Supporting Information

Synergistic remediation of PCB-contaminated soil with nanoparticulate zero-valent iron and alfalfa: Targeted changes in the root metabolite-dependent microbial community

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Supporting Information consists of 20 pages, including 8 texts, 14 figures, and 2 tables.

Supporting Methods

Text S1. Characterization of soil and materials.

The soil pH (soil: water = 1: 2.5, m/v) was measured with a pH meter (S220, Mettler Toledo, Switzerland). The soil texture was analyzed by Mastersizer 2000 (Malvern, UK). The elemental content of soil was obtained by an X-ray fluorescence spectrometry (PW2403, PANalytical B.V., Netherlands) and the organic-associated element content (C, N, S, H, O) was measured with an elemental analyzer (FlashSmart, ThermoFisher, Germany).

In order to add PCBs and nanoparticles into soil homogeneously, a 10-fold dilution method was used.[1,2] First, a fraction of soil was saturated with the acetone solution of PCB28 or PCB180 in a fume hood. After solvent volatilization, the contaminated soil was diluted twice to reach the target concentration of about 1.0 $mg \cdot kg^{-1}$ PCB.[3] Then, the calculated amounts of nZVI powder were added and mixed mechanically for 10 min within 20 L plastic cylinder to achieve the target doses of 0, 10, 100, and 1000 $mg \cdot kg^{-1}$ and Fe_3O_4 nanoparticles were mixed into soil in the same way.

Text S2. Iron determination.

Plant issues and soil samples (0.1 g) were used to measure the active and total Fe content. The active Fe was extracted by 0.1 M HCl of 10 mL at 25 ℃ and 200 rpm for 4 h. For the total Fe measurement, the alfalfa tissues were digested with a mixture of HNO_3 (6 mL) and H_2O_2 (2 mL) in a microwave digestion system (Mars 4, CEM, USA), and the soil samples were digested with a mixture of HNO_3 (6 mL) and HF (2 mL). Following the microwave digestion, the resultant solutions were diluted with 50 mL of 0.1 M HNO_3 to ascertain total Fe content. Then, 1 mL of solution was pipetted, and the Fe(Ⅲ) was reduced to Fe(Ⅱ) with hydroxylamine hydrochloride ($NH_2OH \cdot HCl$). Then 1,10-phenanthroline was used to react and quantify Fe(Ⅱ) using the absorbance at 510 nm.[4] The root tissues for SEM-EDS analysis were previously fixed with 2.5% glutaraldehyde for 4 h, post-fixed with osmium tetroxide for 2 h, dehydrated by a graded series of ethanol (30%, 50%, 70%, 80%, 90%, and 95%) for 15 min at each step, and further dehydrated in a critical point dryer (HCP-2, Hitachi, Japan).

Text S3. Extraction and analyses of PCBs and metabolites.

PCBs in soil were extracted according to the previous method.[1] Briefly, 1.0 g of soil was thoroughly extracted with a mixture of hexane (5 mL) and acetone (5 mL) by ultrasonication at 35 ℃ for 30 minutes twice. The extracts were mixed and enriched under a gentle nitrogen stream into 1 mL. PCBs in liquid culture were purified with 2 mL of sulfuric acid, demulsified with 1 g of ammonium sulfate, and extracted by 5 mL of hexane.[5] The organic phase containing PCBs was dehydrated by anhydrous sodium sulfate and measured by gas chromatography (GC, 7890A, Agilent, USA) equipped with a ^{63}Ni-microelectron capture detector (μECD, Agilent, USA). The congeners were determined by GC (7890B, Agilent, USA) linked to a mass spectrometer (MS, 5977A, Agilent, USA). Sample separation was done with a DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm, Agilent, USA). The column temperature was programmed as follows: initial temperature was held at 80 °C for 2 min, increased to 196 °C with a gradient of 10 °C·min^{-1}, and then raised to 280 °C at 15 °C·min^{-1} and held for 1 min.

In the simulation process, the intermediate products of PCB28 were analyzed according to the methods of Hong et al.[6] The reaction solution was extracted with 10 mL of ethyl acetate twice and the supernatants were mixed and dried with nitrogen blowing. After that, a mixture (100 μL) of N,O-bis(trimethylsilyl)trifluoroacetamide(BSTFA) and trimethylchlorosilane(TMCS) at a ratio of 99:1 were added and incubated at 60 °C for 15 min. The obtained mixtures were then transferred to a vial and brought to volume of 1.0 mL with hexane. The intermediate products were analyzed by the GC-MS. The chromatographic column was an Agilent DB-5MS capillary column (30 cm × 0.25 mm, 0.25 μm). Helium (purity 99.99%) was used as a carrier gas; the injection volume was 1 μL; the oven temperature program was initially 60 °C and held for 2 min, and then increased to 280 °C at 10 °C·min^{-1} and maintained for 8 min. The ion source and quadrupole temperature of mass spectrometer were 230 °C and 150 °C, respectively.

Text S4. Metabolomics analysis.

Ten mg of root powder was ultrasonically extracted in a mixture of chloroform: water: methanol

(1.2 mL, 2:2:5, v/v/v) at 4 °C for 1 h and then centrifugated at 10000*g* for 10 min. Then 400 μL of supernatant was collected and freeze-dried for derivatization. Briefly, 50 μL of methoxyamine hydrochloride in pyridine (20 $mg \cdot L^{-1}$) was added to the vial and incubated at 37 °C for 90 min; 80 μL of MSTFA were then added and incubated at 37 °C for 30 min. To prevent the decomposition of organic matter, all vials were tested within 24 h. Root metabolites were analyzed as six biological replicates. The metabolites were analyzed by the GC-MS. The column temperature was programmed as follows: initial temperature was set at 70 °C for 4 min, raised to 300 °C with the gradient of 15 $°C \cdot min^{-1}$, and held for 5 min. The m/z values were monitored in full scan mode ranging from 33 to 600.

Text S5. Illumina-based 16S rDNA gene sequencing.

The PCR was conducted in triplicate using the following program: 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s for annealing at 55 °C, and 45 s for elongation at 72 °C, and a final extension at 72 °C for 10 min. The reaction solution (20 μL) contained 4 μL of 5× FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of the template DNA. The resulting PCR products were extracted with a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA). Quantification was done using QuantiFluor-ST according to the manufacturer's protocol. Finally, purified amplicons were pooled in equimolar amounts and were paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd., China.

Text S6. Soil microbial community functions.

