0.01$), and total soil Pb concentration ($P = 0.05$) (Table S1).

## 4. Discussion

Six years of SRC management with willow reduced both the total and available HM concentrations as compared to grassland management, likely due to exportation of the HM absorbed by willow plants, as the willow clone Tora is known to accumulate high HM concentrations in the leaves. However, reductions of the soil HM concentrations were significant ($P < 0.05$) only for the EDTA-extractable Pb and Zn (Table 2). The EDTA extractable HM fraction accounted for 57% of the total Cd, 84% of the total Pb and 44% of the total Zn concentrations in line with previous results. Considering the high proportion of HM extracted by EDTA compared to the total concentrations of HM, the reduction of the EDTA-extractable TE fractions in the SRC soil could be the result of both the reduction of the total HM total concentration and availability due to willow absorption. In previous work on the efficiency of willow trees for heavy metal phytoextraction it was reported a Zn removal capacity of more than 11 000 and 274 g $ha^{-1}$ for Zn and Cd, respectively after 3 y of Tora willow cultivation. Therefore, although part of the excessive metals may have been reduced by leaching, willow HM uptake surely played an important role in reducing the HM total concentration and bioavailability. The general lower HM solubility was in agreement with the BioTox test that showed how the soil toxicity was reduced by the SRC as compared to the grassland management.

In general, the functional gene richness and diversity were slightly higher in soils under SRC than grassland (Table 3). Epelde et al. (2010) reported a significant increase in the number of genes detected by the GeoChip in severely HM polluted soils planted with the metal hyperaccumulator plant *Taphrina caerulescens* as compared to unpolluted and non planted soils, whereas in our study the differences were not significant probably because of the low level of pollution and low soil toxicity. This result paralleled that of high overlap of functional microbial groups in soils

