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Combined amendment of immobilizers and the plant growth-promoting strain *Burkholderia phytofirmans* PsJN favours plant growth and reduces heavy metal uptake

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ABSTRACT

In the surroundings of a former Pb/Zn smelter in Arnoldstein (Austria) heavy metal concentrations in planted crops exceed thresholds for usage as food and feed. The aim was to study the effects of a plant growth-promoting bacterial strain in combination with immobilizing soil amendments on plant growth, heavy metal uptake and on microbial community structure. Pot experiments were performed whereby two maize cultivars were grown in different contaminated soils and treatments consisted of *Burkholderia phytofirmans* strain PsJN with and without addition of gravel sludge and siderite bearing material. Inoculation with strain PsJN significantly improved root and shoot biomass of maize independent of immobilizer addition. Analysis of heavy metal content of the rhizosphere and leaves indicated that immobilizing amendments had significant reducing effects on NH_4NO_3 extractable Zn and Pb in soil and in plants grown in treated soils. Microbiomes were analysed by cultivation-independent pyrosequencing analysis of 16S rRNA genes. The results showed clear effects on community composition in response to the immobilizer amendments, whereas inoculation with *B. phytofirmans* affected microbiome diversity only to a minor extent.

1. Introduction

Soil contamination is a global problem with associated risk for the environment and human health. Heavy metals and mineral oils are among the most problematic contaminants contributing to around 60% of soil contamination (Panagos et al., 2013). Some heavy metals such as zinc (Zn) are essential for life in low quantities while others like cadmium (Cd) and lead (Pb) are without known biological function. Both essential and non-essential metals can be toxic to living organisms in excessive concentrations and may affect soil quality, agricultural production, human health and the environment (Kumpiene et al., 2008). A gentle soil remediation approach represents in-situ immobilization combined with phytoexclusion. This method consists of the addition of amendments and the use of metal excluding cultivars to reduce the bioavailability of heavy metals. This approach can be a promising remediation option, especially in large areas with high and multi-elemental contamination (Mench et al., 2006; Friesl-Hanl et al., 2009). It allows production of only moderately contaminated plant biomass, which – depending on the heavy metal contents – may find application e.g. for bioenergy production.

The behaviour and bioavailability of metals depend on the metal, the soil type, the nature of organic matter and soil pH (Walker et al., 2004). The bioavailability of cationic heavy metals to plants is higher in acidic than in alkaline soils. Consequently, the application of organic amendments to adjust soil pH has been widely practiced in acidic soils. For instance, leaching of Cu, Zn and Pb is strongly pH dependent, with lowest mobility around neutral to slightly alkaline conditions; therefore, particular attention should be paid to changes in soil pH induced by the amendments (Kumpiene et al., 2008).

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The use of appropriate plants is vital in immobilization and *in-situ* stabilization techniques. Metal tolerant plants, which are able to subsist under these arduous conditions and excluder plants, which are able to retain the metal at the root system to avoid the transfer into the food chain, are commonly used in metal stabilization strategies (Baker, 1981; Kidd et al., 2009). Plant roots secrete exudates that can modify the availability of metals in soil (Puschenreiter et al., 2005; Kidd et al., 2009) and have developed mechanisms to translocate and store heavy metals.

The use of plant-associated microorganisms, especially bacteria, in phytoremediation strategies has become more important during the last years as they can influence both heavy metal mobility and immobilization. Moreover, bacteria can promote plant growth, plant tolerance to metals and decrease the bioavailability of some metals by influencing plant uptake (Kidd et al., 2009). These mechanisms have been proven important for phytoremediation showing an increase in plant metal tolerance and an increase in plant biomass after inoculation of plants with plant growth-promoting bacteria (PGPB) (Kuffner et al., 2008; Rajkumar and Freitas, 2008).

Plants are associated with complex microbiomes, particularly in the rhizosphere as well as in the plant interior. Plant-associated microbiomes are mostly shaped by the soil environment, the plant genotype/physiology and environmental or agronomic conditions (Peiffer et al., 2013; Philippot et al., 2013; Edwards et al., 2015). Also, the presence of heavy metals in high concentrations is a well-known factor affecting microbial growth and survival, their enzymatic activity and the microbial community structure and diversity (Bååth, 1989; Gremion et al., 2004). Microbial biodiversity and biochemical properties have been even used to monitor the impact of heavy metals and phytoremediation techniques on soil bacterial communities because of their more sensitive and faster reaction to soil contamination than other measurable parameters such as chemical and physical properties (Nannipieri et al., 1997; Gołębiewski et al., 2014).

The aim of this study was to evaluate the potential of a phytostabilization approach, which combines the cultivation of maize in combination with an immobilizer (gravel sludge combined with siderite) and/or a PGPB (*Burkholderia phytofirmans* strain PsJN) to reduce heavy metals uptake and increase plant growth in a Zn/Cd/Pb-contaminated soil. The establishment of the inoculant strain itself may affect the plant microbiome. Alternatively, the treatment may alter soil and plant (physiological) characteristics (e.g. reduced availability of heavy metals) and thereby lead to changes in plant-associated microbiota due to the stress-alleviating conditions. This may potentially indicate a positive effect of the treatment. Therefore, an additional objective of this study was to assess any impact of the applied treatments on microbiomes associated with different plant compartments.

2. Material and methods

2.1. Experimental design

To evaluate the effect of combined immobilizer and plant growth promoting bacteria (PGPB) on trace elements availability a greenhouse pot experiments with two maize cultivars, *cv.* NK Falkone (Syngenta, Basel, CH) and Fuxxol (Societe RAGT, Rodez Cedex, FR) and two Cd, Pb and Zn contaminated soils were performed. Soils were taken from two polluted sites (moderately contaminated soil B and heavily contaminated soil D) close to a smelter located in Arnoldstein, Carinthia, in the southern part of Austria (Table 1). Soil B originates from an arable land on a cambisol, soil D from a grassland on a leptosol. Soil B lies on the top of a hill, in a distance to the former emission center of app. 1000 m, whereas soil D is situated at the bottom of the hill and the distance to the emission center is less than 300 m. Both soils were collected from the top 15 cm of the soil horizon; dried and sieved (2 mm mesh). The amendments applied in this experiment were tested previously by Friesl-Hanl et al. (2006, 2009). An amount of 3% (w/w) amendment, containing 5 parts of gravel sludge and 1 part of siderite bearing material, was added to the soils and mixed using a concrete mixer. The physicochemical properties and the metal concentration of the gravel and siderite material are given in Table 2.

Table 1
General soil properties, total and ammonium-nitrate-extractable metal concentrations.