For the metabolic function analysis of soil microbial community, 1.0 g of soil was added into 100 mL of sterilized PBS solution, and the mixed solution was shaken at 200 rpm and 25 ℃ for 30 min. After 30 min of settling, 180 μL of supernatant were added to microplates. Biolog ECO microplates (Biolog Inc., USA) with 96 wells containing 31 carbon sources in triplicates and three wells without carbon sources were used. The carbon sources consisted of six carbon substrate groups

(carbonhydrates, carboxylic acids, amino acids, polymers, phenols, and amines) and are shown in Table S2. Each microplate well also contained a colorless tetrazolium dye, which can be reduced to a purple formazan when the carbon source is utilized by microorganisms. The different color shades reflect the differences in the utilization of carbon sources. The Biolog ECO microplates were incubated at 25℃ for 7 days before evaluation. Absorbances were recorded at 590 nm (color + turbidity) and 750 nm (turbidity) with a microplate spectrophotometer (infinite M200 pro, Tecan, Switzerland). The two indexes were calculated by equations 1 and 2, where C_i is the difference in absorbance between 590 and 750 nm from the wells containing carbon sources, and R is the difference of the blank without carbon sources.

$$U = \sqrt{\left(\sum (C_i - R)^2\right)} \quad (1)$$

$$H' = -\sum \left(\frac{C_i - R}{\sum (C_i - R)} \times ln \frac{C_i - R}{\sum (C_i - R)} \right) \quad (2)$$

Text S7. Isolation and identification of PCB-degrading bacteria.

The microorganisms used in this study were isolated from the rhizosphere of alfalfa which had grown for 4 months in the PCB28-contaminated soil. According to the previous method,[7,8] a mixture of 1 g of soil, 100 mg of biphenyl, and 100 mL of liquid mineral medium (MM) was incubated (150 rpm) at 28 °C. The bacterial suspension was transferred every 7 days for three times to ensure purity. The MM solution consists of 0.20 g·L^{-1} $MgSO_4$, 0.02 g·L^{-1} $CaCl_2$, 0.36 g·L^{-1} KNO_3, 2.8 g·L^{-1} KH_2PO_4 and 6.7 g·L^{-1} $K_2HPO_4{\cdot}3H_2O$. The isolated microorganisms were then introduced into Luria-Bertani (LB) broth and cultured in an incubator shaker (150 rpm, 37°C) for 24 h. The LB broth consisted of 5 g·L^{-1} yeast extract, 10 g·L^{-1} tryptone, and 10 g·L^{-1} NaCl. The bacteria in the broth were collected after centrifugation (3000*g*, 10 min) and cleaned with PBS solution three times for the following assays. The PBS solution consisted of 6.71 g·L^{-1} $K_2HPO_4{\cdot}3H_2O$ and 2.80 g·L^{-1} KH_2PO_4. The synthetic root exudates consist of D-fructose (9.0 mg·L^{-1}), D-glucose (9.0 mg·L^{-1}), sucrose (17.1 mg·L^{-1}), succinic acid (3.0 mg·L^{-1}), L-malic acid (3.4 mg·L^{-1}), L-arginine (2.2 mg·L^{-1}), L-serine (1.3

mg·L^{-1}) and L-cysteine (1.5 mg·L^{-1}).[9] All solutions were sterilized by autoclaving at 121 °C for 20 min before the assays were conducted.

Bacterial physiological, morphological, and biochemical characteristics were measured using a published method.[8] The gram staining picture was taken with an optical microscope (DM6MLIBS, Leica, Germany). Transmission electron microscopy (TEM) images were obtained from a JEM-1230 tem (JEOL, Japan). 16S rDNA was amplified and analyzed for identification. The bacterial cells were collected by centrifugation at 5000*g* for 3 min and washed twice with sterile water and the genomic DNA was extracted using an E.Z.N.A. bacterial DNA kit (Omega Bio-Tek, Norcross, GA). 16S rDNA was amplified by polymerase chain reaction (PCR) with oligonucleotide primers 27F (50-AGAGTTTGATCCTGGCTCAG-30) and 1492R (50-TACGGCTACCTTGTTACGACTT-30). The PCR were performed with denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR product was sequenced by the Invitrogen Corporation Shanghai Representative Office. Sequences were compared with the 16S rDNA gene database with BLAST at National Center for Biotechnology Information (NCBI) public database (http://blast.ncbi.nlm.nih. gov/Blast.cgi). A phylogenetic tree was generated by the neighbor-joining method using MEGA version 7.

Text S8. Cell viability and hydroxyl radical measurements.

Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenynyl tetrazoliumbromide (MTT) assay kit (Nanjing Jiancheng, China).[10] Cells were seeded in triplicate on 96-well plates at a density of 1.0×10^4 cells per well, followed by addition of 10 μL of 1 mg·L^{-1} MTT and incubation at 37 °C for 4 h. After discarding the media, 200 μL of dimethyl sulfoxide were added to solubilize the blue MTT-formazan product. The optical density of the culture solution in the plate was measured at 570 nm using a microplate reader (infinite M200 pro, Tecan, Switzerland). The production of •OH was determined using the fluorescence probing technique with coumarin.[11] Briefly, 5 mL of the collected samples were added to 5 mL of 1.5 mM coumarin. After 0, 2, 4, 8, 16, and 24 h, 0.2 mL of methanol was added to terminate the reaction. The concentration of •OH was

obtained by measuring 7-hydroxylcoumarin, a strongly luminescent product, using a microplate reader (infinite M200 pro, Tecan, Switzerland) at 350/460 nm of the excitation/emission wavelengths. The total amount of •OH produced was estimated as [7-hydroxylcoumarin]/14.5%.[12] The detection limit of •OH using this method was 0.09 μM. All reagents were chromatographic grade and obtained from J&K Scientific Ltd, China.

References

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Supporting Figures

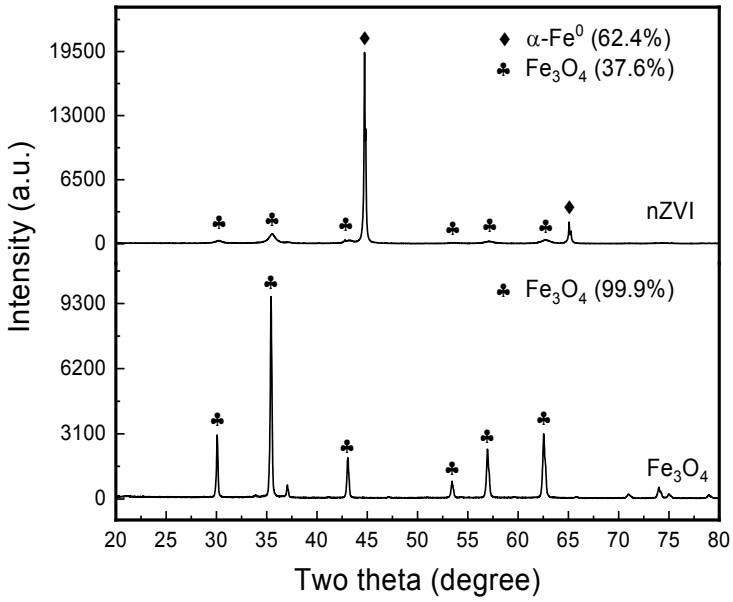


Fig. S1 The crystal structures of nZVI and Fe_3O_4 nanoparticles. The number after each Fe species in the figure is its percentage content.

