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Original Article

Influence of heavy metals on rhizosphere microbial communities of Siam weed (*Chromolaena odorata* (L.)) using a 16S rRNA gene amplicon sequencing approach

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ABSTRACT

A 16S rRNA amplicon sequencing approach was used to assess the impacts of cadmium (Cd) and zinc (Zn) contamination on populations of rhizobacteria on Siam weed (*Chromolaena odorata* (L.)). Bacterial communities were characterized using the Illumina MiSeq platform and the V6 hypervariable region of the 16S rRNA gene. Among the 54,026 unique operational taxonomic units (OTUs) identified, 99.7% were classified as bacteria and the rest were classified as archaea. Several dominant bacterial phyla were observed in all samples—*Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes* and *Bacteroidetes*. These five phyla accounted for 89.2% of all OTUs identified among all sites, and only two OTUs could not be classified to a phylum. Comparison among samples containing low and high levels of Cd contamination using nonparametric Shannon and Shannon diversity indices showed that soils with low levels of diversity had a higher level of Cd ($p < 0.05$). These results indicated that levels of Cd may significantly alter bacterial species selection. The Cd- and Zn-resistant bacteria from each sample were subjected to heavy-metal minimum inhibitory concentration (MIC) analyses. The MIC values obtained from 1152 isolates were used to individually analyze the pattern of gene function using the BioNumerics software. The results of this analysis showed that 26.7% of the bacteria were resistant to Cd concentrations up to 320 mg/L and only 2.3% of bacteria were resistant to Zn at concentrations up to 3200 mg/L. The MIC analyses indicated that the number of resistant bacteria decreased with increasing metal concentrations and those bacteria resistant to Cd and Zn may contain more than one group of metal-resistance genes.

Introduction

Cadmium (Cd) is widespread in the environment and has been recognized as one of the most harmful heavy metal pollutants (Christine, 1997). It may easily move from soil to food plants through root absorption and accumulate in plant tissues (Oliver, 1997). In this way, it may detrimentally affect human health. It has been documented that soils and food plants located nearby a zinc mine in the Mae Sot District of Thailand were contaminated with toxic levels of Cd and Zn (Simmons et al., 2005). Cd is of concern not only because of its potentially harmful effects on humans and animals, but also due to possible adverse effects on microorganisms, which play an important role in the soil ecosystem services, including nutrient cycling and soil fertility (Yao et al., 2000). Cd has been shown to cause changes in the size, composition and activity of the soil microbial community (Giller et al., 1998).

However, many studies have reported that some bacteria can reduce the toxic effects of Cd via several mechanisms as microbial survival in polluted soils is due to innate biochemical, genetic, and structural properties or physiological adaptation. Genetic changes, due to selection pressure, can also occur and the environment can modulate metal speciation (Wuertz and Mergeay, 1997). Heavy metal pollution of soils places a strong stress on soil microbes, resulting in an increase in numbers of resistant bacteria. To survive under metal-stressed conditions, some bacteria have evolved

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mechanisms to efflux metal ions outside of the cell, accumulate metal ions once inside the cell, and reduce heavy metal ions to a less toxic state (Laila et al., 2011; Simmons et al., 2005).

Some rhizosphere microorganisms, which are closely associated with roots, have been termed plant growth-promoting rhizobacteria (Glick et al., 1995), which include a diverse group of free-living soil bacteria that can improve host plant growth and development in heavy metal contaminated soils, often by mitigating toxic effects of heavy metals on plants (Belimov et al., 2004). The use of rhizobacteria in combination with plants could increase the efficiency of phytoremediation systems (Abou-Shanab et al., 2003; Whiting et al., 2001). The microbial ecology of the rhizosphere microbial communities of many plants is not yet fully understood. Siam weed (*Chromolaena odorata*) is recognized as one of the world's worst tropical weeds and has been studied extensively with respect to its Cd/Zn hyperaccumulation characteristics. The plant shows high accumulation levels of soil Cd and Zn and thus, it has great potential for use in phytoextraction technologies (Phaenark et al., 2009). However, the effect of Cd and Zn contamination on rhizosphere microbial communities and activities of *C. odorata* and their associated rhizosphere bacteria have not been elucidated. Siam weed was chosen for this study because it was found in the local area, and also grows well in both areas with uncontaminated as well as contaminated soil.

This study investigated the influence of Cd and Zn on the rhizosphere soil microbial community and the diversity of Siam weed and also investigated the distribution of the Cd/Zn-resistant soil microbes isolated.