Site		Soil B	Soil D
pH_{CaCl_2}	–	4.7 ± 0.1	5.5 ± 0.3
DOC[a]	mg L^{-1}	14.4 ± 0.5	39.7 ± 0.13
C_{org}[a]	g kg^{-1}	26.0 ± 1.3	67.0 ± 0.6
Sand[a,b]	g kg^{-1}	486	662
Silt[a,b]	g kg^{-1}	359	243
Clay[a,b]	g kg^{-1}	155	95
Cd_T	mg kg^{-1}	5.6 ± 0.2	15.1 ± 1.2
Pb_T	mg kg^{-1}	903 ± 34	1744 ± 86
Zn_T	mg kg^{-1}	535 ± 16	1884 ± 130
$Cd_{NH_4NO_3}$	mg kg^{-1}	1.4 ± 0.1	0.9 ± 0.1
$Pb_{NH_4NO_3}$	mg kg^{-1}	11.2 ± 2.0	3.4 ± 0.4
$Zn_{NH_4NO_3}$	mg kg^{-1}	71.3 ± 5.7	60.7 ± 8.4

[a] Data taken from the publication of Puschenreiter et al. (2013).
[b] … single determination.

After amendment equilibration, two maize cultivars Falkone and Fuxxol were grown on amended soil B and soil D in a greenhouse. These cultivars were chosen because of their capacity to accumulate (Falkone) or exclude Cd (Fuxxol) (Friesl-Hanl and Horak, 2011). The maize seeds were surface sterilized before inoculation with 70% ethanol and 5% sodium hypochlorite for 1 min each followed by 4 times washing with distilled water. For bacterial inoculation *Burkholderia phytofirmans* PsJN (Sessitsch et al., 2005) was chosen because of its well-known plant growth promoting traits (Mitter et al., 2013a), which have been particularly demonstrated with maize (Naveed et al., 2014). The strain tolerates moderate heavy metal concentrations, minimal inhibitory concentrations (MIC) were 6 mM Pb, 2 mM Zn and 0 mM Cd, respectively. A derivative of strain PsJN, i.e PsJN::*gusA*11 (Compant et al., 2005), which was transformed with the beta-glucuronidase reporter gene *gusA*, was applied to allow simple detection of the strain in maize shoots.

Maize seeds were incubated with 10^9 cfu/mL of a Luria Broth overnight culture of PsJN::*gusA*11 (Naveed et al., 2014) for 90 min; controls were soaked in Luria Broth. Prior to pot experiments, nursery plantation was performed and transplanted to the corresponding treatments after 14 days of germination. Each treatment was replicated four times and compared to non-amended, non-inoculated controls in both cultivars. As fertilizer Wuxal Super (N, P, K fertilizer with trace elements, Kwizda Agro, Wien) was added (0.05%, bimonthly) to the plants. The temperature in the greenhouse was 25–30 °C and 16 h artificial light was supplied daily.

2.2. Influence of *B. phytofirmans* PsJN::gusA11 on growth and metal uptake of two maize cultivars

Plants were harvested for subsequent analysis 69 days after planting, in the flowering stage. The rhizosphere and the shoots were collected for DNA isolation and inoculant identification from 48 plants (selected at random), three pots per treatment. The rhizosphere was collected shaking the root system and collecting the soil intimately attached to the roots and then this soil was

Table 2
Characteristics of immobilizer amendments.

Amendment	SiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	LOI[a]	Cu	Pb	Zn
	%	%	%	%	%	%	%	%	ppm	ppm	ppm
Siderite-bearing material	12.3	4.3	39.4	2.12	3.4	9.4	0.1	28.2	56	11	78
Gravel sludge	40.5	12.1	6.9	0.17	6.8	12.5	1.2	16.5	85	54	116

Percentages are given per weight.
[a] LOI loss on ignition at 1000 °C.

sieved using a 2 mm sterile sieve. Root and shoot biomass was measured to evaluate the growth effect of bacteria.

Soil physicochemical analysis and plant trace metal accumulation were analysed as described by Friesl-Hanl et al. (2009). Plant material (2 g) was digested using a mixture of concentrated 20 mL HNO_3 and 4 mL $HClO_4$. Soil samples were extracted with NH_4NO_3 (Austrian Standard L 1094-1) and with *aqua regia* (Austrian Standard L 1085). The metal concentrations in the NH_4NO_3 extracts were determined by inductively coupled plasma mass spectrometry (ICP-MS Perkin Elmer Elan 6100), in the plant extracts and *aqua regia* by inductively coupled plasma emission spectrometry (ICP-AES, Varian Saturn Liberty II).

2.3. Microbial community analyses

2.3.1. DNA isolation

After harvesting, shoot and rhizosphere samples were stored at 4 °C for inoculant identification and at −20 °C for DNA isolation.

DNA extraction from rhizospheric soil was based on the protocol provided by the manufacturer of the FastDNA®SPIN Kit for Soil (BIO101, Vista, CA) with some modifications. Briefly, 0.5 g of soil were treated with sodium phosphate buffer, TM buffer (FastDNA®SPIN Kit) and PCI (Phenol–chloroform–isoamyl alcohol) prior to bead beating. The soil was subjected to three bead beating cycles (FastPrep FP120 disrupter, QBiogene, Irwine, CA). Supernatants were pooled and extracted twice with PCI (25:24:1) and once with chloroform–isoamyl (24:1). After these initial modified steps the extraction procedure was continued according to manufacturer instructions.

The DNA isolation from shoots was done using the FastDNA™ Plant/Seed DNA Kit (BIO101, Vista, CA) following the instructions provided by the manufacturers. Finally DNA was stored at −20 °C.

2.3.2. Inoculant detection

2.3.2.1. Culture-based detection of B. phytofirmans PsJN::gusA11. For the inoculant detection 5 g of rhizosphere soil were shaken at 250 rpm with 50 mL 0.9% NaCl solution during 1 h. Tenfold dilution of rhizosphere suspensions was plated into selective LB agar containing spectinomycin (100 g mL^{-1}), 5-bromo-4-chloro-3- indolyl-_-d-glucuronide (XGlcA) (100 g mL^{-1}), and isopropyl-D-galactopyranoside (IPTG) (100 g mL^{-1}) to re-isolate the inoculant *B. phytofirmans* PsJN::*gusA*11 based on GUS reporter. Plates were incubated at 28 °C for 2 days and blue colonies were counted to analyse rhizosphere colonization.

2.3.2.2. PCR based detection of B. phytofirmans (PsJN::gusA11). The amplification of the *gusA* reporter gene was carried out using the primers gusAF (5′-GGTGGGAAAGCGCGTTACAAG-3′) and the gusAR (5′-TGGATTCCGGCATAGTTAAA-3′). For 50 μl PCR reactions the following reactive concentrations were used: 2.5 units FIREPol® DNA Polymerase (Solis Byodine), 0.2 mM of each dNTP, 0.45 μM of each primer, and 2.25 mM $MgCl_2$ and a volume of 0.5 μl DNA extract. The gene fragments were amplified with a T-Gradient thermocycler (BIOMETRA) using an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 61 °C and 1 min and 30 s extension at 72 °C, and a final elongation step of 10 min at 72 °C. The presence and correct size of PCR product was checked in a 1.2% agarose gel and verified by sequencing.

