**Changes induced by heavy metals in the plant-associated microbiome of *Miscanthus* x *giganteus***

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**ABSTRACT**

*Miscanthus* x *giganteus* is a high biomass producing plant with tolerance to heavy metals. This makes *Miscanthus* interesting to be used for phytoremediation of heavy metal contaminated areas coupled with energy production. Since plant performance in metal polluted areas is impaired, their growth and phytoremediation effect can be improved with bacterial assistance. To identify positive and negative responders of *M*. x *giganteus* associated microbiome influenced by Cd, Pb and Zn stress compared to non-contaminated controls, we designed a greenhouse experiment. Structure of the bacterial community in three rhizocompartments, namely rhizosphere, rhizoplane and root endosphere was analysed using an isolation independent molecular approach based on 16S rRNA gene barcoding. Furthermore, quantitative PCR (qPCR) was used for bacterial biomass estimation. Our results indicated that biomass and total bacterial diversity in rhizosphere, rhizoplane and root endosphere did not significantly change despite of substantial root uptake of heavy metals. Overall, we detected 6621 OTUs, from which 171 were affected by metal addition. Whereas *Streptomyces* and *Amycolatopsis* taxa were negatively affected by the heavy metal treatment in endosphere, taxa assigned to *Luteolibacter* in rhizosphere and rhizoplane (log2 fold change 1.9-4.1) and *Micromonospora* in endosphere (log2 fold change 10.2) were found to be significantly enriched and highly abundant (0.1-3.7 % relative abundance) under heavy metal stress. Those taxa might be of key importance for *M*. x *giganteus* performance under heavy metal pollution and might be interesting candidates for the development of new bioinocula in the future to promote plant growth and phytoremediation in heavy metal contaminated soils.

**Keywords**: heavy metal contamination, rhizosphere, rhizoplane, endosphere, *Luteolibacter*, *Micromonospora*

**1. INTRODUCTION**

All over the world, industrial activities have contaminated extensive areas over the last century (Nagajyoti et al., 2010) with heavy metals (HM) being the most frequent type of contamination. In the European Union, it has been estimated that 28.3 % of the total land surface is affected by HM (Tóth et al., 2016). It is well accepted that HM contaminated land not only imposes a risk for the environment but also strongly affects human health. Because of air driven deposition, not only areas in the direct surroundings of industry are subjected to HM contamination, but also soils under agricultural use, which are often not in the direct neighbourhood of the polluter. In addition, even in remote areas with no or only few industrial activities, due to use of fertilizers and other organic field applications, increased HM values in soil have been reported (Alloway, 2013).

Although HM contaminated soils have been banned in many regions as sites for food and feed production, they can be used for the cropping of bioenergy crops. A positive side effect of this form of management compared to leaving those sites open as bare land is the coupling with biomass production and phytoremediation (Li et al., 2014; Schröder et al., 2018). A perennial grass andenergy plant, *Miscanthus* x *giganteus* J. M. Greef & Deuter ex Hodk. & Renvoize*,* has repeatedly been proposed as a good candidate for heavy metal phytoremediation due to its metal tolerance and phytoextraction capacity combined with high biomass production (Barbosa et al., 2015; Kocoń and Matyka, 2012; Pogrzeba et al., 2013). In contrast to hyperaccumulating plants, which can efficiently transfer metals from the soil through roots to the shoots, *Miscanthus* species protect their photosynthetic system by regulating and limiting that transfer. Thus *M*. x *giganteus* generally does not readily translocate metals to above ground organs but accumulates them mostly in roots and rhizomes (Arduini et al., 2004; Nsanganwimana et al., 2014). Hence, *M*. x *giganteus* is a good candidate for phytostabilization due to its capacity to reduce Cd, Zn, Pb and Cu mobility and availability in soil, as well as to improve soil carbon stocks as a consequence of high exudation rates and rhizodeposition (Al Souki et al., 2017; Barbosa et al., 2015; Iqbal et al., 2013).