Materials and methods

Sample collection

Twelve soil samples were collected in March 2012 from the rhizosphere of Siam weed obtained from the Padaeng zinc mine and Baan Pha De village, in the PhraThat PhaDaeng district of Mae Sot, in Tak province, Thailand. The soil samples were collected from the 0–15 cm soil depth, dried at room temperature for 4 d and sieved through a 2 mm metal mesh sieve. The samples were characterized for pH (1:1 soil:water) and electrical conductivity (EC) in a 1:5 soil:water suspension using pH and EC meters, respectively. Soil organic matter was measured using the Walkley-Black titration method (Walkley and Black, 1934), and cation exchange capacity (CEC) was evaluated by leaching with NH_4OAc at pH 7, followed by distillation (Chapman, 1965). To determine the total concentrations of Cd and Zn in soil samples, 1 g of each sample was digested in aqua regia (1:2 HNO_3:$HClO_4$ by volume according to Amacher (1996) and Hesse (1971). The total Cd and Zn concentrations were measured by using a Varian AA240 flame atomic absorption spectrophotometer.

Sample processing, DNA extraction and amplification

Each rhizosphere soil sample was processed using a modified version of the protocol recommended in the PowerSoil™DNA Isolation Kit (MO BIO Laboratories; Carlsbad, CA, USA). Briefly, rhizosphere soil was taken from the roots of Siam weed by shaking softly to remove adhering root-zone soil and then submitted to DNA extraction and purification using the PowerSoil™DNA Isolation Kit. Polymerase chain reaction (PCR) was performed to amplify the V6 region of the 16SrRNA by using the primers 967F (5′-CAACGCGAAGAACCTTACC) and 1046R (5′–ID-CGACAGCCATGCANCACCT) (Sogin et al., 2006), where “ID” indicates a six bases-multiplexing identification barcode unique to each sampling site (Yozwiak et al., 2010). Previous study found that the V6 region works well to characterize microbiota in environmental samples in the laboratory (Staley et al., 2013). Illumina adapter sequences were also included on the 5′-end of both primers. Gel electrophoresis was performed and subjected to gel purification.

Sequence data processing

Purified DNA was submitted for paired-end sequencing on the Illumina MiSeq platform at the University of Minnesota Genomics Center (Saint Paul, MN, USA). The sequencing outputs were trimmed of primer sequences and multiplexing barcodes specific to each sample. The trimmed sequences were quality screened using the Mothur version 1.27.0 software as previously described (Huse et al., 2007; Schloss et al., 2009). Sequences having abundance <1 were removed. The identification of operational taxonomic units (OTUs) was done against the SILVA taxonomic database containing only the V6 region (Huse et al., 2008). Samples from each site were subsampled to 500,000 sequence reads. OTUs were assigned at the 97% similarity using the furthest-neighbor algorithm. Rarefaction and principal coordinate analyses (PCoA) were performed using the Mothur software and graphed in the Excel software package (version 2007; Microsoft Corp.; Redmond, WA, USA).

Statistical analyses

All statistical analyses were conducted at a significance level (α) = 0.05. Calculations of abundance-based coverage (ACE), Chao, Shannon, non-parametric Shannon, and Simpson diversity indices were performed using the Mothur version 1.27.0 software (Schloss et al., 2009).

Isolation of Cd- and Zn-resistant bacteria from rhizosphere soil of Siam weed

Both Cd- and Zn-resistant bacteria were isolated from each rhizosphere soil of Siam weed after suspension in 9 mL sterile 0.85% NaCl and vigorous shaking on an orbital shaker (200 rpm) for 30 min. After allowing for settling for 1 min, bacteria were plated and grown on R2A medium containing 20 μg/mL $CdCl_2$, and incubated at 30 °C for 48 h. Ninety-six colonies from each sample were randomly picked and re-streaked onto R2A agar medium for purity. Strains were stored in 50% glycerol in 96-well microplates at −80 °C until used.