2.3.3. DNA amplification, library preparation and pyrotag sequencing

For the identification of bacteria, 16S rRNA gene amplicons were obtained by PCR using the fusion primer pairs specific for the V5–V9 region (799-forward) (Chelius and Triplett, 2001) and 1520-reverse pH primer (Massol-Deya et al., 1995). The forward fusion primer contained: the Lib-L Primer A sequence specific for the Lib-L chemistry and the One-Way Reads sequencing method (Roche, Branford, CT), the key sequence TCAG, the barcode Multiplex Identifier (MID) sequence specific for each DNA sample, and the 799-forward sequence. The reverse primer contained the Lib-L Primer B sequence (Roche), the key sequence TCAG and the 1520-reverse sequence.

PCR products were generated by amplifying 5 μl of extracted DNA using the FastStart High Fidelity PCR System (Roche) with 0.25 mM dNTPS, 0.5 mg mL^{-1} BSA, 4% (V/V) DMSO, 0.3 μM of each primer and 2.5 U of FastStart High Fidelity DNA polymerase (Roche). The PCR protocol consisted of denaturation at 95 °C for 5 min; followed by 30 cycles of 95 °C for 30 s; amplification for 1 min at 53 °C and 51 °C for bacteria and fungi, respectively, and elongation at 72 °C for 2 min, followed by a final elongation at 72 °C for 10 min.

The PCR products were analysed by gel electrophoresis and cleaned using the AMPure XP beads kit (Beckman Coulter, Brea, CA, USA) following the manufacturer's instructions. Products of the different DNA samples were quantified by a quantitative PCR using the Library quantification kit Roche 454 Titanium (KAPA Biosystems, Boston, MA) and pooled in equimolar amounts in a final amplicon library. The 454 pyrosequencing was carried out on the GS FLX+ system (Roche) using the XL+ chemistry (Roche) following the manufacturer's instructions.

2.3.4. 16S rRNA gene sequence processing

Data quality was checked in PRINSEQ (Schmieder and Edwards, 2011). SFF (Standard Flowgram Format) files were analysed using FlowClus (https://github.com/jsh58/FlowClus). Quality filtering consisted of discarding reads <200 nt and >1000 nt, excluding homopolymer runs >6 nt and ambiguous bases >6, accepting 1 barcode correction and 2 primer mismatches. A value of 25 was considered as a minimum average Phred quality score allowed in a sliding window of 50 nt. After the filtering, reads underwent denoising. Denoised sequences were then de-replicated and sorted in USEARCH v7 (Edgar, 2013). Chimeras were removed using both a *de novo* and a reference based approach with UCHIME (Edgar et al., 2011). The ChimeraSlayer's database was used as a gold standard for the reference based chimera checking (Haas et al., 2011). OTU picking was accomplished in USEARCH with the pairwise identity percentage of 0.97. Taxonomy assignment was performed in QIIME

(Caporaso et al., 2010) employing the naïve Bayesian RDP classifier with a minimum confidence of 0.8 (Wang et al., 2007) against the last version of the Greengenes database (08/2013) (McDonald et al., 2012). Good's non-parametric coverage estimator was computed in QIIME to estimate the percentage of the total OTUs that were sequenced in each sample.

2.3.5. 16S rRNA gene-based microbial community analysis and comparison

A data-driven adaptive method for selecting normalization scale quantile was conducted and data normalized by scaling counts by the nth percentile of each sample's nonzero count distribution in the metagenomeSeq Bioconductor package (Paulson et al., 2013; McMurdie and Holmes, 2014). Data were then tested for multivariate normality (Mardia's test) in the MVN R package, and a Box's M-test for homogeneity of covariance matrices performed in the biotools R package. For meeting the criteria for a normal distribution and homogeneity of the covariances, the dataset was split into the rhizosphere and shoot components, subsets were then transformed by a log (x) (for $x > 0$) function in the vegan R package.

An OTU-based analysis was performed to calculate the richness and diversity using the phyloseq R package (McMurdie and Holmes, 2013). The Chao's estimator was calculated to estimate the OTU richness present in the samples. The diversity within each individual sample was estimated using the Simpson's diversity index. For meeting the criteria for a normal distribution and homogeneity of the covariances, the dataset was split into the rhizosphere and shoot components, subsets were normalized in the clusterSim R package. A multivariate analysis of variances (MANOVA) was calculated in order to compare alpha diversity values in treatments and between cultivars, for the rhizosphere and the shoot subsets. If the multivariate F was found significant, then an individual univariate analysis was carried out using a Tukey's HSD tests applied as post-hoc analysis in the agricolae R package. Since the datasets were slightly unbalanced, type I (multivariate) analysis of variance outputs (stats R package) were compared with its type II counterpart in the car R package.

Multivariate analysis of community structure and diversity was performed on the pyrotag-based datasets using: 1) unconstrained ordination offered by Principal Coordinate Analysis (PCoA) (Gower and Blasius, 2005), 2) constrained multidimensional scaling using Canonical Analysis of Principal Coordinates (CAP) (Anderson and Willis, 2003), 3) permutation test for assessing the significance of the constraints and permutational multivariate analysis of variance (PERMANOVA), 4) indicator species analysis of taxa summarised and the genus level associated with the grouping factors used as constraints (Hartmann et al., 2012, 2014). The differences between bacterial communities were investigated using the Bray–Curtis dissimilarity distance and the ordination methods applied to the same distance matrices. All the ordination analyses were computed and plotted in phyloseq (points 1 and 2). The significance of the cultivar and the treatment grouping factors used as constraints in the CAP was assessed via the permutation test (Legendre and Legendre, 1998) in the vegan R package. The null hypothesis of no differences between *a priori* defined groups (*i.e.* assuming no constraints, as for the PCoA) was investigated using the PERMANOVA approach (Anderson, 2001), implemented in vegan as the ADONIS function and applied to the Bray–Curtis dissimilarity distances. The rhizosphere and the shoot-based datasets were considered separately for meeting the PERMANOVA's assumption of multivariate spread among groups. Indicator species analysis was calculated using the indicspecies R package (De Cáceres and Legendre, 2009), with the aim of identifying taxa summarized at genus level associated with the Falkone rather than the Fuxxol cultivar or with one particular treatment (or treatments).

A Mantel test was used to compare Bray–Curtis dissimilarity distance matrices based on the rhizosphere and shoot OTU tables collapsed at genus level. A confirmatory Procrustes analysis was performed in the vegan R package on PCoA ordinations based on the same distance matrices for comparing the samples distribution between rhizosphere and shoot (Peres-Neto and Jackson, 2001).

2.4. Statistical analysis of physicochemical data

Data from rhizosphere soil was analysed using a conventional analysis of variance (one-way ANOVA). The data which were non-uniformly distributed were tested using a non-parametric test, the Mann–Whitney U test. Results presented are means of four replicates; mean values were compared with the LSD test ($P \leq 0.05$). For correlation with sequencing data, a data frame based on metal concentration and pH values was checked for multivariate normality and homogeneity and then normalized with the clusterSim R package. Furthermore a MANOVA was used to determine the effects of cultivars and treatments. If the multivariate F was significant, then individual univariate analyses were calculated, eventually followed by Tukey's HSD post-hoc tests. Pearson correlation was also calculated between the metal concentration and the pH values in the Hmisc R package. The BioEnv procedure was then used to individuate the heavy metal elements best correlated (highest Pearson correlation) with the Bray–Curtis dissimilarity distance matrices based on the OTU table summarized at genus level, for the rhizosphere and the shoot sets. Similarly, a Mantel test was computed between the above mentioned matrices and the Euclidean distance-based matrix of metals.

