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Commonwealth of Soil Health: How Do Earthworms Modify the Soil Microbial Responses to CeO₂ Nanoparticles?

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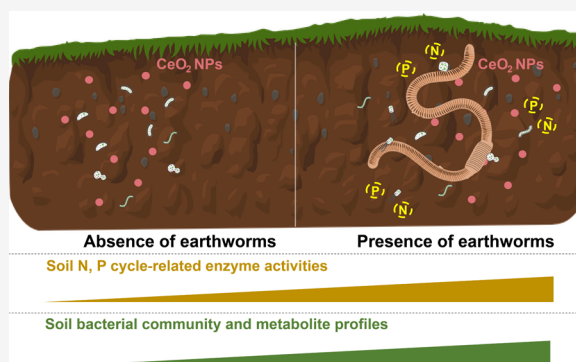
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Supporting Information

ABSTRACT: Soil ecotoxicological assays on nanoparticles (NPs) have mainly investigated single components (e.g., plants, fauna, and microbes) within the ecosystem, neglecting possible effects resulting from the disturbance of the interactions between these components. Here, we investigated soil microbial responses to CeO₂ NPs in the presence and absence of earthworms from the perspectives of microbial functions (i.e., enzyme activities), the community structure, and soil metabolite profiles. Exposure to CeO₂ NPs (50, 500 mg/kg) alone decreased the activities of enzymes (i.e., acid protease and acid phosphatase) participating in soil N and P cycles, while the presence of earthworms ameliorated these inhibitory effects. After the CeO₂ NP exposure, the earthworms significantly altered the relative abundance of some microbes associated with the soil N and P cycles (*Flavobacterium*, *Pedobacter*, *Streptomyces*, *Bacillus*, *Bacteroidota*, *Actinobacteria*, and *Firmicutes*). This was consistent with the pattern found in the significantly changed metabolites which were also involved in the microbial N and P metabolism. Both CeO₂ NPs and earthworms changed the soil bacterial community and soil metabolite profiles. Larger alterations of soil bacteria and metabolites were found under CeO₂ NP exposure with earthworms. Overall, our study indicates that the top-down control of earthworms can drastically modify the microbial responses to CeO₂ NPs from all studied biological aspects. This clearly shows the importance of the holistic consideration of all soil ecological components to assess the environmental risks of NPs to soil health.

KEYWORDS: CeO₂ NPs, earthworm, soil enzymes, bacterial community, metabolite profile



INTRODUCTION

Cerium oxide nanoparticles (CeO₂ NPs) have gained a lot of attention owing to their free radical scavenging activity, as well as their optic and catalytic properties.¹ With a global estimated production of 10,000 metric tons per year, CeO₂ NPs are widely applied in industrial and agricultural products, such as automobile catalyzers, ceramics, abrasives, biosolids, and fertilizers.² As estimated, their annual emission to soil was close to 1,400 metric tons via a diverse number of pathways,² triggering concerns for the potential toxicological risks of CeO₂ NPs to soil ecosystems.

Soil ecosystems, highly complex in their structure and function, are dominated by below-ground communities of soil microbes and fauna.³ Soil microbes play crucial roles in the energy flow, nutrient cycling, decomposition, biodiversity, and stability of soil ecosystems.⁴ Luo et al.⁵ and Pan et al.⁶ reported that CeO₂ NPs had an obvious effect on the soil microbial community structure and inhibited soil dehydrogenase activities at a concentration range of 25–1000 mg/kg. Other NPs, such as Ag NPs,⁷ Cu-based NPs,^{8,9} TiO₂ NPs, and ZnO NPs,^{10,11} also decreased the microbial activity and changed the bacterial community composition. More specifically, some

functional microbes, such as N₂-fixers (*Azotobacter*),¹² nitrifiers (*Nitrosomonas europaea*),¹³ denitrifiers (*Flexibacter* and *Acinetobacter*),¹⁴ anammox bacteria (*Kuenenia*),¹⁵ and P-solubilizing bacteria,¹² were suppressed due to the toxicity of metal-based NPs. Therefore, CeO₂ NPs may impact soil nitrogen and phosphate cycles by changing the soil microbial community structure and activity. NPs also have negative effects on the soil fauna. For example, various NPs (e.g., CeO₂, CuO, ZnO, and Ag) cause osmotic disturbances, oxidative stress, and tissue damage in earthworms (*Eisenia fetida*).^{16–18} The above studies focusing on the toxicity of NPs to individual species may neglect the fact that soil organisms constantly interact with each other at various trophic levels such as symbiosis and competition and often act as a whole to respond to exogenous stimuli. Hence, these interspecific interactions should be

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considered in the risk assessments as they may substantially change the outcome from that obtained in studies investigating individual species. Nevertheless, until now, it still remains unknown to what extent the inclusion of soil fauna may alter the impacts of NPs on the soil microbial community.

