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Title: The impact on the soil microbial community and enzyme activity of two earthworm species during the bioremediation of pentachlorophenol-contaminated soils

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Abstract: The ecological effect of earthworms on the fate of soil pentachlorophenol (PCP) differs with species. This study addressed the roles and mechanisms by which two earthworm species (epigeic Eisenia fetida and endogeic Amynthas robustus E. Perrier) affect the soil microbial community and enzyme activity during the bioremediation of PCP-contaminated soils. A. robustus removed more soil PCP than did E. foetida. A. robustus improved nitrogen utilisation efficiency and soil oxidation more than did E. foetida, whereas the latter promoted the organic matter cycle in the soil. Both earthworm species significantly increased the amount of cultivable bacteria and actinomyces in soils, enhancing the utilisation rate of the carbon source (i.e., carbohydrates, carboxyl acids, and amino acids) and improving the richness and evenness of the soil microbial community. Additionally, earthworm treatment optimized the soil microbial community and increased the amount of the PCP-4-monooxygenase gene. Phylogenic classification revealed stimulation of indigenous PCP bacterial degraders, as assigned to the families Flavobacteriaceae, Pseudomonadaceae and Sphingobacteriacea, by both earthworms. A. robustus and E. foetida specifically promoted Comamonadaceae and Moraxellaceae PCP degraders, respectively.

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Date: 16th February 2015

Dear Editor,

I would like to submit this manuscript, entitled "*The impacts on the soil microbial community and enzyme activity of two earthworm species during the bioremediation of pentachlorophenol contaminated soils*", for the consideration in *The Journal of Hazardous Materials*. This study investigated the difference roles and mechanisms of two ecological earthworms (Epigeic, *Eisenia foetida* and endogeic, *Amynthas robustus E. Perrier*) on soil microbial community and enzyme activities during the bioremediation of pentachlorophenol contaminated soils. The results revealed the different ecological behaviour of earthworms during the PCP bioremediation process, which was first time revealed in this study to our knowledge. To be more precise, the main findings include:

(1) Endogeic *Amynthas robustus E. Perrier* had a better ability on enhancing soil PCP removals through stimulating microbial degradation than epigeic *Eisenia foetida*, while sterile compost as feed had no significantly effect. *Amynthas robustus E. Perrier* could better release the bound residue PCP fixed by sterile compost and soil with its strong bioturbation and humin consumption. Through enhancing the different type of enzyme activity, endogeic earthworm was good at improving the utilization efficiency of nitrogen and soil oxidation process, while epigeic earthworm was adept at promoting the cycle of soil organic matter.

(2) Both earthworms significantly increased the numbers of cultivable bacteria and actinomyces, enhanced the sole-carbon-source (carbonhydrates, carboxy acids, amino acids and amines) utilization ability, and further improved the richness and evenness of soil bacterial community, especially the *Amynthas robustus E. Perrier*.

(3) Soil bacterial community structure was significantly affected by the earthworm addition, and the phylogenetic classification demonstrated that both earthworms stimulated the growth of the indigenous PCP bacterial degraders phylogenetically assigned to families *Flavobacteriaceae, Pseudomonadaceae* and *Sphingobacteriacea*. Futhermore, *Amynthas robustus E. Perrier* had the specific capacities to enhance the bacterial degraders *Comamonadaceae*, whereas *Eisenia foetida* can enhance the bacterial families *Moraxellaceae*.

I affirm that

(1) All of the reported work is original.
(2) All authors have read and approved the final version submitted.
(3) All prevailing local, national and international regulations and conventions, and normal scientific ethical practices, have been respected.
(4) Consent is given for publication in *The Journal of Hazardous Materials*, if accepted.

The total words number is 4836.

We would like to recommend six potential reviewers for your consideration as below:

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I look forward to receiving feedback on this manuscript.

Yours sincerely

Dr. Yongtao Li

Novelty Statement

This study focused on the difference roles and mechanisms of epigeic *Eisenia foetida* and endogeic *Amynthas robustus E. Perrier* on the bioaugmentation of soil PCP degradation. Endogeic worms specifically improved the nitrogen utilization efficiency and soil oxidation process, with better capacity to enhance soil PCP removals through stimulating microbial degradation. Epigeic earthworms could promote the cycle of soil organic matter. Both earthworms significantly changed the soil bacterial community structure and stimulated the growth of the indigenous PCP degraders, phylogenetically assigned to families *Flavobacteriaceae*, *Pseudomonadaceae* and *Sphingobacteriacea*. Particularly, *E. Perrier* enhanced *Comamonadaceae* and *E. foetida* stimulated *Moraxellaceae*.

Highlights

- Endogeic earthworms improve soil PCP removals greater than epigeic one
- Endogeic earthworms enhance nitrogen utilization efficiency and soil oxidation
- Epigeic earthworms promote soil organic matter cycle
- The dominancy of PCP degraders enhanced by both earthworm species

*Abstract

ABSTRACT

The ecological effect of earthworms on the fate of soil pentachlorophenol (PCP) differs with species. This study addressed the roles and mechanisms by which two earthworm species (epigeic *Eisenia fetida* and endogeic *Amynthas robustus* E. Perrier) affect the soil microbial community and enzyme activity during the bioremediation of PCP-contaminated soils. *A. robustus* removed more soil PCP than did *E. foetida*. *A. robustus* improved nitrogen utilisation efficiency and soil oxidation more than did *E. foetida*, whereas the latter promoted the organic matter cycle in the soil. Both earthworm species significantly increased the amount of cultivable bacteria and actinomyces in soils, enhancing the utilisation rate of the carbon source (i.e., carbohydrates, carboxyl acids, and amino acids) and improving the richness and evenness of the soil microbial community. Additionally, earthworm treatment optimized the soil microbial community and increased the amount of the PCP-4-monooxygenase gene. Phylogenic classification revealed stimulation of indigenous PCP bacterial degraders, as assigned to the families *Flavobacteriaceae*, *Pseudomonadaceae* and *Sphingobacteriacea*, by both earthworms. *A. robustus* and *E. foetida* specifically promoted *Comamonadaceae* and *Moraxellaceae* PCP degraders, respectively.

***Response to Reviewers**

Reviewer #1: The authors have done a nice job responding to reviewer comments.

A few minor changes should be performed which would help clarify several points, primarily related to microbial diversity compostion in the initially unspiked soil:

1. Figure S3 shows the DGGE profile of microbial community on day 0 (unspiked soil). There are 10 predominant bands (A1-A10) in such DGGE profile that have been identified as Ralstonia, Pseudomonas and Aeromonas Page 16, line 2-10). However, the authors should include data from these bands (A1-A10) in table 2.

   Answer: Thanks for the comments. Since only seven bands have successful sequence results of all the ten predominant DGGE bands, the author therefore re-number the bands (Fig. S3 and S5) and included their accession number in Table 2

2. Partial 16S rRNA gene sequences (bands A1-A10) should also be submitted to GenBank, and the obtained accession numbers should be cited in table 2 as well. By this way the authors will be able to state that A1-A10 sequences, from spiked soils, are really different than B1-B9 bands which are already described in Table 2.

   Answer: Thanks for the comments and the sequenced bands and their accession number have been included in Table 2. The author also has more discussion related to these 16S rRNA sequences in the main manuscript.

Reviewer #2: Manuscript Number: HAZMAT-D-15-00843R1
Authors: Zhong Lin et al.
Title: The impacts on the soil microbial community and enzyme activity of two earthworm species during the bioremediation of pentachlorophenol contaminated soils

3. I have read the revised version of MS thoroughly. In this version, the authors added more results to convince that the growth of potential PCP degraders can be stimulated by the earthworm treatment. I appreciated the works done in this study. However, the readability of the MS, including the supplementary materials is still low and the overall MS is out of quality for publication in Journal of Hazardous Materials. Although the authors stated that the whole MS was revised with help of native speakers, the writing and editing definitely remain a big room for improvement, which I already indicated in my previous version of comments.

   Answer: Thanks for the comments. The author indeed has asked editing service, provided by Elsevier, to check the grammar and improve the English quality. To address the reviewer's comments, we asked a second Elsevier suggested editing service, and the certificate has been attached as supplementary materials.

4. The quality of data presentation is low. The figures are not aligned vertically. The meanings of the lower case letters (a, b, c) should be clearly specified in the legends of every figure. Due to unknown reasons, two previous comments were missing. I attached them again for authors.

Answer: The author has corrected the figure alignment and solved the problem. For figure 1 to 4, the author has modified the caption as "Bars with different lower case letters (a, b and c) refer to significant differences (ANOVA, Duncan's test, $p<0.05$) between treatments, where the same letter indicates no significant difference." to address their meaning, according to the examples of many published papers.

5. Figure 5 The sample labels in the cluster analysis are not accurate. There are two "CE2-II" in the topology.

   Answer: Thank you for the comments and the author has corrected the figure.

6. Figure 6, the phylogenetic tree is of very poor quality. Consider to merge Figure 6 and Table 3.

   Answer: Thank you for the comments. The author has corrected Figure 6 with more detailed information. From the other reviewer's comments, Table 2 includes more data for Bands A1-A6, and therefore the two figures and tables are not merged.

7. Throughout the MS, many descriptions and explanations for the PCP degraders observed in this study were presented with strong voice, and sometimes, overstatement. For examples, Page 12, line 4-5. "Some indigenous microbes (B1 to B9) might have PCP degrading capabilities and were stimulated by earthworms." The DGGE bands B1 to B9 represented the microbial populations, and their predominance was stimulated with the earthworm treatment. As discussed by the authors, the earthworm treatment can greatly increase the microbial metabolism on the organic maters from soil, compost and substances produced by the earthworms. If I am not wrong, the PCP concentration in soil is only 40 mg PCP/kg soil-compost. This level is far lower than the soil/compost organic matters that were usually as high as several percent. As a result, the dominant microbial populations represented by these DGGE bands should be more associated with the microbial metabolism of soil organic matters than with the PCP degradation.

   Answer: Thank you very much for the comments. The author agrees with the reviewer that some of the stimulated species might be related to organic matter degradation and the addition of PCP cannot support the significant growth of targeting bacteria. The author therefore corrected the whole manuscript to avoid overstatement, and the corrected parts have been marked in yellow color.

8. I have more comments and concerns for authors; however, I cannot indicate them sentence by sentence because the exact line number was not provided with the MS. Several drawbacks are provided below. How did the authors do soil sampling? Will soil sampling affect the results, since the two earthworms have varied ecological behaviors, in particular E. foetida usually in the top soil?

   Answer: The author has added the soil sampling methods in Section 2.2. We have considered the heterogeneous properties of soil and earthworm ecological behaviors, and therefore used soil column cylinder auger to collect the thorough soil from the bottom to the top soils in the pot. The samples were therefore mixed well for further chemical and biological analysis.

9. The primer set (341F and 534R) used for qPCR analysis of bacterial 16S rRNA gene copies is very weird, because there is a GC-rich fragment present in the 5' end of the primer 534R.

   Answer: Thank you for noticing this mistake. The author misused the GC-338F primer sequence data in this part. The 534R primer should be 5'-ATTACCGCGGCTGCTGGC-3') and has been corrected in the revised Supplementary Material.

10. In the preparation of the calibration curves for qPCR, a series of concentrations of plasmid DNA were prepared in terms of copies/reaction. However, the Y axis of Fig. S4 was presented with log (copies/g soil). The two types of data cannot be included in a single figure.

    Answer: Thanks for the comments. The author used the calculated data to illustrate the qPCR calibration curve in the previous version. The original data in terms of copies/reaction has been provided in the supplementary materials.

11. Page 12, 3.8 section, line 1, From qPCR results illustrated in Fig. "S4", is the DGGE image of Fig. S3 produced in this study or from the reference 19? What do the bands that are labeled with A1 to A10 stand for?

    Answer: Thanks for the comments. The author has corrected the manuscript according reviewer's suggestion. Besides, the DGGE figure S3 is the original work of this study. The sequence and Genbank accession number has been added in Table 2 with more discussion in the manuscript.

12. Page 12, " Both endogeic and epigeic earthworms stimulated the bacteria,…. with high similarity to Flavobacteriaceae and Pseudomonadaceae." The Flavobacteriaceae and Pseudomonadaceae are taxonomic names. The accurate description should be "Both endogeic and epigeic earthworms stimulated the bacterial populations represented by the Band 1, Band 2, Band 4, and Band 5 with high sequence similarity to the members related to the families Flavobacteriaceae and Pseudomonadaceae."

