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REGULAR ARTICLE

Effects of a bacterial consortium from acid mine drainage on cadmium phytoextraction and indigenous soil microbial community

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Abstract
Background and aims A major concern in developing microbially-assisted phytoextraction (MAP) is that the effects of introduced microbes on indigenous soil microbial community are profound and irreversible. To date, however, the microbial properties of soils subjected to MAP remain poorly understood. Therefore, we explored the effects of inoculation with a bacterial consortium enriched from acid mine drainage on not only the cadmium (Cd) phytoextraction efficiency of *Averrhoa carambola* but also the microbial properties of the Cd-contaminated soil.
Methods We conducted a field experiment and characterized the microbial community in the contaminated soil using next generation sequencing technology (Illumina MiSeq).
Results The bacterial inoculation increased the Cd concentration in *A. carambola* shoot tissues by 20%–65%, leading to a relatively high Cd removal efficiency (4.63% annually). Meanwhile, there were no significant differences between the treatments in soil bacterial diversity and community composition one year after the initiation of the bacterial inoculation treatment. The most abundant genera of the introduced bacteria were found to either disappear from, or be present in similar relative abundance, in the soils of the different treatments, except *Sulfobacillus*.
Conclusions Collectively, our results provide evidence that MAP could be practiced with minor effects on indigenous soil microbial community.

Keywords Microbially-assisted phytoextraction · Indigenous soil microbial community · Cd contamination · High biomass phytoextractor · Field evaluation

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Introduction

Cadmium (Cd) is ranked as one of the most toxic pollutants in the environment and is classified as a human carcinogen (WHO 1992a). The background concentration of Cd in soils worldwide is generally low, with the median ranging from 0.2 to 0.4 mg kg^{-1} (WHO 1992b). However, as a consequence of intensive human activities over the last century, elevated levels of Cd in soils have been widely reported in many parts of the world (WHO 1992b; Adriano 2001; Zhao et al. 2015). Cadmium in agricultural soils is readily accumulated by crops (McLaughlin et al. 1999), thereby entering the human food chain and posing significant health risks to human

beings (WHO 1992a). An important approach to reduce human exposure to dietary Cd is therefore to clean up the agricultural soils contaminated by Cd.

Due to its low cost and eco-friendly nature, phytoextraction, the use of plants to remove toxic heavy metals from soils, holds promise for in situ clean-up of Cd-contaminated agricultural soils (Salt et al. 1995). Currently, however, full-scale applications of this technique are very limited (Robinson et al. 2015). In most cases, poorly available heavy metals in soils and low biomass yield of phytoextractor plants are among the main bottlenecks limiting successful phytoextraction (Krämer 2005). As a strategy to overcome these two constraints, microbially-assisted phytoextraction (MAP) has received increasing attention over the last decade (Lebeau et al. 2008; Sessitsch et al. 2013). The fundamental principle in this strategy is straightforward: introducing microbes to the roots and/or rhizosphere of phytoextractor plants for enhancing plant biomass yield and/or availability of heavy metals in the rhizosphere (Lebeau et al. 2008; Sessitsch et al. 2013).

A large number of microbes, in particular plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi (AMF), have been reported to be able to enhance plant biomass yield and/or heavy metal accumulation of phytoextractor plants (Lebeau et al. 2008; Sessitsch et al. 2013). However, most of these microbes are not indigenous to the contaminated soils and the introduction of exogenous microbes may result in profound and irreversible effects on indigenous soil microbial community, which has increasingly become a major concern in further developing MAP (Teixeira et al. 2014). Indeed, the potential environmental impact dealing with this aspect has been recognized for some time (Lebeau et al. 2008). However, most previous studies on MAP have concentrated on the effects of microbial inoculation on plant growth, plant nutritive status and uptake of heavy metals from contaminated soils, leaving the potential effects on indigenous soil microbial community largely uninvestigated (e.g. Zaidi et al. 2006; Langella et al. 2014; Phieler et al. 2015). To date, there have been a few attempts to address such an important concern (Di Gregorio et al. 2006; Braud et al. 2009; Chen et al. 2014). In a previous study, Di Gregorio et al. (2006) conducted a 10-week microcosm experiment to explore the effects of inoculation with an indigenous plant growth-promoting rhizobacterium on the soil bacterial community of a phytoextractor (*Brassica juncea*). Subsequently, Braud et al. (2009) examined the effects of inoculation with siderophore-producing bacteria on the phytoextraction of a heavy metal-contaminated soil by pot-grown *Zea mays* for 56 days. In a more recent study, Chen et al. (2014) performed a 2-month microcosm phytoextraction experiment to investigate the effects of inoculation with an endophytic bacterium on the total bacterial number and microbial biomass in a Cd-contaminated soil phytoextracted by *Solanum nigrum*. Together, these studies have contributed to our understanding of the potential effects of MAP on indigenous soil microbial community. However, it is obvious that they are laboratory-based studies wherein conventional microbiological approaches were employed. Therefore, the critical next step towards a better understanding of such an important issue requires researchers to take advantage of modern molecular microbiological tools (e.g. next generation high throughput sequencing; Shokralla et al. 2012) to characterize the microbial properties of contaminated soils subjected to MAP under field conditions.