BioProject accession number

Pyrosequencing data obtained in this study were submitted to NCBI under BioProject number PRJNA274231.

3. Results

3.1. Plant and root biomass

The contaminated soils B and D showed clear differences in total heavy metal content (Table 1). In the less polluted soil B the inoculation with *B. phytofirmans* PsJN::*gusA*11 significantly increased the growth of root and shoot biomass of the two maize cultivars (Fig. 1). Also, the combination of immobilizer amendment and *B. phytofirmans* PsJN::*gusA*11 had a clearly pronounced influence on the plant and root biomass growth in both cultivars. At higher total heavy metal concentrations (soil D), the immobilizer had an influence on the plant biomass of both cultivars, whereas the addition of strain *B. phytofirmans* PsJN::*gusA*11 or the combination treatment had just a small effect on growth of the cultivar Fuxxol.

3.2. Effects on heavy metal availability in soil and plant uptake

The pH effect is one of the main factors influencing metal availability. The immobilizer increased the acidic pH in the less polluted soil B and had less pronounced effects in the slightly acidic soil D. In soil B the immobilizer addition increased the pH from 4.7 to 6.2 in the rhizosphere of cv. Falkone and from 4.9 to 6.1 in the rhizosphere of the cv. Fuxxol (Table 3). The immobilizer in soil D caused only a slight pH increase of pH (Table 3).

The metal availability and metal uptake by the plant are shown in Fig. 2. In soil B, Cd, Pb and Zn availability were reduced by at least 82%, 81% and 92%, respectively, due to the immobilizer, independently of the bacterial inoculation. The uptake of Pb and Zn by the plant was reduced in both cultivars by the immobilizer and the

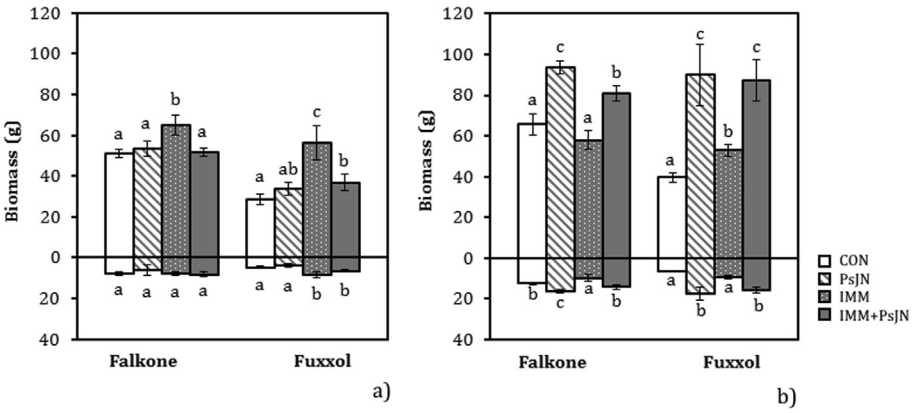

Fig. 1. Shoot and root biomass (mean ± SD) of two maize cultivars in a) soil D and b) soil B. The different letters indicate significant differences between treatments ($p < 0.05$).

combination treatment (Supplemental Table 1, Fig 2). However, we could not observe this effect with Cd, where despite a clear decrease in the available metal in soil B, the plant uptake was not decreased in neither of the cultivars (Fig. 2). *B. phytofirmans* PsJN alone did not mobilize heavy metals in the rhizosphere, but depending on the plant cultivar and the type of heavy metal, inoculation led to increased or decreased plant heavy metal uptake (Fig. 2). In the more contaminated site, soil D, the differences between treatments were less pronounced and the immobilizer treatments were not as effective as in soil B (data not shown).

3.3. Re-isolation of *B. phytofirmans* PsJN::*gusA11*

To evaluate the persistence of the inoculant strain, plants and rhizosphere soils were evaluated for the presence of *B. phytofirmans* PsJN::*gusA*11 at the end of the experiment by selective plating as well as by PCR. Results of this experiment confirmed qualitatively the presence of the inoculant strain in the rhizosphere and shoot endosphere of all three treatments, in which *B. phytofirmans* PsJN::*gusA*11 had been applied.

3.4. Culture-independent analysis

For a more comprehensive assessment on treatment effects on the plant microbiome, culture-independent analysis of rhizosphere and shoot samples was performed using 454 pyrosequencing. The results showed a clear effect on community composition in response to immobilizer addition and an effect of the maize cultivar. Shoot and rhizosphere from both plant cultivars in soil B and all different treatments described above were used for this culture-independent analysis in triplicates and a total set of 46 samples (2 missing replicates) was pyrosequenced. After quality filtering and chimera removing, 688,087 high-quality 16S V5–V9 sequences remained for community analysis. This corresponds to an average of 14,958 ± 6782 pyrotags per sample, with an average read length of 460 bp and a min. and max. of 190 bp and 763 bp, respectively. Sequence clustering yielded 522 (248 ± 176 per sample) OTUs, with a Good's coverage of 99%. Rarefaction analysis confirmed the latest result by reaching the saturation phase (data not shown). The rhizosphere dataset yielded a total of 229,307 reads, with an average of 9969 ± 2056 (n = 23) sequences per sample. This corresponds to an average of 419 ± 11 observed OTUs per sample. The endophytic population reached a total of 458,780 pyrotags, with an average of 19,946 ± 6163 (n = 23) reads and 77 ± 61 OTUs per sample.

OTU abundance data were clustered at each major taxonomic level, that is, phylum, class, order, family and genus. This identified 21, 50, 86, 147 and 201 archaeal and bacterial taxa, respectively. Twenty-five % of the total pyrotags were identified at the genus level. Overall, Proteobacteria (39%), Actinobacteria (21%), Acidobacteria (11%), Chloroflexi (7%), Firmicutes (6%) and Gemmatimonadetes (5%) were the predominant diverse bacterial phyla (Supplemental Table 2, Fig. 3). Among the Proteobacteria, 48% Alpha-, 19% Beta-, 19% Gamma- and 14% Deltaproteobacteria were found. Among others, four OTUs (0.8%) were identified as belonging to the Archaea kingdom. The archaeal community was mostly represented by Nitrososphaeraceae.

Although on higher taxonomic level Actinobacteria, Firmicutes and Alpha-, Beta- and Gammaproteobacteria were found both in the rhizosphere as well as in shoot samples with an abundance of more than 1% a clear differentiation could be observed.