Determination of minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of metal that completely inhibited growth of each bacterium. Isolates were streaked onto R2A agar plates containing 20 μg/mL $CdCl_2$ and single colonies were grown in R2A broth and incubated at 30 °C for 24 h. The MICs of Cd and Zn were determined by replica plating onto R2A agar medium containing 20 μg/mL, 40 μg/mL, 80 μg/mL, 160 μg/mL or 320 μg/mL of $CdCl_2$ and 200 μg/mL, 400 μg/mL, 800 μg/mL, 1600 μg/mL or 3200 μg/mL of $ZnCl_2$. The MIC values of each isolate were entered into the BioNumerics version 3.5 software (Applied Maths Company; Saint-Martens-Latem, Belgium). The data were analyzed using simple matching analysis with binary coefficients. Dendrograms were produced to show the relationship of bacterial strains based on cadmium and zinc resistance.

Results and discussion

Soil characterization

As shown in Table 1, the soil texture at all 12 sampling sites varied from fine-to coarse-textured soils. The soils from the 12

Table 1
Characteristics of soils from 12 studied sites.

Site	Information	Location (easting northing)	Characteristic						
			Texture	pH (1:1)	ECe (dS/m)	OM (%)	CEC (cmol/kg)	Total Cd (mg/kg soil)	Total Zn (mg/kg soil)
THA1	Mining area	0464405 1842146	Sandy loam	8.1 ± 0.3	1.0 ± 0.1	1.0 ± 1.1	6.0 ± 0.7	898.2 ± 79.7	19,516.3 ± 3334.9
THA2	Horticultural crop area	0460516 1843492	Loam	7.1 ± 0.1	1.0 ± 0.0	2.9 ± 0.2	19.4 ± 0.4	103.0 ± 9.8	3886.0 ± 522.6
THA3	Paddy field	0457186 1843026	Clay loam	7.8 ± 0.0	0.3 ± 0.0	3.9 ± 1.2	21.2 ± 0.7	97.4 ± 1.6	3780.1 ± 54.4
THA4	Mining area	0459178 1843080	Sandy clay loam	7.7 ± 0.0	0.3 ± 0.0	5.8 ± 0.5	26.5 ± 1.1	76.9 ± 4.5	3414.4 ± 440.0
THA5	Maize cultivated area	0453750 1848128	Loam	7.4 ± 1.1	1.3 ± 0.0	2.9 ± 0.0	14.8 ± 0.6	72.9 ± 6.2	2437.3 ± 152.5
THA6	Cassava cultivated area	0460345 1843653	Loam	7.7 ± 0.0	1.2 ± 0.0	3.0 ± 0.1	16.2 ± 0.7	74.9 ± 8.0	3130.2 ± 108.1
THA7	Paddy field	0460366 1843197	Sandy clay	6.9 ± 0.0	4.2 ± 0.2	1.8 ± 0.8	12.1 ± 1.0	34.3 ± 4.5	1326.0 ± 85.4
THA8	Paddy field	0459184 1842403	Clay	7.1 ± 0.1	11.3 ± 0.0	3.6 ± 0.2	25.2 ± 2.9	33.9 ± 0.7	1467.9 ± 72.0
THA9	Abandoned mine land	0464410 1842490	Sandy loam	7.6 ± 0.1	0.4 ± 6.8	2.0 ± 0.0	13.1 ± 0.2	28.2 ± 2.9	1217.1 ± 134.5
THA10	Paddy field	0459175 1843083	Loam	7.5 ± 0.1	0.9 ± 0.0	2.8 ± 0.1	19.6 ± 0.8	23.9 ± 0.7	1024.8 ± 41.6
THA11	Paddy field	0459239 1842508	Clay	7.0 ± 0.0	10.5 ± 0.2	2.8 ± 0.0	18.6 ± 0.4	9.7 ± 0.8	557.7 ± 26.3
THA12	Mine reclamation area	0464575 1841575	Sandy clay loam	6.3 ± 0.0	0.9 ± 0.0	0.9 ± 0.3	14.1 ± 0.9	0.1 ± 0.0	586.9 ± 37.6

sampling sites were slightly acid to moderately alkaline (pH 6.3–8.1). Most of the soil samples were non-saline, while some were moderately saline (0.3–11.3 dS/m). The soil organic matter content of all samples was low to very high (0.9–5.8%). The THA4 soil had the greatest amount of organic matter (OM) at 5.8%.

