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# 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.

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## 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.



## 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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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 × 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