Despite the genetic properties of *M*. x *giganteus*, which allow its growth in HM contaminated soils, numerous reports indicate a positive effect of plant growth promoting bacteria on *Miscanthus* yield, mainly in soils with high degrees of contamination (Babu et al., 2015; Schmidt et al., 2018; Straub et al., 2013). Microorganisms can be beneficial for the plant when grown in HM contaminated soils because of their ability to promote acquisition of nutrients, formation of hairy roots, photosynthesis, transpiration, defence against phytopathogens, modulate metal solubility as well as uptake and alleviate metal phytotoxicity (Babu et al., 2015; Pogrzeba et al., 2017; Visioli et al., 2015; Wang et al., 2019). However, the effects of HMs on the plant-associated microbiome are still only poorly understood, mainly how response pattern differ in different plant compartments, e.g. rhizosphere or endosphere. Therefore, in the frame of this study we performed a greenhouse experiment using *M*. x *giganteus* and investigated the response of bacteria in the rhizosphere (soil close to the root surface), rhizoplane (root surface) and root endosphere (root interior) to Cd, Pb and Zn short-term pollution using a molecular barcoding approach. We analysed the bacterial community structure 14 weeks after the contamination was induced and compared the data to microbiomes of plants grown in a non-contaminated control soil. Since *M*. x *giganteus* accumulates metals in belowground parts, we expected to observe the biggest impact of metal contamination on the bacterial community in the root endosphere.

**2. MATERIAL AND METHODS**

**2.1 Experimental setup**

We collected 500 kg of fresh soil for our greenhouse experiment from a long-term agro-ecological research farm in Scheyern, Germany (48°29’57’’ N, 11°26’32’’ E; (Auerswald et al., 2001)) from the upper 20 cm in early spring 2014. The soil was characterized as a Luvisol with silty loam texture, consisting of 22.50 % clay, 58.60 % silt and 18.90 % sand; the pH was 7.1 (0.01 mol L-1 CaCl2). To remove plant residues and gravel, the soil was sieved (< 2 mm). To increase the soil porosity, 6 vol% quartz sand (0.6-1.2 mm, Dorsilit) was added to the soil.

In April 2015, plastic pots (5 L) were filled with 5.7 kg (dry weight)of the soil-sand mixture and planted with *Miscanthus* x *giganteus* rhizomes (Energene Ltd., Poland; approximately 10 cm length). The rhizomes were prepared as rhizome cuts of the mother plants and came from one plantation, indicating very low genetic variability. The plants were grown in a greenhouse at 25/20 °C day/night temperature, relative humidity 66 % and a photoperiod of 15 h. Plants were watered with approximately 600 mL tap water per pot twice a week throughout the experiment. After 5 weeks of preadaptation, Pb-acetate (54.7 mg Pb kg-1 *dw*), Zn-nitrate (217.5 mg Zn kg-1 *dw*) and Cd-acetate (2.1 mg Cd kg-1 *dw*) were applied (HM treatment) on the soil surface with prior excessive watering to initiate the drainage in pots. As a control (Con) plants received acetic acid and ammonium nitrate (43.7 and 267.2 mg kg-1 *dw,* respectively) using the same watering procedure to exclude the effect of acetate and nitrate, amended with metal salts. Each treatment was performed in triplicates.

Sampling was carried out 14 weeks after HM treatment, when the plants had reached flowering stage. For the determination of HM concentrations and physicochemical soil characteristics, bulk soil was sampled from the pots with a soil corer and the cores were divided into three parts of proximately 5 cm and stored at 4 °C. Each depth layer was analyzed separately.

For sampling of the rhizosphere, rhizoplane and root endosphere a protocol from Edwards et al. (2015 was used. In short, excess soil was manually shaken from roots, leaving only strongly attached soil. For the extraction of rhizosphere soil, roots were placed into sterile 250 mL centrifugation tubes with sterile phosphate buffered saline (PBS) solution, and vigorously shaken and vortexed. After centrifugation (10000 x g) supernatant was discarded and the soil that was washed from the roots was considered as rhizosphere. Roots were then placed into fresh sterile PBS, sonicated for 30 s (50-60 Hz, output frequency 45 kHz, power 80 W, VWR International, Lutterworth, UK) on ice and centrifuged (4700 x g). PBS was discarded and the remaining soil was considered as rhizoplane. Roots were finally sonicated two more times using fresh PBS, surface sterilized (2 min in Tween 80, 4x washed with sterile MilliQ water, 2 min in 70 vol% ethanol, 3x washed, 10 min in 8 vol% NaOCl, 8x washed) and dried on a sterile filter paper in a laminar hood. Rhizosphere, rhizoplane soil and roots were snap-frozen in liquid nitrogen and stored at -80 °C for DNA extraction. To prove efficiency of root surface sterilization the water from the last washing step was used as a template for a PCR reaction using universal primers for the 16S rRNA gene (see method section 2.5) and checked on 2 % (*w/v*) agarose gel for presence of bands. No amplification was detected.