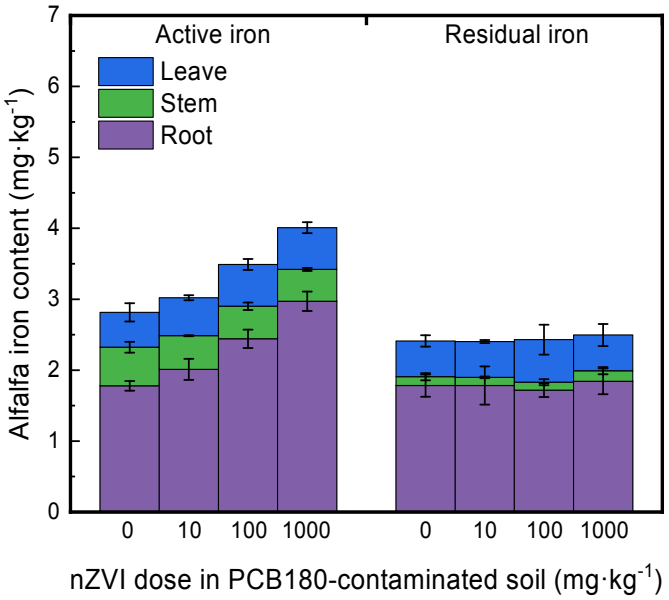


Fig. S2 The effects of nZVI (0, 10, 100, and 1000 mg kg^{-1}) on the content of active and residual Fe in the leaves, stems, and roots of alfalfa cultivated in PCB180-contaminated soil for 120 days. The bars indicate the mean ± standard deviation.

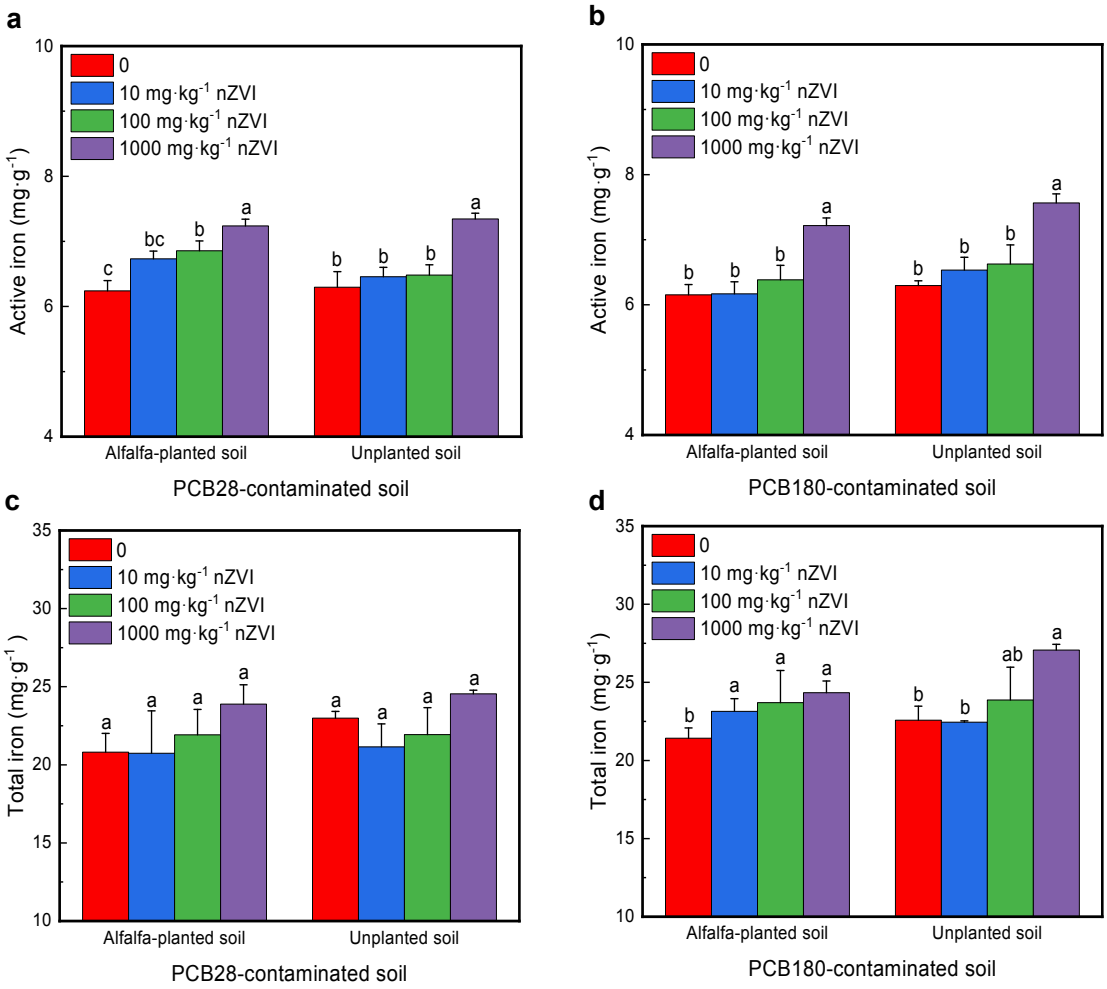


Fig. S3 The effects of nZVI on (a and b) active and (c and d) total Fe content in (a and c) PCB28- and (b and d) PCB180-contaminated soils after 120 d. The Fe concentrations are given as mean ± standard deviation. Different letters indicate significantly differences at $p<0.05$ (one-way ANOVA).

