PHYTO2ENERGY

Phytoremediation driven energy crops production on heavy metal degraded areas as local energy carrier

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PROPRIETARY RIGHTS STATEMENT

Definition of a system of biological indicators to assess soil quality in contaminated soils after phytoremediation

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v1	2017/06/02	M.Schloter	Document creation
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Definition of a system of biological indicators to assess soil quality in contaminated soils after phytoremediation

OVERVIEW OF THE TECHNICAL DOCUMENT

WP:	**2**
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Title :	D.2.2 HMGU_ Definition of a system of biological indicators to assess soil quality in contaminated soils after phytoremediation

This technical document provides a theoretical framework how to analyze the improvement of soil quality in heavy metal contaminated soils after phytoremediation

INTRODUCTION

Given their often large and complex microbiomes, soils can be considered as hotspots for microbial biodiversity on Earth. As a result, soils provide a large number of biological processes that are essential for life on Earth. Such processes have been coined life support functions (LSF). These LSF include: (1) the provision of 'fertile ground' as a basis for a sustainable bio economy including the growth of food, feed and bioenergy crops, (2) the maintenance of a natural unthreatened plant biodiversity at sites which are not used for agricultural production; (3) the safeguarding of clean drinking water, by filtering and degrading pollutants in soil before they enter the groundwater body; and (4) the potential of soils to act as a sink for atmospheric CO_2. However, these functions of soils are strongly impacted by contamination as often the diversity of soil biota and their activity pattern are strongly reduced, which strongly impacts the multi-functionality of soils. In this respect mainly heavy metal (HM) contaminated soils have been considered as critical. Thus in the frame of this project several bioremediation strategies have been developed to reduce the HM content in contaminated soils. To determine the success of these strategies for soil quality and the related ecosystem services, we propose a framework of biological indicators. This system will be described in the following technical document. The described system is a framework of methods that can be used for the assessment of soil quality depending on the respective on site situation and the capacities provided by the contributing laboratories

As significant improvements of soil quality of HM contaminated sites by bioremediation strategies take longer than the project period, the proposed indicator system was not tested under field conditions, but important steps forward towards the detection of important indicator species were made in green house trials carried at HMGU by Phyto2Energy fellows .

METHODOLOGY

Indicator concept

We propose a hierarchical concept to assess the impacts of bioremediation strategies on soil quality. This indicator system is based on biological indicators, as soil biota are considered as important drivers for many ecosystem functions. The indicators include “black-box methods”, analytical tools to describe microbial community structure and some indicators species, as well as approaches to describe microbial activity pattern as well as important functional traits.

Microbial biomass – The black box approach

The soil microbial biomass can be defined as organisms living in soil that are generally smaller than approximately 10 µm. Most attention is given to fungi, archaea and bacteria, these groups of microbes being the most important with reference to energy flow and nutrient transfer in terrestrial ecosystems. Fungi and bacteria are generally dominating within the biomass. The microbial biomass consists of dormant and metabolically active organisms. However, the presently widespread biomass estimates, either direct or indirect (biochemical) techniques, were not properly valid and checked for separating these fractions. Microbial biomass content is an integrative signal of the microbial significance in soils because it is one of the few fractions of soil organic matter that is biologically meaningful, sensitive to management or pollution and finally measurable. With the development of the four now widespread indirect methods, fumigation-incubation (FI), substrate-induced respiration (SIR), fumigation-extraction (FE) and ATP content (Jenkinson and Powlson, 1976; Anderson and Domsch, 1978; Jenkinson and Ladd, 1981; Vance et al., 1987), a great deal of effort has gone into the measurement of the size of the microbial biomass and its associated nutrient pools. Recently also the measurement of DNA content has been considered as a good microbial biomass parameter (Gschwendtner et al., 2016).

All these methods are designed to quantify the microbial biomass carbon in different soil samples, soil horizons, soil profiles and sites. However, it must be realized that between different soil samples different biomass may occur without direct correlation to soil quality. Nevertheless, the soil microbial biomass is the eye of the needle through which all organic matter needs to pass through. As a susceptible soil component, the biomass may be therefore a useful indicator since pollution may reduce this pool as, e.g. demonstrated by Fritze et al. (1996) for heavy metal contaminated soils.