**2.2 Total and CaCl2-extractable metal concentrations**

For determination of total metal concentrations, soil samples and roots were dried (90 °C for 24 h and 48 h, respectively); soil samples were sieved (< 2 mm) and roots were pulverized. Homogenized plant samples were subjected to pressure digestion using nitric acid and a high-pressure digestor (Seif, Unterschleißheim, Germany). The digests were heated for 10 h at 170 °C. Soil samples were subjected to *aqua regia* extraction according to ISO 11466 (1995). Total Pb, Zn and Cd concentrations were subsequently measured in digests or *aqua regia* extracts by inductively coupled plasma – atomic emission spectrometry (ICP-AES, Spectro Ciros Vision system, SPECTRO Analytical Instruments, Kleve, Germany). As described by Novozamsky et al. (1993) 0.01 mol L-1 CaCl2 is the best single extractant for the assessment of the availability of HM to plants. Therefore, CaCl2 extracts were prepared from 5 g of dried (90 °C for 24 h) soil using 0.01 mol L-1 CaCl2 (1:4, *w/v*). Samples were shaken on an overhead shaker for 45 min and filtered through 0.45 µm pore-size Millex-HV filters (Merck, Darmstadt, Germany). In extracts, CaCl2-extractable metal concentrations were measured by ICP-AES.

For analytical quality control (AQC) three reference materials were determined: ERM CD 281 for plant material and BCR 141R and BCR 142R for soil. AQC measurements in mg/kg were: ERM CD 281 (own / certified): Cd: 0.125 ± 0.005 / 0.120 ± 0.007; Pb: 1.52 ± 0.05 / 1.67 ± 0.11; Zn: 29.3 ± 0.75 / 30.5 ± 1.1. BCR 141R (aqua regia: own / certified): Cd: 14 ± 0.5 / 14 ± 0.4; Pb: 55.8 ± 3.2 / 54.3 ± 2.0; Zn: 270 ± 9.4 / 270 ± 8.0. BCR 142R (aqua regia: own / certified): Cd: 0.248 ± 0.02 / 0.25 ± 0.01; Pb: 25.9 ± 0.42 / 25.7 ± 1.6; Zn: 91.9 ± 2.8 / 93.3 ± 2.7.

**2.3 DNA extraction**

DNA was extracted using PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA) according to manufacturer’s instructions from 0.5 g of rhizosphere and rhizoplane soil and 0.04 g of air-dried roots after a bead beating homogenization step of 30 s-1 for 2x 3 min (Precellys24 Instrument, PeqLab, Erlangen, Germany). An extraction with no sample template served as a blank extraction control. Quality and quantity of extracted DNA was checked spectrophotometrically (NanoDrop, PeqLab, Erlangen, Germany).

**2.4 Quantification of bacterial 16S rRNA genes**

Quantitative PCR (qPCR) was used to determine the abundance of bacterial 16S rRNA gene copies in rhizosphere and rhizoplane using SYBR Green-based detection with an ABI 7300 Cycler (SYBR Green I; Life Technologies, Darmstadt, Germany). To avoid PCR inhibition due to substances co-extracted during DNA extraction, DNA was prior to amplification diluted 1:1000 as tested in pre-experiments (data not shown). Samples were quantified in triplicates using the primers FP16S/RP16S (Bach et al., 2002). Each 25 µL reaction contained 1x Power SYBR Green PCR Master Mix (Life Technologies, Darmstadt, Germany), 0.04 % (*w/v*) bovine serum albumin (Sigma Aldrich, Taufkirchen, Germany), 0.2 µM of each primer (Metabion, Martinsried, Germany) and 2 µL of 1000-times diluted template DNA. Thermal cycling was initiated with a denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 45 s (denaturation), 58 °C for 45 s (annealing), 72 °C for 45 s (elongation) and melting curve analysis to confirm the specificity of amplification. No-template reactions were included as negative controls. To generate standard curves serial dilutions from 101 to 106 copies µL-1 of plasmid DNA (ZeroBlunt TOPO cloning kit, Invitrogen AG, Carlsbad, USA) containing a fragment of the 16S rRNA gene of *Pseudomonas putida* was used (Bach et al., 2002). The calculated efficiency (E = 10(-1/slope)-1) was 92 %.

