Supporting Information

Title: Unique rhizosphere micro-characteristics facilitate phytoextraction of multiple metals in soil by the hyperaccumulating plant *Sedum alfredii*

*Dandi Hou[1], Kai Wang[2], Ting Liu[1], Haixin Wang[1], Zhi Lin[1], Jie Qian[2], Lingli Lu[1], and Shengke Tian[1]**

[1]Ministry of Education Key Laboratory of Environmental Remediation and Ecological Health, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China

[2]School of Marine Sciences, Ningbo University, Ningbo, 315211, China

***Corresponding author.** [1]Ministry of Education Key Laboratory of Environmental Remediation and Ecological Health, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China. E-mail: tiansk@zju.edu.cn Tel: +86-571-88982515 Fax: +86-571-88982907

Summary of the numbers in supporting information:

- The number of pages: 14
- The number of figures: 5
- The number of table: 3

Additional Details on Material and Methods.

Plant culture

The general properties of soil contaminated with heavy metals were as follows: pH 7.4, total C (21.37 $g \cdot kg^{-1}$), total P (725.30 $\mu g \cdot g^{-1}$), total K (4.21 $mg \cdot g^{-1}$), total Cd (5.76 $\mu g \cdot g^{-1}$), total Zn (1985.10 $\mu g \cdot g^{-1}$), total Pb (667.47 $\mu g \cdot g^{-1}$), and total Cu (698.76 $\mu g \cdot g^{-1}$). The soils were air-dried and sieved through a 2 mm mesh. The soils were air-dried, and ground to pass through a 2 mm mesh. Rhizoboxes were designed according to Li *et al.*, [1] with the sizes of 120×120×180 (length × width × height, mm). The rhizobox was divided into three sections, a rhizosphere zone (20 mm in width), which was surrounded by nylon mesh (300 mesh), and left and right bulk zones (non-rhizosphere zones, 50 mm in width). Soils of 0.4 kg and 1.8 kg were placed in the rhizosphere and non-rhizosphere zone, respectively. Three two-week old seedlings of the HE and NHE *S. alfredii* were transplanted to the rhizoboxes, with each treatment replicated three times. The plants were watered to maintain soil moisture at approximately 65% of the maximum water-holding capacity. The plants were grown in a greenhouse with natural light and average day/night temperatures of 30/24ºC, and day/night humidity of 70/85%.

Plant harvesting and soil sampling

After six months of growth, the rhizosphere and bulk zone of the rhizoboxes were separated. *In situ* zymography was carried out immediately as described below. Subsequently, the excess soil was manually shaken from the roots, leaving

approximately 1 mm of soil still attached to the roots. The 1 mm of adhering soil is washed off in 20 ml of phosphate-buffered saline and kept as the rhizosphere compartment. Five soil subsamples were collected from bulk zones, homogenized in 50 mL Falcon tubes, and immediately flash frozen in liquid nitrogen and maintained at -80ºC prior to DNA extraction. Another portion of fresh soil samples was passed through a 2-mm sieve, sealed in a plastic bag, and stored at 4°C to preserve moisture status for later microbial analysis.[2] To assess soil properties and for elemental analyses, soils in the rhizosphere and non-rhizosphere zones were sampled both at the beginning and end of the experiment.

At harvest, the plants were separated into roots and shoots, washed thoroughly, and rinsed with distilled water. The plant samples were then oven dried at 65ºC, weighed, and ground to pass through a 60 mesh. Plant samples (0.1 g) were digested with 5 mL HNO_3 and 1 mL H_2O_2 at 180ºC for 8 hours, and the digest was transferred to a 50-mL volumetric flask, made up to volume with water and filtered for elemental analysis.