Assessment of microbial community structure

The measurement of the microbial biomass is a black box approach, without differentiating the heterogeneity of the microbial community. Molecular methods, independent from

cultivation, based on the genotype (Amann et al., 1995) and phenotype (Zelles, 1996) of the microbes allow a deeper understanding of the composition of microbial communities. Based on such studies, it can be estimated that 1 g of soil consists of about 10,000 different microbial species. High throughput techniques mostly based on next- or second generation sequencing allow nowadays even the assessment of such huge diversities levels. Mainly for the monitoring of contaminations, microbial diversity parameters are often used as an indicator for the assessment of soil quality. Muller et al. (2001) for example, investigated the long-term effects of long-term exposure to mercury on the soil microbial community along a gradient of pollution. It could be shown that bacterial diversity was reduced in the contaminated soils. Due to the mentioned complexity of the whole microbial community it might be useful to define for each site indicator organisms which respond to the type of contamination in a very characteristic matter. Those organisms can be then included in any routine analysis program and accessed via quantitative real time PCR of marker genes like the 16S rRNA gene, which is the “Golden standard” in this field of analytics.

Assessment of microbial activity

Microbial activities are the driver for the ecosystem services provided by soils. Microbial activities are not only of crucial importance for biogeochemical cycling leading to the liberation of nutrients available for plants but also for the mineralization and mobilization of pollutants and xenobiotics. The group of methods on soil microbial activities embraces biochemical procedures revealing information on metabolic processes of microbial communities. To estimate the soil microbial activity, two groups of microbiological approaches can be distinguished.

First, experiments in the field that often require long periods of incubation (i.e. Hatch et al., 1991; Alves et al., 1993) before significant changes of product concentrations are detected, i.e. 4–8 weeks for the estimation of net N mineralisation. In this case, variations of soil conditions during the experiment are inevitable, i.e. aeration, and may influence the results). Short-term laboratory procedures that are usually carried out with sieved samples at standardized temperature, water content and pH value. Short-term designs of 2–5 h minimize changes in biomass structure during the experiments. Such microbial activity measurements include enzymatic assays that catalyse substrate-specific transformations and may be helpful to ascertain effects of bioremediation. However, it is important to mention, that laboratory results refer to microbial capabilities, as they are determined under optimized conditions of one or more factors, such as temperature, water availability and/or substrate.

The green house experiment

To identify bacterial phyla, which are most sensitive to HM contamination in soil, which could be used as indicators for an improved soil quality as a result the bioremediation process a

greenhouse experiment was performed at HMGU (Photo 1). The experiment has been based on the following settings:

***Photo 1**: Green house experiment with Miscanthus x giganteus at HMGU*

Soil:	Luvisol (Agricultural origin) 19 % sand, 59 % silt, 22 % clay; pH 7,1; 0.12 mg/g DOC; 0.01 mg/g TNb; sieved soil
Plants:	*Miscanthus* x *giganteus*
Pot size:	5 L
Heavy metal (HM) treatment:	54.7 mg Pb-acetate/kg, 217.5 mg Zn-nitrate/kg, and 2.1 mg Cd-acetate/kg
Acetate-nitrate (AN) treatment:	43.7 mg acetic acid/kg and 267.2 mg ammonium nitrate/kg
Watering:	600 mL/pot, 2-times a week
Incubation:	12h, 25 ^{o}C day; 12h night, 20 ^{o}C
Incubation time:	14 weeks
Replicates per treatment:	3 pots

At the end of the experiment DNA was extracted from bulk soil samples, rhizosphere and endosphere using the MoBio extraction kit. DNA was stored at – 20 ^{o}C. Molecular barcoding of bacterial communities was performed according to Gschwendtner et al.(2016).