The cation exchange capacity (CEC) of all samples varied from 6.0 cmol/kg to 26.5 cmol/kg. The CEC in the THA4 soil had the highest value of 26.5 cmol/kg. The total Cd concentration in the soils was correlated with the total Zn concentration (coefficient of determination, $R^2 = 0.988$). The total Cd and Zn concentrations in the surface soil samples from THA1 through to THA6 were relatively high with a range of 72.9–898.2 mg/kg Cd and 2437.3–19,516.3 mg/kg Zn, respectively. In contrast, sites THA7 to THA10 had medium Cd and Zn levels (23.9–34.3 mg/kg Cd and 1024.8–1467.9 mg/kg Zn), and sites THA11 and THA12 had low Cd and Zn levels (9.7–0.1 mg/kg Cd and 557.7–586.9 mg/kg Zn). These results indicated that many agricultural areas contained high amounts of heavy metals. Those areas have become contaminated with cadmium and zinc through receiving irrigated water which has flowed through the zinc-mineralized area associated with the Padaeng deposit (Phaenark et al., 2009; Simmons et al., 2005).

Analysis of bacterial communities in soils

In total, 54,026 OTUs were observed among all samples with a mean of 16,528.8 ± 2328.4 identified in individual samples (Fig. 1). Of these, 99.74% were classified as bacteria and 0.26% were archaea. Five dominant phyla were observed—*Proteobacteria, Actinobacteria, Acidobacteria, Firmicutes* and *Bacteroidetes* (Fig. 2). These five phyla accounted for 87.2% of all OTUs identified among all sites, and only two OTUs were not classified into any phylum.

Diversity and physicochemical parameters

Analysis of variance, using Bonferroni's Multiple Comparison Test, and based on the information of non-parametric Shannon and

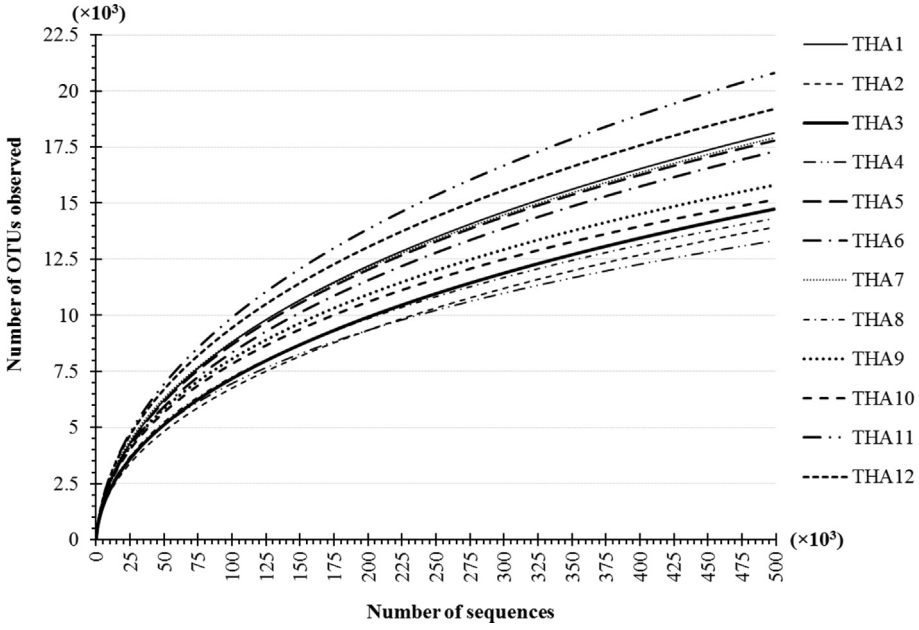

Fig. 1. Rarefaction curves (number of operational taxonomic units (OTUs) versus number of sequences) for each of the 12 sampling sites.