Soil physicochemical and biological properties

Soil pH was measured with a glass electrode in samples with a soil:water ratio of 1:2.5. To analyze the total concentrations of heavy metals, soil samples (0.2 g) were digested with 7.0 mL HNO_3:$HClO_4$:HF (at a ratio of 5:1:1, v/v/v) at 180ºC for 10 h. Bio-available heavy metals were extracted by DTPA (diethylene triamine pentaacetic acid) extracting agent (0.005 mol L^{-1} DTPA, 0.01 mol L^{-1} $CaCl_2$, and 0.1 mol L^{-1} TEA,

pH 7.3). Soils were evaluated for DTPA-extractable heavy metals in a 1:2 soil to solution ratio, which was obtained after shaking for 2 h. Concentrations of Cd, Zn, Pb, and Cu in the digestive and extractive solutions of plant and soil samples were determined by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7500a, USA). Sample replicates, reagent blanks, rice flour (IRMM-804, Sigma) and soil (GBW07429, the National Research Center for Certified Reference Materials of China) standard reference materials were included in each batch of analysis to ensure the quality of analysis. The recovery of standard for each element ranged between 90 and 110%.

Soil microbial biomass C was measured by the fumigation–extraction method and organic C concentration was determined using an automated total organic C analyzer (Analytikjena MultiN/C 3100, Germany).[2] To determine the soil urease and acidic phosphatase activities, fresh soil samples were incubated with 10% urea and disodium phenyl phosphate solution, then quantified colorimetrically using a spectrophotometer at 578 nm and 510 nm, respectively.[3] Soil zymography, an *in situ* method for imaging enzyme activities in soils,[4] was applied to compare the protease and amylase activities in the rhizosphere and bulk soils of *S. alfredii.* It is based on a gel screen containing the enzyme's substrate that is incubated attached to undisturbed soil.[5] All gels were scanned together on a graphic scanner (Epson Expression 10000XL, Japan). The digital gel images were analyzed using MatLab (The MathWorks, USA).

Processing of pyrosequencing data

All sequences were run through the QIIME pipeline (version 1.7.0).[6] The sequences were assigned to each sample according to the barcodes and quality controlled using the *split_libraries.py* script. Chimeric sequences were detected and removed using Usearch.[7] The remaining sequences were clustered into OTUs using the *pick_de_novo_otus.py* script with the UCLUST method at a threshold of 97% similarity and singletons were discarded. After this, archaea and chloroplast sequences were removed. To reduce the influence of sequencing depth on treatment effects, samples were then randomly resampled to the same sequence depth, based on the least number of sequences (28,100 sequences per sample).