Overall, the number of different bacterial phyla was higher in the rhizosphere than in the shoot. In the rhizosphere, Alphaproteobacteria with representatives from the Rhizobiales, *Kaistobacter*, and *Rhodoplanes*, Beta- and Gammaproteobacteria, Acidobacteria, Actinobacteria with representatives of Actinomycetales, Gaiellales and *Mycobacterium* as well as Firmicutes (*Bacillus*, *Solibacillus*) were the classes and OTUs with highest counts. In addition Crenarchaeota, Acidobacteria, AD3, Chloroflexi, Gemmatimonadetes, *Bradyrhizobium* sp. and Deltaproteobacteria were enriched in the rhizosphere as compared to shoots (Supplemental Table 2, Fig. 3). Moreover, representatives of Nitrososphaera sp. (Archaea) were enriched in rhizosphere samples. While the different cultivars had little influence on bacterial composition, the immobilizer, independent of *B. phytofirmans* PsJN addition, led to an increase of Actinobacteria, Bacteroidetes, Chlorobi (Green sulphur bacteria)

Table 3
pH values (mean ± SD) from Fuxxol and maize cultivars in soil B and soil D. Different letters indicate significant differences at $p < 0.05$.

	Falkone				Fuxxol			
	Con	PsJN	Imm	Imm + PsJN	Con	PsJN	Imm	Imm + PsJN
Soil B	4.7 ± 0.1 *a*	4.7 ± 0.2 *a*	6.1 ± 0.1 *b*	6.2 ± 0.0 *b*	4.9 ± 0.1 *a*	4.8 ± 0.0 *a*	5.9 ± 0.3 *b*	6.1 ± 0.0 *b*
Soil D	5.4 ± 0.4 *a*	5.3 ± 0.0 *a*	6.1 ± 0.1 *b*	6.3 ± 0.1 *b*	5.7 ± 0.1 *a*	5.7 ± 0.0 *a*	5.9 ± 0.0 *b*	5.7 ± 0.1 *a*

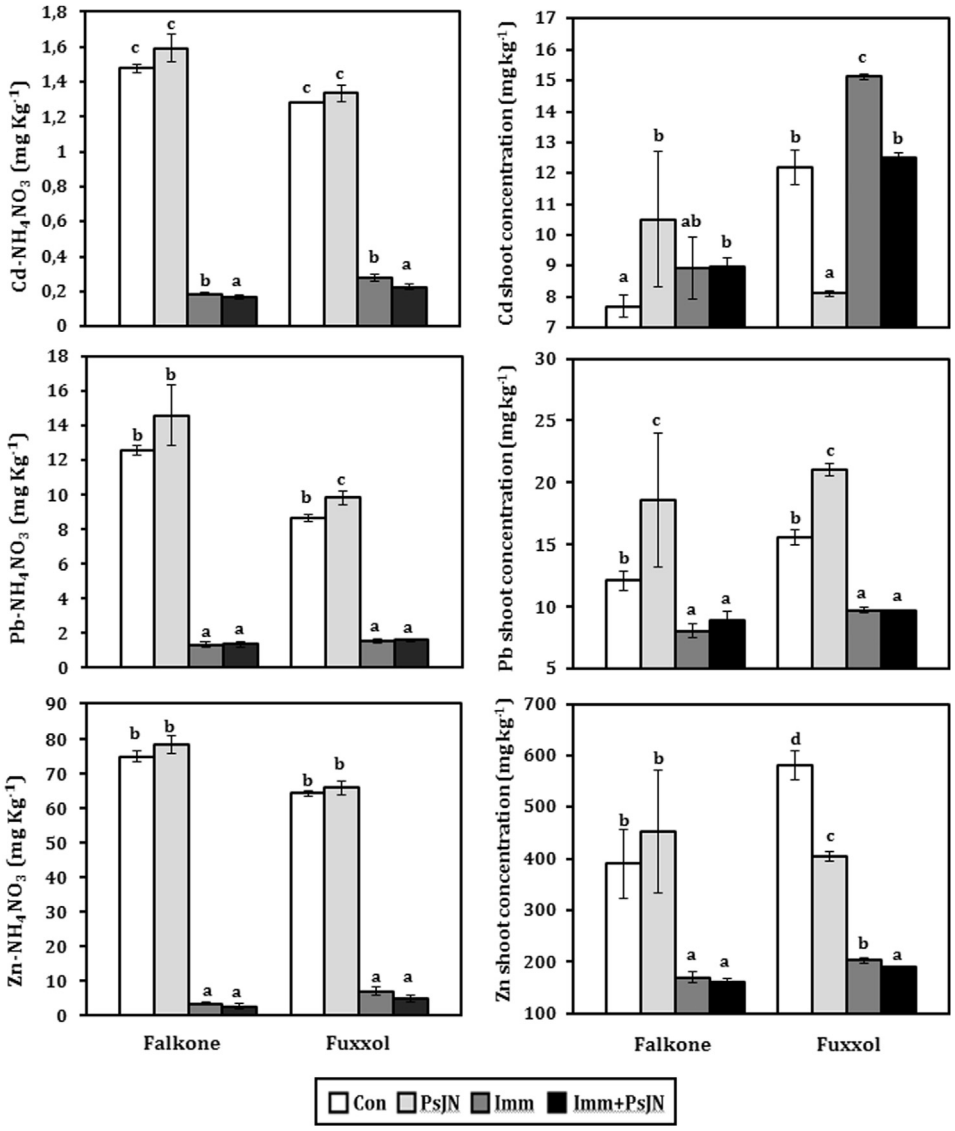

Fig. 2. Available Cd, Pb and Zn extracted from soil B with NH_4NO_3 (left) and total metal concentration in shoots (mean ± SD) of both cultivars (right). Bars with different letters in each contamination level indicate significant differences ($p < 0.05$).

and Betaproteobacteria, whereas the abundance of Bacilli decreased. Specifically, OTUs of the order Actinomycetales of the Actinobacteria, Chitinophagaceae of the Bacteroidetes, OPB56 of Chlorobi and Alcaligenaceae, Comamonadaceae (Genus *Variovorax*) and Oxalobacteraceae of the Betaproteobacteria showed higher abundance after immobilizer addition.

The prevalence of abundant OTUs differed clearly in shoot and the rhizosphere samples. In shoots, the most abundant phylum was Proteobacteria, OTUs corresponding to Pseudomonadaceae and Enterobacteriaceae (both Gammaproteobacteria) and *Achromobacter* (Betaproteobacteria) dominated the community. OTUs of Alcaligenes sp. (Burkholderiales, Betaproteobacteria) and *Stenotrophomonas* sp. (Gammaproteobacteria) were also enriched (Supplemental Table 2, Fig. 3). After immobilizer addition we could observe changes in the root microbiome of the cultivar Falkone. The amendment addition induced a strong shift in the abundance of Proteobacteria, Firmicutes, Actinobacteria, and Archaea representatives. Specifically, OTUs of *Mycoplana* sp., *Kaistobacter* sp., *Sphingomonas* sp. (Alphaproteobacteria), *Burkholderia* sp., *Variovorax* sp. and some members of Oxalobacteraceae (Betaproteobacteria), *Acinetobacter* sp., *Erwinia* sp. (Enterobacteriaceae) and certain OTUs corresponding to Pseudomonadaceae (Gammaproteobacteria), *Bacillus* sp., *Staphylococcus* sp., *Aerococcus* sp. and *Streptococcus* sp. (Firmicutes), members of Micrococcaceae family and *Deinococcus* sp. (Actinobacteria), members of Chitinophagaceae family (Bacteroidetes) and *Candidatus Nitrososphaera* (Archaea) showed higher abundance in shoots after immobilizer addition. This differentiation could not be observed in the shoot microbiome of the cultivar Fuxxol. No clear effects were found in the rhizosphere and the shoot microbiome when only *B. phytofirmans* PsJN::*gusA*11 was applied, except of Actinobacteria (f. Actinobacteriaceae) in the shoot of cv. Falkone.