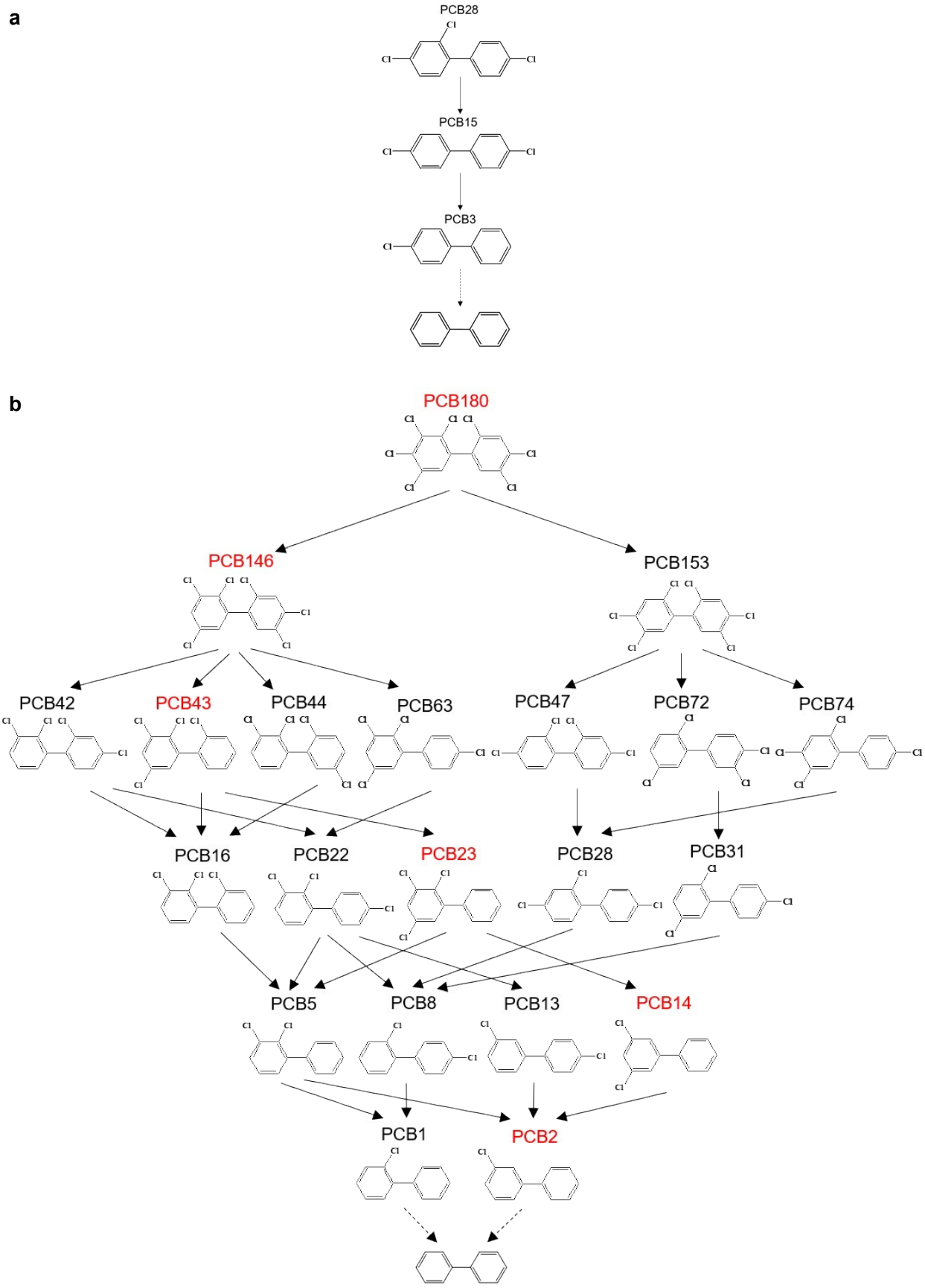


Fig. S4 Proposed PCB28 and PCB180 dechlorination pathways in soil.

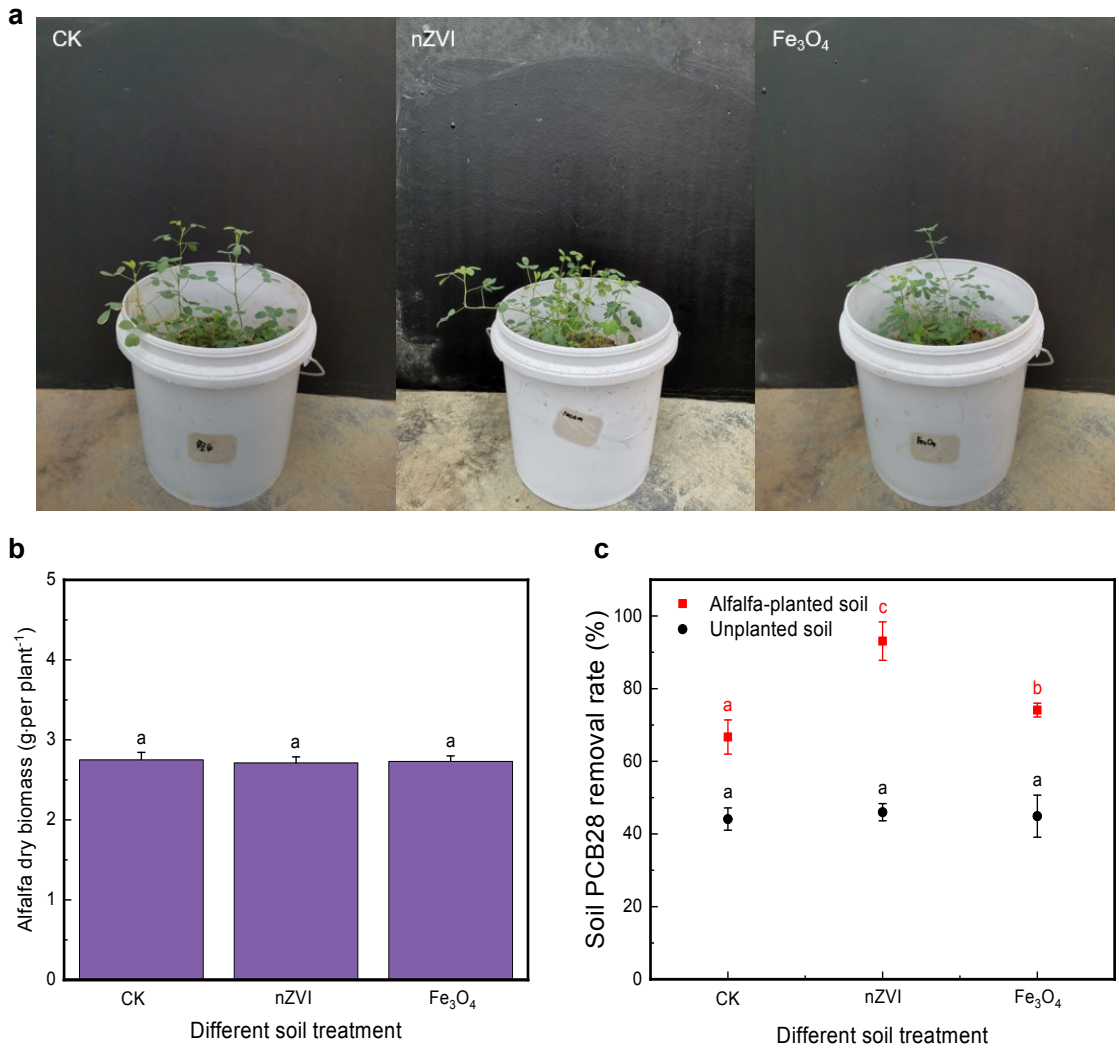
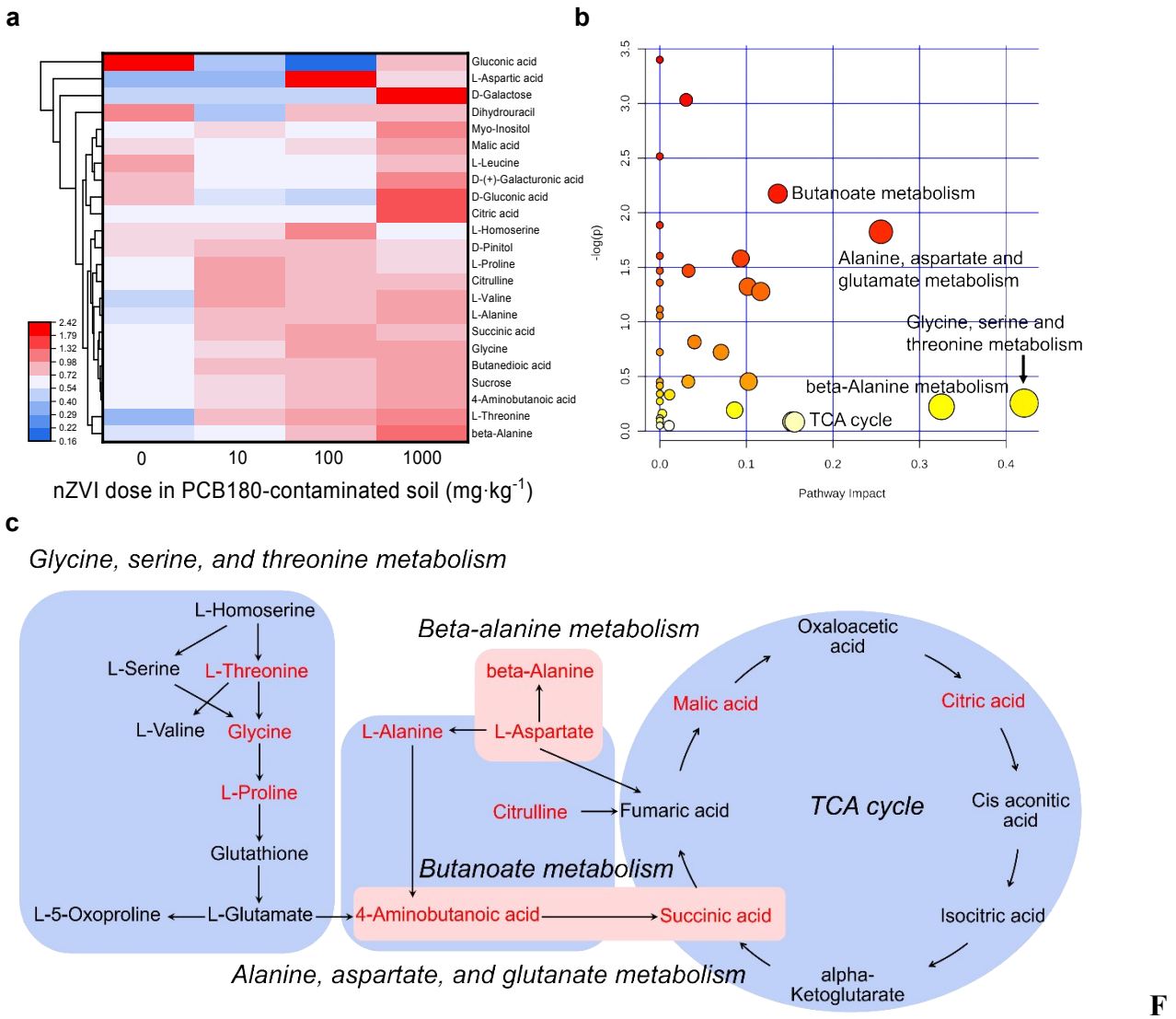