Soil fauna can affect soil microbes through decomposing, mineralizing, and burrowing activities.¹⁹ Earthworms, known as “ecosystem engineers”, are critical invertebrates in the soil ecosystem. They are known to promote the soil microbial biomass,^{20–22} increase the abundance of denitrifying microbes and cumulative N₂O emissions,²¹ and affect the distribution of the soil microbial community due to their burrowing activities.^{23,24} For example, the ingestion and egestion of soil through the digestive system of earthworms increase the activity and abundance of beneficial microbes, while decreasing the pathogenic microorganisms.²⁵ Earthworm mucus, rich in carbohydrates and protein-like materials, provides organic materials to support extensive microbial population growth and activity.²⁶ A previous study showed that Ag₂S NPs caused a slight reduction in the growth of earthworms and changed their gut microbiota, which resulted in the reduction of soil N₂O emission.²⁷ This finding suggests that NPs can indirectly impact the functioning of the soil microbial community by impacting the growth of earthworms and modifying their gut microbiome. Therefore, we presume that earthworm activities such as the digestion of the soil organic matter in the gut and epidermal mucus secretion may play a key role in governing the NP toxicity to the soil bacterial community structure and function.

Alterations in the microbial community structure can indicate changes in the microbial community function and metabolism in soil ecosystems.²⁸ Soil enzyme activities have a close relation with the soil microbial community, and they affect the soil metabolism.⁶ In addition, reductions in the soil microbial diversity are accompanied by the inhibition of the microbial metabolic activity.^{29,30} However, a collective response of soil microbial systems, including the microbial community structure and function and microbial metabolic processes, to NP stress in the presence of earthworms has rarely been reported. Soil metabolomics is an emerging technology with the potential to characterize soils and evaluate the metabolic status of the soil microbial community by measuring the metabolites of important metabolic pathways.³¹ Jones et al.³² reported that soil metabolites could be used as biomarkers for soil contamination. Therefore, the employment of phenotypic traits and multiomics (microbiomics and soil metabolomics) data can serve as an integrative approach to understand the mechanisms by which the soil microbial community responds to NPs.

Based on this background, the aims of this study are as follows: (1) to investigate the changes in the soil enzyme activities, community structures and functions, and metabolite profiles in response to CeO₂ NP exposure in soil and (2) to determine how the presence of earthworms (*E. fetida*) affects the soil microbial responses to NPs. To achieve these aims, MiSeq sequencing and soil metabolomics were applied to evaluate the alterations in the soil bacterial community and metabolite profiles. To the best of our knowledge, this is the first study to couple the soil enzymes, microbial community, and soil metabolomics to systematically reveal the responses of soil microorganisms to CeO₂ NP stress in the presence and absence of earthworms. We anticipate that our findings will provide new insights into the NP effects in a more realistic

setting as compared to the classic studies just focusing on individual species without considering species interactions.

MATERIALS AND METHODS

Reagents, Test Soil, and Earthworms. CeO₂ NPs with the size <25 nm were purchased from Sigma-Aldrich. The test soil was collected from an agricultural land in Hailun city, Heilongjiang Province, China. The collected soil sample was first air-dried and sieved through a 2 mm mesh, subsequently homogenized thoroughly, and stored in the dark at room temperature for further use. Earthworm *E. fetida* was used as it is the recommended species in the OECD 222 guideline. The specimens were bought from an earthworm farm in Jurong city, Jiangsu Province, China. Details of the reagents, test soil, and earthworms are provided in Text S1 and in Table S1 in the Supporting Information.