RESULTS AND CONCLUSION

The application of the heavy metals in the green house experiment resulted in a significant increase of total HM concentrations in the top soil of the pots. Bioavailable HM concentrations however were not significantly increased at the end of the experiment (Table 1)

	TOTAL HM concentrations			BIOAVAILABLE HM concentrations		
Treatment	**Pb**	**Zn**	**Cd**	**Pb**	**Zn**	**Cd**
	(mg/kg)	(mg/kg)	(µg/kg)	(µg/g dw)	(µg/g dw)	(ng/g dw)
H_2O root	17.5±0.3	68.0±7.7	651.7±30.7	LOQ	0.01±0	LOQ
H_2O bulk	17,0	56,4	617,0	LOQ	0,01	LOQ
AN root	17.2±0.2	70.1±15.2	654.5±38.9	LOQ	LOQ	LOQ
AN bulk	16,6	57,1	562,0	LOQ	LOQ	LOQ
HM root	59.2±7.4	444.3±214.6	3153.3±1276.6	LOQ	0.15±0.18	13.26±11.81
HM bulk	138.3	952.6	701211	LOQ	1,33	7,73

Table 1: *Total and bioavailable HM concentrations in the different treatments in bulk soil- and in root samples. The treatments included two controls (soils spiked with acetate-nitrate (AN) and H_2O) as well as one treatment with a mixture of heavy metals (HM) (n=3)*

The microbial biomass as measured by the extraced DNA from the samples was significanly affected in the rhizosphere. The application of the HMs resulted in an decrease of microbial biomass. In bulk soil and the root interior no significant effects were measured (Table 2).

Extracted DNA	BULK	RH	END
	ng per g of soil	ng per g of soil	ng per g of roots
H_2O	97.31 ± 1.02	171.16 ± 33.01	215.14 ± 124.90
AN	98.18 ± 16.87	195.64 ± 90.31	335.10 ± 42.74
HM	103.80 ± 13.45	116.07 ± 28.55	183.73 ± 13.53

Table 2: *Microbial biomass in the different treatments in bulk soil (BULK) - rhizosphere (RH) and in samples from the root interior (END). The treatments included two controls (soils spiked with acetate-nitrate (AN) and H_2O) as well as one treatment with a mixture of heavy metals (HM) (n=3)*

As expected bacterial diveristy pattern (alpha diveristy) decresed from bulk soil to the root interior. However, the impact of the heavy metal contamination on the bacterial diversity was low in all plant compartments (Figure 1).