Fig. S5 (a) Photographs of alfalfa growth, (b) the alfalfa biomass, and (c) PCB28 removal rates in the PCB28-contaminated soil (control, 1000 mg·kg^{-1} nZVI, and 1000 mg·kg^{-1} Fe_3O_4 treated). In panels (b and c), the bars indicate the mean ± standard deviations; different letters indicate significant differences at p<0.05 (one-way ANOVA).



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ig. S6 (a) Hierarchical clustering heatmap, (b) metabolism pathway analysis, and (c) schematic diagram of root metabolites in alfalfa cultivated in PCB180-contaminated soils with 0, 10, 100, or 1000 mg·kg^{-1} nZVI. In panel (b), every circle represents a metabolic pathway, and the log of the *p*-value was from the enrichment analysis (the redder the circle, the larger the difference) and the pathway impact value was from the topology analysis (the larger the circle, the more impact). In panel (c), red and blue represent metabolites that increased and decreased in 1000 mg·kg^{-1} nZVI-treated roots compared with the control, respectively.

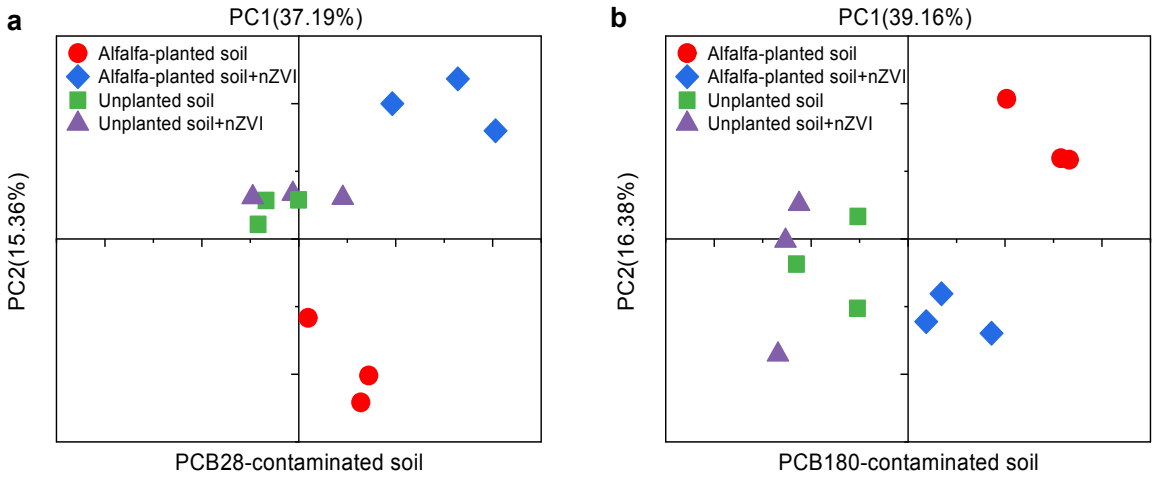

Fig. S7 PCoA analysis of bacterial communities in (a) PCB28- and (b) PCB180-contaminated soils.