Experimental Design. Approximately 500 g of dry soil was weighed into each polyethylene container (1000 mL). The specified amount of dry CeO₂ NPs was then mixed in with the soil to obtain the following six treatments: soil (control); soil + 50 mg of CeO₂ NPs/kg dry soil (NP50); soil + 500 mg of CeO₂ NPs/kg dry soil (NP500); soil + worms (worm); soil + 50 mg of CeO₂ NPs/kg dry soil + worms (NP50 + worm); and soil + 500 mg of CeO₂ NPs/kg dry soil + worms (NP500 + worm). The two test concentrations of CeO₂ NPs were selected to differentiate the dose-dependent responses, and they also fell in the concentration range reported in the previous toxicological studies of CeO₂ NPs.^{5,6,18} Each treatment had six replicates. Ultrapure water was added into the soil to reach a moisture content of 60% of the maximum water holding capacity (WHC), after which the soil was mixed again. The soils were allowed to equilibrate for 1 week in the laboratory (20 °C, 80% humidity, and 24 h dark) before introducing the earthworms. The earthworms were rinsed with pure water to remove the adhered soil particles before being weighed. Twenty-five healthy adult earthworms with similar mass (350–450 mg) were transferred into each container. The 36 test containers were then placed in a growth chamber at 20 °C, 80% humidity, and a 16 h/8 h light–dark cycle (600 Lux light intensity) for 28 days. During the 28 day exposure, the moisture content of soil was maintained at 60% of the maximum WHC by replenishing the weight loss with ultrapure water. Containers were constantly checked, and dead earthworms were removed from the soil if present. After 28 days, the remaining earthworms were sampled (Table S2), weighed, and depurated on wet filter paper for 24 h to void their gut contents to determine the bioaccumulation of Ce.

Detection of Ce and Mineral Elements in the Earthworms and Soil. Earthworms were freeze-dried, weighed, and individually digested with HNO₃ using a graphite block digestion at 90 °C for 2 h. The concentration of Ce in the digest was measured by inductively coupled plasma–optical emission spectrometry (ICP-OES). Soil samples were air-dried and sieved (<0.149 mm), microwave-digested in a mixture of HNO₃, HF, and H₂O₂ at 180 °C for 40 min, and analyzed by ICP-OES for total concentrations of K, Ca, Na, Mg, Al, Si, Fe, Mn, Ce, Zn, Cu, and Ni.

Analysis of Soil Microbial Biomass C and Enzyme Activities. Soil microbial biomass C and enzyme activities (β -glucosidase, catalase, peroxidase, acid protease, acid phosphatase) were determined in triplicate for each treatment. Soil microbial biomass C and enzyme activities were expressed as milligrams C per kilogram of dry soil and unit activity per