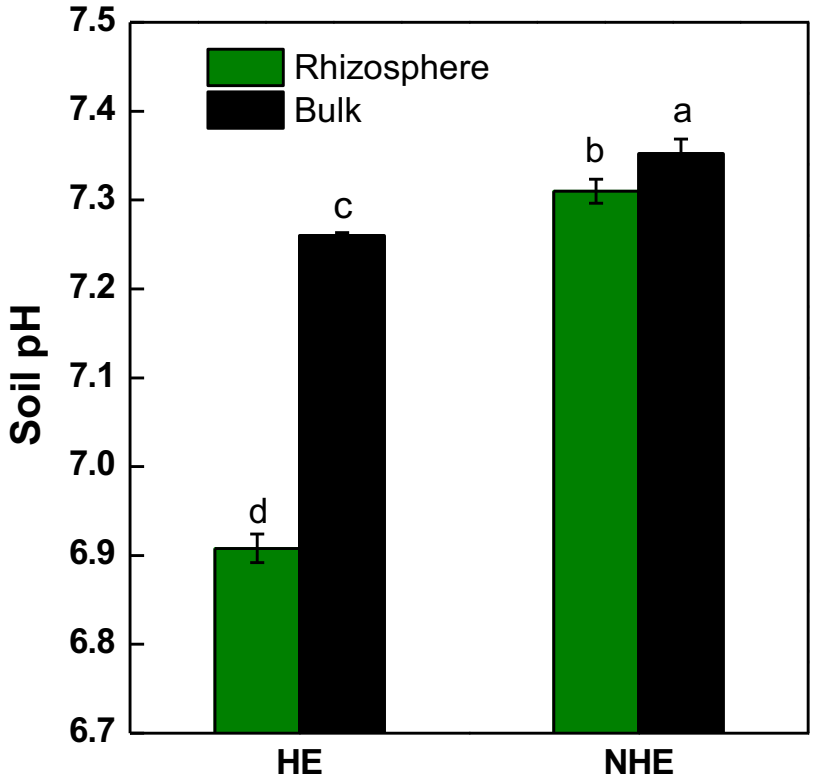
Statistical analysis

Data of plant and soil properties，such as plant biomass, metal concentrations, and enzyme activities are presented as mean ± standard deviation of the mean based on three replicates. Significant differences ($P \leq 0.05$) among treatments were analyzed using a protected Fisher's least significant difference (LSD) test after a one-way analysis of variance (ANOVA). These statistical analyses were conducted using SPSS 18.0.

References

1. Li, T. Q.; Di, Z. Z.; Islam, E.; Jiang, H.; Yang, X. E., Rhizosphere characteristics of zinc hyperaccumulator *Sedum alfredii* involved in zinc accumulation. *J. Hazard. Mater.* **2011**, *185* (2-3), 818-823.
2. Yang, W.; Zhang, T.; Li, S.; Ni, W., Metal removal from and microbial property improvement of a multiple heavy metals contaminated soil by phytoextraction with a cadmium hyperaccumulator *Sedum alfredii* H. *J. Soils Sediments* **2014**, 14 (8), 1385-1396.

3. Cang, L.; Zhou, D. M.; Wang, Q. Y.; Wu, D. Y., Effects of electrokinetic treatment of a heavy metal contaminated soil on soil enzyme activities. *J. Hazard. Mater.* **2009**, *172*, (2-3), 1602-1607.
4. Spohn, M.; Carminati, A.; Kuzyakov, Y., Soil zymography - A novel in situ method for mapping distribution of enzyme activity in soil. *Soil Biol. Biochem.* **2013**, 58, 275-280.
5. Spohn, M.; Kuzyakov, Y., Distribution of microbial- and root-derived phosphatase activities in the rhizosphere depending on P availability and C allocation - Coupling soil zymography with C-14 imaging. *Soil Biol. Biochem.* **2013**, *67*, 106-113.
6. Caporaso, J. G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F. D.; Costello, E. K.; Fierer, N.; Pena, A. G.; Goodrich, J. K.; Gordon, J. I.; Huttley, G. A.; Kelley, S. T.; Knights, D.; Koenig, J. E.; Ley, R. E.; Lozupone, C. A.; McDonald, D.; Muegge, B. D.; Pirrung, M.; Reeder, J.; Sevinsky, J. R.; Tumbaugh, P. J.; Walters, W. A.; Widmann, J.; Yatsunenko, T.; Zaneveld, J.; Knight, R., QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7* (5), 335-336.
7. Edgar, R. C.; Haas, B. J.; Clemente, J. C.; Quince, C.; Knight, R., UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **2011**, *27* (16), 2194-2200.



123

124 **Figure S1** The pH differences in rhizosphere and bulk soil after planting HE and NHE

125 *Sedum alfredii* for 6 months. Data points represent the mean ± SD (n=3). Asterisks

126 indicate values are significantly different from rhizosphere and bulk soils. (** $P <$

127 0.01).