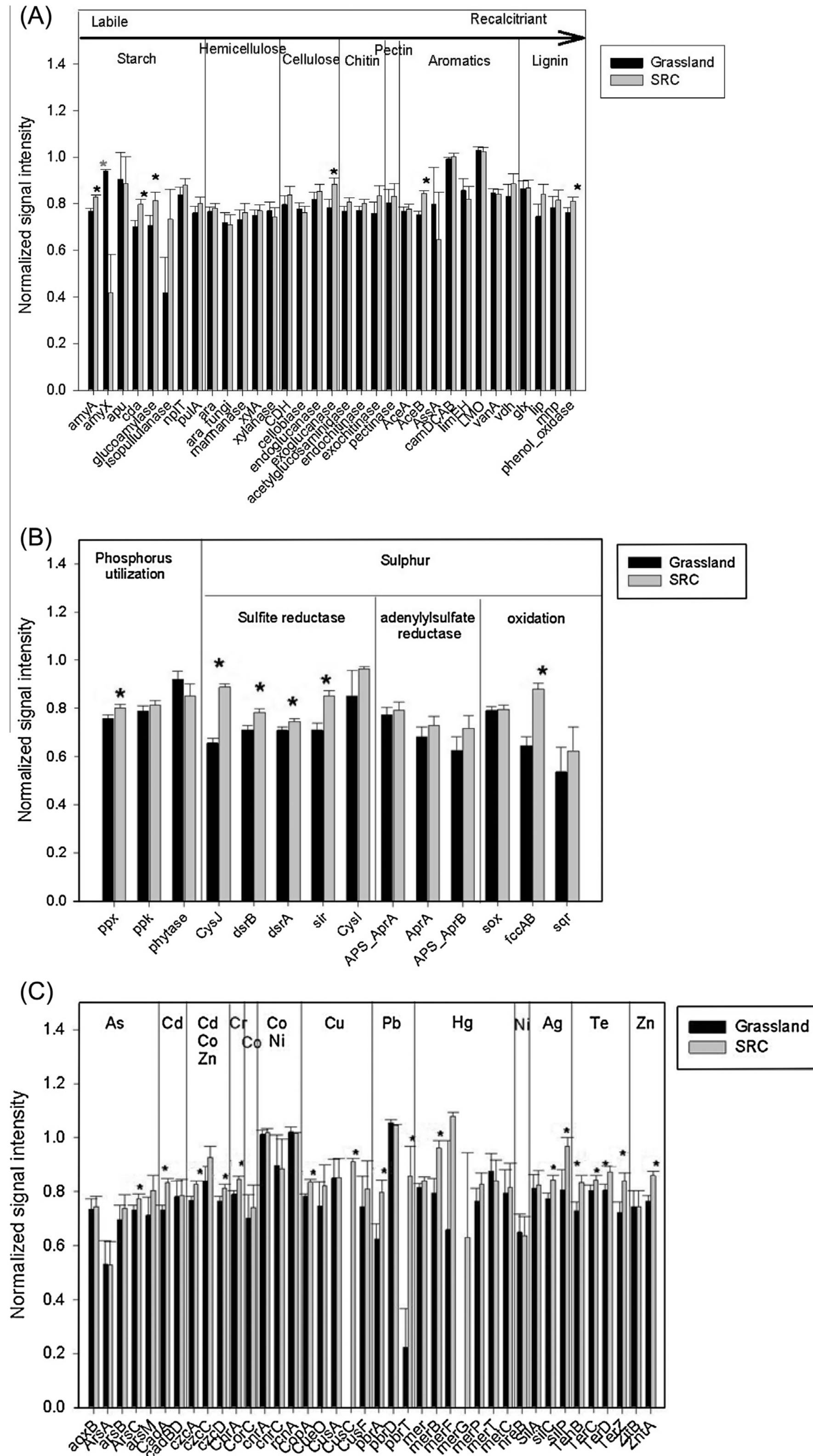


**Fig. 2.** Normalized signal intensity of functional genes encoding enzymes involved in degradation of C polymers (A), of functional genes encoding enzymes involved in S and P cycles (B) and of genes encoding for metal resistance (C) in the soils under grassland or SRC regimes. The complexity of carbon is presented in order from labile to recalcitrant from left to right. Error bars represent standard error. Symbols ** and * indicate significant differences at $P < 0.05$ and $<0.10$, respectively. The * symbols in black indicate that SRC > Grassland and * symbols in gray indicate that SRC < Grassland.

under grassland or SRC management, although the functional gene compositions could be separated in the DCA profile (Fig. 1). This indicated that the distinct compositions were mainly driven primarily by abundance differences among common genes, rather than the presence or absence of selected unique genes. Consistently, at the functional gene level, no genes involved in C,

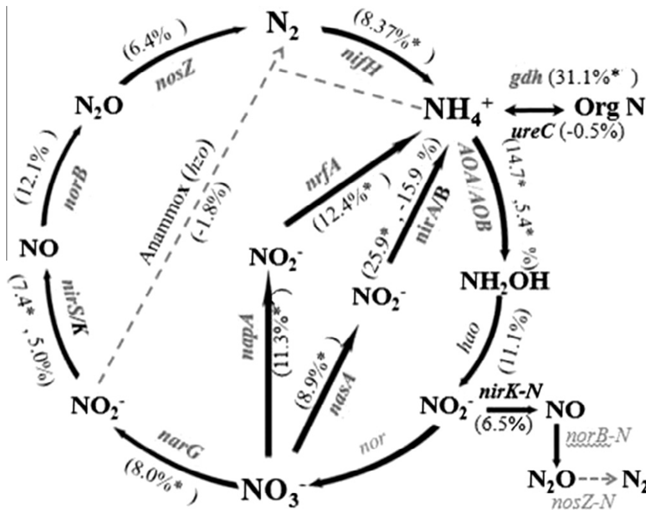


**Fig. 3.** Percentage change of normalized signal intensity from detected functional genes involved in nitrogen (N) cycling in the soils under SRC to grassland regime. Symbols ⁎⁎ and ⁎ indicate significant differences at $P < 0.05$ and $<0.10$, respectively. Symbols ⁎⁎ and ⁎ indicate significant differences at $P < 0.05$ and $<0.10$, respectively. The genes in black font indicate that SRC > Grassland and gene symbols in gray font indicate that SRC < Grassland. Genes norB-N and nosZ-N were not present on the used GeoChip version.