    Answer: Thanks for the comments and the author has corrected this and other relative sentences in accordance with the suggestion.

13. Page 13, " With phylogenic relationship to PCP-……..in the microbial community structure." I cannot understand this. If I am not wrong, no phylogenetic relationship was studied in this study.

    Answer: Thanks for the comments. The author mispresented this sentence and corrected it as "Since the PCP-4-monooxygenase *pcpB* gene was found in the family *Sphingobacteriaceae*".

14. Page 15-Page 16, " The 16S rRNA……..or earthworm's intestinal microbiota [19]." How can the five families in this study be compared to the three genera in the previous study [19]? Actually, the genus Pseudomonas is under the family Pseudomonadaceae.

Answer: Thanks for the comments and the author has corrected the sentence as “From our microbial community analysis on uncontaminated soils with or without earthworms (Fig. S3), all the dominant bands in clean soils (with high sequence similarity to the families *Aeromonadaceae*, *Burkholderiaceae*, *Xanthomonadaceae* and *Opitutaceae*) were different from the identified PCP degraders or earthworm's intestinal microbiota”. The dominant bacteria in the clean soils were aligned with high similar bacterial families, and the genus Pseudomonas was a mistake, corrected by *Aeromonadaceae*. The detailed sequences of clean soil bands were illustrated in Table 2 and Figure S3.

15. Page 16, " These results agree with previous research in that "these bacteria" have the ability to ……". Do "these bacteria" refer to the microbial populations represented by the DGGE bands or the genera Ralstonia, Aeromonas, and Pseudomonas? The description is not accurate.

    Answer: These bacteria refer to the identified PCP degraders from DGGE bands in PCP additive treatments. The author has corrected the sentence for a clearer description.

16. Page 16, " From the bacterial diversity evolution…….. Sphingomonas were dominant [31]." I cannot understand the sentence. Please re-word it.

    Answer: Thanks for the comment. “Our study found the predominance of *Comamonadaceae* and *Sphingomonas* after PCP degradation, similar to previous investigation on the evolution of microbial diversity during in PCP-degrading activated soils [31], and the results suggested that bacteria with the ability to metabolise PCP would be dominant in soils with PCP contamination.”

17. Page 16, "Sphingobacteriaceae is also known to use polycyclic or monocyclic aromatic hydrocarbons as a sole carbon source [44]". To my knowledge, the populations related to the Sphingobacteriaceae can use a wide spectrum of substrates.

    Answer: Thanks for the comment. The author does not mean *Sphingobacteriaceae* can “only” use polycyclic or monocyclic aromatic hydrocarbons, but can use them as the “sole” carbon source. For a clearer description, the sentence has been corrected as “*Sphingobacteriaceae* is also known to utilise a wide spectrum of substrates, including polycyclic or monocyclic aromatic hydrocarbons like PCP, as the sole carbon source”.

18. Page 16, " However, there might be other……..such as Flavobacteriaceae and Comamonadacea." The argument is poorly supported by the results provided in this study.

    Answer: Thank you for the comments. The author has rewritten the whole paragraph for the better description with new and more relative references.

19. Page 16-17, " The PCP oxygenase genes…….in the PCP degradation enhancement." Please make the discussion clear in connection with the context.

    Answer: Thank you for the comments. The author has rewritten the whole paragraph for the better description with new and more relative references.

20. Page 17, "With 16S rRNA stable isotope…. biodegradation in soils [51]. Check the grammar.

    Answer: Thanks for the comments and the author has corrected this sentence as “By 16S rRNA stable isotope probing technique, Dallinger and Horn have revealed a strong impact of earthworms on the active microbial community, in which the families *Pseudomonadaceae*, *Flavobacteriaceae* and *Comamonadaceae* played the key roles in 2,4-dichlorophenol (2,4-DCP) biodegradation”.

21. Page 17, " Some fungal species were also……..to accelerate soil PCP degradation." Please make the discussion clear in connection with the context, or a separate paragraph of discussion.

    Answer: Thanks for the comments. The author would like to summarize the impacts of earthworm on soil PCP degraders (both bacteria and fungi) in this paragraph. The author has modified the whole paragraph for a better description in accordance with the comments.

22. Page 18, "Change in the soil bacterial community and PCP degradation genes……..were stimulated by earthworms." I can partially agree with the conclusion. Microbial activities on the soil/compost organic matters can play a major role, and should not be ignored.

    Answer: Thanks for the comments and the author has modified the sentences for better description, as “The change in the soil bacterial community and PCP degradation genes in this study therefore suggested that the dominancy and activities of indigenous PCP bacterial degraders were stimulated by both the rich organic matters from compost additives and the direct soil microenvironment improvement by earthworms.”

*Revised Manuscript
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The impact on the soil microbial community and enzyme activity of two earthworm species during the bioremediation of pentachlorophenol-contaminated soils

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ABSTRACT

The ecological effect of earthworms on the fate of soil pentachlorophenol (PCP) differs with species. This study addressed the roles and mechanisms by which two earthworm species (epigeic *Eisenia fetida* and endogeic *Amynthas robustus* E. Perrier) affect the soil microbial community and enzyme activity during the bioremediation of PCP-contaminated soils. *A. robustus* removed more soil PCP than did *E. foetida*. *A. robustus* improved nitrogen utilisation efficiency and soil oxidation more than did *E. foetida*, whereas the latter promoted the organic matter cycle in the soil. Both earthworm species significantly increased the amount of cultivable bacteria and actinomyces in soils, enhancing the utilisation rate of the carbon source (i.e., carbohydrates, carboxyl acids, and amino acids) and improving the richness and evenness of the soil microbial community. Additionally, earthworm treatment optimized the soil microbial community and increased the amount of the PCP-4-monooxygenase gene. Phylogenic classification revealed stimulation of indigenous PCP bacterial degraders, as assigned to the families *Flavobacteriaceae*, *Pseudomonadaceae* and *Sphingobacteriacea*, by both earthworms. *A. robustus* and *E. foetida* specifically promoted *Comamonadaceae* and *Moraxellaceae* PCP degraders, respectively.

1. Introduction

Pentachlorophenol (PCP) is extensively used as a wood preservative, herbicide, pesticide, and broad-spectrum biocide [1]. Because of its stable aromatic ring, high chloride content and significant toxicity to human health, PCP is listed as a priority pollutant by the U.S. Environmental Protection Agency and by similar agencies in many other countries [2]. Although PCP has been prohibited for future use, a considerable amount of PCP residue is found in water, soils and sediments due to its chemical stability and low biodegradability. Its toxicity is expected to last for a long time [3]. Indigenous microbes play an important role in the detoxification and clean-up of PCP contaminated soil [4, 5]. PCP degraders are ubiquitous at contaminated sites with wide spread PCP contamination [6], but their degradation rates are relatively low in soil due to low solubility/bioavailability of PCP, poor nutrient level and inappropriate soil redox conditions [7, 8]. The natural attenuation therefore often takes years or even decades to occur. Bioaugmentation [5, 9] and biostimulation [10], aiming at the addition of exogenous degrading strains or growth substrates, respectively, are viewed as cost-effective and environmentally friendly methods for organic pollution mitigation at industrial sites [11]. These two strategies could significantly improve the existence or activities of PCP degraders, and further accelerate PCP bioremediation. However, there are disadvantages to normal biostimulation or bioaugmentation treatment, such as low substrate effectiveness, die-off of the inoculated degrader by starvation and inhibition due to antibiotic-producing soil microbes, and insufficient oxygen supply for aerobic biodegradation [12-15]. This is the reason that bioaugmentation or biostimulation is the protagonist of laboratory successes but a spectator in field applications.

Earthworm treatment strategies could effectively improve bioremediation efficiency,

reduce risk and decrease management expenditures. Earthworms are highly adaptable and have a large reproductive capacity, showing high tolerance and resistance to organic pollutants [13, 16]. Through mucilaginous secretions and soil organic matter transformation, earthworms increase soil microbial activity and nutrient availability [17]. It is reported that earthworms can stimulate both bacterial [18] and fungal [19, 20] species related to PCP degradation. Furthermore, soil aeration is strengthened by bioturbation, which improves transportation and dispersal of microorganisms and enhances cell-mineral interactions [17, 21, 22]. These ecological functions allow earthworms to effectively ameliorate disadvantages in soil properties and offset the limitations of bioaugmentation. According to their ecological behaviour, earthworms are categorised as epigeic and endogeic, and have variable biological, chemical and physical effects on soil; ultimately, they influence the fate of organic contamination in soils differently [13, 22]. Despite numerous studies on the bioremediation capability of domesticated earthworms, little is known about the effect of different earthworm species on the soil microbial community and enzyme activity during bioremediation of PCP contaminated soils.

This study determined the role of two ecologically distinct earthworms (epigeic *E. foetida* and endogeic *A. robustus*) in the treatment of PCP contaminated soils. Their effect on soil enzyme activity and biomass of cultivable microorganisms during PCP bioremediation was studied. Additional analyses revealed the influence of the earthworms on changes in bacterial function and community diversity. The sequence and phylogenic classification of PCP degraders was discussed to demonstrate the role of these earthworm species in bioaugmentation improvement.

2. Materials and Methods

2.1 Sites and sampling

Surface soil samples (0-20 cm) were collected from the Teaching and Research Base of the South China Agricultural University (23°14'22"N, 113°37'51"E) in Zengcheng City, China. All samples were air dried, passed through a 2 mm sieve, and adjusted to 60% moisture prior to use. Composts were collected from the cattle ranch of the South China Agricultural University (23°09'29"N, 113°21'37"E), aerobically fermented at approximately 40°C for 20 d and then passed through a 2 mm sieve before use. No PCP was detectable in either soil or compost samples. The chemical properties of soil and compost samples are shown in Table S1. The epigeic earthworm, *E. foetida*, and the endogeic earthworm, *A. robustus*, were purchased from the South China Agricultural University Research Base in Jiangmen City and Yingde City, China, respectively. Pentachlorophenol, acetone and ethanol were analytic grade and purchased from Sigma-Aldrich (USA), and all the other chemicals were purchased from Chengshuo Company (China).

2.2 Experimental design and procedure

As shown in Table 1, four treatments were created to evaluate earthworm-associated bioremediation in pots as follows: neither compost nor earthworms, *CK*; 4.5% sterile compost without earthworms, *C;* and 4.5% sterile compost and one of two earthworm species, *CE1* and *CE2*, respectively. The treatment of unpolluted soils with or without compost and earthworms has been used in our previous research [19] to identify the source of key PCP degraders from soils or earthworm intestinal microbiota. Given PCP contamination levels in China [23, 24] and the PCP lethal dosage for earthworms (LD_{50}, 73 mg kg^{-1} for *Eisenia fetida* and 150 mg kg^{-1} for *Amynthas robustus E. Perrie*, Fig. S1), 40 mg kg^{-1} (wet soil weight) was set as the initial PCP contamination

concentration. This concentration did not significantly affect earthworm activities. The preparation of artificial PCP contaminated soils followed previous protocols [17], as described in the Supplementary Material. For soil sampling at different time points, approximately 10.0 grams of soil sample (from bottom to top) were collected using a soil column cylinder auger, and then mixed well for chemical and biological analysis.

2.3 Chemical analysis of soils and composts

Total nitrogen was determined by Kjeldahl digestion [25]. After digestion with perchloric acid and hydrofluoric acid, total phosphorus and potassium were measured by colourimetric assay (ammonium molybdate) in a spectrophotometer and by atomic absorption spectrometry, respectively [14, 25]. The measurement of soil pH (combined glass electrode with soil/water=1/2.5), maximum water holding capacity (cutting ring method) and organic matter (potassium dichromate oxidation-volumetric method) followed previously described methods [14, 22]. The fractionation of humic substances followed Nieman et al. [26] and Scelza et al. [27].

2.4 Residual soil PCP analysis

Soil extractable PCP was obtained following the method of Khodadoust [28]. The soil samples (2 g, dry weight) were weighed in polycarbonate centrifuge tubes and suspended in 40 mL water:ethanol (50:50, v/v). The tubes were sealed, placed on an orbital shaker at 180 rpm for 1 h, and then centrifuged at 3,000 g for 15 min. The supernatants were passed through a 0.45 μm filter and the precipitate was separated as humic acid, fulvic acid and humin fractions using 0.5 mol L^{-1} NaOH and HCl, respectively [26]. The earthworm-accumulated PCP was extracted according to Parrish et al. [29], and the bound PCP residues were extracted from the three fractions as described by Nieman et al. [26] and Scelza et al. [27].

