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Seasonal change of microbial activity in microbially aided bioremediation

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ABSTRACT

Microbial community patterns and their potential substrate utilization were examined to test for sustainability in metal polluted soil. The acid mine drainage (AMD) influenced test field was characterized for total soil respiration and the functional diversity of the soil bacterial communities using BIOLOG EcoPlate assays. Inoculation with the mycorrhizal fungus *Rhizophagus irregularis* and two streptomycetes led to an altered metabolic diversity and soil vitality, with cell numbers increased by one to three orders of magnitude. The change in metabolic activity was stable even after one winter with severe frost periods. The inoculation thus resulted in enhanced microbial activities. This vitalization resulted in enhanced formation of soil organic matter which, in turn, can sustain higher microbial cell numbers. We therefore conclude that inoculation with indigenous bacteria and a versatile mycorrhizal fungus results in improved vitality suitable for plant growth at heavy metal polluted soils. This holds a huge potential for the remediation of the legacies of mining activities and allows for land-use strategies on metal contaminated sites the world-over.

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1. Introduction

Pollution with toxic heavy metals is a widespread ecological problem in various ecosystems with pollution resulting from anthropogenic activities such as ore mining and smelting, industrial and municipal waste disposal, fossil fuel burning or agricultural activities (Adriano, 2001; Nriagu, 1979). More than 300,000 metal contaminated sites are known in Western Europe alone, which still await remediation measures translating to 4.2 potential contaminated sites per 1000 inhabitants or 5.7 verified contaminated sites per 10,000 inhabitants (McGrath et al., 2006). The formation of acid mine drainage (AMD) adds to metal toxicity, in that under acidic conditions prevalent at former mining sites, metals are increasingly soluble and thus bioavailable with resulting enhanced ecotoxicity (e.g., Heikkinen et al., 2002; Johnson and Hallberg, 2005; Rohwerder et al., 2003).

Conventional techniques for remediation of the soils at AMD sites are often expensive with 1 ha of metal polluted soil excavated to a depth of 50 cm needing at least 1 million US $ for excavation, transport and storage of hazardous waste (Salt et al., 1995). Additionally the surrounding ecosystems at sites of disposal are harmed. Clean-up costs as well as threat to ecosystems might be reduced by more than 75% with the application of phytoremediation, provided the soil supports plant growth (Salt et al., 1995). As heavy metal pollution changes the soil microbial communities, functioning of the soil–plant ecosystem is disrupted with long-term effects on fertility and plant growth (Brookes, 1995). To overcome this drawback, microbially assisted phytoremediation has been devised (see Gadd, 2000 and citations therein). However, plant–bacteria interactions might also be detrimental, which is why the associated mechanisms need to be unraveled in detail in order to choose the inocula with care and to provide inocula capable of coping with the site specific conditions (Mani and Kumar, 2014; Zhuang et al., 2007).

Both, bacteria and fungi influence rhizosphere processes. These microorganisms not only influence plant physiology, growth, defense mechanisms and nutrient uptake (see Gianinazzi-Pearson and Gianinazzi, 1983; Smith and Read, 1997) but also alter pollutant availability, uptake, translocation, chelation, and for organics: degradation and volatilization (Alloway, 1990; Audet and Charest, 2008, 2013; Davies et al., 2001). To evaluate interactions not only between selected microbes and a specific plant, but to take into account microbe–microbe interactions within the soil, the microbiological processes in soil need to be examined. Therefore, the aims of this study were to provide inocula tolerating the effects of heavy metal contamination and to observe the changes in the microbial community after an entire season. The key aspect of this study was to improve soil quality and productivity for agricultural land-use. A suitable proxy to evaluate soil quality (Killham, 1994), and therefore also to productivity of agricultural systems is the microbial activity in soil (Girvan et al., 2003; Odonnell et al., 1994). This microbial activity

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can be quantified by two independent approaches — either estimating the abundances of soil organisms or measuring carbon dioxide (CO_2) release from bulk soil (Moyano et al., 2008; Trumbore, 2009).

The former uranium mining area in Eastern Thuringia has been exploited from 1949 through 1989, after which technical and radiological remediation ensued. Heap material was replaced in a former open mining pit and groundwater levels were allowed to raise to restore anoxic conditions. However, at the surface where formerly heaps had been situated, the remaining contamination still leads to visibly retarded plant growth. Specifically at a former leaching heap, the effects were prominent and led us to establish a test filed site. The heap had been leached with uranium-rich, acidic pit waters (pH 2.7–2.8) and later with sulfuric acid to precipitate uranium in a low pH setting. After heap removal and excavation of approximately 6 m, sandy silt soil was applied to a depth of approximately 40 cm (Schindler et al., 2012). Soil, groundwater characteristics and residual contamination of the area were analyzed in former examinations (Carlsson and Büchel, 2005; Grawunder et al., 2009). After several years of horizontal infiltration and capillary rise, the soil contains heavy metals at concentrations above national threshold values. Some places were affected by pyrite oxidation and AMD. Thus, this test field site provided excellent conditions to test whether amendment with soil microorganisms could positively influence soil microbiology, generation of humic substances, and ultimately plant growth. Our specific hypotheses were that on both short and medium term, soil microbial activities would increase due to microbial inoculation, and that the alteration of the microbial community composition is sustainable even over longer periods of time.