More field experiments are also needed to improve the applicability of MAP, given that only a few field evaluations of this remediation approach have been made (Wang et al. 2005, 2007a; Farwell et al. 2007; Jankong et al. 2007; Phieler et al. 2015; Prapagdee and Khonsue 2015). The results of these studies provide some evidence that microbial inoculation was able to enhance phytoextraction efficiency through increasing plant biomass yield and/or shoot heavy metal concentration. It should be noted, however, that the efficiencies of MAP observed in these evaluations seemed to be low (< 1%). For example, the Cd phytoextraction efficiencies observed by Wang et al. (2007a) and Phieler et al. (2015) were estimated to be 0.85% and 0.03%, respectively.

In this study, the effectiveness of a bacterial consortium enriched from acid mine drainage (AMD) in enhancing Cd phytoextraction by *Averrhoa carambola* was examined under field conditions. *A. carambola* was employed due to its high efficiency in Cd phytoextraction (Li et al. 2009, 2012); while the bacterial consortium from AMD was chosen because it is efficient in mobilizing Cd in contaminated soils (Marhual et al. 2008; Xu 2012). Meanwhile, we characterized the microbial community in the contaminated soil using next generation sequencing technology (Illumina MiSeq). To our knowledge, this study is the first attempt to adopt next generation sequencing technology to analyze the microbial community structure of heavy metal-contaminated soils subjected to MAP.

Materials and methods

Preparation of bacterial consortium

The bacterial consortium used in this study was enriched from AMD as described by Xiang et al. (2010). Briefly, a variety of AMD samples were collected from a mine located in Shaoguan, southern China (25°02′ N, 113°39′ E) and were combined as the initial inoculum for the bacterial consortium. The modified 9 K medium (Silverman and Lundgren 1959), supplemented with 1% pyrite as the sole energy source (Rodriguez-Leiva and Tributsch 1988), was used to enrich the bacterial consortium. Cultures were incubated on a rotary shaker (150 rpm) at 25 °C. The top five dominant genera in the enriched bacterial consortium included *Leptospirillum*, *Sulfobacillus*, *Acidithiobacillus*, *Ferrimicrobium* and *Acidisphaera* (characterized using 16S rRNA gene-clone library analysis; Xu 2012). Their relative abundances were 50.1%, 20.7%, 12.2%, 9.76% and 1.22%, respectively. The final cell density of the enrichment culture was approximately 5×10^6 cells mL^{-1}, which was counted by using a phase-contrast microscopy (Olympus BX50, Olympus Optical Co, Ltd., Japan) and a Hawksley bacterial counting chamber (Dopson et al. 2004).

Field experiment

The present field experiment was carried out at a paddy field located in Shaoguan, southern China (24°40′ N, 113°20′ E). In this area, the average annual temperature and rainfall are approximately 19.5 °C and 1520 mm, respectively. The field soil was contaminated by Cd (Table 1) and a high-biomass Cd accumulator (*A. carambola*) has been cultivated as part of a field trial for Cd phytoextraction since June 2008. The field trial comprised five blocks and each block was divided into five plots (4 m × 2 m each; planting density: 20 seedlings m^{-2}). To determine the plant growth and shoot Cd concentration, the phytoextractor has been harvested at 20 cm above ground level twice a year (in January and July) since July 2010. Such an undestructive harvest approach (i.e. roots were not harvested) was applied because it allowed repeated harvests of shoot biomass based on one planting and was desirable for reducing cost of phytoextraction. Note also that the Cd concentrations in *A. carambola* roots were generally lower than those in shoots (Li et al. 2009). The harvested plant materials of each plot were divided into three fractions (stem, twig and leaf), dried and weighed. For analysis of shoot Cd concentration, three subsamples of each fraction of the shoot dry matter from each plot were collected to form a composite sample. Immediately after each harvest in July, a composite soil sample consisting of three subsamples was also collected from each plot to examine the potential changes in soil chemical properties. Taking advantage of the high regrowth ability of these phytoextractor plants, the present field experiment was initiated directly in these pre-existing plots on 1st August 2013 (the plant's active growing season) and consisted of three treatments: *A. carambola* uninoculated (CK1), *A. carambola* inoculated with the bacterial consortium sterilized by autoclaving (CK2) and *A. carambola* inoculated with the bacterial consortium (Inoculated). Each treatment was replicated three times and each replicate was arranged at random in a plot of the three blocks that were selected randomly from the five blocks set up since June 2008. For the plots of CK2 and Inoculated, the soils in the plant root zone were directly sprayed with the suspensions (pH = 2) of the enriched bacterial inoculum (approximately 5×10^6 cells mL^{-1}) either sterilized or not sterilized at a dose of 0.5 L m^{-2}, respectively. This dose was selected because our preliminary experiment showed that a higher dose (1 L m^{-2}) did not lead to a greater Cd availability in the soil. To exclude the potential effects associated with low pH of the suspensions, the plots of CK1 were treated with ultra-pure water (adjusted to pH 2 with HCl) in a similar manner as described above. During the duration of the present field experiment (August 2013 to July 2014), phytoextractor plants were harvested twice and soil samples were collected once as described above. To evaluate the potential effects of the bacterial inoculation on indigenous soil microbial community, additional soil samples (a composite sample consisting of three subsamples was collected in each plot) were taken from the plant root zone immediately after the plant sampling in July 2014.