Soil PCP was quantified using high-performance liquid chromatography (HPLC, Waters 1525/2487, USA), supplemented with a Waters 1525 binary pump, an analytical reversed-phase column (5 μm Pinnacle II C18, 4.6 mm (i.d.) and 25 cm long, Waters, USA) and a Waters 2487 dual λ absorbance UV/vis detector. The mobile phase consisted of 80% methanol and 20% water (1% acetic acid) and was pumped at a rate of 1.0 mL min^{-1}. Measurements were taken under isocratic conditions at 40±1°C at a wavelength of 220 nm. Soil PCP concentration was determined according to the standard curves from five external PCP standards.

2.5 Microbial analysis

The total numbers of cultivable bacteria, fungi, and actinomyces were counted as colony forming units (CFUs) [30]. Microbial biomass, activities and soil enzyme activities were analysed in accordance with previous instructions [22-24]; the details are listed in the Supplementary Materials. The microbial community was further assessed by the following two indices according to the results of the Biolog EcoPlate™ (Supplementary Materials): Average Well Colour Development (*AWCD*, to evaluate the ability to use different carbon sources) and Richness (*S*, the number of positive wells on the EcoPlate™, to evaluate utilisation of carbon sources). *AWCD* is calculated using the following Equation (1), where A_i represents the absorbance at 590 nm of the *i*th well and A_1 is the 590 nm absorbance of control wells on the Biolog EcoPlate™:

$$AWCD = \sum(A_i - A_1)/31 \quad (1)$$

The soil microbial community structure on day 42 was evaluated by Denaturing Gradient Gel Electrophoresis (DGGE) with 16S rRNA amplification of the V3 region (Supplementary Material). Representative bands in the DGGE gels were purified and

re-amplified until no multiple bands were observed on the DGGE gel. The targeting bands were subsequently purified with an Omega microElute gel extraction kit (Omega Biotek, USA) and cloned into *Escherichia coli* JM109 supplied with the TA cloning kit (Takara, Japan). The positive *E. coli* clones were selected on Luria-Bertani (LB) agar with 100 μg L^{-1} ampicillin after 16 h incubation at 37°C. Plasmids were extracted with a plasmid mini kit (Omega Biotek, USA) and checked by gel electrophoresis. The appropriate insertions were sequenced by the Beijing Genomics Institution (BGI, China) and further compared to the GenBank database from the National Centre for Biotechnology Information (NCBI) using BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The neighbour-joining phylogenic tree of the present community was analysed with MEGA (Version 4) and evaluated using bootstrap values based on 1000 replicates. Quantitative polymer chain reaction (qPCR) was carried out to determine the amount of total bacteria (16S rRNA) and PCP-4-monooxygenase gene (*pcpB*) [31] after PCP degradation treatments (Supplementary Material).

2.6 Data analysis

Variance analysis was used to distinguish significant differences between each treatment. All statistics were carried out in SPSS (Version 13.0), and significance was set at $p<0.05$ with differences between treatments marked with different letters of the alphabet.

3. Results

3.1 PCP biodegradation process

Fig. 1a shows a gradual decrease in extractable PCP residues for all treatments during the 42 days of bioremediation. There was no difference ($p>0.05$) among treatments at

0, 7 and 14 d. However, by the end of the incubation period (42 days), CE1 (endogeic *A. robustus* earthworm) removed more PCP (83.4%) than CE2 (epigeic *E. fetida* earthworm, 49.2%), and these were both higher than CK (28.3%) and C (28.1%). The removal of soil-bound PCP residues was similar; the highest in *C* (1.66 mg kg^{-1}), followed by *CK* (1.34 mg kg^{-1}), *CE2* (1.45 mg kg^{-1}) and *CE1* (1.00 mg kg^{-1}). PCP accumulation was small in both epigeic (0.04 mg kg^{-1}) and endogeic (0.05 mg kg^{-1}) earthworms, only accounting for 0.10% and 0.14% of initial PCP concentrations (Fig. 1b). The survival rate of epigeic *E. foetida* and endogeic *A. robustus* was 100% and 87%, respectively. Earthworms, especially the endogeic species (*A. robustus)*, significantly improved the removal efficiency of soil extractable and bound residual PCP.

3.2 Soil chemical properties

After 42 days of bioremediation, the chemical properties of the soil differed depending on the treatments (Table S2). The soil pH in *CE1* and *CE2* was higher than in *C* and *CK*; the latter had the lowest pH. The addition of sterile compost increased the content of organic matter in *C*, *CE1* and *CE2*. Although total nitrogen was equal between treatments (p>0.05), the amount of NH_4-N and NO_3-N in earthworm treatments (*CE1* and *CE2*) was higher than in *CK* and *C*. There was no difference in fulvic acid between treatments, whereas humic acid was higher in *C* and *CE1* and humin was higher in *CK* and *C*.

3.3 Cultivable soil microorganisms

The number of bacteria, fungi, and actinomyces for different treatments is shown in Fig. 2. There were more cultivable bacteria in the earthworm treatments (*CE1* and *CE2*) than in *C* and *CK*. After 42 days of bioremediation, the number of cultivable

bacteria per treatment was 29.0×10^5 CFU g^{-1} (*CE1*), 25.6×10^5 CFU g^{-1} (*CE2*), 19.6×10^5 CFU g^{-1} (*C*) and 14.9×10^5 CFU g^{-1} (*CK*). Cultivable fungi showed a dramatic increase from 0 to 14 d (Fig. 2b), with no difference between treatments throughout the bioremediation process. The final amount of cultivable fungi in *CE1*, *CE2*, *C* and *CK* treatments was 5.0×10^4 CFU g^{-1}, 4.8×10^4 CFU g^{-1}, 5.3×10^4 CFU g^{-1} and 4.5×10^4 CFU g^{-1}, respectively. As shown in Fig. 2c, endogeic *A. robustus* stimulated cultivable actinomyces and after 42 days of bioremediation, they were higher (5.2×10^5 CFU g^{-1}) than for the other treatments (*CE2*, 2.9×10^5 CFU g^{-1}; *C*, 1.5×10^5 CFU g^{-1}; and *CK*, 1.3×10^5 CFU g^{-1}) (Fig. 2c). The contribution of endogeic and epigeic earthworms to cultivable actinomyces and bacteria was identified, indicating their roles in the microbial community and their function in PCP degradation.

3.4 Soil enzyme activity

Soil enzyme activity represents the capacity to metabolise carbon and nitrogen in soils. Fig. 3 shows urease, catalase, invertase and cellulase activities in the treatment soils. Earthworm treatments (*CE1* and *CE2*) enhanced urease activity from 0.13-0.14 mg g^{-1} to 0.32-0.46 mg g^{-1}, which did not change in *C* and *CK* (Fig. 3a). Similarly, catalase activity did not change in *C* and *CK*, whereas in *CE1* and *CE2* it increased from 0.22-0.23 mg g^{-1} at 0 d to 0.76-0.95 mg g^{-1} at 28 d, and then stabilised (Fig. 3b). Fig. 3c shows an increase in invertase activity after 42 days of bioremediation in *C*, *CE1* and *CE2*, of 7.7 mg g^{-1}, 5.5 mg g^{-1} and 6.0 mg g^{-1}, respectively. Earthworm treatments remarkably enhanced soil cellulase activity, 2.2 mg g^{-1} in *CE1* and 2.7 mg g^{-1} in *CE2*, both over twice as high as initial cellulase activities in *C* and *CK* (Fig. 3d). Soil enzyme activity was significantly improved by earthworms.

3.5 Total microbial activity and biomass

Soil respiration rates varied throughout the bioremediation process. The highest rates occurred from 7 to 28 d and were 1.2, 3.6, 7.1 and 4.7 mg CO_2 kg^{-1} soil for *CK*, *C*, *CE1* and *CE2*, respectively (Fig. 4a). The biomass carbon in *CE1* (447.2 mg kg^{-1}) and *CE2* (367.3 mg kg^{-1}) was much higher than in *CK* (146.1 mg kg^{-1}) and *C* (205.2 mg kg^{-1}) (Fig. 4b), which was similar to biomass nitrogen, in which *CE1* (99.9 mg kg^{-1}) and *CE2* (96.3 mg kg^{-1}) were higher than *C* (62.0 mg kg^{-1}) and *CK* (40.1 mg kg^{-1}) (Fig. 4c). Both microbial activity and biomass were enhanced by earthworm treatment, indicating an effect of earthworms on the microbial community.

3.6 Soil bacterial functional diversity

Fig. S2 illustrates the variation in *AWCD*, representing the carbon utilization functional diversity of soil bacteria. The two earthworm treatments *CE1* and *CE2* (1.87 ± 0.39 and 1.72 ± 0.39) had similar *AWCD* values, which were higher than the non-earthworm treatments *C* and *CK* (1.34 ± 0.73 and 1.31 ± 0.58, $p<0.05$). Compared to the behaviour of PCP bioremediation, the results indicated that earthworms increased the carbon utilization capacities of indigenous microbes and therefore improved their PCP degradation performance. The higher richness (*S*) in *CE1* (endogeic *A. robustus*) than *CE2* (epigeic *E. fetida*), *C* and *CK*, suggested a significantly stronger effect of endogeic earthworms on microbial carbon utilization. The carbon source utilisation efficiency data (Table S3) did not reveal any impacts on miscellaneous or polymer metabolism. The indigenous microbes in earthworm treatments (*CE1* and *CE2*) used carbohydrates, carboxyl acids, amino acids and amines more efficiently, indicating that earthworms can stimulate the use of small molecule carbon sources, attributing to the enhanced carbon utilization capacities.

3.7 Analysis of microbial community structure

The change in soil microbial community diversity (16S rRNA DGGE) in earthworm treatments is shown in Fig. 5. There was no difference within replicates (I, II and III for each treatment), whereas the dominant bands between treatments were different. Cluster analysis showed more similarity between the two earthworm treatments (*CE1* and *CE2*), than between these and the cluster formed by the control (*CK*) and sterile compost (*C*). Thus, earthworm addition had a significantly greater impact on the soil microbial community than compost addition. Some indigenous microbes (B_1 to B_9, Fig. 5) were stimulated by earthworms, significantly different from the bands (A_1 to A_7, Fig. S3) in the uncontaminated treatments (Table 2). It is possible that they might have PCP-degrading capacities or associate with compost organic matter metabolism to accelerate PCP degradation. From the sequence and phylogenic classification via GenBank BLAST analysis and RDP classifier, their closest known relatives are listed in Table 2 and Fig. 6. Both endogeic and epigeic earthworms stimulated the bacterial populations represented by band B_1 (KM284689), B_2 (KM284690), B_4 (KJ137169) and B_9 (KM284692), with high sequence similarity to the families *Flavobacteriaceae* and *Pseudomonadaceae*. Two strains (KM284691 and KM284693) were only promoted by endogeic earthworm treatment and were close to the families *Comamonadaceae* and *Sphingobacteriaceae*. Epigeic earthworm addition stimulated the PCP-degrading or organic matter metabolism microorganisms (KM284694, KJ137165 and KJ137166) with high sequence similarity to the families *Moraxellaceae*, *Flavobacteriaceae* and *Sphingobacteriaceae*. Compared to our previous research of the effect of earthworms in PCP-contaminated and sterile soils [19], these species are not related to the PCP degraders in the earthworm intestinal microbiota (*Cupriavidus* and *Aeromonas*), and they are therefore the indigenous

microbes responsible for PCP degradation or organic matter metabolism to accelerate PCP degradation.