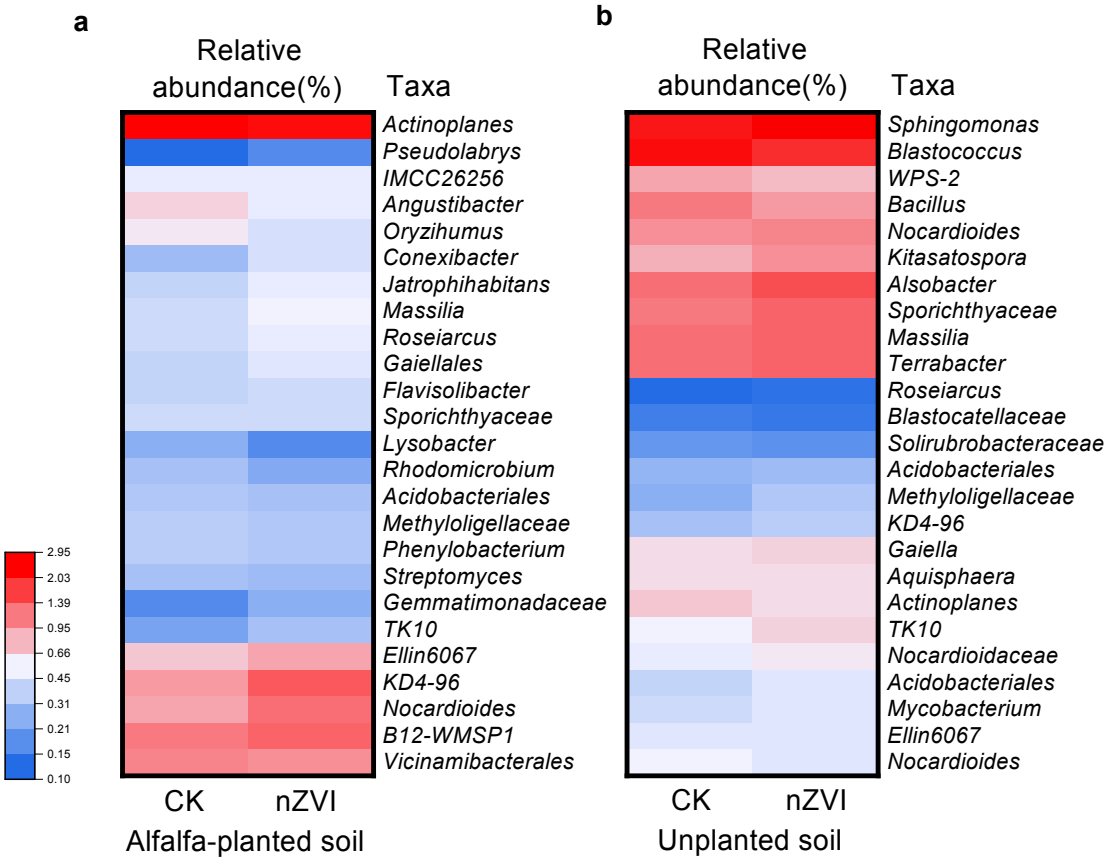

Fig. S8 Heatmap of significantly altered bacteria at the genus level in (a) rhizosphere and (b) unplanted PCB28 contaminated soil with control (CK) and 1000 mg·kg^{-1} nZVI. Red indicates increased bacterial taxa, and blue indicates decreased bacteria taxa.

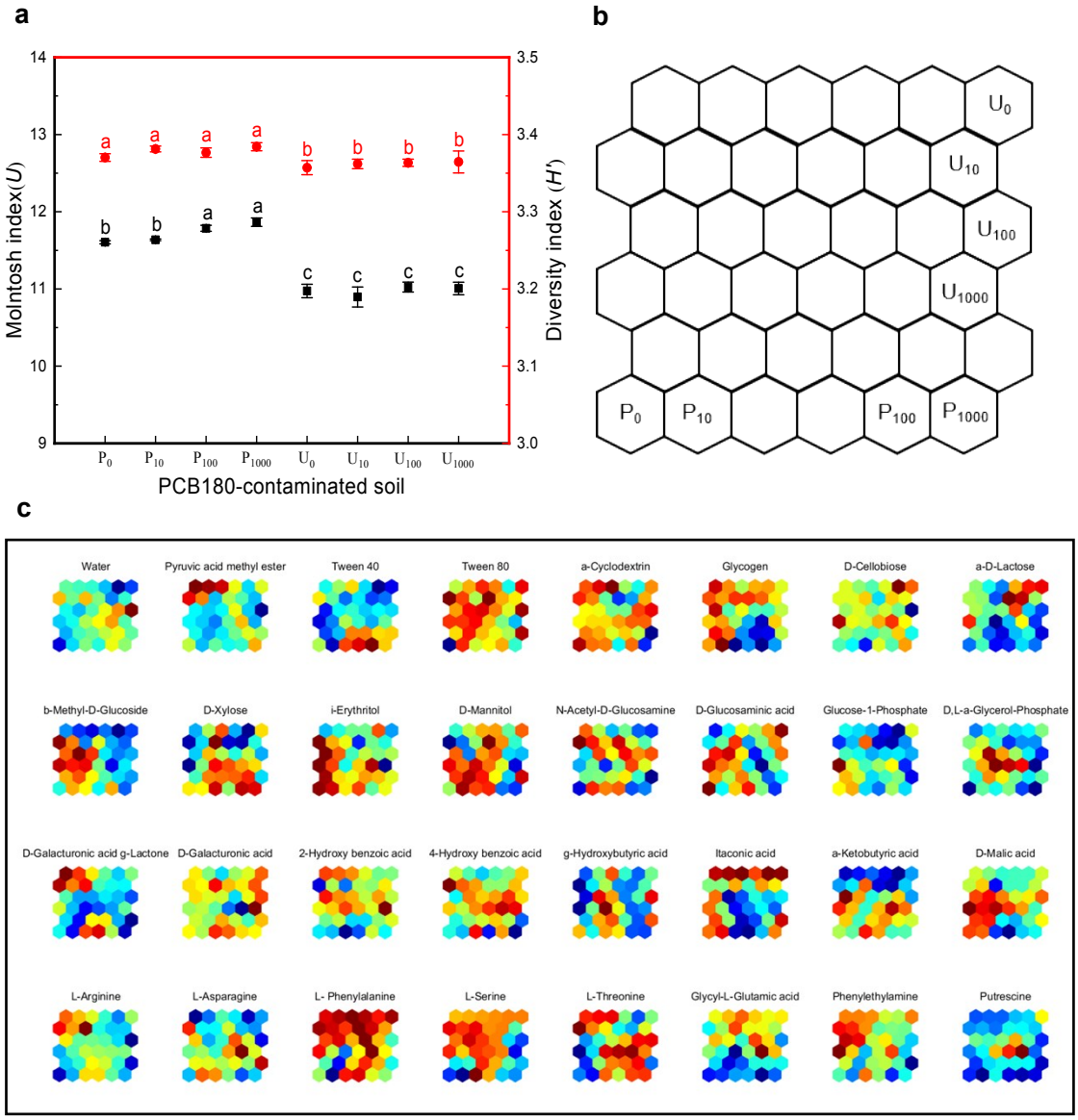

Fig. S9 (a) The McIntosh index and Shannon-Wiener diversity index, (b) a trained neural network, and (c) the ability of the microbiome to metabolize each carbon source in PCB180-contaminated soil. In panel (a), the bars indicate the mean ± standard deviation; different letters indicate significant differences at $p<0.05$ (one-way ANOVA). In panels (a, b), alfalfa-planted soil is labeled as P, unplanted soil is labeled as U, the numbers following P or U represent nZVI concentrations. In panel (c), red and blue indicate absorbance value increases and decreases, respectively.