2. Materials and methods

2.1. Study site

The test site “Gessenwiese” (50.854434° N, 12.147119° E) was established at the base area of the former leaching heap “Gessenhalde”, southeast of Ronneburg, Germany. In 2004, three fields were established at the test site, each divided into nine subplots of 4 m × 4 m (Büchel et al., 2005) and microbial assisted phytoremediation was investigated in the following years (Reinicke et al., 2013; Schindler et al., 2012). The nine subplots were either inoculated with different microorganisms or left unamended to examine the microbial influence on plant growth and bioremediation (Schindler et al., 2012).

The microbial inoculations were performed with three control subplots each: without, with mycorrhiza (*Rhizophagus irregularis* Sy 167) and with both, mycorrhiza and *Streptomyces* (*Streptomyces acidiscabies* E13 and *Streptomyces tendae* F4) inoculation (Fig. 1).

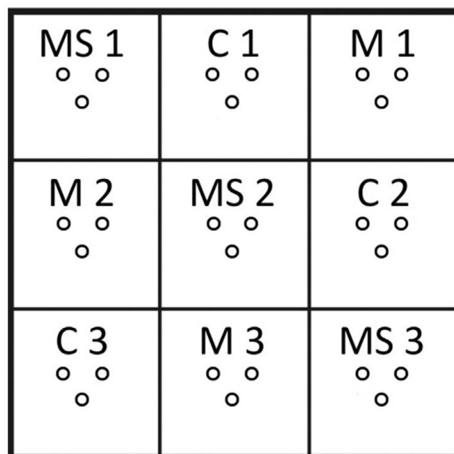

Fig. 1. Test field site design with CO_2 efflux measurement collars. C, untreated control; M, mycorrhiza inoculation; MS, mycorrhiza and *Streptomyces* inoculation; rings, CO_2 measurement collars.

Commercially available arbuscular mycorrhiza inoculum, consisting of expanded clay containing *R. irregularis* Sy 167 spores was mixed with the first 5 cm of the soil. The control plots were treated with expanded clay lacking fungal inoculum. The heavy metal resistant bacteria were mixed in equal proportions. *S. acidiscabies* E13 and *S. tendae* F4 had been isolated from the area (Sineriz et al., 2009). On the untreated control (C) plots and mycorrhiza (M) plots, autoclaved bacteria were amended to control for addition of nutrients with the bacterial inoculum. The inoculation was performed yearly in May from 2004 through 2010.

2.2. Sampling

Soil samples (0–5 cm depth) for microbiological analyses were with a sterile spatula next to the CO_2-efflux measurement collars and immediately stored in 50 ml Falcon-tubes at 4 °C. For all measurements, monthly sampling was performed during 2009 and 2010.

Processing under sterile conditions consisted of grinding in a mortar and sieving through a 2 mm mesh, adding 9 ml 0.9% NaCl and sterile glass beads to one gram of soil. Incubation at 28 °C for 1 h in a rotary shaker (250 rpm) followed by 1 h settling time before most probable number (MPN) counting (Oblinger and Koburger, 1975) in 96-well microtiter plates (PS-Microplate, 96 well, flat bottoms, Greiner Bio-One, Germany). The plates were filled with 200 µl liquid Standard Nutrient Broth I (Carl Roth GmbH, Germany) following a ten-fold serial dilution of the samples. All wells, containing 3 × 3 parallels of one sample as well as three controls with sterile media were incubated at 28 °C for five days. Growth was detected with a VersaMax tunable multiplate reader (Molecular Devices, Sunnyvale, California) by comparing the values of each well with the sterile control at 600 nm. MPN per gram dry soil was calculated (Hurley and Roscoe, 1983) with x being MPN estimate, k the number of dilutions, n_i the number of replicate samples tested per dilution, p_i the number of positive samples per dilution, d_i the dilution factor at level i, and v_i the amount of samples tested per replicate following Eq. (1).

$$\sum_{i=1}^{k} \frac{v_i d_i p_i}{1-e^{-v_i d_i x}} = \sum_{i=1}^{k} v_i d_i n_i \tag{1}$$

2.3. CO_2 measurements

According to the experimental design, three measurement collars per subplot (PVC, ⌀ 10 cm and 7 cm high) were installed approximately 2 cm into the mineral soil. Soil CO_2 release was measured using a closed manual chamber system with an infrared gas analyzer (LI-6400 and LI-6400-09, LiCor Inc., Lincoln, NE, USA) on soil sampling days. After placement of the chamber on the previously installed collar, the CO_2 concentration inside the chamber was slightly reduced below ambient concentration (380 ppm) and allowed to rise above ambient content. Three measurement cycles with standard deviation below 10% were performed for each collar. The average of all efflux measurements of each treatment and each day was used for further analysis. Every CO_2 flux measurement was accompanied by measurements of soil water content at 5 cm soil depth (ThetaProbe, Delta-T Devices, Cambridge, U.K.) and soil temperature in the upper soil horizon at 5, 10 and 15 cm depth, adjacent to each respiration collar.

2.4. Physiological profiles of microbial communities

BIOLOG EcoPlates were used to investigate the metabolic diversity using 10 mg soil shaken in 100 ml K_2HPO_4 (50 mM) for 2 h to extract the soil community. The supernatant was mixed with distilled water for a final 1:100 or 1:1000 dilutions after 30 min settling.

The samples were dispensed into the wells of a BIOLOG EcoPlate according to manufacturer instructions (Biolog Inc., Hayward, CA, USA).

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