**2.5 Diversity of bacterial 16S rRNA genes**

To profile bacterial communities of *M.* x *giganteus* rhizocompartments, amplicon-based barcoding was performed using the 16S rRNA gene as a marker and next-generation sequencing. For samples derived from the rhizosphere and rhizoplane the hypervariable regions V3-V4 of the bacterial 16S rRNA gene were amplified using S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 (341F/B805R) primers (Klindworth et al., 2013) containing ‘3 end overhanging adapters compatible with index primers. To avoid the amplification of plastid DNA in root endosphere a primer pair S-D-Bact-0335-a-S-17/S-D-Bact-0769-a-A-19 (335F/769R) (Dorn-In et al., 2015) amplifying the same region was used instead. Each 25 µL reaction contained 1x NebNext High Fidelity PCR Master Mix (New England Biolabs, Frankfurt am Main, Germany), 0.2 µM of each primer and 5 ng of DNA template. PCR amplifications were carried out in a PeqSTAT 96 universal thermal cycler (Peqlab Biotechnologie, Erlangen, Germany) using the following conditions: initial denaturation at 98 °C, 5 min, followed by 23 cycles of denaturation (98 °C, 10 s), annealing (53.5 °C, 30 s) and elongation (72 °C, 30 s), finishing with final elongation at 72 °C, 5 min. The amplicon size was checked on a 1 % (*w/v*) agarose gel and PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany; DNA to beads ratio 1 to 1.3). Fragment sizes and concentrations were measured by an Agilent DNA 7500 chip with a Bioanalyzer 2100 device (Agilent, Waldbronn, Germany) and quantified with a Quant-iT Pico Green dsDNA Assay Kit (Thermo Fisher Scientific, Dreieich, Germany). Indexing PCR reaction was carried out as follows: 98 °C for 30 s, followed by 8 cycles of 98 °C, for 10 s, 55 °C for 30 s and 72 °C for 30 s, ending with 72 °C for 5 min. Each indexing PCR reaction contained 1x NebNext High Fidelity PCR Master Mix (New England Biolabs, Frankfurt am Main, Germany), 2.5 µL of each index primer (N7xx, S5xx; Nextera XT DNA Library Preparation Kit, Illumina, San Diego, USA) and 10 ng amplicon DNA as a template. Samples were purified using Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany), quality checked using an Agilent DNA 7500 chip with a Bioanalyzer 2100 and quantified with a Quant-iT Pico Green dsDNA Assay Kit (Thermo Fisher Scientific, Dreieich, Germany). Finally, the libraries were pooled to a final concentration of 4 nM and pair-end sequenced on the MiSeq platform using MiSeq Reagent Kit v3 for 600 cycles (Illumina, San Diego, USA) and PhiX as internal control.

**2.6 Sequence data analysis**

In pre-processing steps, first adapter sequences were removed and the forward and reverse reads merged using the tool AdapterRemoval 2.1.7 (Lindgreen et al., 2012) with a minimum trimming read length of 50 bp and Phred quality score of 15. Second, PhiX related reads and chimeric sequences were excluded using DeconSeq 0.4.3 (Schmieder and Edwards, 2011) and usearch61 algorithm (Edgar, 2010), respectively. Decontaminated and adapter-trimmed sequences were then analysed with QIIME 1.9.1 (Caporaso et al., 2010). Assembled read pairs with an acceptable quality (Phred score ≥ 3) and length (420-480 bp), as recommended by Bokulich et al. (Bokulich et al., 2013) were clustered into operational taxonomic units (OTUs) at 97 % pairwise sequence identity using the open reference strategy with UCLUST (Edgar, 2010) Taxonomy was assigned using RDP Classifier 2.2 (Wang et al., 2007) trained with the SILVA database (release 123). Unassigned Micromonosporaceae sequences were further matched against the NCBI database (BLASTN). Sequences with top scores, at least 97 % identity and E value 0 were used for assignment as genus *Micromonospora*. From the final OTU count table singletons and rare OTUs were filtered using a relative abundance cut-off 0.001 %. Chloroplast sequences were as well removed. OTUs present in negative extraction and PCR controls were rare and all the reads were filtered during analysis, indicating that the samples analyzed were free of contamination.