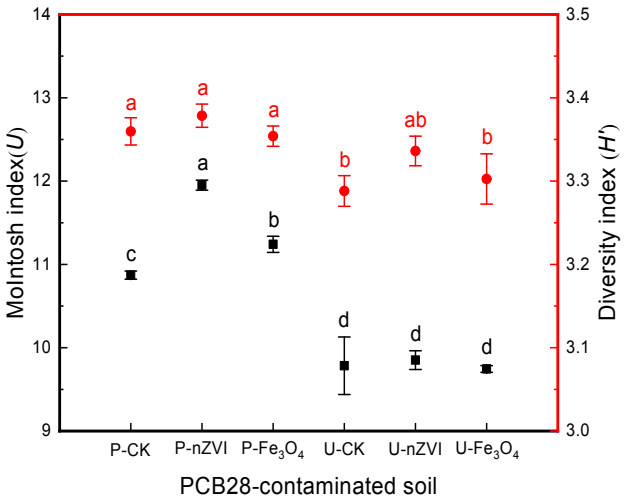

Fig. S10 The abilities of microbial communities to metabolize carbon source in PCB28-contaminated soil with control (CK), 1000 mg·kg^{-1} nZVI, and 1000 mg·kg^{-1} Fe_3O_4 nanoparticles. In panel, alfalfa-planted soil is labeled as P, unplanted soil is labeled as U. The bars indicate the mean ± standard deviations; different letters indicate significant differences at $p < 0.05$ (one-way ANOVA).

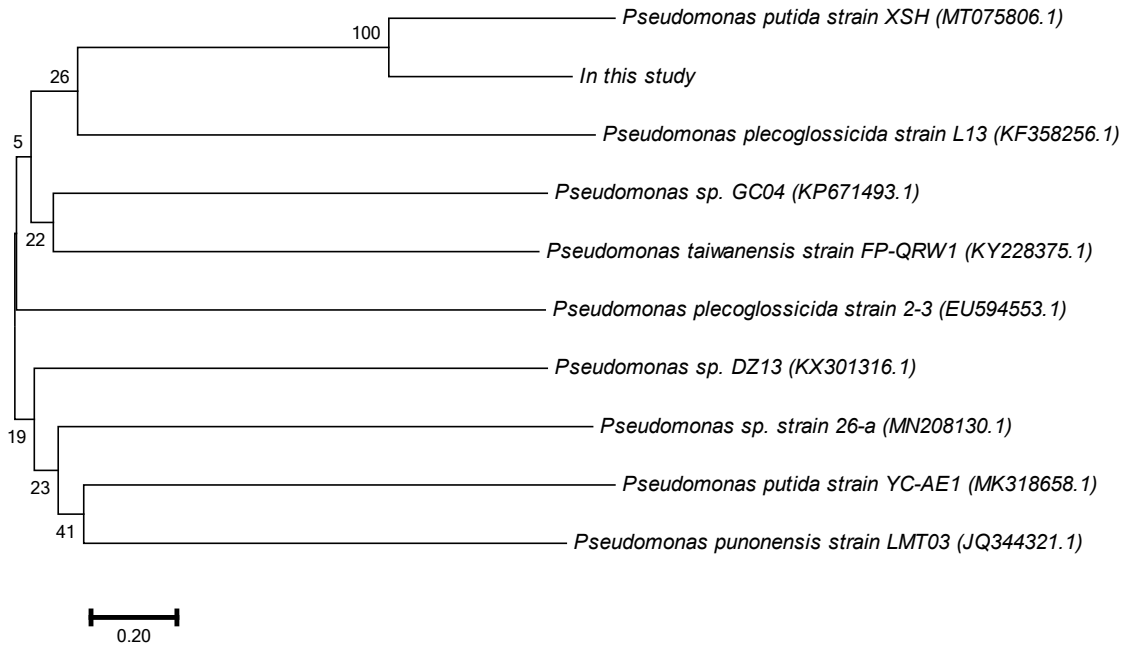

Fig. S11 Neighbor-joining algorithm tree of partial 16S rDNA gene sequences. The numbers at the branch nodes are bootstrap values based on 1000 re-samplings for maximum likelihood.

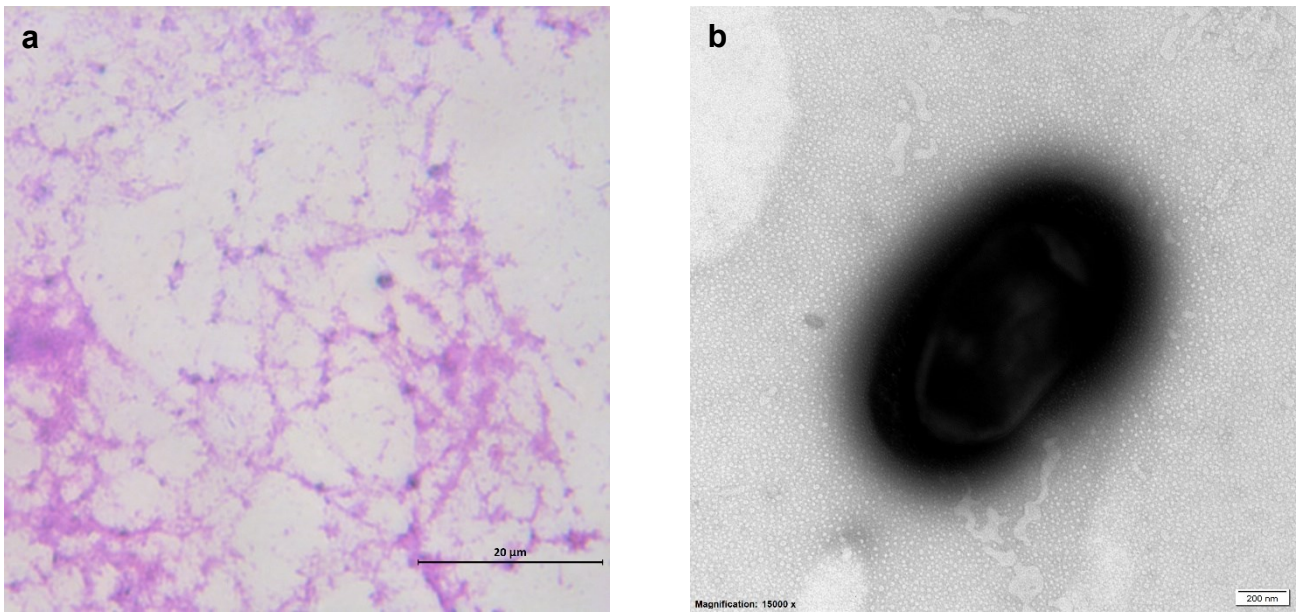

Fig. S12 (a) Gram staining and (b) TEM image of the degradation bacteria cells.