3.8 PCP-4-monooxygenase gene (pcpB)

Using the qPCR calibration curve illustrated in Fig. S4, the copy numbers of 16S rRNA and the PCP-4-monooxygenase (*pcpB*) gene were quantified for each treatment. There was no significant difference ($p>0.05$) in total bacterial 16S rRNA between *CK* ($3.27\pm0.15 \times10^{10}$ copies g^{-1}), *C* ($3.22\pm0.09 \times10^{10}$ copies g^{-1}), *CE1* ($2.37\pm0.17 \times10^{10}$ copies g^{-1}) and *CE2* ($3.09\pm0.01 \times10^{10}$ copies g^{-1}) treatment. *CE1* treatment had the highest *pcpB* gene abundance ($5.97\pm0.23 \times10^{6}$ copies g^{-1}), followed by *CE2* treatment ($4.16 \pm 0.01 \times10^{6}$ copies g^{-1}). They were both significantly higher (approximately 1.73 to 2.16 times) than the *C* and *CK* treatments ($2.41\pm0.01 \times10^{6}$ copies g^{-1} and $2.22 \pm 0.01 \times10^{6}$ copies g^{-1}, respectively). Because the PCP-4-monooxygenase *pcpB* gene was found in the family *Sphingobacteriaceae* [31], the results provide additional evidence that the PCP degrader *Sphingobacteriaceae* was enriched in the *CE1* and *CE2* systems, similar to our conclusion from the microbial community structure.

4. Discussion

The role of earthworms in soil PCP biodegradation was studied using extractable and bound residual PCP during 42 days of bioremediation. In sterile compost (*C*) and control (*CK*) treatments, there was limited PCP degradation and no difference ($p>0.05$) after 42 days. Therefore, sterile compost did not affect PCP bioremediation by indigenous microbes. Both endogenic (*CE1*) and epigeic (*CE2*) earthworm treatments enhanced soil PCP removal, endogeic more than epigeic earthworms (Fig. 1a). This

enhancement could be due to bioturbation and promotion of bacterial-mineral interactions by the earthworms [17, 21, 22]. The microbial activity and biomass data in this study (Fig. 2 and Fig. 3) suggest the stimulation of indigenous microbes, especially cultivable bacteria and actinomyces, in the earthworm treatment groups. Key environmental variables for PCP bioremediation included soil moisture, oxygen and nutrient levels, which may be maintained and stabilised by earthworm activities. Earthworm movements augment soil porosity and oxygenation, increasing soil surface area and bioavailability of PCP, therefore enhancing its biodegradation [32-34]. Additionally, the mucus, urine and cast of earthworms are nutritionally rich and have the potential to stimulate indigenous microorganisms and aid in PCP bioremediation [35]. Our results showed that earthworm treatments could neutralise soil pH and increase available carbon and nitrogen (dissolved organic matters, NH_4-N and NO_3-N) for soil microorganisms (Table S2). A higher consumption of humin was another way earthworms improved the health and fertility conditions of acidic soils. Similarly, Luepromchai et al. reported accelerated removal of polychlorinated biphenyl via earthworm remediation, and the distribution and abundance of polychlorinated biphenyl-degrading microorganisms were improved [21]. Dechlorination of soil dichlorodiphenyltrichloroethane (DDT) was also enhanced by earthworm-stimulated aerobic degradation by soil microorganisms [22]. The addition of sterile compost provided food for earthworms to support their growth and reproduction, and avoided the risks of exogenous microbes. Several previous studies indicated that the addition of organic material was positively correlated to the survival of earthworms in organic contaminated soil (i.e., weight gain, reproduction and presence of cocoons or juveniles) [36]. Contreras-Ramos et al. reported that *E. fetida* had a low survival rate and pronounced weight loss when they did not add organic matter during the

bioremediation of phenanthrene- and anthracene-contaminated soils [33].

This work found an increase in the enzymatic activity of urease, catalase and cellulase in earthworm treatments (*CE1* and *CE2*). Specifically, urease and catalase activities in *CE1* were higher than in *CE2*, whereas cellulase activity was higher in *CE2* (Fig. 3). Soil enzyme activity, which represents the level of substrate metabolism in soils, shows the biological features of soil quality [37]. Urease improves the utilisation efficiency of nitrogen and promotes the soil nitrogen cycle; catalase stimulates the oxidation process and enhances soil fertility. Invertase and cellulase promote the conversion of carbohydrates and participate in the soil organic matter cycle, respectively [37, 38]. This study suggests that endogeic earthworms (*A. robustus*, *CE1*) enhanced nitrogen utilisation efficiency and soil oxidation, whereas epigeic earthworms (*E. fetida*, *CE2*) promoted the soil organic matter cycle. Less bound residual PCP in *CK* than *C* indicates that sterile compost has a role in absorbing PCP. After 42 days of bioremediation, *CE1* had less bound residual PCP than *CE2*, showing that endogeic earthworms disrupt bound residual PCP more effectively. Because PCP is mainly entrapped in soil organic matter fractions, it cannot be accessed by soil microbes and has a lower bioavailability for remediation. These results agree with the different ecology and habits of the two earthworm types that were studied. Epigeic *E. foetida* usually live in topsoils and the litter layer, with a preference for rich organic matter but relatively poor burrowing ability [22, 33]. By crushing organic debris into tiny particles, therefore accelerating the decomposition of organic matter, and secreting metabolic products, *E. foetida* favours the growth of indigenous soil microorganisms [13, 33]. PCP degradation was thus improved because of higher nutrient availability in the soil. Endogeic *A. robustus* improved bacterial-mineral interaction through bioturbation and movement [13, 32], thus

enhancing the release of PCP residues. Due to its strong burrowing capacity, *A. robustus* increases soil porosity and provides more oxygenation under the soil surface [39]. By feeding on both decayed organic matter and soil particles, the absorbed PCP was degraded by the intestinal flora and digestive enzymes in the gut of endogeic earthworms [18, 22, 40]. Therefore, *A. robustus* enhances soil PCP degradation more than *E. foetida* via the increase in PCP bioavailability, soil aeration and intestinal digestion.

The abundance and activity of appropriate microorganisms are key to successful PCP biodegradation [41]. Our results indicated that earthworms improved soil respiration and microbial biomass (Fig. 4), further enhancing the use of small molecule carbon sources and soil microbial richness and evenness (Table S3 and Fig. 4). Indigenous bacterial functional and community diversity in earthworm treatments also changed. The 16S rRNA phylogenic trees revealed that the dominant DGGE bands belonged to the five families *Flavobacteriaceae*, *Pseudomonadaceae*, *Comamonadaceae*, *Sphingobacteriaceae* and *Moraxellaceae* (Table 2). From our microbial community analysis of uncontaminated soils with or without earthworms (Fig. S3 and Fig. S5), all the dominant bands in clean soils (with high sequence similarity to the families *Aeromonadaceae*, *Burkholderiaceae*, *Xanthomonadaceae*, *Enterobacteriaceae* and *Opitutaceae*) were different from the identified microbes responsible for PCP degradation or organic matter metabolism (Table 2). From previous research, all of the identified bacteria in the DGGE bands with PCP addition have the ability to degrade various organic pollutants, such as PCP, chlorinated phenols, polycyclic aromatic hydrocarbons (PAHs) and benzoic acid [31, 42, 43]. Crawford and Ederer isolated the following four PCP degrading bacteria from geographically diverse areas: *Arthrobacter*, *Flavobacterium*, *Pseudomonas* and *Sphingomonas* [42]. Lange et al.

identified PCP-4-monooxygenase in *Flavobacterium*, which catalyses the oxygenolytic removal of the first chlorine from PCP [43]. Our study found a predominance of *Comamonadaceae* and *Sphingomonas* after PCP degradation, similar to previous investigations on the evolution of microbial diversity in PCP-degrading activated soils [31]. The results suggested that bacteria with the ability to metabolise PCP would be dominant in soils with PCP contamination. *Sphingobacteriaceae* is also known to utilise a wide spectrum of substrates, including polycyclic or monocyclic aromatic hydrocarbons, such as PCP, as their sole carbon source [44]. From our analysis of the quantitative change of the PCP-4-monooxygenase gene, the copy number of the *pcpB* gene was enriched approximately 2 times in earthworm treatments (*CE1* and *CE2*), compared to the control treatments (*C* and *CK*). However, this might be underestimated due to the existence of other functional PCP oxygenase genes [45] in other microbial species in this study, such as the families *Sphingobacterium* and *Comamonadacea*. For example, two new PCP-degrading genes (*pcpA* and *pcpC*) were identified in *Sphingobium chlorophenolicum* [46], which could not be amplified with *pcpB* primers. Some recent evidence also suggested that there is huge diversity of *pcpB* genes within different *Sphingobium* species [47] or even across other species via horizontal gene transfer [48]. Furthermore, most of the PCP oxygenase genes were characterized in cultivable PCP degraders [49, 50], but the majority of uncultivable microorganisms (>90%) might contain new types of PCP-degrading genes hidden from current techniques. Culture-independent approaches [6, 51] are therefore suggested for future research to quantify all the functional PCP-degrading genes and reveal their diversity *in situ*, for a deeper understanding of the roles of earthworms in PCP degradation enhancement.

Vermicomposting can simulate either bacterial or fungal activities to improve PCP

degradation [40, 52, 53]. Using a 16S rRNA stable isotope probing technique, Dallinger and Horn have revealed a strong impact of earthworms on the active microbial community, in which the families *Pseudomonadaceae*, *Flavobacteriaceae* and *Comamonadaceae* played key roles in 2,4-dichlorophenol (2,4-DCP) biodegradation [52]. *Pseudomonas* was one of the dominant atrazine degraders in earthworm burrow linings [53]. Bernard's work showed that endogeic earthworms affected bacterial functional communities and organic matter metabolism, strongly stimulating the growth of several bacterial families, such as *Flavobacteriaceae*, *Chitinophagaceae* and *Sphingobacteriaceae* [40]. Some fungal species responsive to cellulose or PCP degradation were also previously identified in soil PCP degradation with earthworms, including *Mucoraceae*, *Tremellaceae*, *Trichocomaceae* and *Hypocreaceae* [19]. These fungal species had roles in cellulose decomposing (*Mucoraceae* and *Tremellaceae* for plant-derived cellulose degradation) [54] or PCP biodegradation (*Trichosporon* for chlorpyrifos mineralization) [55]. Thus, earthworm treatment could improve the bacterial and fungal activities for compost decomposing, secrete small molecular metabolites to benefit the soil microbial community, and directly stimulate PCP degraders (both bacteria and fungi) to accelerate soil PCP degradation.

5. Conclusions

Both endogeic *A. robustus* and epigeic *E. foetida* earthworms accelerated PCP degradation. Cultivable bacteria and actinomyces were promoted, increasing their ability to use small molecule carbon sources. The soil microbial community was also changed by the earthworm treatments. Endogeic *A. robustus* had a better PCP degradation performance than epigeic *E. foetida*, due to its strong bioturbation and humin consumption, which released bound residual PCP absorbed by sterile composts

and soils. Because of their differing effects on soil enzyme activity, endogeic *A. robustus* and epigeic *E. foetida* preferentially enhanced the soil nitrogen and carbon cycle, respectively. The change in the soil bacterial community and PCP degradation genes in this study therefore suggested that the dominance and activities of indigenous PCP bacterial degraders were stimulated by both the rich organic matter from compost additives and the direct soil microenvironment improvement by earthworms. The dominant functional microbes were assigned to the families *Flavobacteriaceae*, *Pseudomonadaceae* and *Sphingobacteriacea*. In particular, endogeic *A. robustus* stimulated *Comamonadaceae*, whereas epigeic *E. foetida* stimulated *Moraxellaceae*. Consequently, these two types of earthworms have different ecological roles in the PCP bioremediation process, which, to our knowledge, was revealed for the first time in this study.

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7. Table

Table 1. Earthworm treatments.

Treatment	PCP (mg kg^{-1})	Soil (kg)	Sterile compost (%)	Earthworm (individuals)	
				Endogeic *Amynthas robustus E. Perrie*	Epigeic *Eisenia fetida*
CK	40	1	–	–	–
C	40	1	4.5	–	–
CE1	40	1	4.5	8	–
CE2	40	1	4.5	–	15

Note: *CK*, blank control (without compost or earthworms); *C*, sterile compost treatment (no earthworms); *CE1*, *Amynthas robustus E. Perrie* with sterile compost; *CE2*, *Eisenia fetida* with sterile compost.

Table 2. Closest phylogenic matches for soil bacterial 16S rRNA gene sequences.