**2.7 Statistical analysis**

All statistical analyses were performed in R version 3.4.3 (R Core Team, 2017) using the RStudio interface (version 1.0.143), including packages “plyr” (Wickham, 2011), “dplyr” (Wickham et al., 2017), “reshape2” (Wickham, 2007) “stringr” (Wickham, 2018) and “ggplot2” (Wickham, 2009) for data manipulation and graphical visualizations. Alpha-diversity was calculated using Observed species richness, Shannon’s diversity index and Abundance-based coverage estimator (ACE) implemented in the R package “phyloseq” (McMurdie and Holmes, 2013; McMurdie and Holmes, 2014); statistical significance was determined using analysis of variance (ANOVA; *p* < 0.05) and Tukey HSD posthoc test (R package “stats”). Statistical significance of treatments on β-diversity metrics was determined by two-way PERMANOVA analysis (function adonis2 from R package “vegan” (Oksanen et al., 2018)) based on unweighted UniFrac distance matrices (Lozupone and Knight, 2005). The same distance matrices was also used to generate principal coordinates analysis plots in R using package “ape” (Paradis et al., 2004). Differentially abundant bacterial taxa (bacterial responders to metal treatment) were assessed with R package “DESeq2” (Love et al., 2014) within a “phyloseq” toolbox (McMurdie and Holmes, 2013). From non-rarefied OTU tables the negative binomial Wald test (alpha < 0.05) was calculated and *p*-values adjusted for multiple testing using Benjamini-Hochberg multiple-inference correction. The main responders to metal stress were selected according to significant log2 fold change (*p* < 0.05), considerable relative abundance (> 0.1 %) and consistent response in all replicates. Further, statistical significance of bacterial responders to metal treatment on genus level using t-test (*p* < 0.05) was calculated. Statistical significance of treatment affecting bacterial 16S rRNA gene copies (qPCR) was analysed by Kruskal-Wallis non-parametric test (kruskal.test).

**3. RESULTS**

**3.1 Heavy metal concentrations in the soil and in the roots of *M.* x *giganteus***

Total HM concentrations measured in HM treated pots were the highest in the top soil layer (Table 1). CaCl2-extractable metal fractions, regarded as bioavailable, were low. We measured 0.15 ± 0.18 mg Zn kg-1 *dw* and 13.26 ± 11.81 µg Cd kg-1 *dw* in the top soil layer, while Pb and metal concentrations in deeper layers were under the limit of quantification (LOQ). In roots from HM treated pots high concentrations of HM were determined, especially of Cd, where 1.8 mg kg -1 (*dw*) Cd was detected, which corresponds to 88.7 % of the applied Cd. Zn and Pb concentrations in roots represented 6.9 and 0.6 mg kg -1 (*dw*), which is 28.9 % and 19.4 % of the applied Zn and Pb, respectively (Table 1).

**3.2 Effect of heavy metals on bacterial biomass and total bacterial community**

Bacterial biomass was estimated in rhizosphere and rhizoplane using qPCR, targeting the bacterial 16S rRNA gene (Fig. S1). The presented values are more estimates on bacterial abundance and do not represent absolute numbers, as a result of i) variable ribosomal gene copy number among bacterial taxa, ii) DNA extracted from dead cells, iii) DNA extraction efficiency as well as iv) 16S rRNA gene presence in chloroplasts and mitochondria of plants. For the latter reason, qPCR assays were not done for root endophytes (Bach et al., 2002; Beckers et al., 2016). Nevertheless, the qPCR results provide a reproducible metric for demonstration of shifts in bacterial community size as shown in other studies (Fierer et al., 2005; Rousk et al., 2010). Compared to the untreated control, HM treatment did not cause a significant decrease in bacterial biomass in any of the analysed compartments. This result asserts that differences in relative abundance of taxa between the treatments, determined by amplicon-based barcoding, also represent absolute changes.

Bacterial α-diversity expressed as observed species richness metric and Shannon’s diversity index was the highest in rhizosphere (2,377 for mean observed species richness) and the lowest in root endosphere (1,561 for mean observed species richness). At the applied concentrations, the HM treatment did not have significant effects on species richness or diversity in any of the compartments (Fig. S2).