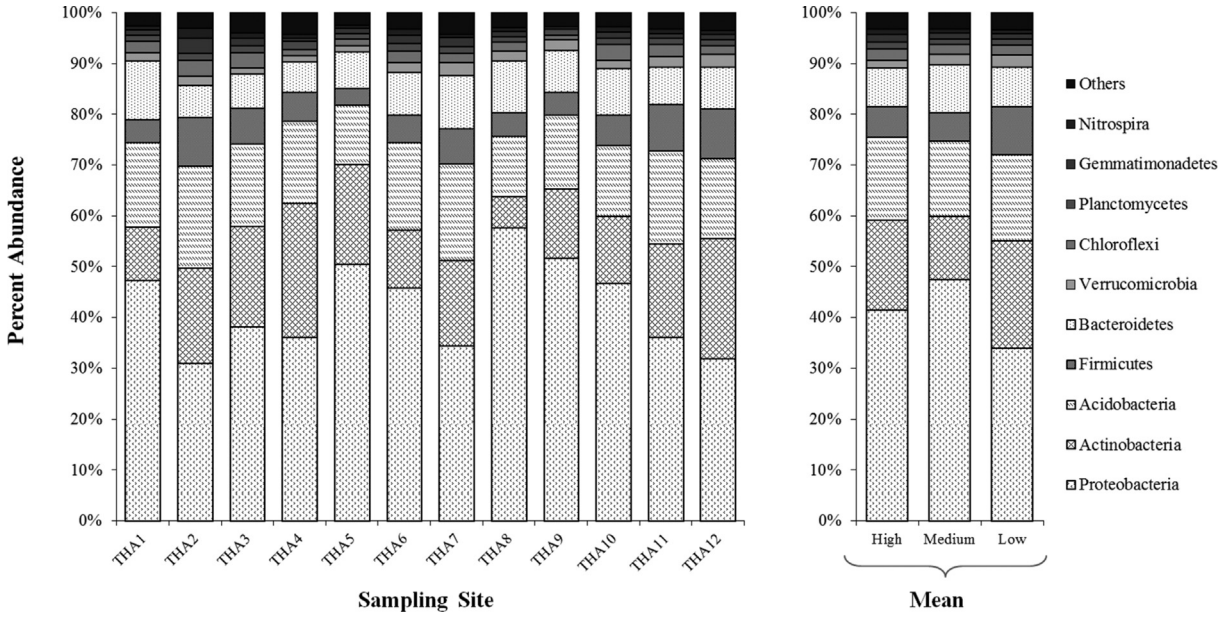

Fig. 2. Relative amounts of the most abundant phyla at each sampling site.

Shannon's indices, showed that the biological diversity was significantly higher in the rhizosphere soil of the low Cd and Zn level group, compared to the high Cd and Zn level group (Table 2). In addition, uniformity of the distribution of each species in a sample was found in the group with the most consistently low Cd and Zn levels. As a result, soils with highly elevated levels of Cd and Zn significantly influenced the biodiversity of bacteria. The non-parametric estimation of Shannon's index of diversity is used when the number of species and species abundances are unknown (Chao and Shen, 2003). Rhizosphere soils in the low Cd and Zn level group had a unique structure in terms of the distribution of biodiversity (Fig. 3).

Comparison of microbial community structure

The relative abundance of the five most abundant phyla did not differ significantly among all sites. The structure of the microbial community in soil samples was compared using PCoA. Analyses of the first two dimensions (axes) showed that soils with low Cd and Zn concentrations were distributed in the same quadrant, and these microbes formed a similar microbial community structure. In contrast, microbes in soils of moderate to high Cd and Zn levels had microbial communities distributed in several quadrants, and were separated from the group with low levels of Cd and Zn, except for the THA4 soil. The results of this analysis showed that soils with increasing Cd and Zn levels affected the microbial community (Fig. 3).

Isolation and characterization of cadmium and zinc resistant bacteria

Ninety-six bacterial isolates from each sample were used to analyze the pattern of gene function by testing for MICs. The MIC values obtained from all the 1152 isolates were analyzed using the BioNumerics software. Dendrograms of each sample were produced to analyze the distribution pattern of Cd- or Zn-resistant bacteria or both. The results showed that more than 26.7% were resistant to Cd at a concentration up to 320 μg/mL and only 2.3% were resistant to Zn at concentration up to 3200 μg/mL. From the MIC analyses, the number of resistant bacteria decreased with

Table 2
Diversity indices for all sites.

Diversity measure	Sampling site											
	High Cd level (>37 mg/kg soil)						Medium Cd level (10–37 mg/kg soil)				Low Cd level (<10 mg/kg soil)	
	THA1	THA2	THA3	THA4	THA5	THA6	THA7	THA8	THA9	THA10	THA11	THA12
Number of OTUs observed	18,123	13,925	14,731	13,317	17,783	17,295	17,910	14,316	15,801	15,148	20,801	19,196
OTU richness estimators												
Chao	28,452	21,665	22,750	19,188	27,120	27,459	27,828	21,477	23,544	21,553	32,679	28,535
ACE	34,985	27,528	28,473	22,761	32,966	34,889	34,761	26,129	28,327	25,302	40,711	34,812
Jackknife	38,576	27,829	30,444	25,338	36,340	35,247	35,372	27,535	30,003	27,329	47,529	37,221
Nonparametric-Shannon's index[a]	7.03	6.86	6.88	6.90	6.89	6.98	7.27	6.50	7.05	7.02	7.40	7.41
Shannon's index (H′)[a]	6.98	6.81	6.83	6.86	6.83	6.93	7.22	6.46	7.00	6.98	7.34	7.36
Shannon evenness index (E)	0.71	0.71	0.71	0.72	0.70	0.71	0.74	0.67	0.72	0.72	0.74	0.75
Simpson's index (D)	0.007	0.004	0.006	0.007	0.015	0.009	0.004	0.016	0.009	0.007	0.003	0.003
Good's coverage	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.98	0.98