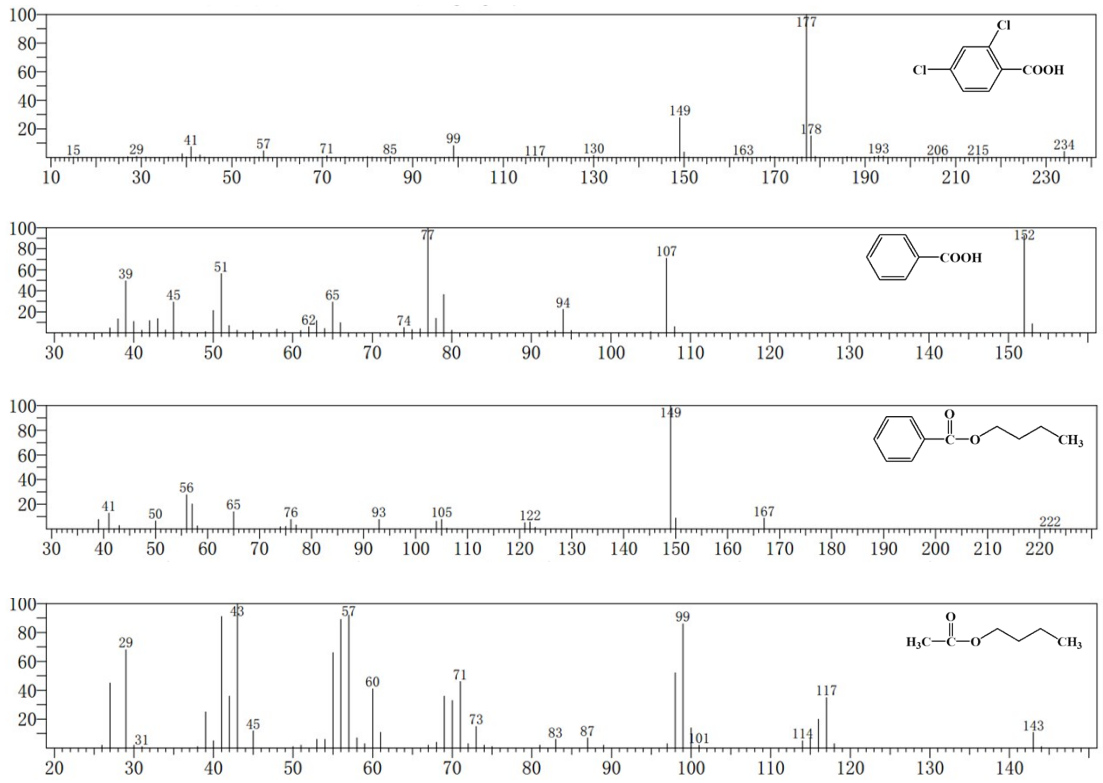

Fig. S13 The oxidation products of PCB28 identified by gas chromatography-mass spectrometry.

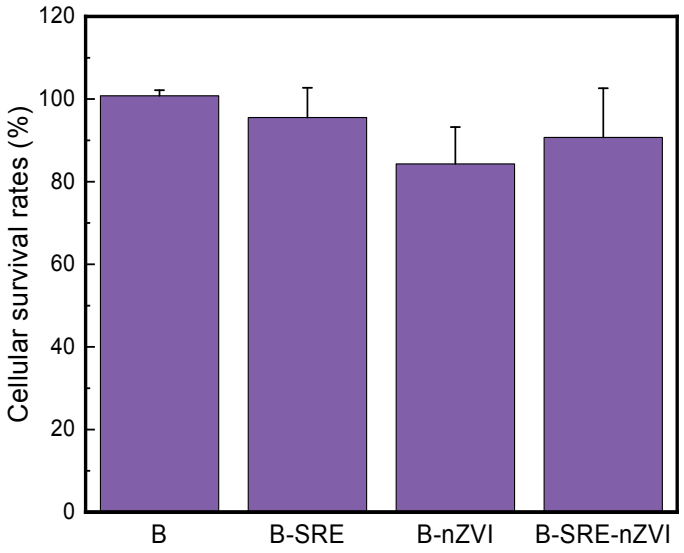

Fig. S14 Cellular survival rates in the simulated environment with PCB28 contamination. The bars indicate the mean ± standard deviation

Supporting Tables

Table S1. Chemical composition of sampled agricultural soil.

Parameters	Mean	Standard deviation
pH	6.08	0.01
N (%)	0.20	0.01
C (%)	1.84	0.07
S (%)	0.05	0.00
H (%)	0.27	0.03
O (%)	5.46	0.20
MgO (%)	0.52	0.00
Al_2O_3 (%)	12.04	0.03
SiO_2 (%)	71.43	0.03
K_2O (%)	3.19	0.01
CaO (%)	0.65	0.01
Fe_2O_3 (%)	3.56	0.01

Table S2. Distribution and classification of the 31 types of carbon sources in Biolog ECO microplate.

Serial number	1, 5, and 9	2, 6, and 10	3, 7, and 11	4, 8, and 12
A	Water	β-Methyl-D-glucoside[3]	D-Galactonic acid γ-lactone[3]	L-Arginine[2]
B	Pyruvic acid methyl ester[4]	D-Xylose[3]	D-Galacturonic acid[4]	L-Asparagine[2]
C	Tween 40[6]	i-Erythritol[3]	2-Hydroxy benzoic acid[5]	L-Phenylalanine[2]
D	Tween 80[6]	D-Mannitol[3]	4-Hydroxy benzoic acid[5]	L-Serine[2]
E	α-Cyclodextrin[6]	N-Acetyl-D-glucosamine[3]	γ-Hydroxy butyric acid[4]	L-Threonine[2]
F	Glycogen[6]	D-Glucosaminic acid[4]	Itaconic acid[4]	Glycyl-L-Glutamic acid[2]
G	D-Cellobiose[3]	Glucose-1-phosphate[3]	α-Keto butyric acid[4]	Phenylethylamine[1]
H	α-D-Lactose[3]	D, L-α-Glycerol phosphate[3]	D-Malic acid[4]	Putrescine[1]

Note: A-H and 1-12 are the column and line numbers of the 96-well Biolog ECO microplate, respectively; the superscript numbers 1-6 in the table classify the corresponding carbon sources into amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds, and polymers, respectively.