To investigate effects of the HM application on bacterial community structures of each rhizocompartment, β-diversity was analysed using permutational analysis of variance and principal coordinates analysis (PCoA) (Fig. 1). The application of heavy metals did not significantly impact the composition of bacterial communities in rhizosphere, rhizoplane nor root endosphere (*p* > 0.05), as is also shown on the PCoA, where no clear grouping of replicates of one treatment was observed. Only in root endosphere the samples are visually separated according to principal component 2, however the difference was not significant.

**3.3 Bacterial responders to metal treatment**

A total of 7,015,656 raw sequences were obtained. After merging and decontamination on average 91.2 % of the original reads were retained, after quality and length filtering 74.9 %, after OTU (97 % identity) calling and taxonomic assignment 62.4 %, after removal of chloroplast sequences 60.1 % and after application of abundance filter 55.9 %. Finally, 1,960,462 paired reads, ranging from 215,679 to 23,994 paired reads per sample, were assigned to 6,621 unique OTUs (97 % identity). The data was rarefied to 23,994 paired reads per sample to enable comparison across samples with different library sizes. Rarefaction analysis showed a tendency to saturation of bacterial richness rarefaction curves, indicating that most of the bacterial diversity was covered in all samples, which was confirmed with high similarity of Abundance-based coverage estimator (ACE) and Observed species richness values.

From the 6,621 OTUs detected in this study, 171 OTUs were identified (DESeq2 analysis) as significantly differentially abundant according to the treatment (all differentially abundant taxa are shown in Fig. 2). Similar response patterns were detected for the rhizosphere and rhizoplane compartments, where 31 and 28 OTUs from 3 phyla differentially responded to the HM treatment, respectively. Interestingly, in the root interior (endosphere) we identified the highest number of responders: 112 differentially abundant OTUs from 5 phyla. Despite the high number of significant responders (all differentially abundant taxa determined by DESeq2 analysis), we considered the main responders to heavy metal treatment to be those with significant log2 fold change, mean relative abundance > 0.1 % and consistent response in all replicates. As a result, the main positive responders (more abundant in HM treated samples) were identified in rhizosphere (OTU3941 and OTU4046) and rhizoplane (OTU3941 and OTU3859), all assigned to the genus *Luteolibacter*. The main positive responder identified in endosphere (OTU4379) was assigned to the genus *Micromonospora.* The main responders to HM treatment were highly abundant in the corresponding compartment. A single *Luteolibacter* OTU3941 represented 3.7 % of mean relative abundance under HM treatment in the rhizoplane compartment. The same OTU in HM treated rhizosphere contributed 2.7 % of mean relative abundance. Similarly, *Micromonospora* OTU4379 in the root endosphere of HM treated pots represented 2.3 % of mean relative abundance. Main OTUs that negatively responded to HM treatment in endosphere were assigned to genera *Streptomyces* (OTU5781, OTU5802, OTU6549, OTU6477 and OTU5765) and *Amycolatopsis* (OTU5631).

To test whether the main responders to metal stress show the same trend as on the OTU level (shown in Fig. 2) also on the genus level, a t-test was applied. For that, the reads of all OTUs assigned to the genus to which a responder belongs were summed up for control and HM treated samples. Genus *Luteolibacter* in rhizosphere (12 OTUs, *p* = 0.042) had a significant response to HM treatment also on the genus level (Fig. 3). This genus was among the 10 most abundant genera in the rhizosphere and rhizoplane samples (Fig. S3). One of the main positive responders, *Luteolibacter* OTU3941 represented 65.1 % of total *Luteolibacter* genus relative abundance in rhizosphere and 65.8 % in rhizoplane under HM treatment. Genus *Micromonospora* also showed a trend of increased relative abundance under HM treatment, although the difference was not significant (*p* > 0.05).