Alpha diversity characteristics such as the observed OTUs, the Chao's estimator and the Simpson's index are described in Table 4. In the rhizosphere, the observed OTUs and Simpson's index turned out to be significantly correlated with the treatment. Both, immobilizer and the combined treatment led to a higher number of OTUs and diversity values. All indices of the shoot microbiome were significant for the cultivar category. The cultivar Falkone always showed the highest richest and most diverse microbial

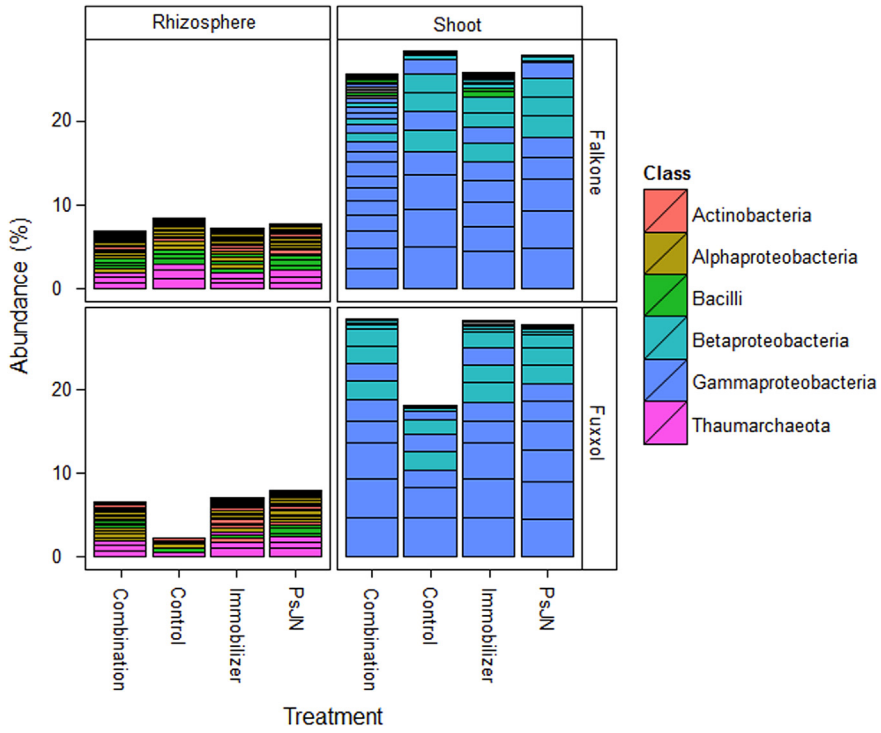

Fig. 3. Taxonomic composition and relative abundance of OTUs summarized at class level. Each barplot represents a treatment and is located in a facet defined by the plant organ and the cultivar. Barplots are chunked in OTUs piled-up in order of relative abundance and colours represent classes. Only taxa with a variance $>1e^{-4}$ were considered.

communities. Besides, the Simpson's index was found to be significant also for the treatment and the interaction cultivar × treatment. Again, immobilizer and the combined treatment differed from the control and *B. phytofirmans* PsJN::*gusA*11 alone.

Table 4
Alpha-diversity calculated by MANOVA for the rhizosphere (a) and the shoot (b). Significant differences using Tukey's Post hoc test are indicated by different letters. Significance values in ANOVA are given, NS: not significant.

Rhizosphere (a)	Observed OTUs	Chao	Simpson
Cultivar			
Falkone	399.75 ± 10 a	432.22 ± 15.7 a	0.983 ± 0.004 a
Fuxxol	401.91 ± 8.7 a	430.35 ± 11.5 a	0.984 ± 0.005 a
Treatment			
PsJN + Imm	408 ± 6.2 b	440.6 ± 14 a	0.987 ± 0.001 b
Con	390.6 ± 8.6 a	420.78 ± 4.2 a	0.978 ± 0.005 a
Imm	405.67 ± 6.4 b	432.41 ± 13.6 a	0.986 ± 0.002 b
PsJN	397.17 ± 5.8 ab	429.75 ± 14.1 a	0.983 ± 0.002 ab
ANOVA Pr (>F)			
Cultivar (C)	NS	NS	NS
Treatment (T)	<0.01	NS	<0.01
C × T	NS	NS	NS
Shoot (b)	**Observed OTUs**	**Chao**	**Simpson**
Cultivar			
Falkone	80.5 ± 60.6 b	120.22 ± 79.8 b	0.76 ± 0.09 b
Fuxxol	32.64 ± 14.6 a	42.38 ± 16.2 a	0.70 ± 0.03 a
Treatment			
PsJN + Imm	70.33 ± 53.8 a	97.32 ± 67.2 a	0.78 ± 0.11 b
Con	30.6 ± 12.7 a	41.91 ± 18.8 a	0.70 ± 0.04 a
Imm	71.5 ± 68.4 a	90.79 ± 77.2 a	0.74 ± 0.07 ab
PsJN	53.5 ± 47.6 a	95.09 ± 92.5 a	0.70 ± 0.02 a
ANOVA Pr (>F)			
Cultivar (C)	<0.05	<0.001	<0.01
Treatment (T)	NS	NS	<0.05
C × T	NS	NS	<0.001

The findings were confirmed by the unsupervised exploratory ordination offered by PCoA (data not shown) and its constrained counterpart represented by CAP, used to reveal beta-diversity patterns in the microbial rhizosphere and shoot dataset. In particular, the CAP plot showed a clear differentiation between all treatments in the rhizosphere (Fig. 4). No significant differences between the cultivars were noticed. In the shoots on the other hand, the cultivar Falkone treated with immobilizer differentiated from Fuxxol and only immobilizer addition resulted there in a clear separation (Fig. 4).

The PERMANOVA applied to the Bray–Curtis dissimilarity matrix used for both the ordinations confirmed (9999 permutations) that in the rhizosphere only the treatment category was found significant ($F = 3.8407$, $R^2 = 0.37204$, $p = 0.0001$). Furthermore, in the shoot both the cultivar ($F = 9.0244$, $R^2 = 0.20763$, $p = 0.0023$) and the treatment categories ($F = 3.0868$, $R^2 = 0.21305$, $p = 0.0243$) turned out to be significant, as well as their interaction ($F = 3.3933$, $R^2 = 0.23421$, $p = 0.0150$). When a confirmatory permutational test was applied to the CAP scale after 9999 reiterations, only the treatment appeared significant for both the rhizosphere ($p < 2e\text{-}16$) and the shoot ($p = 0.006901$).