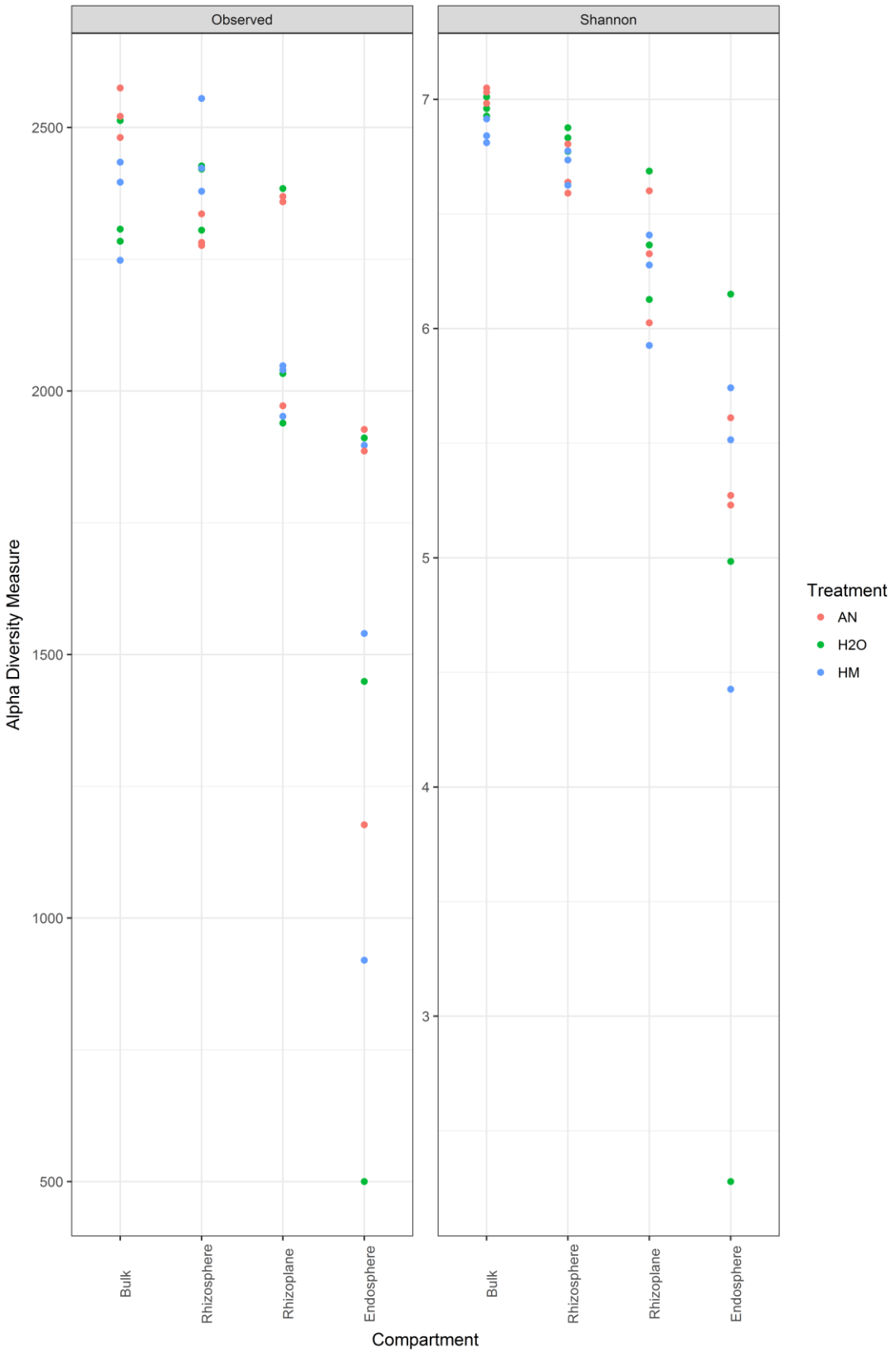
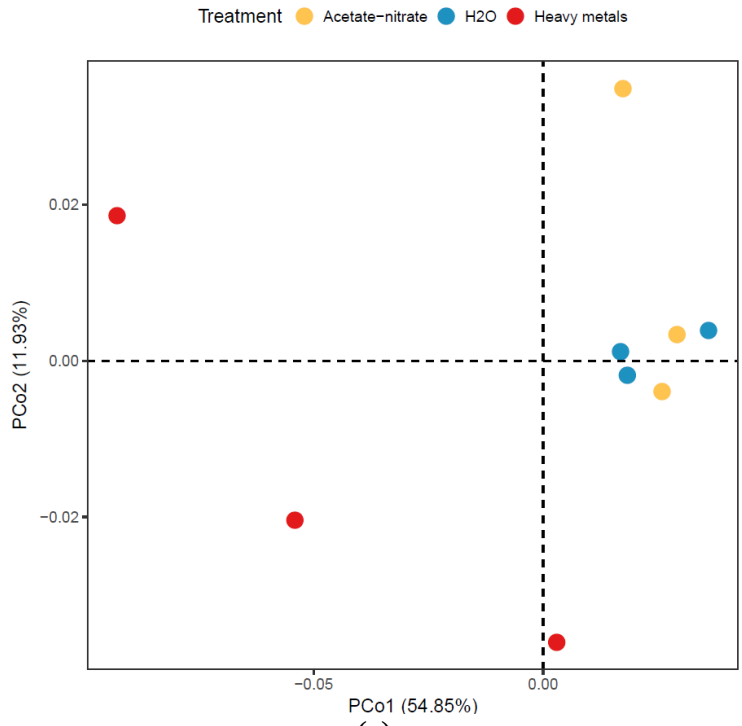
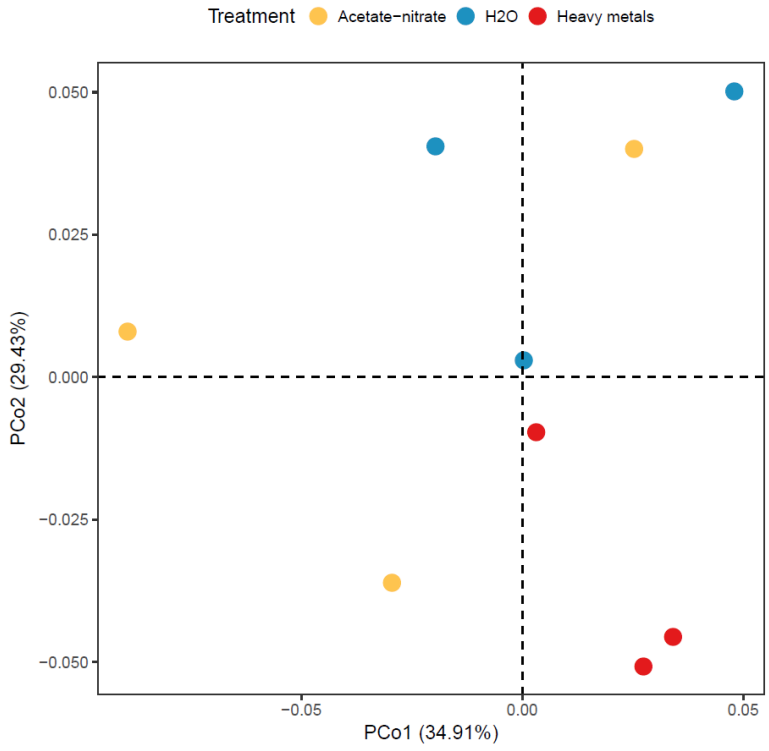