OTU = operational taxonomic unit; ACE = abundance-based coverage estimator.
OTU richness in soils was calculated based on three estimators (Chao, ACE, and Jackknife) and was not significantly different among the soil groups.
[a] Indicate bacterial diversities in soils from high Cd level area were significantly lower than the diversities in soils from low Cd level at α level = 0.05.

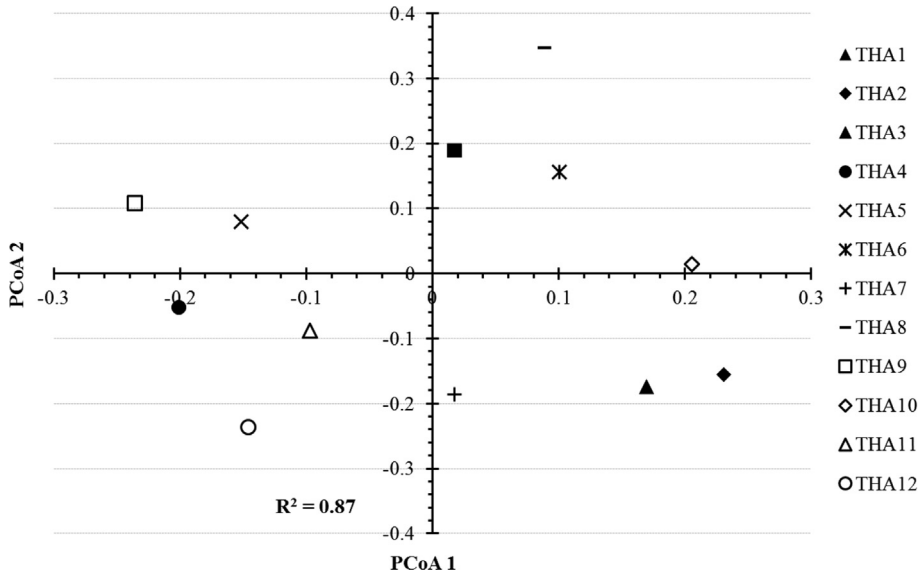

Fig. 3. Principal components analysis (PCoA) plot of microbial community structure at all sites and the associated coefficient of determination (R^2).

increasing Cd and Zn concentrations. Bacteria that are resistant to Cd and Zn in the same concentration range may contain the same group of resistance genes (Abou-Shanab et al., 2007).

Taken together, the current results indicate that soils with low levels of Cd and Zn had higher species diversity values than soils with high levels of Cd and Zn. Additionally, bacteria found in soils with high levels of Cd and Zn had greater resistance levels to Cd and Zn than those found in soils with low levels of Cd and Zn. These data support the contention that the addition of heavy metals to soils likely will have lasting effects on the soil microbial structure and function.

Conflicts of interest

The authors declare there are no conflicts of interest.

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References

Abou-Shanab, R.A., Angle, J.S., Delorme, T.A., Chaney, R.L., van Berkum, P., Moawad, H., Ghanem, K., Ghozlan, H.A., 2003. Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. New Phytol. 158, 219–224.

Abou-Shanab, R.A., van Berkum, P., Angle, J.S., 2007. Heavy metal resistance and genotypic analysis of metal resistance genes in Gram-positive and Gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*. Chemosphere 68, 360–367.

Amacher, M.C., 1996. Nickel, cadmium and lead. In: Spark, D.L., Page, A.L., Helmke, P.A., Loeppert, R.H., Soltanpour, P.N., Takatabai, M.A., Johnson, C.T., Summer, M.E. (Eds.), Method of Soil Analysis Part 3: Chemical Methods. ASA and SSSA Inc, Madison, WI, USA, pp. 739–768.