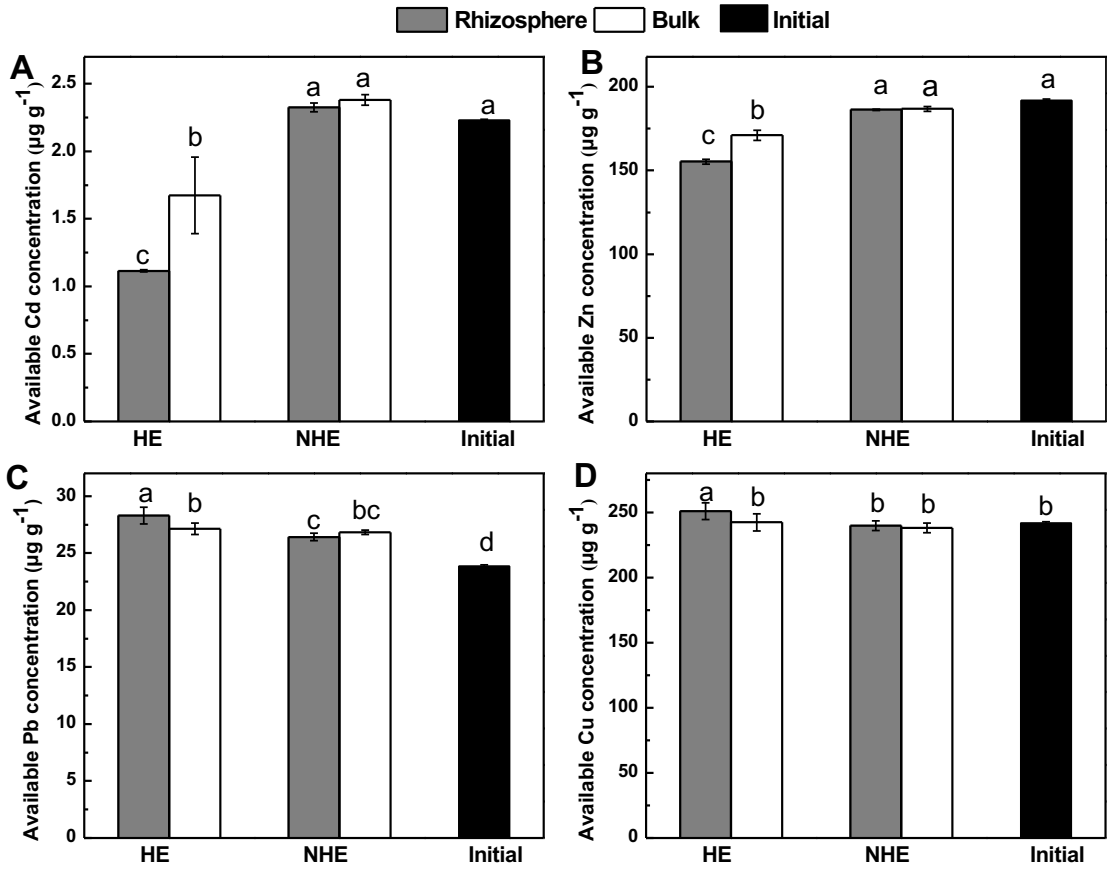


Figure S2 The available concentrations of (A) Cd, (B) Zn, (C) Pb, and (D) Cu in the rhizosphere and bulk soil after 6-months of *S. alfredii* growth. Data points represent means ± SD (n=3). Different letters indicate significant differences among ecotypes at $P < 0.05$.

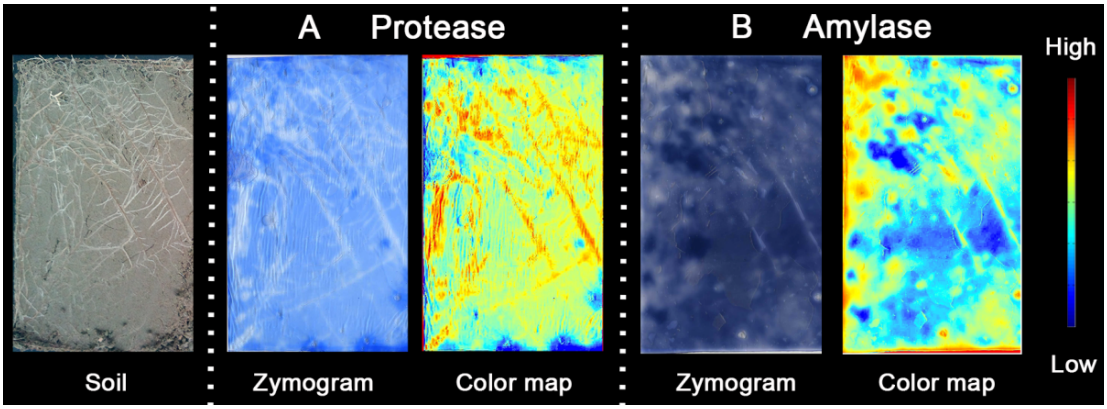


Figure S3 Soil zymography to map distribution of (A) protease and (B) amylase in rhizosphere after the 2-month growth period of HE *S. alfredii.*