Figure 1: *Bacterial alpha diversity in the different treatments in bulk soil, rhizosphere and in samples from the root interior. The treatments included two controls (soils spiked with acetate-nitrate (AN) and H_2O) as well as one treatment with a mixture of heavy metals (HM) (n=3)*

PCoA analysis indicated differences in bacterial beta diversity between the control treatments and the treatments where HMs were added to the soil for all analyzed compartments (bulk soil, rhizosphere and root interior; Figure 2).



(a)



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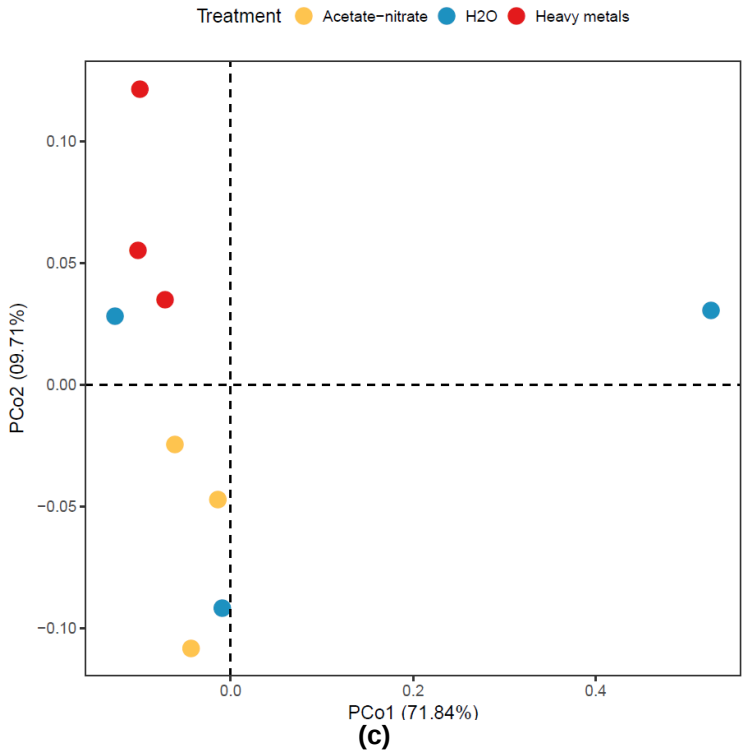

Figure 2: *Bacterial beta diversity in the different treatments in rhizosphere (a) bulk soil (b) and in samples from the root interior (c). The treatments included two controls (soils spiked with acetate-nitrate (AN) and H_2O) as well as one treatment with a mixture of heavy metals (HM) (n=3)*

For bulk soil these differences resulted from a significant decrease of Cyanobacteria in the HM treated soils and an increase in the relative abundance of Proteobacteria. The differences between both controls are a result of a reduced abundance of Nitrospirae in the treatments which received acetate-nitrate (Figure 3). **Thus we propose the abundance of Cyanobacteria as a good indicator to assess the progress of bioremediation in bulk soil**