Band[a,b]	Taxonomic group[c]	Closest relatives (accession no.)[d]	Similarity (%)	Accession no.
B_1	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae*	*Myroides* sp. from soil (KC456556)	100	KM284689
B_2	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae*	*Myroides* sp. from soil (KC456517)	99	KM284690
B_3	*Proteobacteria, Betaproteobacteria, Comamonadaceae*	*Comamonas* sp. from sludge (KC011355)	99	KM284691
B_4	*Proteobacteria, Gammaproteobacteria, Pseudomonadaceae*	*Pseudomonas sp.* (X93997)	100	KJ137169
B_5	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae*	Uncultured *Flavobacterium* sp. from rhizosphere (JX500585)	100	KM284692
B_6	*Bacteroidetes, Sphingobacteriia, Sphingobacteriaceae*	*Sphingobacterium* sp. from soil (HQ860323)	100	KM284693
B_7	*Proteobacteria, Gammaproteobacteria, Moraxellaceae*	*Acinetobacter* sp. from soil (JX979119)	100	KM284694
B_8	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae*	*Myroides sp.* from a swine lagoon (EF640606)	99	KJ137165
B_9	*Bacteroidetes, Sphingobacteriia, Sphingobacteriaceae*	Uncultured *Mucilaginibacter sp.* from soil (FJ037541)	99	KJ137166
A_1	*Bacteroidetes, Sphingobacteriia, Sphingobacteriaceae*	*Nubsella sp.* from soil (EF179856)	100	KJ137168
A_2	*Proteobacteria, Gammaproteobacteria, Xanthomonadaceae*	*Dyella* sp. from soil (NR_044540)	100	KJ137171
A_3	*Proteobacteria, Gammaproteobacteria, Aeromonadaceae*	*Aeromonas* sp. (AB698740)	99	JX566688
A_4	*Proteobacteria, Betaproteobacteria, Burkholderiaceae*	Uncultured *Ralstonia* sp. (GQ417854)	99	JX566687
A_5	*TM7, TM7_genera_incertae_sedis*	Uncultured bacterium from soil (HM651874)	99	KJ137167

A_6	*Proteobacteria, Gammaproteobacteria, Enterobacteriaceae*	Uncultured *Klebsiella* sp. (HQ264076)	99	KJ137172
A_7	*Verrucomicrobia, Opitutae, Opitutaceae*	*Alterococcus* sp. from earthworms gut (HM459619)	99	KJ137170

[a] Corresponding DGGE bands illustrated in Fig. 5 and Fig. S3.

[b] Bands B_1 to B_9 are the DGGE bands in PCP contaminated soils, and A_1 to A_7 are those in uncontaminated soils.

[c] The phylogenic assignment follows the sequence analysis by the RDP classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp) or the GenBank database from NCBI (http://www.ncbi.nlm.nih.gov), given the phylum and the lowest predictable phylogenic rank.

[d] Partial sequencing of 16S rRNA and comparison with previous reports in GenBank by BLAST.

8. Figure legends

Fig. 1. Soil extractable residual PCP concentration (A) and profiles (B) in different treatments during the PCP degradation experiment. Data are mean±SD (n=3). Bars with different lower case letters (a, b and c) refer to significant differences (ANOVA, Duncan's test, $p<0.05$) between treatments, where the same letter indicates no significant difference.

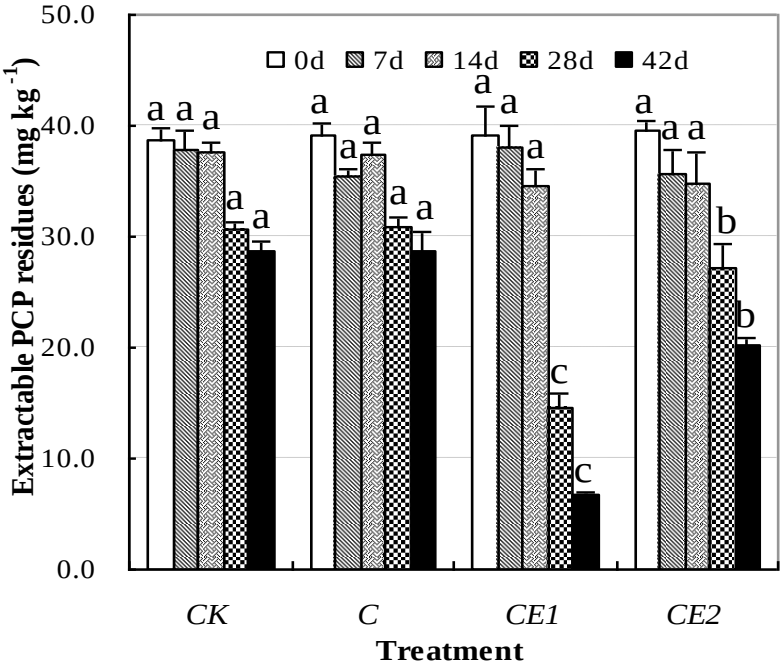
Fig. 2. Colony forming units (CFU) of cultivable bacteria (A), fungi (B) and actinomyces (C) for PCP degradation treatments. Data are mean±SD (n=3). Bars with different lower case letters (a, b and c) refer to significant differences (ANOVA, Duncan's test, $p<0.05$) between treatments, where the same letter indicates no significant difference.

Fig. 3. Soil enzyme activity for PCP degradation treatments. Data are mean±SD (n=3). Bars with different lower case letters (a, b and c) refer to significant differences (ANOVA, Duncan's test, $p<0.05$) between treatments, where the same letter indicates no significant difference.

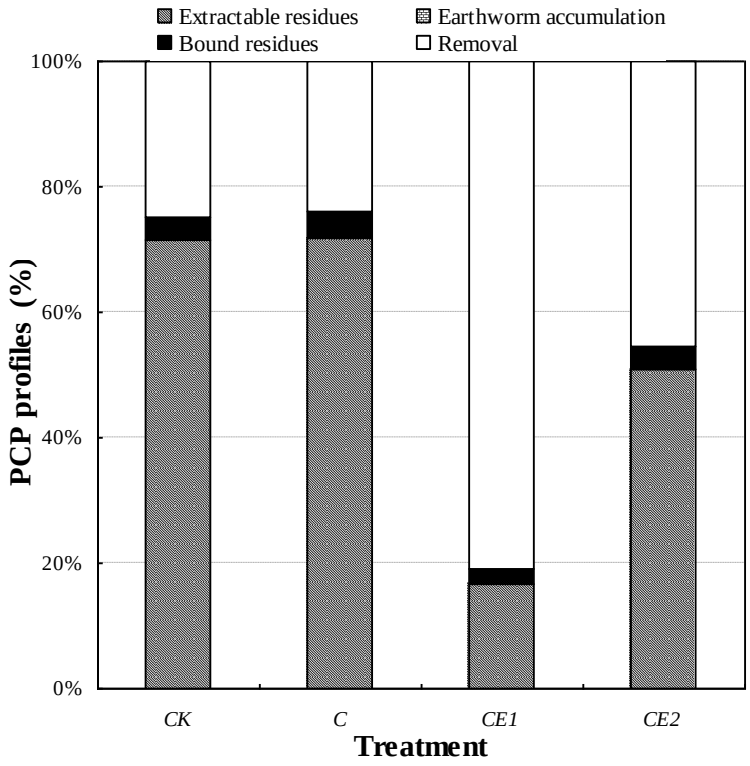
Fig. 4. Soil respiration (A), microbial biomass carbon (B) and nitrogen (C) for PCP degradation treatments. Data are mean±SD (n=3). Bars with different lower case letters (a, b and c) refer to significant differences (ANOVA, Duncan's test, $p<0.05$) between treatments, where the same letter indicates no significant difference.

Fig. 5. Microbial community structure (16S rRNA DGGE) for different treatments after 42 days of PCP biodegradation. I, II and III represent individual biological replicates for each treatment.

Fig. 6. The neighbor-joining phylogenic tree of bacteria 16S rRNA after 42 days of PCP biodegradation. The analysis is based on their closest relatives from NCBI database and by ClustalX software. Scale of bar indicated 5% sequence divergence via a bootstrap analysis with 1000 trials.

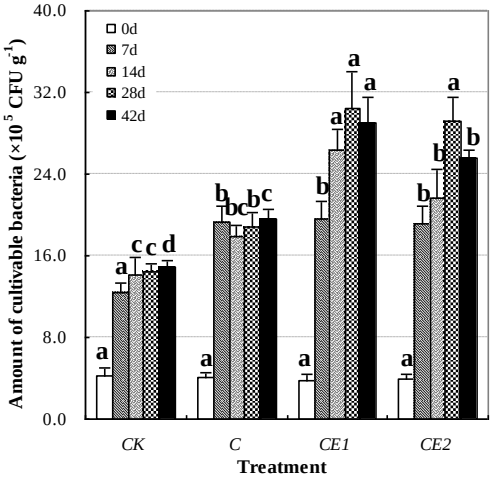

50.0
40.0
30.0
20.0
10.0
0.0
0d
7d
14d
28d
42d
Extractable PCP residues (mg kg-1)
a a a a a
a a a a a
a a a c c
a a b b
CK
C
CE1
CE2
Treatment

(A)

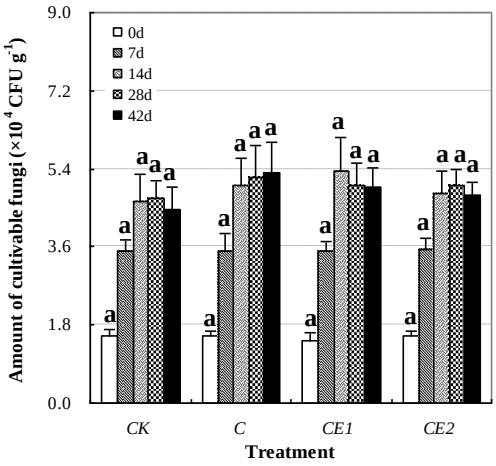

Extractable residues
Earthworm accumulation
Bound residues
Removal
100%
80%
60%
40%
20%
0%
PCP profiles (%)
CK
C
CE1
CE2
Treatment

(B)

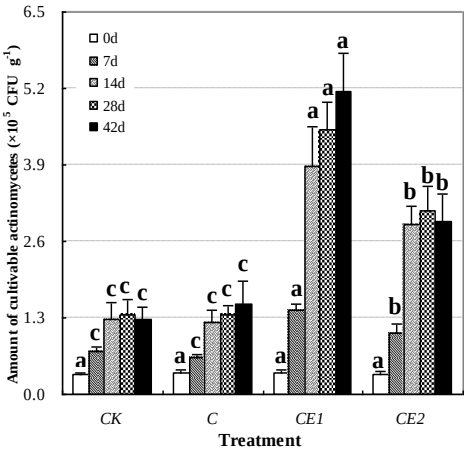
Fig. 1.


Amount of cultivable bacteria (×10^5 CFU g^{-1})
0d
7d
14d
28d
42d
CK
C
CE1
CE2
Treatment

(A)


Amount of cultivable fungi (×10^4 CFU g^{-1})
0d
7d
14d
28d
42d
CK
C
CE1
CE2
Treatment

(B)


Amount of cultivable actinomycetes (×10^5 CFU g^{-1})
0d
7d
14d
28d
42d
CK
C
CE1
CE2
Treatment

(C)

Fig. 2.