N, P and S cycling were unique to either the SRC or grassland soils. In total, there were 7 and 21 unique genes for grassland and SRC, respectively (Table S2).

The SRC regime slightly increased the soil microbial biomass and soil respiration phosphatase and β-glucosidase activities, and significantly increased arylsulfatase, protease and urease activities as compared to the grassland management. These results could be due to the combined effects of significantly reduced HM availability and soil toxicity under the SRC management (Ascher et al., 2009; Renella et al., 2008; Kumpiene et al., 2009; Epelde et al., 2008), and release of root exudates and litter by fast growing willow, which sustain a larger and more active microbial biomass in the SRC soils compared to the grassland soil (Grayston et al., 1997; Renella et al., 2007).This hypothesis was also supported by the greater number of functional genes detected under SRC than in grassland. In fact, among the soil chemical and biochemical properties, most of the variation in functional genes was explained by changes in soil metal concentrations, P and N concentrations and soil toxicity (Fig. 4). Nitrification is essential for the N cycle and at sub-neutral pH value both ammonia oxidizer bacteria and Archaea play an important role in nitrification. It is long known that soil HM pollution can reduce the soil ammonification and nitrification potentials and in our work we found that the SRC management increased the diversity of genes involved in nitrification and the significantly higher soil $NO_3^-$-N concentration (Table 1), showing a similar trend for both the N cycling and the relative enzyme activities in the soils under SRC management. Although the relationship between the frequency of functional genes and enzyme activity in soil is still not completely understood, the significantly higher richness of genes involved in S- and N-cycles (Figs. 2 and 3) in the SRC soils compared to the grassland soils could be related to their significantly higher arylsulfatase, protease and urease activities, that may have induced higher $SO_4^{2-}$ and inorganic N availability in the SRC than in grassland soils. Nitrogen dynamics in soil depend depends on the mineralization of organic N (e.g. soil organic matter, plant residues, insect hexoskeleton), catalyzed by urease, protease and chitinase activities (Adamczyk et al., 2010; Bach and Munch, 2000). The significantly higher urease and protease activities in soil under SRC than grassland management, could be responsible for the faster N turnover in these soils and also related to the higher richness of genes involved in nitrification (Fig. 3). Though not significant, the higher richness of N-acetylglucosaminidase (chitinase) encoding genes (Fig. 3) could also play an important role in the faster N turnover in soil under the SRC as chitinase activity releases low molecular weight N-sugars from which N in rapidly mineralized to inorganic N (Gooday, 1994). Overall, the results of higher protease and urease enzyme activities and higher richness of genes coding for chitinase and nitrification indicate a faster N turnover and higher N availability to plants in soils under SRC than grassland management, likely due to the higher release of root exudates (Renella et al., 2008) of the fast growing willow plants. These results also indicate that an effective ecological restoration of the soils with a minimal foreseen maintenance.

The higher richness of five genes involved in the degradation of plant C-polymers was detected in soils under SRC than grassland