Belimov, A.A., Kunakova, A.M., Safronova, V.I., Stepanok, V.V., Yudkin, L.Y., Alekseev, Y.V., Kozhemyakov, A.P., 2004. Employment of rhizobacteria for the inoculation of barley plants cultivated in soil contaminated with lead and cadmium. Microbiology (Moscow) 73, 99–106.

Chapman, H.D., 1965. Cation exchange capacity. In: Black, C.A. (Ed.), Methods of Soil Analysis. Part II. Chemical and Microbiological Properties. ASA and SSSA Inc, Madison, WI, USA, pp. 891–901.

Chao, A., Shen, T.J., 2003. Nonparametric estimation of Shannon's index of diversity when there are unseen species in sample. Environ. Ecol. Stat. 10, 429–443.

Christine, C.C., 1997. Cd bioaccumulation in carp (*Cyprinus carpio*) tissues during long-term high exposure: analysis by inductively coupled plasma-mass spectrometry. Ecotox. Environ. Safe 38, 137–143.

Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil. Biol. Biochem. 30, 1389–1414.

Glick, B.R., Karaturovic, D.M., Newell, P.C., 1995. A novel procedure for rapid isolation of plant growth promoting pseudomonads. Can. J. Microbiol. 41, 533–536.

Hesse, P.R., 1971. Textbook of Soil Chemical Analysis. William Clowes and Sons Limited, London, UK.

Huse, S.M., Huber, J.A., Morrison, H.G., Sogin, M.L., Welch, D.M., 2007. Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biol. 8, R143.

Huse, S.M., Dethlefsen, L., Huber, J.A., Mark, W.D., Relman, D.A., Sogin, M.L., 2008. Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. PLoS Genet. 4, e1000255.

Laila, M.A., Wagdy, K.B., Thanaa, H.A., Karima, F.M., 2011. Heavy metal resistance and gene expression analysis of metal resistance genes in Gram-positive and Gram-negative bacteria present in Egyptian soils. J. Appl. Sci. Environ. Sanit. 6, 201–211.

Oliver, M.A., 1997. Soil and human health: a review. Eur. J. Soil. Sci. 48, 573–592.

Phaenark, C.P., Pokethitiyook, M., Kruatrachue, M., Ngernsansaruay, C., 2009. Cd and Zn accumulation in plants from the Padaeng zinc mine area. Int. J. Phytoremediation 11, 479–495.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., et al., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541.

Simmons, R.W., Pongsakul, P., Saiyasitpanich, D., Klinphoklap, S., 2005. Elevated levels of cadmium and zinc in paddy soils and elevated levels of cadmium in rice grain downstream of a zinc mineralized area in Thailand: implications for public health. Environ. Geochem. Health 27, 501–511.

Sogin, M.L., Morrison, H.G., Huber, J.A., Mark, W.D., Huse, S.M., Neal, P.R., Arrieta, J.M., Herndl, G.J., 2006. Microbial diversity in the deep sea and the under explored "rare biosphere". Proc. Natl. Acad. Sci. U. S. A. 103, 12115–12120.

Staley, C., Unno, T., Gould, T.J., Jarvis, B., Phillips, J., Cotner, J.B., Sadowsky, M.J., 2013. Application of Illumina next-generation sequencing to characterize the bacterial community of the Upper Mississippi River. J. Appl. Microbiol. 115, 1147–1158.

Walkley, A., Black, C.A., 1934. An examination of degradation methods for determining soil organic matter: a proposed modification of the chromic acid titration method. Soil Sci. 37, 29–35.

Whiting, S.N., de Souza, M.P., Terry, N., 2001. Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environ. Sci. Technol. 35, 3144–3150.

Wuertz, S., Mergeay, M., 1997. The impact of heavy metals on soil microbial communities and their activities. In: Elsas, J.D., Wellington, E.M.H., Trevors, J.T. (Eds.), Modern Soil Microbiology. Marcel Decker, New York, NY, USA, pp. 1–20.

Yao, H.Y., He, Z.L., Wilson, M.J., Campbell, C.D., 2000. Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use. Microb. Ecol. 40, 223–237.

Yozwiak, N.L., Skewes, C.P., Gordon, A., Saborio, S., Kuan, G., Balmaseda, A., Ganem, D., Harris, E., Derisi, J.L., 2010. Human enterovirus 109: a novel interspecies recombinant enterovirus isolated from a case of acute pediatric respiratory illness in Nicaragua. J. Virol. 84, 9047–9058.