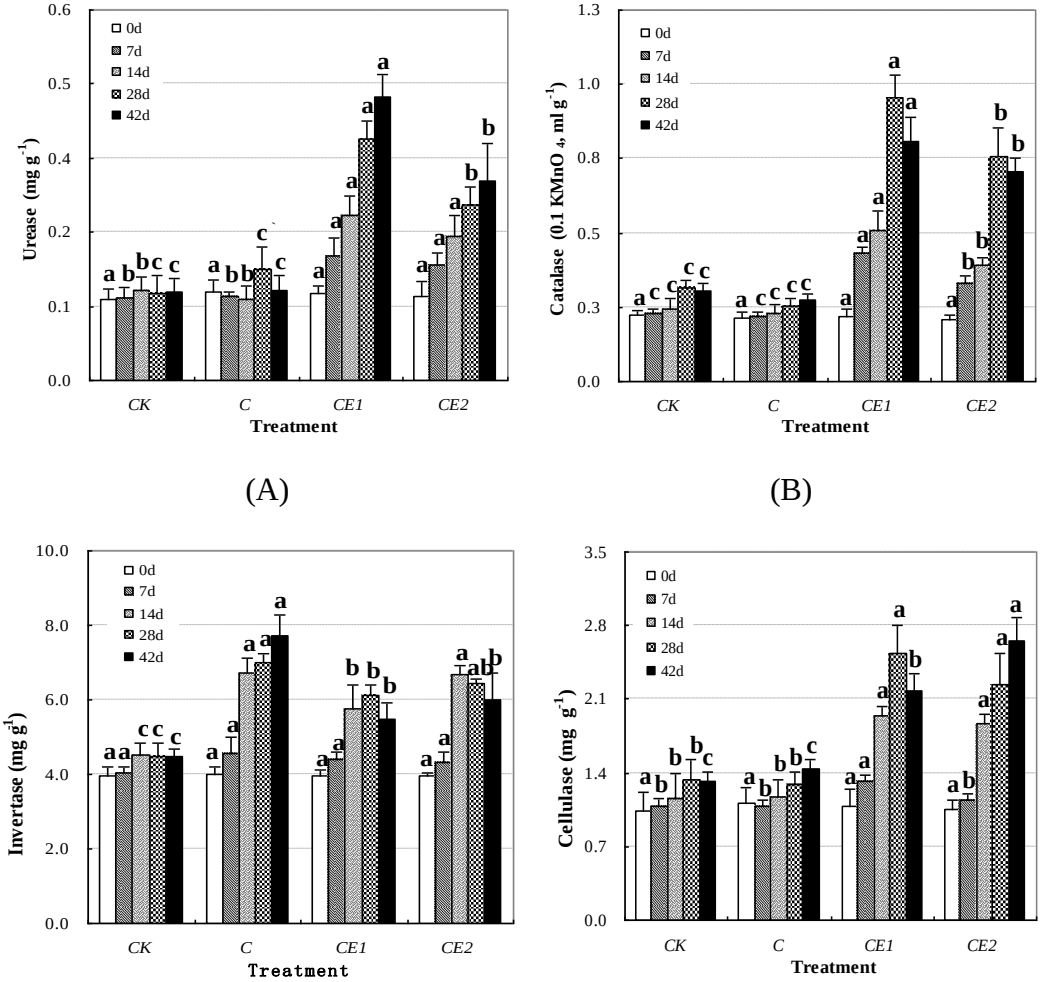
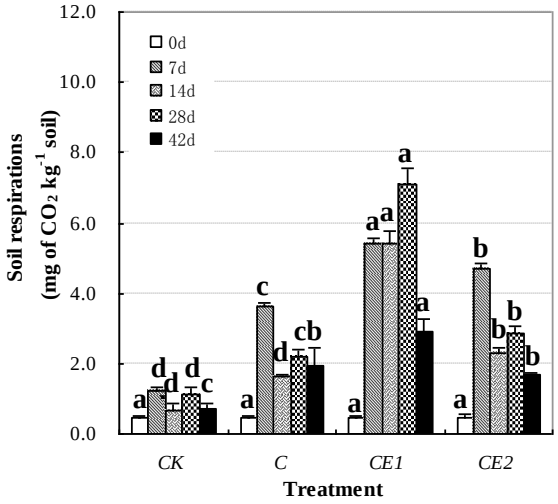
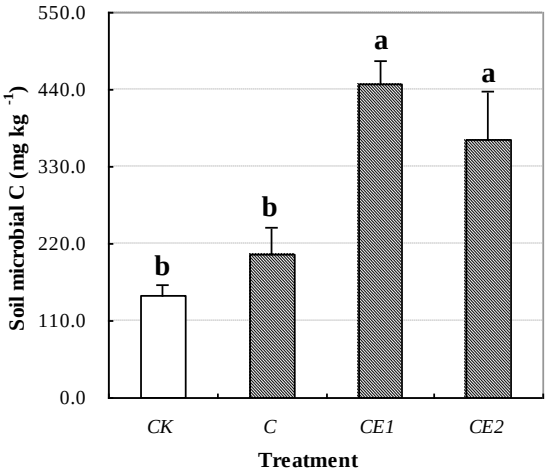

0d
7d
14d
28d
42d
Urease (mg g-1)
Catalase (0.1 KMnO 4, ml g-1)
Invertase (mg g-1)
Cellulase (mg g-1)
CK
C
CE1
CE2
Treatment
(A)
(B)
(C)
(D)

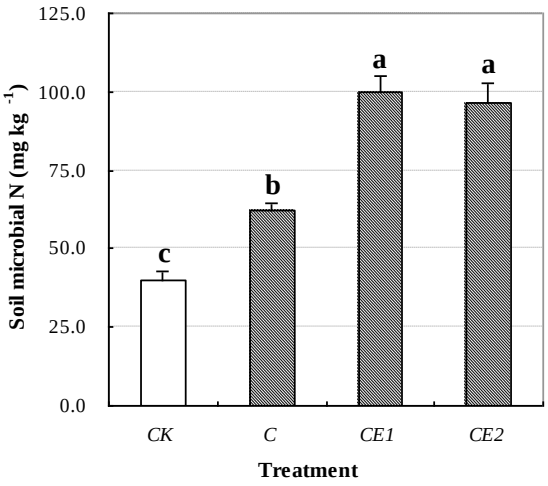
Fig. 3.


12.0
10.0
8.0
6.0
4.0
2.0
0.0
0d
7d
14d
28d
42d
Soil respirations
(mg of CO2 kg-1 soil)
a
d
d
d
c
a
c
d
cb
a
a
a
a
a
b
b
b
b
CK
C
CE1
CE2
Treatment

(A)


550.0
440.0
330.0
220.0
110.0
0.0
Soil microbial C (mg kg -1)
b
b
a
a
CK
C
CE1
CE2
Treatment

(B)


125.0
100.0
75.0
50.0
25.0
0.0
Soil microbial N (mg kg -1)
c
b
a
a
CK
C
CE1
CE2
Treatment

(C)

Fig. 4.

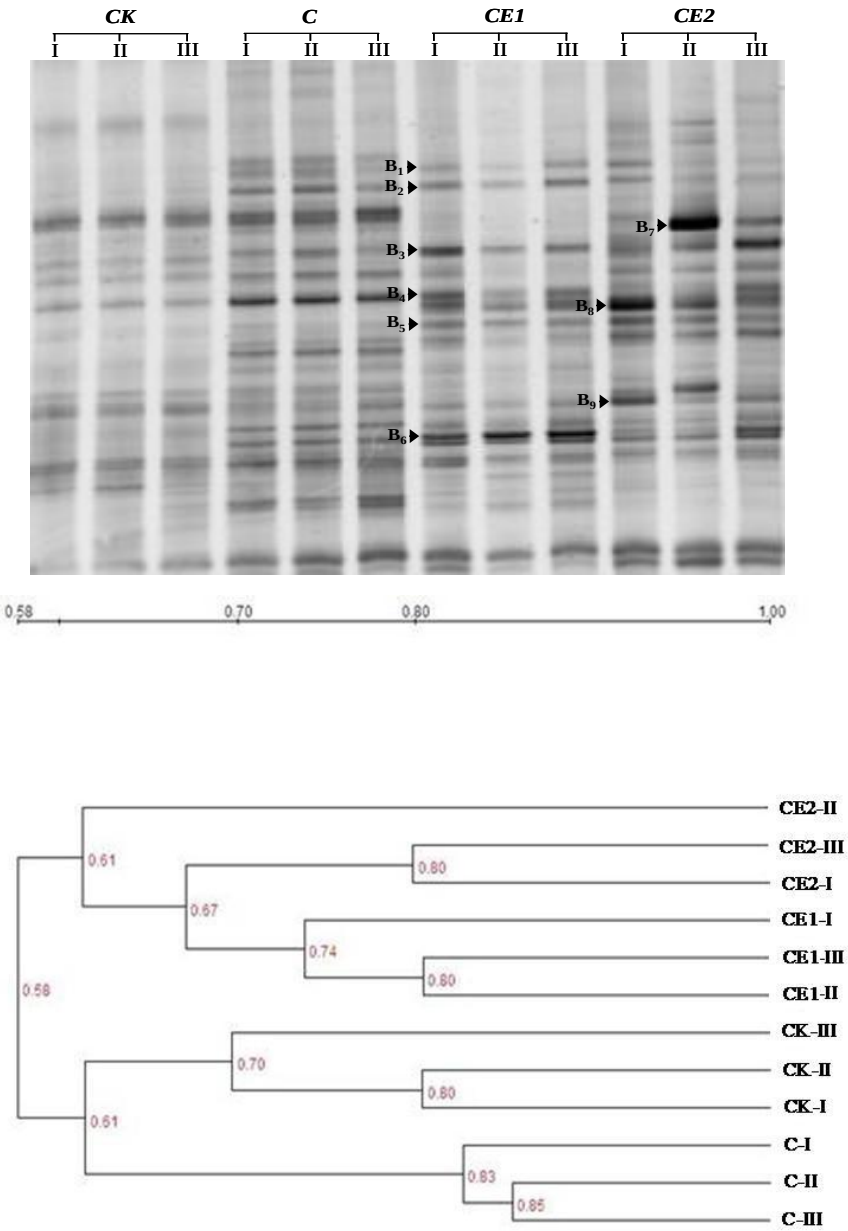

CK
C
CE1
CE2
I II III I II III I II III I II III
B1
B2
B3
B4
B5
B6
B7
B8
B9
0.58
0.70
0.80
1.00
CE2-II
CE2-III
CE2-I
CE1-I
CE1-III
CE1-II
CK-III
CK-II
CK-I
C-I
C-II
C-III
0.61
0.80
0.67
0.74
0.80
0.58
0.70
0.80
0.61
0.83
0.85

Fig. 5.

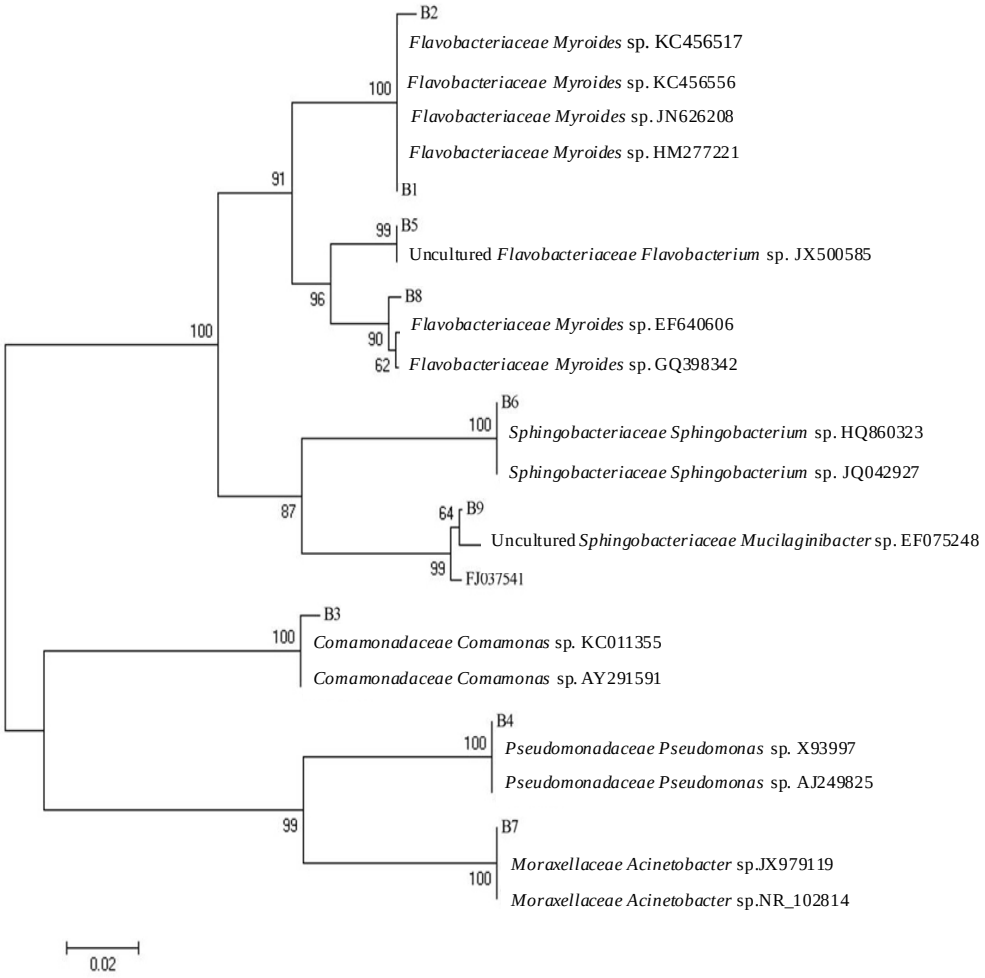

B2
Flavobacteriaceae Myroides sp. KC456517
100
Flavobacteriaceae Myroides sp. KC456556
Flavobacteriaceae Myroides sp. JN626208
Flavobacteriaceae Myroides sp. HM277221
91
B1
99
B5
Uncultured Flavobacteriaceae Flavobacterium sp. JX500585
96
B8
100
Flavobacteriaceae Myroides sp. EF640606
90
62
Flavobacteriaceae Myroides sp. GQ398342
B6
100
Sphingobacteriaceae Sphingobacterium sp. HQ860323
Sphingobacteriaceae Sphingobacterium sp. JQ042927
87
64
B9
Uncultured Sphingobacteriaceae Mucilaginibacter sp. EF075248
99
FJ037541
B3
100
Comamonadaceae Comamonas sp. KC011355
Comamonadaceae Comamonas sp. AY291591
B4
100
Pseudomonadaceae Pseudomonas sp. X93997
Pseudomonadaceae Pseudomonas sp. AJ249825
99
B7
Moraxellaceae Acinetobacter sp.JX979119
100
Moraxellaceae Acinetobacter sp.NR_102814
0.02

Fig. 6.