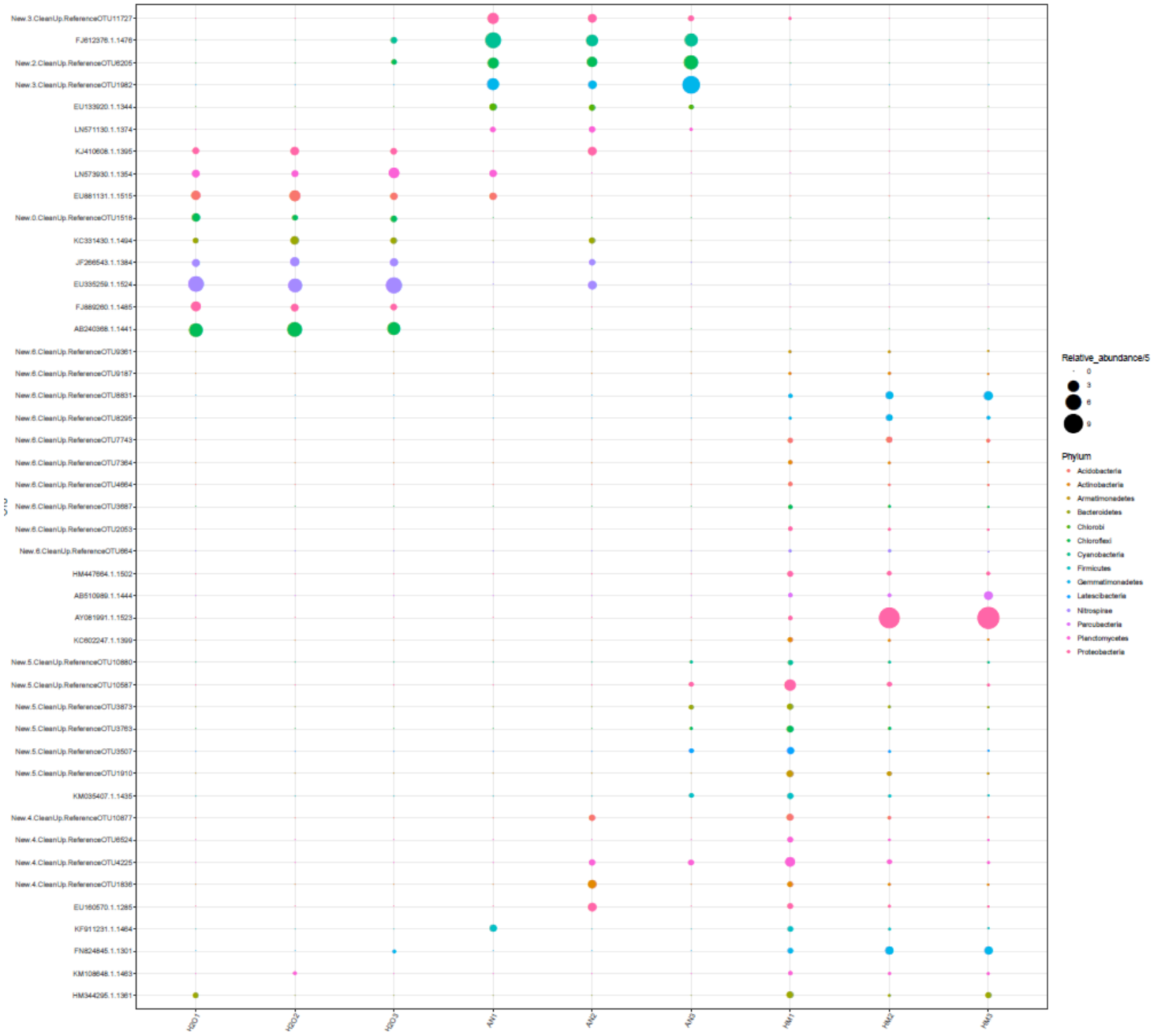

Figure 3: *Bacterial responders to the different treatments in bulk soil. The treatments included two controls (soils spiked with acetate-nitrate (AN) and H_2O) as well as one treatment with a mixture of heavy metals (HM) (n=3)*

For the rhizosphere all bacterial species which responded to the HM addition (e.g. Saccharibacteria) were also affected by the addition of ammonia nitrate, **thus a clear indicator definition for the rhizosphere was not possible** (Figure 4)