Chemical analysis

Soil samples were air-dried and ground to pass a nylon sieve (0.15 mm for total Cd concentration and 1 mm for the other chemical properties) before analysis. Method 3052 recommended by the US EPA (1996) was used to determine the total Cd concentration in the soils. The diethylenetriaminepentaacetic acid (DTPA) method of

Table 1 Selected chemical properties (means ± s.e.; $n = 3$) of the soils collected from different treatments before and 1-y after treatment application

	Before treatment application (July 2013)			1-y after treatment application (July 2014)		
	CK1	CK2	Inoculated	CK1	CK2	Inoculated
pH	6.70 ± 0.22 a	7.01 ± 0.12 a	7.16 ± 0.14 a	6.89 ± 0.05 a	7.20 ± 0.21 a	7.17 ± 0.02 a
Total-Cd	5.18 ± 0.30 a	4.71 ± 0.34 a	4.37 ± 0.35 a	4.94 ± 0.22 a	4.27 ± 0.41 a	4.33 ± 0.65 a
DTPA-Cd	2.02 ± 0.13 a	2.10 ± 0.15 a	1.73 ± 0.11 a	1.37 ± 0.03 b	1.73 ± 0.11 ab	2.00 ± 0.05 a
NH_4^+-N	67.7 ± 3.90 a	77.4 ± 8.34 a	71.4 ± 6.63 a	14.2 ± 0.88 a	12.3 ± 0.29 a	13.4 ± 1.52 a
NO_3^--N	11.2 ± 3.55 a	5.56 ± 1.31 b	16.5 ± 3.52 a	7.05 ± 1.32 b	5.17 ± 1.31 b	24.7 ± 18.9 a
Available P	32.8 ± 1.86 a	37.4 ± 2.50 a	40.8 ± 2.86 a	21.0 ± 1.04 a	31.0 ± 0.89 a	37.4 ± 1.29 a
Available K	133 ± 7.04 a	123 ± 5.89 a	127 ± 22.2 a	171 ± 23.5 a	104 ± 20.0 a	140 ± 17.2 a

Data are expressed as mg kg^{-1} of dry weight soil except those for pH. Different lowercase letters in the same row indicated significant difference ($P < 0.05$, LSD) between the treatments on the same sampling date

Lindsay and Norvell (1978) was employed to evaluate Cd availability in these soils. Soil pH was measured in a 1:5 soil:deionized water suspension using a pH meter (pH 510, Eutech Instruments Pvt. Ltd., Singapore). Ammonium-nitrogen (NH_4^+-N) and nitrate-nitrogen (NO_3^--N) were extracted with 2 M KCl at a soil:solution ratio of 1:5 and determined using an automatic chemical analyzer (Smart Chem 200, AMS, Italy). Available phosphorus (P) and potassium (K) were extracted using the Bray-1 and ammonium acetate extraction method (Lu 2000), and determined photometrically and by a flame photometer (Model 52-A, Perkin-Elmer, USA), respectively.

Microbiological analysis

Genomic DNA was extracted from each of the soil samples using the Power Soil DNA Extraction Kit (MoBio, USA) according to the manufacturer's instructions. The universal primers of F515 and R806 were used to amplify the V4 region of bacterial and archaeal 16S rRNA genes, with the reverse primer modified to contain a sample-specific 8-bp barcode. All PCRs were conducted in a total volume of 20 μL consisting of 0.4 μL of 10 μM each primer, 2 μL of 10× Ex Taq Buffer (Mg^{2+} Plus), 0.4 μL of 20 mg mL^{-1} bovine serum albumin (Takara, Japan), 0.1 μL of 5 U *Taq* DNA polymerase (Takara, Japan) and 2 μL of 200 μM dNTP mix. Cycling conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles consisting of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 45 s, and a final extension step at 72 °C for 10 min. For each soil sample, PCR products were amplified from three subsamples, pooled and purified with a QIAquick gel extraction kit (Qiagen, USA). The purified PCR products from each sample were mixed in approximately equimolar proportions to form a composite sample that was sequenced using Illumina MiSeq platform (San Diego, CA, USA). The Illumina sequencing data have been deposited in the NCBI Sequence Read Archive database (accession number: SRR2924889).

The raw Illumina data were processed using Mothur v.1.30.2 (Schloss et al. 2009) and QIIME v.1.8.0 (Caporaso et al. 2010). Sequences were clustered into operational taxonomic units (OTUs) based on a 97% sequence similarity. Taxonomy for each OTU was assigned with the RDP classifier version 2.6 (Wang et al. 2007b) at an 80% confidence threshold. The diversity and structure of the soil microbial communities were assessed after resampling of the sequences to the same depth (6853 sequences per community, i.e. the minimum number of quality sequences per sample in this study). The Bray-Curtis coefficient-based principal coordinates analysis (PCoA) was performed at OTU level to evaluate the similarity among the soil microbial communities of different treatments.

Statistical analysis

Statistical analyses were conducted with the SPSS version 18.0 for Windows (SPSS Inc., Chicago, USA). Differences between the treatments in soil properties, plant biomass yield, Cd accumulation, soil microbial diversity and composition were analyzed using the one-way Analysis of Variance (ANOVA). The least significant difference test (LSD) was used to identify significant ($P < 0.05$) differences between means.

Results

Plant biomass yields

The potential effects of bacterial inoculation on the biomass yield of *A. carambola* were evaluated by harvesting shoot tissues from different treatments before the initiation of treatments and 0.5 and 1 year after the treatments. No significant ($P > 0.05$) differences in shoot biomass yield were observed between the plots for the three treatments before the initiation of the present study (Fig. 1a). The bacterial inoculation did not lead to significant differences in shoot biomass yield between treatments 0.5 and 1 year after treatment application (Fig. 1b, c).