The impact on the soil microbial community and enzyme activity of two earthworm species during the bioremediation of pentachlorophenol-contaminated soils

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1. Preparation of Artificial PCP Contaminated Soils

Artificial PCP contaminated soils were prepared following existing protocols [27]. To minimize the toxicity of acetone and reduce its impacts on soil microbial population/activity, the acetone-PCP was added to a small proportion of soil (<20%) and then mixed thoroughly with the majority of the soil. Briefly, 120 and 360 mg of PCP were dissolved in 5 mL and 10 mL acetone, respectively, and then mixed thoroughly with 0.5 kg soil and a 1.0 kg soil-compost mixture for 0.5 h. After the acetone was volatilised overnight, the microbial population (>5.0×10^5 CFU g^{-1}) and activities (urease activity > 0.12 mg g^{-1}) remained in over 95% of the original soils and recovered to 100% after 3 days. Subsequently, the artificial PCP-contaminated soils were thoroughly mixed with 2.5 kg uncontaminated soil or an 8.0 kg soil-compost mixture, respectively, to make homogeneous soil samples for PCP degradation experiments. For each treatment in triplicate, 1 kg of artificial PCP contaminated soil was added to a flowerpot (12 cm height × 10 cm upper diameter × 6 cm bottom diameter). Sterile compost was pre-treated with a total dose of 60 kGy of γ-irradiation [56], and prepared with 4.5% compost as supplemental nutrition for the earthworms. Both epigeic and endogeic earthworms were incubated in uncontaminated soils for 1 month before use in the experiment. Mature earthworms (*E. foetida*, 0.23 ± 0.09 g each; *A. robustus*, 0.57 ± 0.26 g each) were washed with deionised water and stored on moistened filter paper at ambient temperature for 24 h in a dark room. They were then added to the experimental treatments at 30 individuals kg^{-1} and 15 individuals kg^{-1}, respectively (after ensuring that the earthworm weights were the same). The soil samples were moistened with sterile deionised water every three days to maintain moisture at 60%. The flowerpots were covered with perforated aluminium foil and inoculated in a dark ventilated incubator at 25°C. Approximately

30 g of soil were sampled at 0, 7, 14, 28 and 42 d, and immediately stored at 4°C for laboratory analysis.

2. Microbial Analysis Methods

2.1 Colony forming units of cultivable microorganisms

The total number of cultivable bacteria, fungi, and actinomyces were counted as colony forming units (CFUs) on agar plates by serial dilution. The cultivation media for bacteria, fungi, and actinomyces were beef extract peptone medium, Czapek's medium, and Gause's medium No. 1, respectively [57].

2.2 Microbial biomass and activities

Total soil microbial biomass carbon and nitrogen were determined by the fumigation-extraction method [24]. Total microbial activity was measured by basal respiration [25].

2.3 Enzyme activity analysis

Urease and catalase activity were determined by the phenol-sodium hypochlorite colourimetric method and titration, respectively [26]. Invertase and cellulase activity were determined by the 3,5-dinitrosalicylate [25] and 3,5-dinitrosalicylate colourimetric methods [26].

2.4 Functional diversity analysis

Soil bacterial functional diversity was evaluated using the method of Garland and Mills [58]. Briefly, 10 g of fresh soil was suspended in 100 mL sterile deionised water and shaken on an orbital shaker for 30 min at 200 rpm. After a 1,000 x dilution in sterile deionised water, 150 µL of soil supernatant was added into each well of the Biolog EcoPlate^{TM} (Biolog, Hayward, CA). The plates were incubated at 25°C in the

microplate reader (Emax, Molecular Devices, USA) and the absorbance at 595 nm was measured every 24 hours for 7 days.

2.5 DNA extraction and 16S amplification

Briefly, total soil DNA was extracted with a Powersoil DNA extraction kit (MoBio, USA) following the manufacturer's instructions. The V3 region of 16S rRNA gene was amplified by touchdown polymerase chain reaction (PCR) with the primer set 518R (5'-ATTACCGCGGCTGCTGG-3') and GC-338F with the GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGA GGCAGCAG-3'). The PCR reaction system contained 25 μL of Premix Taq (Takara, Japan), 1.5 μL of each primer and 2 μL of the DNA template, brought to 50 μL with sterile Milli-Q water. Amplification was conducted in a PTC-200 thermal cycler (BioRad, USA) using the conditions reported by Cea et al. [5]. The initial DNA denaturation was performed for 5 min at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; 10 cycles of 30 s at 92°C, 30 s at 55°C, and 45 s at 72°C; and a final extension at 72°C for 10 min.

2.6 Denaturing Gradient Gel Electrophoresis

DGGE was performed in the DCode universal mutation detection system (BioRad, USA). Approximately 1 μg of 16S rRNA PCR products was loaded in each lane of an 8% polyacrylamide (37.5:1 acrylamide:bisacrylamide) gel in Tris-acetate-EDTA (TAE) buffer with the denaturing gradient ranging from 40% to 60%. Gel electrophoresis was performed at 60°C for 13 h at 80 V. The DGGE gel was subsequently stained with SYBR Green I nucleic acid staining solution (GenScript, USA) for 30 min before being photographed and analysed by a Molecular Imager Gel Doc XR System (BioRad, USA).

2.7 Quantitative PCR

Quantitative PCR was carried out to quantify the number of 16S rRNA and pcpB genes in soil samples after treatment with the CFX96 Real-Time PCR Detection System (BioRad, USA). Each 10 µL reaction system included 5 µL iTAQ SYBR-green supermix (BioRad, USA), 1.0 µL of each primer, 1.0 µL DNA template and 2 µL molecular water. The primer pair for 16S rRNA was 341F (5'-CCTACGGGAGGCAGCAG-3') and 534R (5'-ATTACCGCGGCTGCTGGC-3'). The primer set for the PCP-4-monooxygenase (*pcpB*) gene included pcpB-G (5'-GG{C/G}TTCAC{C/G}TTCAA{C/T}TTCGA-3') and pcpB-D (5'-TCCTGCAT{G/C}CC{G/C}AC{A/G}TTCAT-3'). The qPCR program for the two primer pairs were: 95°C 4 min, 40 cycles of 95°C 60 s, 58°C 60 s, 72°C 60 s and 80°C 10 s (fluorescent signal detection), and a final melting curve from 65°C to 95°C at 0.5°C intervals.

For the calibration curve, the 16S rRNA and *pcpB* genes were first amplified from soil DNA samples. The amplification was undertaken in a C1000 Thermal Cycler (BioRad, USA), and the 50 µL reaction system contained 2.5 µL of each primer, 0.5 µL DreamTaq DNA polymerase, 5 µL DreamTaq green buffer, 1 µL DNTPs (5 mM), 1 µL DNA template and 41 µL molecular water. The amplification program was the same as for qPCR, except for no 80°C fluorescent detection in each cycle, and replacing the melting curve by a final extension at 72°C for 5 min. All the amplification products were subsequently purified with the Gel Extraction Kit (Qiagen, USA), and then cloned into the pGEM®-T vector (Promega, USA) following the manufacturer's instructions. Plasmid DNA was extracted and purified with a Minipreps Kit (Promega, USA). The plasmid concentration was determined using a ND-2,000 UV-Vis spectrophotometer (NanoDrop Technologies, USA). The

plasmid copy number of each DNA insert was determined by the amount and molecular weight of the targeting double-stranded plasmid. All the plasmids were diluted in the series of 3×10^8, 3×10^7, 3×10^6, 3×10^5, 3×10^4, 3×10^3, 3×10^2, 3×10^1 and 3 copies/reaction, and quantified together with targeting soil DNA in the same qPCR program to obtain the linear standard curve, as illustrated Fig. S4.

Table

Table S1. Chemical properties of the soils and composts.

	pH	Total nitrogen (g/kg)	Total phosphorus (g/kg)	Total potassium (g/kg)	Organic matter (g/kg)
Soil	4.71	1.13	1.91	9.72	22.50
Compost	7.54	8.97	13.25	17.68	198.35

Table S2. The pH value and key carbon/nitrogen sources in different treatments after 42 d PCP degradation.

Treatment	pH	Organic matter (g/kg)	Dissolved organic matter (g/kg)	Nitrogen (g/kg)	NH_4-N (mg/kg)	NO_3-N (mg/kg)	Humus		
							Humic acid (g/kg)	Fulvic acid (g/kg)	Humin (g/kg)
CK	4.90 ± 0.05 c	23.46 ± 2.57 b	1.19 ± 0.10 a	1.39 ± 0.22 a	12.14 ± 2.44 a	5.35 ± 1.43 a	0.86 ± 0.14 b	2.90 ± 0.05 a	5.05 ± 0.04 a
C	6.09 ± 0.15 b	36.45 ± 1.65 a	1.07 ± 0.16 b	1.46 ± 0.19 a	14.24± 3.12 a	5.89 ± 1.94 a	1.48 ± 0.24 a	2.99 ± 0.07 a	5.26 ± 0.06 a
CE1	6.80 ± 0.09 a	37.53± 0.67 a	1.00 ± 0.05 b	1.51 ± 0.26 a	35.52 ± 3.64 b	8.87 ± 2.01 b	1.21 ± 0.09 a	2.97 ± 0.06 a	4.35 ± 0.30 b
CE2	6.68 ± 0.05 a	37.32 ± 1.67 a	1.43 ± 0.21 a	1.48 ± 0.17 a	27.73 ± 2.87 b	8.73 ± 2.27 b	1.04 ± 0.05 b	2.90 ± 0.14 a	4.42 ± 0.02 b

Table S3. The microbial utilization efficiency of six main carbon sources in EcoPlate in different treatments.