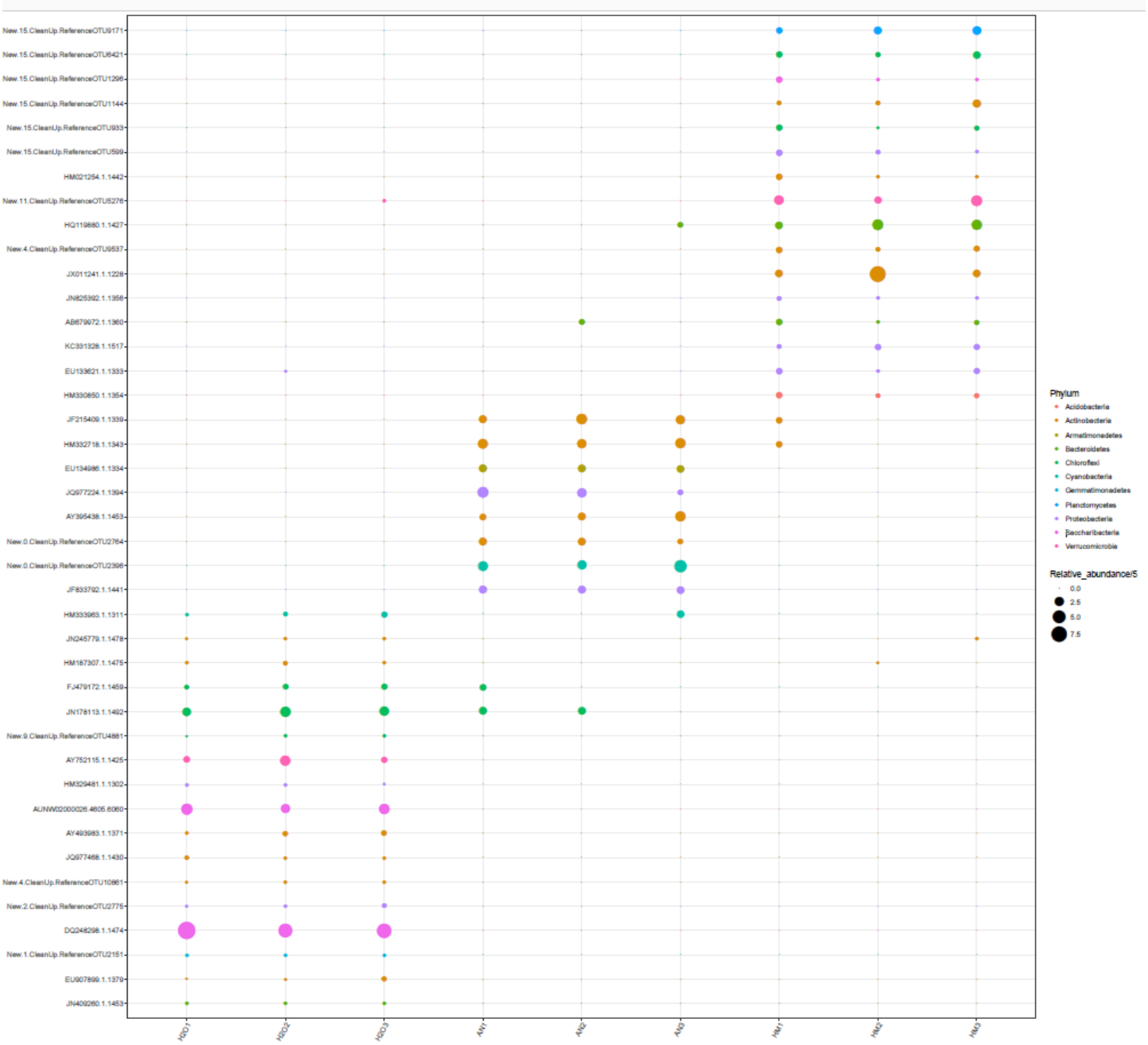

Figure 4: *Bacterial responders to the different treatments in the rhizosphere. The treatments included two controls (soils spiked with acetate-nitrate (AN) and H_2O) as well as one treatment with a mixture of heavy metals (HM) (n=3)*

For the root interior, the high relative abundance of Proteobacteria in plants grown in HM contaminated soils was obvious. Firmicutes, which were frequently occurring in the root samples from plants, where only water had been applied to the soils where also out competed in those treatments with ammonia nitrate application to soil, so they could not be used as an indicator for contamination. **So we propose to use the ratio of Proteobacteria to other phyla as an indicator for the success of the bioremediation process. Lower ratios indicate a successful strategy.**