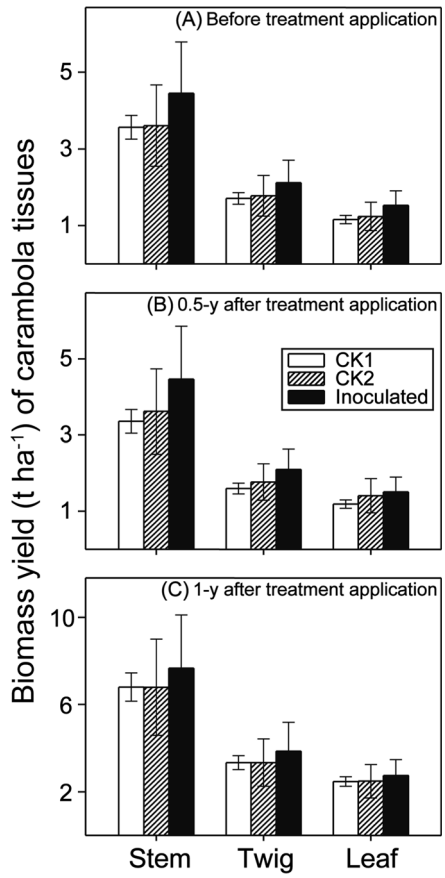

Fig. 1 Biomass yield (t ha^{-1}) of *A. carambola* tissues harvested from different treatments before (A panel), 0.5- (B panel) and 1-y after (C panel) treatment application (i.e. in July 2013, January 2014 and July 2014, respectively). Data are presented as means ± s.e. ($n = 3$) and on a dry weight basis. No significant differences were found ($P < 0.05$, LSD) between the treatments in shoot Cd concentration at the same sampling time

Cd uptake and removal by plants

Before treatment application, there were no statistically significant ($P > 0.05$) differences between the treatments in the concentration of Cd in shoot tissues (Fig. 2a). In contrast, significant ($P < 0.05$) differences were found between the treatments in shoot Cd concentration not only 0.5 but also 1 year after treatment application (Fig. 2b, c). Specifically, the Cd concentrations in individual shoot tissues of *A. carambola* inoculated with the bacterial consortium were significantly ($P < 0.05$) higher than those of the other two control treatments at both of the two sampling time points.

The amounts of Cd removal by the shoot tissues at different sampling times were calculated (Table 2), based on the shoot biomass yields and Cd concentrations. Higher amounts of Cd removal by the individual shoot tissues were observed in the Inoculated treatment (Table 2), which could largely be attributed to the significant higher Cd concentrations in these tissues as compared to those of the other two treatments (Fig. 2b, c).

Soil chemical properties

Before treatment application, the plots for the three treatments did not show significant ($P > 0.05$) differences in the selected soil chemical properties, with the exception of NO_3^--N (Table 1). However, the concentration of DTPA-extractable Cd in the soil of the Inoculated treatment was significantly ($P < 0.05$) higher than that of CK1 1 year after treatment application, besides the significantly ($P < 0.05$) higher NO_3^--N concentration in the soil as compared to those of the other two treatments (Table 1). Conversely, the other selected chemical properties of the soils did not show any significant differences between the treatments 1 year after treatment application (Table 1).

Soil microbial diversity and community composition

A total of 31,459 OTUs were recorded from the 141,214 quality sequences across the soil samples. The three treatments varied in OTU number, but no significant ($P > 0.05$) differences were found between them on any of the two sampling dates (Fig. 3a, b). Similarly, there were no significant ($P > 0.05$) differences between the treatments in the other soil microbial α-diversity measures (including Chao1, ACE, Simpson, Shannon and Faith's PD) considered in the present study (Fig. 3a, b).

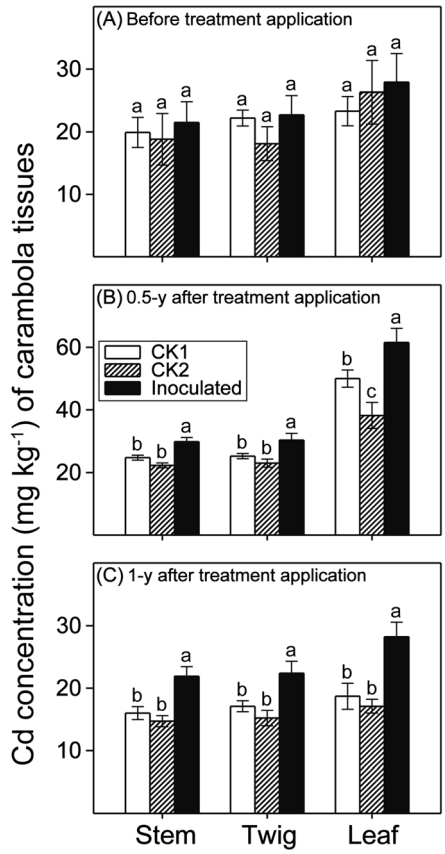

Fig. 2 Cd concentrations (mg kg^{-1}) in *A. carambola* tissues harvested from different treatments before (A panel), 0.5- (B panel) and 1-y after (C panel) treatment application. Data are presented as means ± s.e. (n = 3) and on a dry weight basis. Different lowercase letters above the bars indicated a significant difference ($P < 0.05$, LSD) between the treatments in tissue Cd concentration at the same sampling time

In addition, PCoA analysis of the community composition showed that the Inoculated treatment did not form a cluster that was different from those of the two control treatments either before or 1 year after treatment application (Fig. 4).