Treatment	Polymers	Carbonhydrates	Carboxyl acids	Amino acids	Amines	Miscellaneous
CK	0.92 ± 0.00 b	1.12 ± 0.02 b	0.79 ± 0.01 c	0.48 ± 0.04 b	1.12 ± 0.03 b	0.87 ± 0.05 a
C	1.09 ± 0.04 ab	1.26 ± 0.03 ab	0.92 ± 0.05 b	0.58 ± 0.01 b	1.18 ± 0.04 b	0.95 ± 0.02 a
CE1	1.27 ± 0.05 a	1.42 ± 0.05 a	1.25 ± 0.06 a	0.78 ± 0.05 a	1.51 ± 0.03 a	0.79 ± 0.03 a
CE2	1.17 ± 0.03 ab	1.35 ± 0.03 a	1.24 ± 0.03 a	0.80 ± 0.03 a	1.46 ± 0.07 a	0.87±0.03 a

Figure

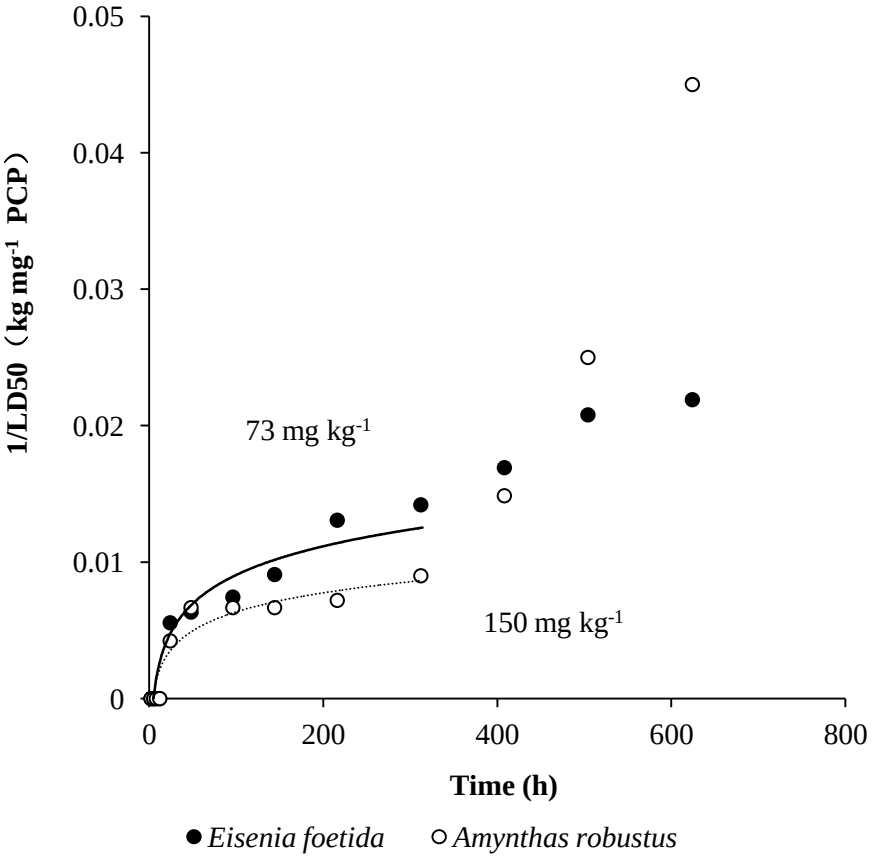


Fig. S1. LD_{50} test of *Eisenia fetida* and *Amynthas robustus E. Perrie* in PCP contaminated soils. PCP concentrations in soils were 10, 20, 40, 60, 80, 100 and 200 mg kg^{-1}. From calculation, the LD50 of *Esenia fetida* was 73 mg kg^{-1} at 300 hours, whereas it was 150 mg kg^{-1} for *Amynthas robustus E. Perrie*. Besides, the survival rate of epigeic *Eisenia fetida* and endogeic *Amynthas robustus E. Perrie* was 100% and 87%, respectively. No significant inhibition was found for either earthworm at the PCP concentration in this study.

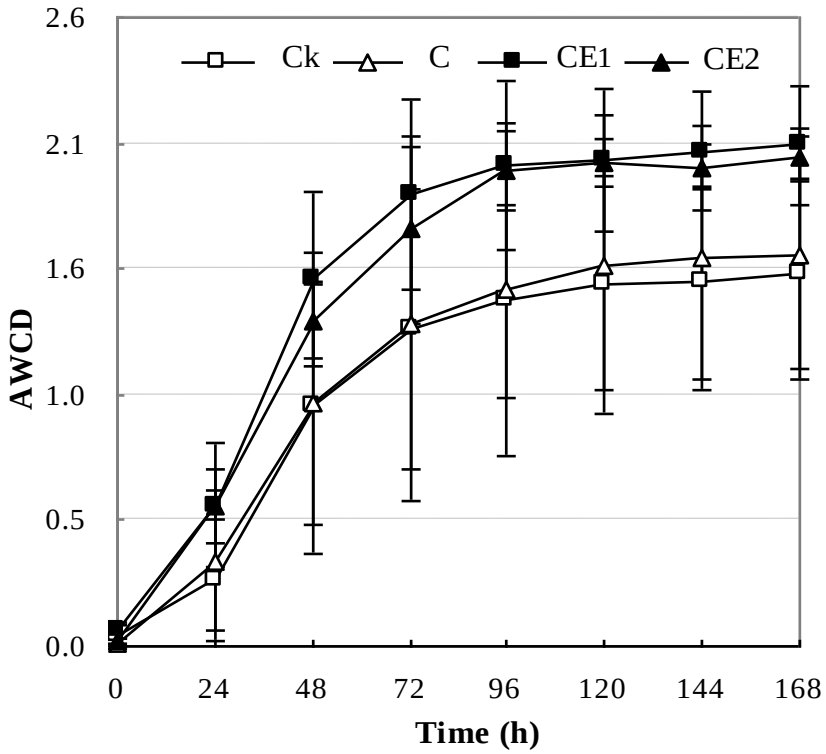


Fig. S2. The Average Well Colour Development (*AWCD*) of soil bacterial community against time. Data are mean±SD (n=3).

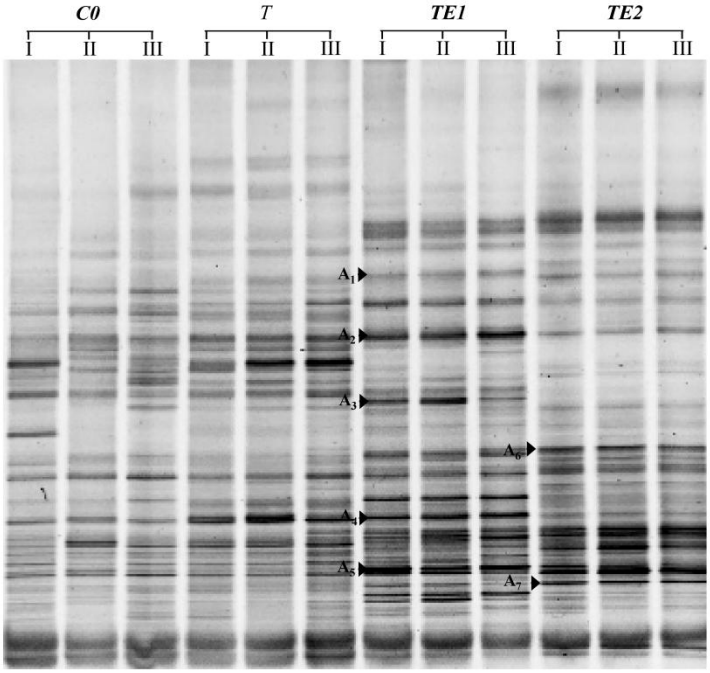
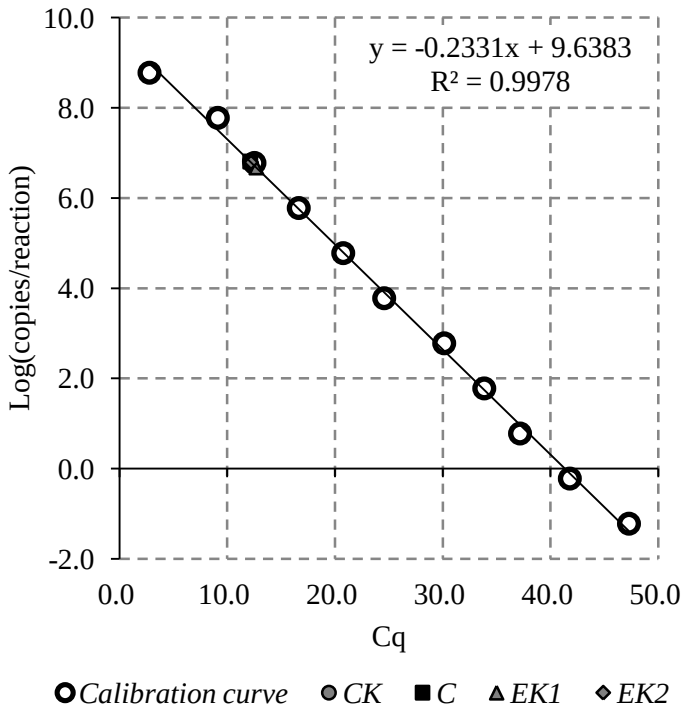
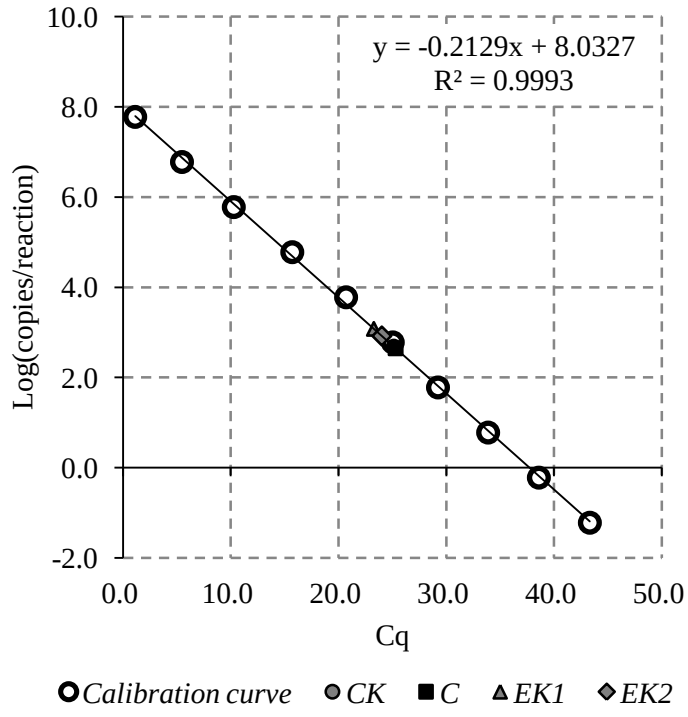


Fig. S3. Microbial community structure (16S rRNA DGGE) on Day 0 for different treatments. I, II and III represent individual biological replicates for each treatment.



(A)



(B)

Fig. S4. qPCR calibration curve and soil genes quantification for 16S rRNA (A) and *pcpB* functional gene (B).

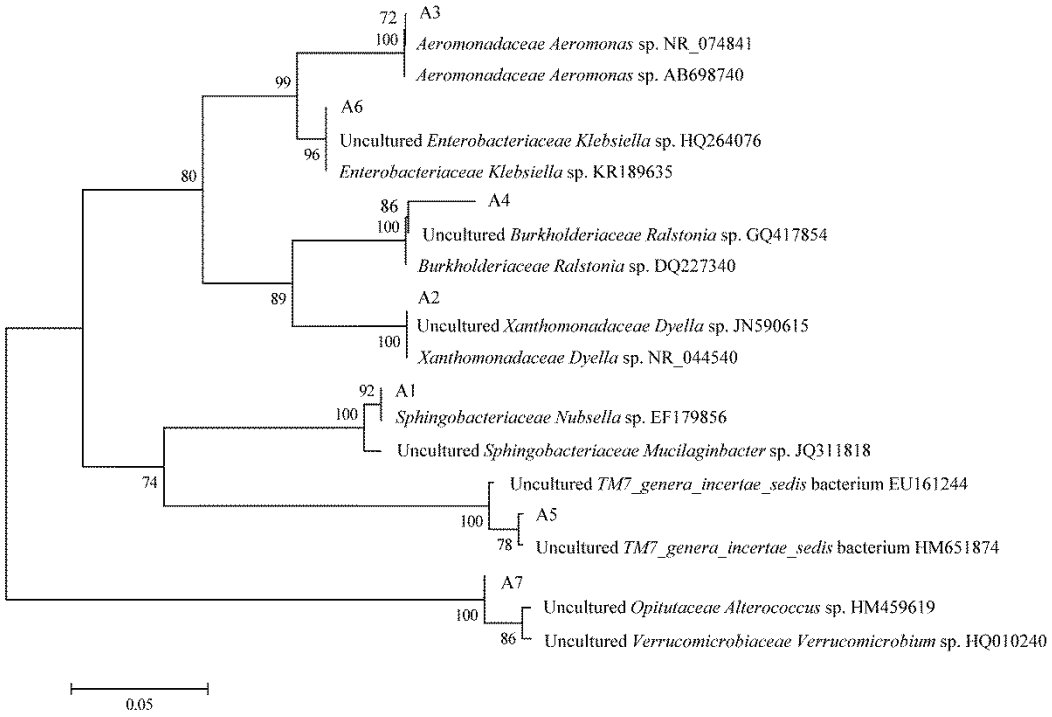


Fig. S5 The neighbor-joining phylogenic tree of bacteria 16S rRNA on Day 0 for different treatments. The analysis is based on their closest relatives from NCBI database and by ClustalX software. Scale of bar indicated 5% sequence divergence via a bootstrap analysis with 1000 trials.

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