**4. DISCUSSION**

The presented data provides a characterization of rhizosphere and also, best to our knowledge, first detailed characterization of rhizoplane and root endosphere bacterial communities under heavy metal and control treatment. We could show that overall richness (Fig.S2) and bacterial 16S rRNA copy numbers (Fig. S1) did not differ between control and heavy metal treatment. This result is surprising, since other studies reported disturbances in rhizosphere communities due to metal stress (Gremion et al., 2004; Pham et al., 2018; Zhang et al., 2012). Although, direct comparisons are due to differences in experimental setups (e.g. different heavy metals and concentrations or the setup of controls) often not possible. In our experimental setup, heavy metal bioavailability was low. This, in combination with the efficient metal exclusion mechanism of *Miscanthus* by increased root exudation (Guo et al., 2017) could contribute to the lack of response of rhizosphere and rhizoplane total bacterial communities. Additionally, applied metal treatment also did not affect *M*. x *giganteus* biomass, which was tested in a separate experiment (data not shown), confirming its metal tolerance. Metal sorption to soil organic matter, oxyhydroxides and clay minerals start soon after application of metals and gradually decreases metal availability over time (Degryse et al., 2009; Welp and Brümmer, 1999). The adsorption and solubility of metals also depends on the physico-chemical properties of the elements. Generally, adsorption of Zn and Cd is medium and the adsorption of Pb is high (Welp and Brümmer, 1999), as we observed in our study. In addition, heavy metal uptake by *M*. x *giganteus* and accumulation in roots (1.9 mg Cd kg-1, 62.9 mg Zn kg-1 and 10.6 mg Pb kg-1) can explain the decrease in total metal concentrations measured in the planted pots. High variability of measured soil metal concentrations could be the consequence of metal application to the soil surface, therefore an excessive soil homogenization after metal treatment is recommended instead.

In an earlier study, it has been reported that metal contamination may influence abundance of specific taxa in *M.* x *giganteus* rhizosphere (Pham et al., 2018). The main responders were *Terrimonas*, *Gemmatimonas*, *Thiobacter*, *Nitrospira*, *Bauldia*, *Levilinea*, Gp5 and Gp6 (Acidobacteria). Although in our study the effect of heavy metal treatment was not observed on the total bacterial community level, application of metals resulted in differential abundance of particular taxa (Fig. 2). *Luteolibacter* was determined as the main responder to heavy metal exposure in rhizosphere and rhizoplane. Its high mean relative abundance (2.7 % and 1.1 % in rhizosphere and 3.7 % and 0.1 % in rhizoplane), presence among 10 most abundant genera (Fig. S3) and significantly increased abundance also on the genus level (Fig. 3) are implying biological importance for the plant and heavy metal resistance/tolerance. Members of the phylum Verrucomicrobia were abundant in acid rock drainage contaminated with multiple heavy metals and moderately acidic conditions (Okabayashi et al., 2005). *Luteolibacter* was also isolated from leek and potato rhizosphere and its rhizosphere competence was shown, since it was more abundant in leek rhizosphere compared to bare soil (da Rocha et al., 2010), but no effect on root growth was observed (Nunes Da Rocha et al., 2011). Literature describing *Luteolibacter* is scarce, since Verrucomicrobia members are difficult to grow in a pure culture, even though that phylum is among the dominant bacterial groups present in soils and rhizospheres[82]. Therefore, more effort should be put on the clarification of the role of *Luteolibacter* sp. associated to *M*. x *giganteus* in metal contaminated soil.

Metals were in roots highly accumulated, especially Cd (88.7 % of applied Cd). However, root endophytic bacterial communities did not show a significant change in structure or diversity (Fig. 1, Fig. S2). Within the plant tissue, endophytes are better protected against environmental stresses compared to rhizosphere communities (Li et al., 2016), which may be valid as well for moderate metal contamination. Many tolerant plants sequester metals preferentially into compartments where they do not interfere with general metabolism. In most cases, this detoxification mechanism is mainly based on active sequestration of metals into the cell wall or vacuoles of the rhizodermis, where they are stored in complexes with organic acids and only lower concentrations are observed within the root cells (Lux et al., 2011; Lyubenova et al., 2013; Mijovilovich et al., 2009). That was shown for *M. sinensis*, where Al was localized in cell walls throughout the root cross-section (Haruma et al., 2018). Therefore, sequestration of metals might not just protect the plant from the toxic effect of metals but also bacterial endophytes residing within cells, in the intercellular space or in the vascular system (Rajkumar et al., 2009). In addition, metal tolerant plants might be selecting for mainly metal resistant/tolerant microbiome. For example, endophytes of hyperaccumulator plant were found more metal resistant than its rhizosphere isolates (Idris et al., 2004).