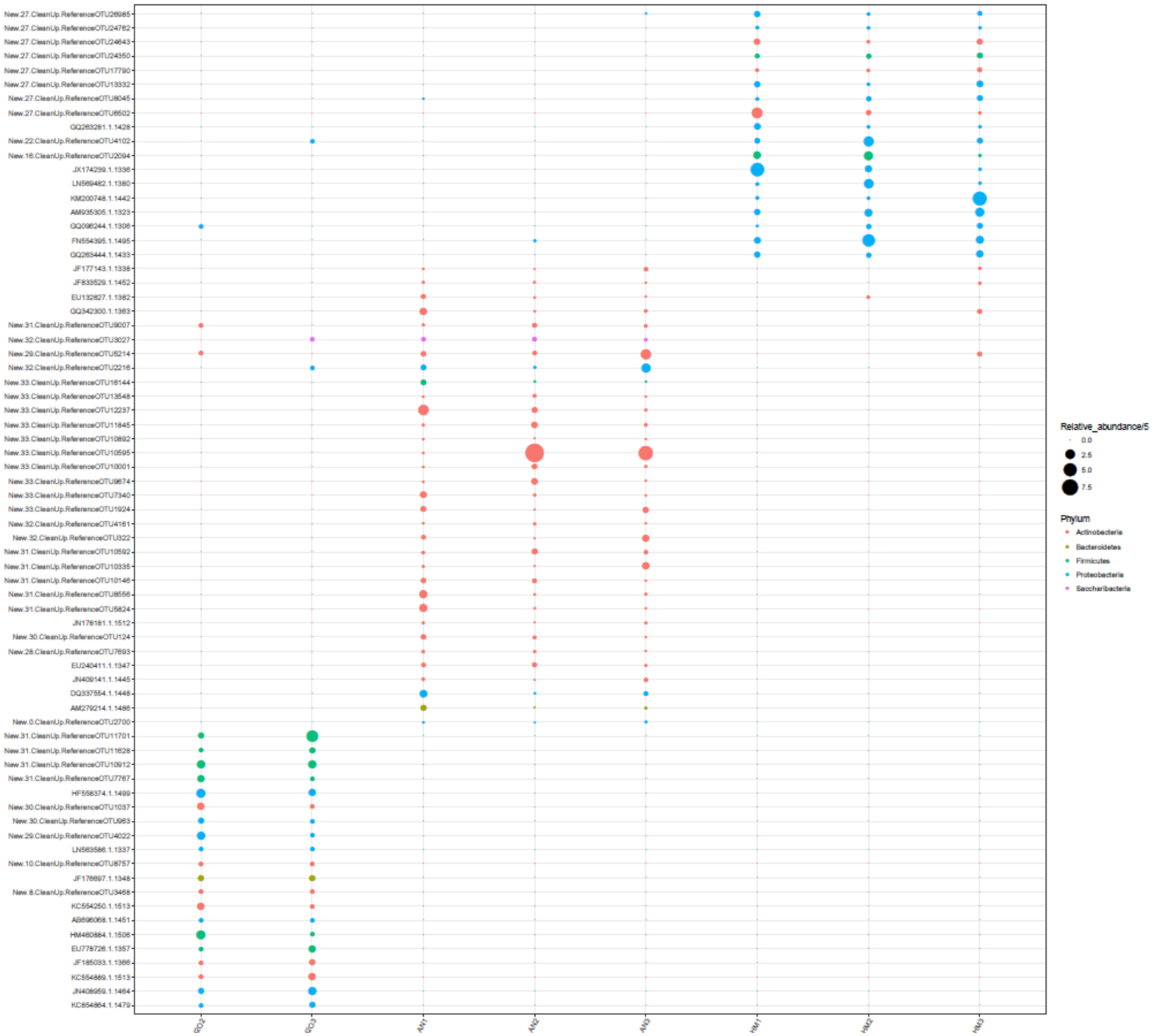

Figure 5: *Bacterial responders to the different treatments in the rhizosphere. The treatments included two controls (soils spiked with acetate-nitrate (AN) and H_2O) as well as one treatment with a mixture of heavy metals (HM) (n=3)*

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