The top 10 dominant microbial phyla in the soil samples collected from different treatments before and 1 year after treatment application were *Acidobacteria*, *Proteobacteria*, *Chloroflexi*, *Crenarchaeota*, *Planctomycetes*, *Nitrospirae*, *Verrucomicrobia*, *Actinobacteria*, *Bacteroidetes* and *Gemmatimonadetes* (Fig. 5). Moreover, there were no significant ($P > 0.05$) differences between the treatments in relative abundance of these dominant phyla at each sampling time point (Fig. 5). Similarly, the top 10 dominant soil microbial genera (*Candidatus Nitrososphaera*, *Rhodoplanes*, *Nitrospira*, *DA101*, *Steroidobacter*, *Pirellula*, *Anaeromyxobacter*, *Gemmata*, *Flavisolibacter* and *Pilimelia*) did not show a significant ($P > 0.05$) difference between the treatments on either sampling date (Fig. 6).

When the top 5 dominant genera in the microbial consortium were considered, they showed four different patterns of occurrence and abundance in the contaminated soils. *Leptospirillum* was present in the soils CK1 and CK2 before treatment application, but significant differences between the treatments in this aspect were no longer observed 1 year after treatment application although enhanced relative abundances were recorded (Fig. 7). *Sulfobacillus* occurred in similar abundance in the soils of different treatments before treatment application, but was persistent only in the Inoculated treatment soil 1 year after treatment application (with an elevated relative abundance; Fig. 7). *Acidithiobacillus* was absent from the soils of different treatments before treatment application, but present in the soils in similar abundance 1 year after treatment application (Fig. 7). *Ferrimicrobium* and *Acidisphaera* were not detectable in all the soil samples examined in this study. Notably, the relative abundances of these genera in the soils were low (< 1%).

Table 2 The amount (g ha^{-1}) of Cd removal by *A. carambola* tissues harvested from different treatments before, 0.5- and 1-y after treatment application (i.e. in July 2013, January 2014 and July 2014, respectively)

	Before treatment application			0.5-y after treatment application			1-y after treatment application		
	CK1	CK2	Inoculated	CK1	CK2	Inoculated	CK1	CK2	Inoculated
Stem	71.0	67.9	95.7	83.3	80.7	134	109	100	168
Twig	38.0	32.2	48.1	40.5	40.7	63.8	56.9	50.6	86.5
Leaf	27.0	32.6	42.7	59.5	53.9	93.0	46.0	42.4	77.3
Total	136	133	186	183	175	291	212	193	332

Data are presented on a dry weight basis. Total = stem + twig + leaf

Discussion

A new type of bacterial consortium was employed in this study to improve Cd phytoextraction by the high biomass Cd-accumulator *A. carambola* in an attempt to demonstrate that MAP can be more efficient at the field scale than previously reported (Wang et al. 2007a; Phieler et al. 2015). Unlike other soil-borne microbes tested by other workers (Lebeau et al. 2008; Sessitsch et al. 2013), this bacterial consortium was enriched from AMD. The top three dominant microbial genera (*Leptospirillum*, *Sulfobacillus* and *Acidithiobacillus*, with relative abundance >80%) in this consortium are thought to be directly relevant for metal sulfide oxidation and may thereby mobilize heavy metals (Marhual et al. 2008; Schippers et al. 2010). Indeed, our pilot study based on a 12-week bench-scale experiment showed that addition of the consortium to the contaminated soil increased the Cd removal efficiency of *A. carambola* by approximately 3-times, which was associated with an apparent decrease in soil pH (from 6.70 to 5.12) for the first 4 weeks and then a steady increase in soil pH (from 5.12 to 5.88) for the subsequent 8 weeks. Further, the existence of the acidophilic bacteria *Leptospirillum*, *Sulfobacillus* and *Acidithiobacillus* (cell number: 3.1, 1.8 and 1.3 × 10^5 cells g^{-1} dry soil, respectively; unpublished results) in the contaminated soil at 12 weeks after addition of the consortium was confirmed using the fluorescent in situ hybridization method as described by Kock and Schippers (2008). These results were reasonable given that many acidophilic microbes in AMD can grow over a relatively broad range of pH. More specifically, members of *Leptospirillum*, *Sulfobacillus*, *Acidithiobacillus*, *Ferrimicrobium* and *Acidisphaera* (i.e. the top 5 dominant genera in the consortium enriched in this study) were reported to adapt to pH values from 1.3 to 5.7, 2.0 to 6.5, 0.5 to 6.0, 1.3–5.5 and 1.5–6.0, respectively (Baker and

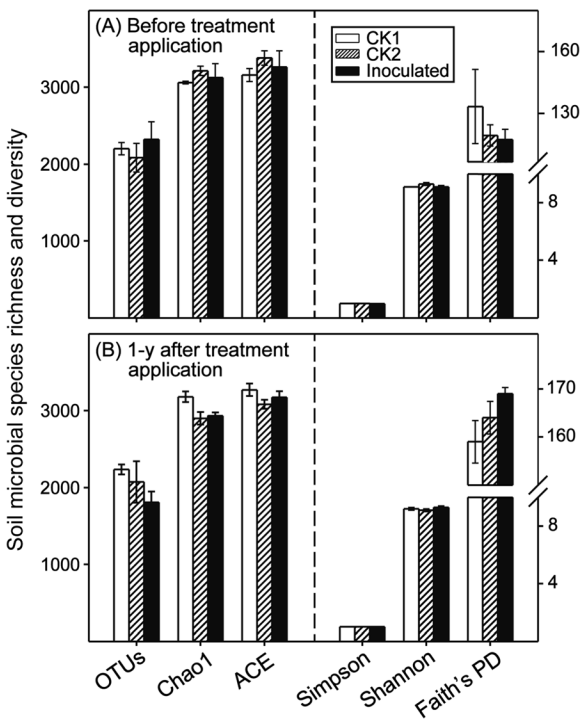

Fig. 3 Microbial species richness and diversity of the soils collected from different treatments either before (A panel) or 1-y after (B panel) treatment application. Data are presented as means ± s.e. (n = 3). No significant differences were found (P < 0.05, LSD) between the treatments in microbial species richness and diversity at the same sampling time