All responders detected in the endosphere compartment belonged to the phylum Actinobacteria. Members of the genera *Streptomyces* and *Amycolatopsis* were lower abundant in the roots of *Miscanthus* plants treated with heavy metals. This was surprising, particularly for *Streptomyces*, as strains of this genus are in general shown to be resistant to heavy metals due to efficient superoxide dismutase and membrane transport system (Schmidt et al., 2005). Therefore, effects on *Streptomyces* might be indirectly caused, e.g. due to changes on plant root exudation known to occur in response to heavy metal treatment (Viehweger, 2014) and, hence, selection of different organisms from the surrounding soil.

*Micromonospora*, which was in the present study highly abundant (2.29 % mean relative abundance) main positive responder to metal stress in the root endosphere, has been identified in Zn-treated soil in significantly higher abundances compared to control (Kou et al., 2018). *Micromonospora* sp. have been isolated from long-term contaminated soils near fertilizer factory (Pečiulytė and Dirginčiutė-Volodkienė, 2009) and from a nickel-mining site (Lin et al., 2015), as well as from endosphere of several plants (Carro et al., 2013; Coombs and Franco, 2003; Trujillo et al., 2007). Genomic analysis of *M. lupini* strain Lupac 08 revealed several strategies to successfully compete with other microorganisms in rhizosphere and also enter and colonize internal plant tissue (Trujillo et al., 2014). Also *in vivo* experiment indicated that inoculation with that strain stimulated clover nodulation and plant growth. Interestingly, genomes of *Micromonospora* strains isolated from diverse habitats encoded the same traits as the endophytic strains. That could give to micromonosporae the capacity to colonize multiple ecological niches, as it was hypothesized in a study comparing whole genomes of 40 *Micromonospora* type strains (Carro et al., 2018). That study also revealed that some, if not all *Micromonospora* strains possess putative genes for synthesis of phytohormones, phosphatases and siderophores, defense against pathogenic fungi and stress response. Since endophytes often express a greater beneficial effect for plants than rhizosphere bacteria, which may be even more evident under stress conditions (Hardoim et al., 2008), *Micromonospora* could be especially valuable for *M. x giganteus* growth promotion in metal contaminated sites.

**5.** CONCLUSION

In our study we identified differentially abundant bacteria in rhizosphere, rhizoplane and root endosphere of *M. x giganteus* grown on artificially heavy metal contaminated soil using 16S rRNA gene based barcoding of bacteria. *Luteolibacter* species were recognised as important bacteria associated with rhizosphere and rhizoplane and *Micromonospora* species with root endosphere. Those taxa were highly abundant in the respective compartment. This indicates that those bacterial responders are well specialized and adapted to the compartment and niche where they reside, which could provide an advantage for more efficient proliferation and colonization. Therefore, *Luteolibacter* and *Micromonospora* might improve the growth and performance of *M*. x *giganteus* under heavy metal contamination. We suggest further targeted approaches for isolating those bacteria, since their use as inocula may improve plant performance in heavy metal contaminated soils.

**Data available**

The sequencing data has been uploaded to NCBI database with BioProject ID: PRJNA490326.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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**FIGURE CAPTIONS**

Fig. 1: Principal coordinates analysis (PCoA) plots on unweighted UniFrac distances showing no significant change in bacterial community structure as an effect of heavy metal (HM) treatment compared to control (Con) in rhizosphere, rhizoplane and root endosphere compartment (n = 3).

Fig. 2: Bacterial responders to heavy metal (HM) treatment analyzed with DESeq2 (p < 0.05; p value correction for multiple testing) in A) rhizosphere, B) rhizoplane and C) root endosphere. Bars represent all differentially abundant OTUs, expressed as a log2 fold change comparing HM and control (Con) treatment. Their mean relative abundance in HM (positive side of the axis) or Con (negative side of the axis) treatment is shown at the end of the bars. The main responders to metal treatment, which were selected according to significant log2 fold change, considerable relative abundance (> 0.1 %) and consistent response in all replicates are indicated with asterisks (\*). The lowest taxonomical annotation is shown. P. abbreviates phylum, C. class, O. order and F. family.

Fig. 3: Relative abundances of responders to applied metals on genus level in heavy metal (HM) and control (Con) treatment in A) rhizosphere, B) rhizoplane and C) root endosphere. Where genus was not assigned, the lowest assignment is shown. (P) abbreviates phylum, (C) class, (O) order and (F) family. Mean relative abundance is depicted with whiskers representing standard deviation (n = 3). Statistical significance (p < 0.05) is indicated with asterisks (\*).