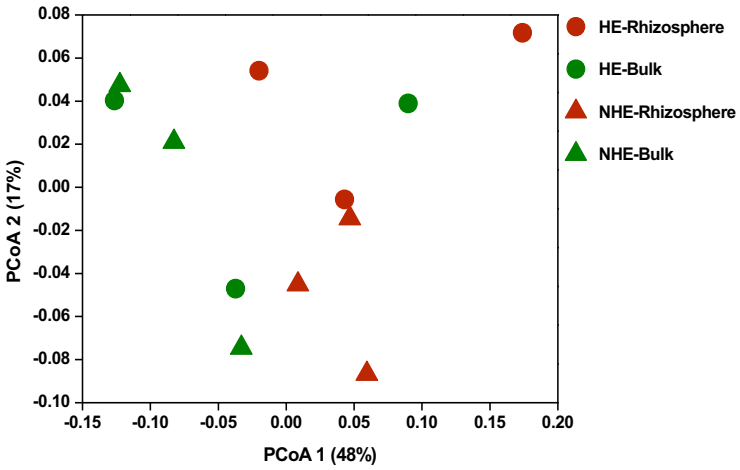


Figure S4 Root-associated bacteria vary by compartment and ecotype of *Sedum alfredii*. Principal coordinates analysis (PCoA) plots to visualize the weighted UniFrac distance among the bacterial communities.