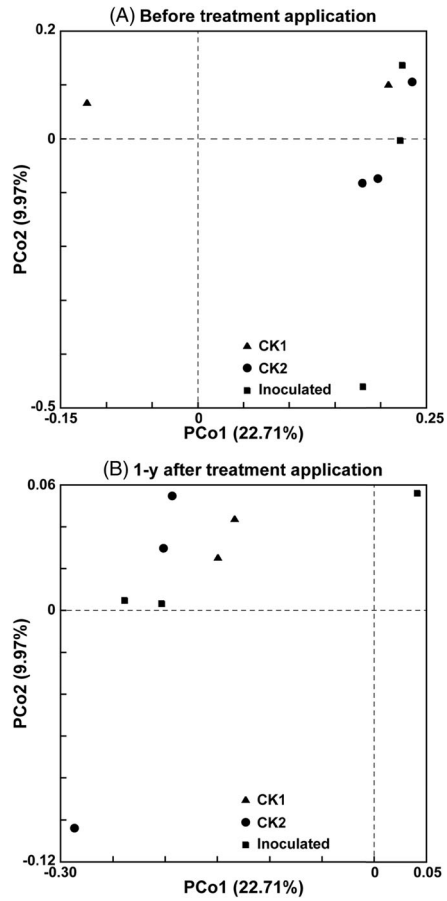

Fig. 4 Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity among soil microbial communities of different treatments either before (A panel) or 1-y after (B panel) treatment application. No obvious clustering was observed with respect to different treatments. Two replicates of CK1 were highly overlapped in both sampling time points

Banfield 2003; Karavaiko et al. 2005; Liu et al. 2007). In addition, acidophilic microbes are considered to be able to create acidic microsites in soils (Chesworth 2008), which, in turn, can provide shelters for acidophilic microbes themselves. It is therefore intuitive to expect that inoculation with such a bacterial consortium can lead to elevated Cd removals by *A. carambola* from the near-neutral pH Cd-contaminated soil under field conditions.

In agreement with our expectation, we found that inoculation with the bacterial consortium enhanced Cd concentrations in shoot tissues of *A. carambola* by approximately 20%–65% (Fig. 2b, c). Such an increment might be considered normal and so could often be observed at the field scale, given that a systematic review of the literature on performance of MAP showed that inoculation with microbes was able to increase metal concentrations in plant shoots by a factor of about 2 on average (Lebeau et al. 2008). However, the data from the currently available field-based studies deviated markedly from the finding of that review, which was likely due to the fact that most studies reviewed therein were laboratory-based. Indeed, the increase of heavy metal concentration in plant shoots associated with MAP under natural field conditions seemed to be

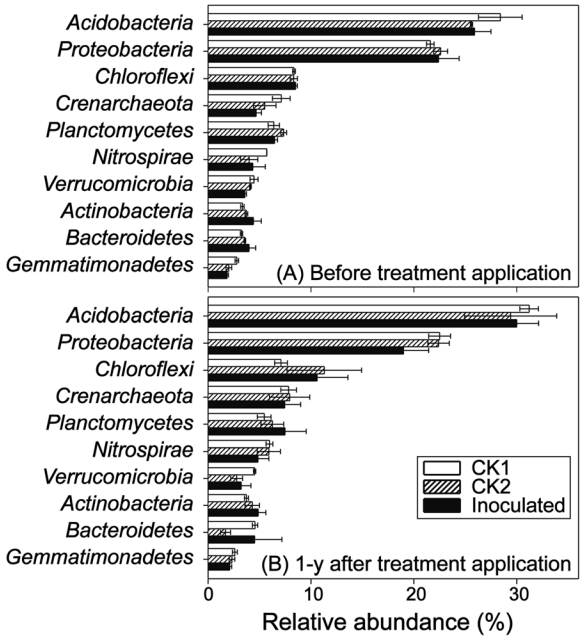

Fig. 5 Relative abundance (%) of the top 10 dominant microbial phyla in the soils collected from different treatments either before (A panel) or 1-y after (B panel) treatment application. Data are presented as means ± s.e. (n = 3). No significant differences were found (P < 0.05, LSD) between the treatments in relative abundance of these phyla at the same sampling time