The association strength of each OTU (summarized at genus level) with a particular cultivar or treatment (and combination thereof) was tested using the indicator species analysis. Only for the rhizosphere compartment was possible to individuate OTUs significantly associated (after FDR correction) with the control + PsJN group (p__Proteobacteria; c__Gammaproteobacteria; stat = 0.978 q-value = 0.0043), with the Combination group (c__Gammaproteobacteria; o__Alteromonadales; stat = 0.932, q-value = 0.0043), with the Combination + Immobilizer group (p__Fibrobacteres; c__Fibrobacteria; stat = 0.932, q-value = 0.0043) and with the Combination + Immobilizer + PsJN group (p__Gemmatimonadetes; c__Gemm-3; stat = 0.966, q-value = 0.0118).

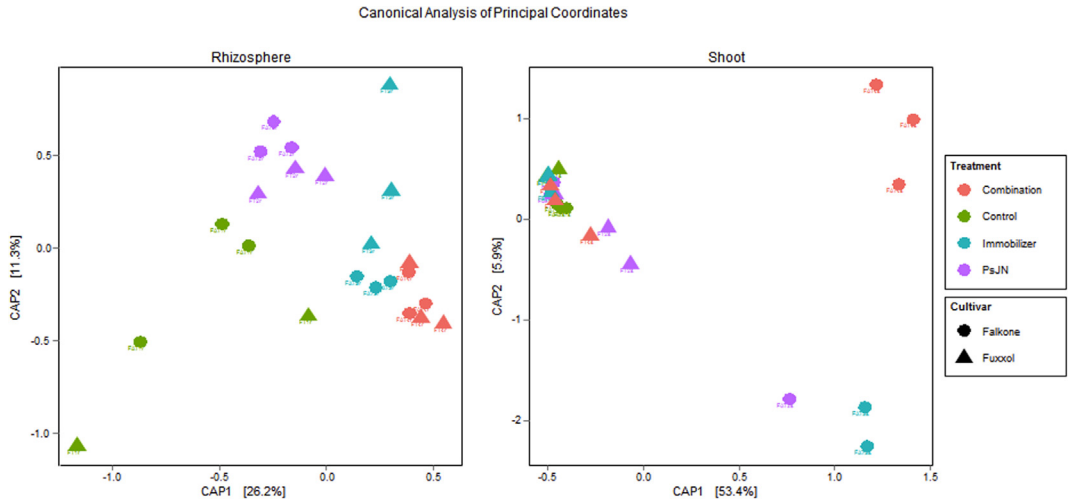

Fig. 4. Canonical Analysis of Principal coordinates (CAP) of Bray–Curtis dissimilarities based on the 16S rRNA gene V5–V9 pyrotags split into the rhizosphere and shoot plant organs and constrained to the cultivar and the treatment grouping factors.

A comparison between rhizosphere and shoot microbial communities was carried out by means of Mantel test and Procrustes analysis (PROTEST). The Mantel test applied to the distance matrices used for the ordinations proved that the microbial structure in the two plant sites are not correlated ($r = -0.02$, $p = 0.5316$). The permutation test based on Procrustes statistics confirmed no association between datasets, giving a high sum of squares value ($m12 = 0.88$, $r = 0.34$) and $p = 0.1533$ for unconstrained multidimensional scaling, after 9999 reiterations.

A Mantel test was applied in order to unveil a possible correlation between the bacterial assemblage dissimilarities (*i.e.* the OTUs tables summarized at genus level as for the indicator species analysis) and the environmental data (heavy metals). The Mantel statistics showed a significant ($p = 0.0001$) Pearson's correlation ($r = 0.45$) for the rhizosphere community and a weak ($r = 0.12$) relation for the shoot endophytes ($p = 0.0491$).

The BioEnv procedure was then used to select the subset of heavy metal data best correlated (highest Pearson correlation) with each rhizosphere and shoot bacterial matrix dissimilarity. The BioEnv routine reported some weak suggestion indicating that the rhizosphere could correlate with the bioavailable Cd and Pb ($r = 0.16$) and the shoot would rather be more influenced by the Zn level inside the plant ($r = 0.04$).

4. Discussion

In the light of the results obtained in this study, the use of bacterial inoculants in combination with immobilizer amendments can be considered as a promising way to reduce the negative effects of heavy metals on plant, soils and microbial communities. Here, we describe that amendments with gravel sludge and siderite bearing materials in combination with inoculation of the plant growth-promoting strain *B. phytofirmans* PsJN had a pronounced effect on the growth of maize plants resulting in an increase of shoot and root biomass. Generally, Pb and Zn concentrations in plants were reduced after immobilizer treatment, whereas Cd behaved differently.

Immobilisation with gravel sludge and siderite bearing material reduced the available Cd, Pb and Zn in soil (Fig. 2). This was considered as an ideal immobilization treatment providing a large mineral surface with calcite and clay minerals added with gravel sludge, sorption onto iron of the siderite and increased cation exchange capacity (Friesl-Hanl et al., 2006; Vangronsveld et al., 2009). Amendments together with soil microorganisms have high potential to reduce the availability of heavy metals, as well as to increase the availability of nutrients and microbial activity in soil (Bolan et al., 2011). Soil pH is one of the most relevant factors in stabilizing metals. Here, the applied immobilizer caused a pH increase resulting in reduced solubility of the heavy metals, which is in agreement with previous findings (Ainsworth et al., 1994; Burgos et al., 2006). pH as a major factor also explains the less pronounced effects of the immobilizer treatment on the availability of heavy metals in soil D as immobilizer treatment effects on pH were less clear (Table 3) and pH levels of soil D were naturally higher. This pH effect could be also observed in considering the ratio between total and extractable heavy metals of soil B and D (Table 1), which clearly showed comparable or even higher values of heavy metals in soil B as compared to soil D, while total levels of heavy metals were 2–3 fold higher in soil D. In addition, other differences between the two soils might have contributed to the different effects of the immobilizer treatment.

The immobilizer treatment led to reduced Zn and Pb uptake, whereas Cd behaved differently. Cd uptake was not decreased at elevated pH in our experimental setup. A potential explanation might be a specific Cd uptake and transport mechanism in plants. Metal homeostasis in plants is regulated by transport proteins and calcium channels, but depending on the bioavailability huge differences in the uptake of metals exists, with Cd and Zn generally being highly available metals (Clemens, 2006). Generally, each transport mechanism is likely to take up a range of ions. Different ionic species may interact during plant uptake (Tangahu et al., 2011) and metals can have synergetic, antagonistic or additive effects (Siedlecka, 1995). This applies also for metals in the same transition groups with similar chemic, geochemical and environmental properties such as Zn and Cd. Together with differences in transporter affinities to specific metals (Pence et al., 2000; Clemens, 2006) and competition effects between similar metals such as Zn and Cd, this complex regulation of metal uptake in plants and changing effects of soil properties and especially pH on the potential functionality of metal transporters could have had an impact on the specific uptake of Cd as observed in our experiment (Fig. 2). At least for the cv. Fuxxol an antagonistic effect of Zn and Cd can be assumed as a strong reduction of the Zn uptake in immobilizer treatments might have led to high Cd uptake. Such a phenomenon

depending on soils and treatment was already observed by Friesl-Hanl et al. (2006) in field experiments. It is well known that Zn interacts with Cd and can increase its uptake in hydroponic (Chaoui et al., 1997) and pot (Sozubek et al., 2015) experiments. In many of these reports soils were spiked with Cd and Zn but the behaviour can also be different in long-term contaminated soils (Friesl-Hanl et al., 2006). In our case Zn uptake in maize shoots was reduced, mobile Zn and Cd pools in soil were reduced and Cd uptake increased possibly due to enhanced phytochelatin production in maize roots for chelating and storing of Cd in vacuols as tolerance mechanism (Marschner, 1995).