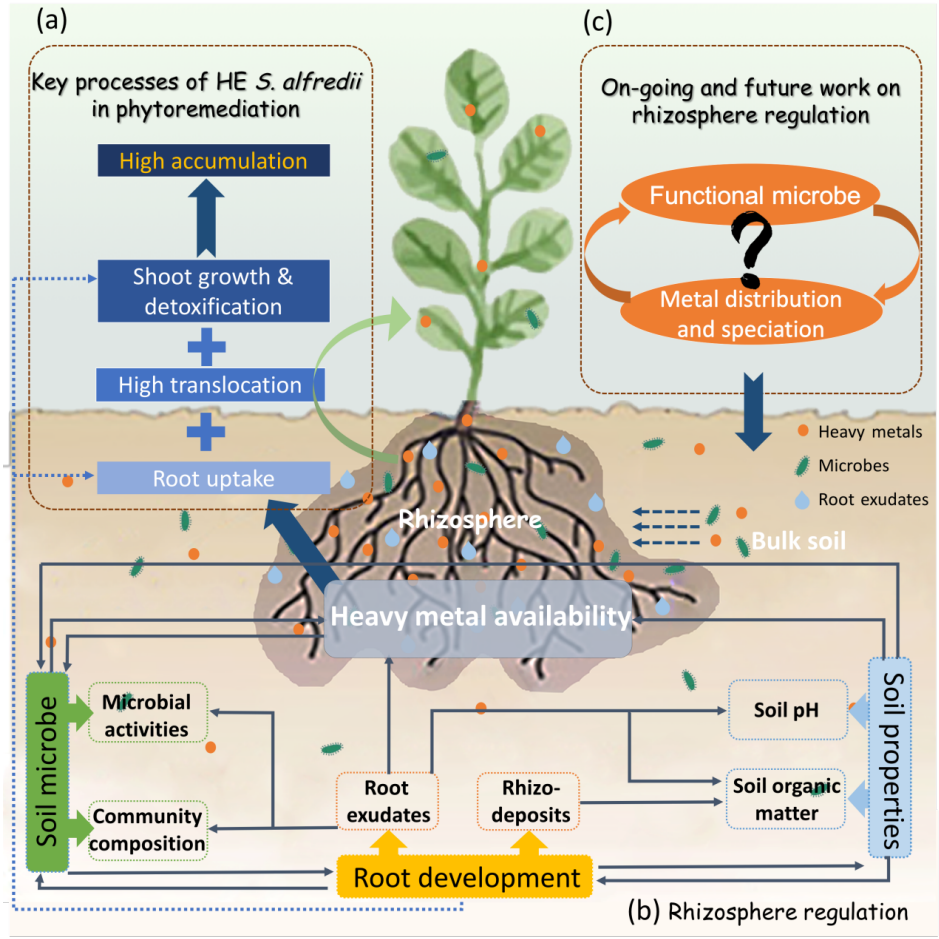


Figure S5 Schematic of possible mechanisms of rhizosphere characteristics of HE *S. alfredii* responsible for heavy metal uptake and accumulation in phytoremediation of metal contaminated soil. (a) Some key processes determining the ability to hyperaccumulate metals reported by our previous studies, including efficient root uptake and loading into the xylem, enhanced root-to-shoot translocation, and detoxification via chelation and subsequent sequestration in cell vacuoles. (b) The complex interaction of root development, soil properties and soil microbes in the rhizosphere of HE *S. alfredii* underlying the activation and uptake of heavy metals. (c) Our on-going and future work will be devoted to confirming the possible rhizosphere mechanisms proposed in this study and exploring the interplay among heavy metal migration, transformation and associated functional microbes.

153 **Table S1** Bacterial richness and α-diversity estimates (per 28,100 sequences) of the

154 bacterial community

Compartments		Richness estimators		Diversity indices	
		Chao1	S_{obs}	Shannon	Simpson
HE	Rhizosphere	5196	3191	9.0	0.988
	Bulk	5195	3168	9.1	0.989
NHE	Rhizosphere	5034	3270	9.3	0.993
	Bulk	4757	3040	9.1	0.991

OTU-operational taxonomic unit

S_{obs}-observed OTUs, observed richness

Data is normalized to the sample with the lowest number of sequences

155

156 **Table S2** Permutational MANOVA results using weighted and unweighted UniFrac as

157 a distance metric.

Weighted UniFrac				
Factor	**% Explained**	**F. Model**	**R^2**	***P* value**
Compartment	25.61	3.55	0.26	0.012
Ecotype	10.46	1.45	0.10	0.200
Ecotype : Compartment	6.20	0.86	0.06	0.494
Residuals	57.74		0.58	
Total			1.00	
Unweighted UniFrac				
Compartment	14.46	1.79	0.14	0.001
Ecotype	12.12	1.50	0.12	0.008
Ecotype : Compartment	8.65	1.07	0.09	0.237
Residuals	64.75		0.65	
Total			1.00	

158

159 **Table S3** The Nearest Sequenced Taxon Index (NSTI) score of each sample to
160 evaluate the accuracy of PICRUSt.

Sample ID	Compartment	Ecotype	Metric	Value
H1	Rhizosphere	HE	Weighted NSTI	0.195
H2	Rhizosphere	HE	Weighted NSTI	0.195
H3	Rhizosphere	HE	Weighted NSTI	0.154
H4	Bulk	HE	Weighted NSTI	0.184
H5	Bulk	HE	Weighted NSTI	0.215
H6	Bulk	HE	Weighted NSTI	0.227
H7	Rhizosphere	NHE	Weighted NSTI	0.200
H8	Rhizosphere	NHE	Weighted NSTI	0.183
H9	Rhizosphere	NHE	Weighted NSTI	0.193
H10	Bulk	NHE	Weighted NSTI	0.220
H11	Bulk	NHE	Weighted NSTI	0.216
H12	Bulk	NHE	Weighted NSTI	0.215

161