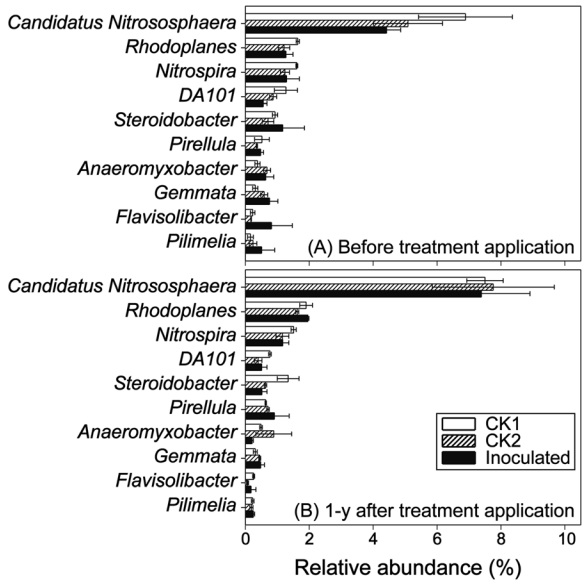

Fig. 6 Relative abundance (%) of the top 10 dominant microbial genera in the soils collected from different treatments either before (A panel) or 1-y after (B panel) treatment application. Data are presented as means ± s.e. (n = 3). No significant differences were found (P < 0.05, LSD) between the treatments in relative abundance of these genera at the same sampling time

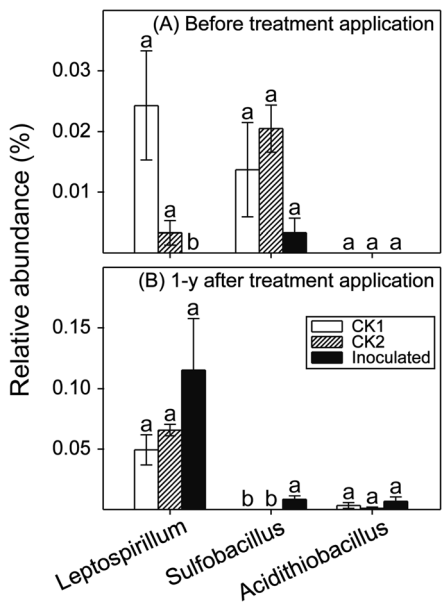

Fig. 7 Relative abundance (%) of *Leptospirillum*, *Sulfobacillus* and *Acidithiobacillus* in the soils collected from different treatments either before (A panel) or 1-y after (B panel) treatment application. Data are presented as means ± s.e. (n = 3). Different lowercase letters above the bars indicated significant difference (P < 0.05, LSD) between the treatments in relative abundance of these genera at the same sampling time

recorded only by two research groups. Wang and his colleagues found that inoculation with AMF enhanced Cu, Zn and Pb concentrations in shoot of *Elsholtzia splendens* by approximately 20–40% (Wang et al. 2005, 2007a), while Prapagdee and Khonsue (2015) showed that inoculation with *Arthrobacter* sp. led to an approximately 43% increase in Cd concentration of *Ocimum gratissimum* shoots. In contrast, no significant effects of microbial inoculation on shoot heavy metal concentration of phytoextractor plants were observed in other two field experiments (Farwell et al. 2007; Phieler et al. 2015). Jankong et al. (2007) reported a negative effect of inoculation with rhizofungi on As concentrations in fronds of *Pityrogramma calomelanos* (24–30% reduction in As concentration). These discrepancies reflect the complex nature of the extrapolation of the laboratory-based results to field conditions, although they may be partly due to the relatively small number of field-scale studies currently available.

The increased shoot Cd concentrations observed in this study could be attributed to a beneficial effect of the bacterial consortia on Cd mobilization in the contaminated soil (Marhual et al. 2008; Schippers et al. 2010). Yet, we found that concentration of DTPA-extractable Cd in the soil inoculated with the bacterial consortium was similar to that of the control soil inoculated with the autoclaved microbial consortium (i.e. CK2) 1 year after treatment application (Table 1). This result could be explained by a scenario that the introduced bacteria had functioned to mobilize soil Cd but the bacterium-induced increase in soil Cd availability was counteracted by the increased Cd uptake by *A. carambola* (Fig. 2a, b). Surprisingly, microbe-induced changes in soil chemical properties have rarely been investigated under field conditions, although microbes are thought to assist phytoextraction through biochemical processes occurring at the soil-root interface (Sessitsch et al. 2013). An exception was a study by Phieler et al. (2015) who examined the effects of inoculation with either *R. irregularis* or a mixture of mycorrhiza and streptomycetes on heavy metal availability of a field soil planted with *S. bicolor*. In agreement with our results, no significant changes in soil heavy metal availability were observed by these authors (Phieler et al. 2015). In a wider context, the lack of a clear connection between the capacities of introduced microbes to mobilize heavy metals and the changes in soil heavy metal availability was frequently observed even in laboratory-based studies on MAP (Sessitsch et al. 2013); this deserves further investigation.

In contrast to our results on shoot Cd concentration, no significant differences were found between treatments in shoot biomass yield of *A. carambola* within 1 year after treatment application (Fig. 1b, c). This finding was reasonable, although most field-scale studies showed that microbial inoculation often led to an increase in shoot biomass yield (Wang et al. 2005, 2007a; Farwell et al. 2007; Jankong et al. 2007; Phieler et al. 2015). Firstly, no characteristics desirable for improving plant growth were reported for the dominant genera of the microbial consortium used in this study (Schippers et al. 2010). Secondly, a recent meta-analysis of 73 papers on MAP revealed that only about 19% of the 738 individual cases (treatments) presented in the papers were associated with an increase in both shoot biomass yield and heavy metal concentration (Sessitsch et al. 2013). Nonetheless, the shoot biomass yields observed here were considerably high, which supported our previous results (Li et al. 2009). Note also that the Inoculated treatment was associated with a higher concentration of soil NO_3^--N (Table 1), which may increase the shoot biomass yield over a long time scale. This observation could be attributed tentatively to the fact that *Nitrobacter* occurred in Inoculated treatment (albeit low relative abundance of approximately 0.1%) but was absent in the other two CKs. Because it is known that almost all acid-tolerant nitrifying bacteria belong to *Nitrobacter* (De Boer and Kowalchuk 2001) and that the existence of acid-tolerant nitrifying bacteria can help non-acid-tolerant nitrifying bacteria (e.g. those belonging to *Nitrospira*, whose relative abundance was approximately 2% in this study, Fig. 6) to oxidize ammonium to form NO_3^- in acidic environments (De Boer and Kowalchuk 2001).