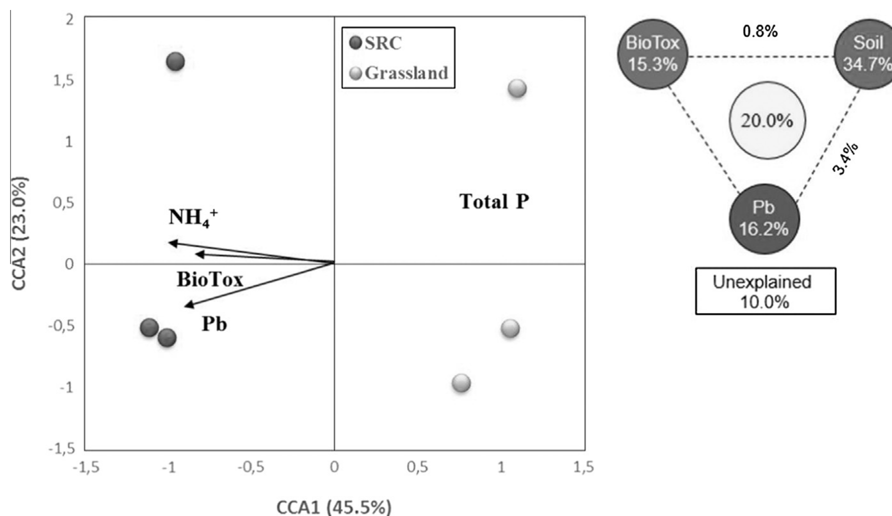


**Fig. 4.** Canonical correspondence analysis (CCA) plot and VIF scores between selected soil parameters and the structure of detected functional genes in the microbial communities of the soils under grassland or SRC regimes. CCA-based variation partitioning analysis (VPA) showed the proportions of community structure variations that can be explained by BioTox, Pb and total P contents. The circles show the variation explained by each group of environmental factors alone. The numbers between the circles show the interactions of the two factors on either side.

soils (Fig. 2). Among the C-polymer degrading genes, those coding for β-1,4 exoglucanase are essential for initiating the cellulose decomposition (Cantarel et al., 2009), leading to the release of cellobiose after the action of β-1,4 glucosidase. Phenol oxidase is also an important extracellular enzyme activity initiating the degradation of highly recalcitrant plant polymers such as lignin (Zibilske and Bradford, 2007). The greater richness of these functional genes in soil under SRC management parallel those of the aceB gene encoding for ligninolytic enzymes (Fig. 3). Overall, these results are in agreement with those by Epelde et al. (2010), showing a richer functional community in TE polluted soils after remediation using the Cd/Zn hyperaccumulator plant *T. caerulescens*. In our experiment, the higher richness of genes involved in the degradation of plant C-polymers detected in soils under SRC (Fig. 2) indicated shifts in microbial representative microbial groups expressing enzymatic activities toward cellulose, hemicelluloses, and lignin, likely related to the SRC management where the plant litter is normally left on site for decomposition after tree harvesting.

Genes encoding HM resistance were detected in higher numbers in soils under SRC. Among these, *merG* for Hg resistance and *CusC* for Cu resistance were unique to soils under SRC. While total and available Cu (Table 2) and Hg concentrations in the Lommel soil were not particularly high in the studied soils (Sonke et al., 2010), there were high levels of EDTA-extractable Cd, Pb and Zn pools (Table 2) detected in the both soils under grassland and SRC, and this chemical HM pool is considered to be available to plants and microorganisms (Sanders et al., 1986; Polettini et al., 2006). The Hg resistance detected in several microbial phylogenetic groups is considered to be an ancient resistance mechanism (Osborn et al., 1997), not necessarily related to severe Hg environmental pollution. Moreover, multiple metal resistance genes are often present in the same organism, and it cannot be excluded that enrichment of organisms resistant to one metal may also enrich for additional metal resistance.

In conclusion, the SRC management of HM polluted soils can reduce HM availability, increase microbial community functional gene diversity and the rate of specific soil functions, as compared to a non-managed grassland cover. Overall, SRC management may have long term beneficial effects on soil fertility and ecosystem services in HM contaminated sites. Monitoring of soil biochemical properties and functional gene diversity can be used to assess the progress of HM site remediation.

## Conflict of interest

None of the authors of the present manuscript have conflict of interest to declare.

## Acknowledgments

The presented work was supported by the EU FP 7 GREENLAND project (FP7-KBBE-266124). We thank two anonymous reviewers for their constructive comments to our manuscript.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.06.062.

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