Plant growth-promoting bacteria can act directly by influencing soil fertility, increasing the availability of essential plant nutrients and producing plant growth-promoting compounds such as phytohormones or by producing antimicrobial compounds, inducing plant stress defence mechanisms (Mitter et al., 2013b). Furthermore, PGPB may cause precipitation, complexation, mobilization or immobilization of heavy metals (Sessitsch et al., 2013). In phytoremediation processes PGPB can be also a very important and additional asset to immobilizer treatments as they might increase plant tolerance to heavy metals but also stimulate biomass growth. An increase of biomass after inoculation with *B. phytofirmans* PsJN has been reported in maize, wheat and *Acacia ampliceps*, although not in heavy metal-contaminated soils (Afzal et al., 2013; Naveed et al., 2014). *B. phytofirmans* strain PsJN has shown high ACC-deaminase activity, produces indole acetic acid and may change plant growth hormone levels (Sessitsch et al., 2005; Kurepin et al., 2015). In addition to these plant growth-promoting characteristics, this strain is able to colonize efficiently various plant compartments and has shown a broad host range (Mitter et al., 2013a). Moreover, microorganisms can also act as heavy metal immobilizing agents by absorbing or precipitating heavy metals (Gadd, 2001; Haferburg and Kothe, 2007) making heavy metals less available to plants. Based on our results we have no indication that *B. phytofirmans* PsJN inoculation alone changes heavy metal availability in the rhizosphere, but it seems that this strain influences heavy metal uptake systems in plants (Fig. 2). No clear tendency was observed, depending on the plant genotype and heavy metals, *B. phytofirmans* PsJN led to a stimulation or inhibition of heavy metal uptake. Nevertheless, *B. phytofirmans* PsJN had a clear influence on plant growth under moderate heavy metal stress in soil B containing moderate levels of Zn, Pb and Cd (Fig. 1). At high heavy metal concentrations in soil D the beneficial effects of *B. phytofirmans* PsJN disappeared (Fig. 1), indicating that the bacterium itself was affected, e.g. due to toxicity of heavy metals as this strain moderates only moderate heavy metal concentrations or altered soil conditions. In our experiments, the combination of bacterial inoculation and immobilizer amendment resulted in a reduction of heavy metal contents in shoots and still could improve plant growth.

By analysing the bacterial communities with next generation sequencing (NGS) we found not surprisingly that the OTU number in the rhizosphere was clearly higher and much more diverse than the shoot microbiome. The rhizosphere presents favourable conditions for the settlement of soil microorganisms and it is an environment that acts also selectively on microbial growth and therefore on the relative abundance of certain groups (Steer and Harris, 2000; Khan, 2005).

Gammaproteobacteria were highly abundant in shoot microbiomes, which is in agreement with previous studies on culturable maize endophytes (McInroy and Kloepper, 1995). Specifically, the shoot endosphere from both cultivars was dominated by *Pseudomonas*. This genus is commonly found in association with maize crops and has been recognized as one of the taxa with a promising level of colonization and persistence in the shoot together with other taxa like *Microbacterium* and *Curtobacterium* (Ryan et al., 2008; Pereira et al., 2011). Members of the genus *Pseudomonas* are well known for plant growth-promoting traits (Rajkumar and Freitas, 2008; Kumar and Patra, 2013).

We found different effects due to the treatment and plant cultivar on microbiome diversity. *B. phytofirmans* PsJN::*gusA*11 was re-isolated from all treatments where the bacteria were inoculated, however, the inoculation on its own did not affect shoot or rhizosphere microbiomes. Changes in the rhizosphere microbiome depended mainly of the soil properties and the immobilizer addition, whereas with shoot-associated microbiomes cultivar-specific effects were encountered. Different cultivars are likely to show physiological differences, in particular in response to heavy metal (stress) potentially leading to differences in associated microbiomes. In our experiment the cultivar Falkone hosted a higher microbial diversity with enriched abundance of Alphaproteobacteria and Firmicutes.

The immobilizer addition had an effect on the shoot microbiome as well as on the rhizosphere microbiome. In the cultivar Falkone the microbial diversity increased after immobilizer treatment. Apart from the reduced stress due to lower heavy metal availability, immobilizer application could result in a specific physiological alteration in this cultivar allowing a higher diversity of microorganisms to colonize. In the cultivar Fuxxol, richness and diversity were significantly lower than in Falkone and immobilizer addition had no pronounced effect on microbial diversity. Generally, the immobilizer increased the abundance of some members of the order Actinomycetales (Actinobacteria), Chitinophagaceae (Bacteroidetes), OPB56 (Chlorobi) and Alcaligenaceae, Comamonadaceae specifically *Variovorax* sp. and Oxalobacteraceae (Betaproteobacteria) in the rhizosphere. The rhizosphere microflora is largely driven by the quality and quantity of root exudates (Dennis et al., 2010) and soil properties such us organic matter, pH, and nitrogen content. In our experiment the immobilizer addition affected the pH, the heavy metal availability and the nutrient status, potentially also leading to altered root exudation. These changes are likely to be responsible for the observed community changes (Rousk et al., 2010; Chen et al., 2013), which together with an increased diversity pinpoint to a stress alleviating effect.

In shoot microbiomes Proteobacteria and Firmicutes were clearly overrepresented and all common individual OTUs of the shoot microbiome were missing or rare in the rhizosphere and vice versa. Endophytic bacteria benefit from inhabiting a specific niche in the plant where they are protected from biotic and abiotic stress as compared to surface bacteria (Hardoim et al., 2008), and it seems evident that the common OTUs of the shoot endosphere are adapted to that niche. The shoot microbiome was more stable than the rhizosphere microbiome within treatments, and it seems that certain taxa have high affinity to the maize endosphere, partly even cultivar specific (Supplemental Table 2, Fig. 3).

We showed that the combined application of immobilizer and *B. phytofirmans* PsJN is a promising approach to reduce heavy metal stress to plants and increase diversity of plant-associated microbial communities. *B. phytofirmans* PsJN has been shown to promote growth of different plant species cultivated in different soils, however, depending on the soil environment or the plant cultivated other microbial strains may be selected as inoculant. Development and testing of appropriate procedures and technologies (e.g. fermentation, formulations) are needed now to scale up the application of both immobilizer and plant beneficial bacteria to enable *in-situ* immobilization and/or phytostabilization under field conditions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.08.038.

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