From a practical perspective, the actual efficiency of MAP under field conditions is more important than the magnitude of an increase in either shoot biomass yield or heavy metal concentration per se, although it has always been neglected in previous studies (e.g. Wang et al. 2005; Farwell et al. 2007; Jankong et al. 2007; Prapagdee and Khonsue 2015). In this study, the shoots of *A. carambola* inoculated with the bacterial consortium were able to remove 623 g Cd ha^{-1} (Table 2) from the contaminated soil (total soil Cd approximately 4.4 mg kg^{-1}; Table 1) within 1 year after treatment application. This figure (623 g Cd ha^{-1}) was higher than not only that (approximately 0.5 g Cd ha^{-1}) of the *S. bicolor* inoculated with mycorrhiza and *Streptomyces* at a low soil Cd level (total soil Cd: 0.72 mg kg^{-1}; Phieler et al. 2015) but also that (155 g Cd ha^{-1}) recorded for the *E. splendens* inoculated

with AMF and *Penicillium* at a high soil Cd level (total soil Cd: 7 mg kg^{-1}; Wang et al. 2007a). Assuming that Cd contamination occurred only in the 0–20 cm layer of the soil and that the soil had a bulk density of 1.3 g cm^{-3} (Zhang et al. 2010), the annual Cd removal by the shoots of inoculated *A. carambola* accounted for 4.63% of the total amount of Cd in the contaminated soil. Such a Cd removal efficiency was about 4–150 times higher than those reported in comparable field-scale studies (Wang et al. 2007a; Phieler et al. 2015). Moreover, if the Cd removal efficiency observed here does not change over time, a 50% reduction in total soil Cd can be achieved within 15 years (a reasonable time span; Huang et al. 1997). During this period, repeated inoculation is needed because the most abundant genera of the introduced bacteria were found to either disappear from, or be present in similar relative abundance, in the soils of the different treatments (as discussed below). It should be noted that the potential environmental risk associated with Cd accumulation in fruits of *A. carambola* can be avoided, since our observation in the past decade showed that the plant individuals did not bear fruit when their shoots were harvested annually. Despite this, further study is required to examine whether the bacterial consortium increases migration of Cd into deeper soil layers and groundwater.

The Illumina next generation sequencing technology employed in this study enabled us to capture more of the soil microbial diversity. For example, the average number of microbial species (OTUs, Fig. 3) observed per soil sample here was higher than that detected using MiSeq in another Cd-contaminated soil phytoextracted by bioenergy crops (Ding et al. 2016). Note, however, that the identities of dominant microbial phyla and genera recorded here (Figs. 5 and 6) were similar to those reported for other contaminated agricultural soils (Liu et al. 2014; Sun et al. 2015; Ding et al. 2016). More importantly, our results revealed no significant differences between the treatments in microbial diversity (both alpha and beta), relative abundance of dominant phyla and that of dominant genera (Figs. 3–6), indicating that introducing the microbial consortium had minor effects on the indigenous soil microbial community of *A. carambola*. This finding seems to be inconsistent with that of Di Gregorio et al. (2006) who reported that inoculation with *Sinorhizobium* sp. Pb002 strongly altered the soil microbial community structure of pot-grown *B. juncea*. Such a discrepancy can be explained in several possible ways. Firstly, the next generation sequencing technology used here is more robust in characterizing the soil microbial community than PCR-DGGE analysis of that study (Shokralla et al. 2012). Secondly, soil microbial communities are able to recover from the disturbances associated with introduced microbes within a period of time, which is longer than the time-scale of the experiment (6 weeks) performed by Di Gregorio et al. (2006) but not that of ours (1 year). Thirdly, laboratory studies do not fully reflect field conditions where the effects of certain environmental variables on soil microbial community may overcome those of introduced microbes.

As to the introduced microbes per se, the top 5 genera were either absent from or present in the soils in similar abundance 1 year after treatment application, except *Sulfobacillus* (Fig. 7b). Moreover, their relative abundances recorded here were low (< 1%) and comparable to those observed in other contaminated agricultural soils (Lin et al. 2010; Sun et al. 2015), suggesting that they are less likely to have a more visible effect on indigenous soil microbial community. It should be noted that the quantity and metabolic activity of soil microbes cannot be unequivocally revealed by the microbiological analysis performed in this study, which is worthy of further exploration.

Conclusions

This study showed that *A. carambola* inoculated with the bacterial consortium enriched from AMD removed an estimated 4.6% of the total Cd in the topsoil, which was much higher than those previously reported. Meanwhile, our results provided evidence for the first time that MAP could be practiced with minor effects on the indigenous soil microbial community. Future studies should conduct more field experiments to test the extent to which our findings are applicable to other similar situations, which will help pave the way towards the full-scale implementation of MAP.

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