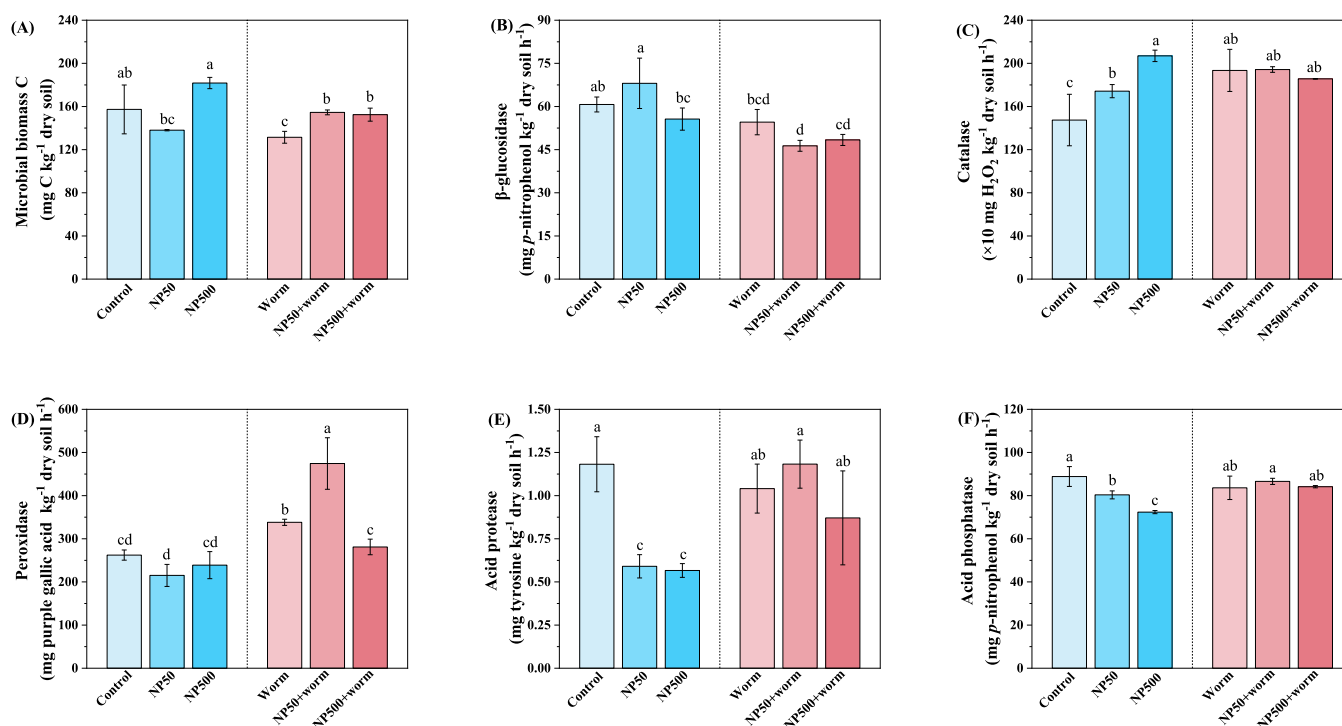


Figure 1. Soil microbial biomass C (A) and activities of β -glucosidase (B), catalase (C), peroxidase (D), acid protease (E), and acid phosphatase (F) in soil spiked with CeO_2 NPs at 50 or 500 mg/kg dry soil and incubated for 28 days with or without earthworms (*E. fetida*). Values are mean $\pm$ standard deviation ($n = 3$), and different letters indicate the statistical difference at the $p < 0.05$ level.

kilogram of dry soil within 1 h, respectively (see Text S2 in the [Supporting Information](#) for details).

Soil Microbial Community Structure and Bioinformatics Analysis. Soil DNA was extracted from 0.5 g fresh soil samples using a E.Z.N.A. soil DNA kit following the manufacturer's instructions. Each soil sample was subjected to two successive DNA extractions, and the duplicates were pooled as a DNA sample. The DNA quality and concentration were analyzed with a NanoDrop 2000. The extracted DNA was stored at -20°C for further use. The detailed PCR amplification and sequencing procedures for the bacterial communities are shown in Text S3 in the [Supporting Information](#). The universal bacterial 16S rRNA gene of the V3–V4 region was used to measure the bacterial community structure. Sequencing libraries were paired-end sequenced (2×300) on the Illumina MiSeq platform according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The sequencing data were submitted to the NCBI Sequence Read Archive database (no. SRP338936).

Soil Metabolite Analysis. After the exposure and after the removal of the earthworms, the soil from each replicate was thoroughly homogenized, and 1 g of soil was frozen in liquid nitrogen and stored at -80°C for later extraction. The soil metabolites were extracted with a mixture of methanol, acetonitrile, and water (2:2:1; v/v/v). An ultrahigh-performance liquid chromatography (UHPLC) system (1290 Infinity LC, Agilent Technologies, USA) coupled to a quadrupole time-of-flight (Q-TOF) system (AB Sciex TripleTOF 6600, Sciex, USA) was used to analyze the soil metabolites. Detailed information on the metabolite extraction and LC–mass spectrometry (MS) analysis is provided in Text S4 in the [Supporting Information](#).

Statistical Analyses. One-way analysis of variance (ANOVA) with Duncan's test in SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was used to analyze for the significance of differences among treatments. Nonmetric multidimensional scaling (NMDS) and Adonis analysis, Spearman's correlation analysis, and partial least-squares discriminant analysis (PLS-DA) were performed using “vegan”, “corrplot”, and “mixO-mics” packages, respectively, in R 3.6.0 for soil bacterial community analysis. Data in soil metabolomics were normalized (Pareto scaling) and transformed (log-transformed). Principal component analysis (PCA) and PLS-DA were performed using SIMCA 13.0 (Umetrics, Umeå, Sweden) for soil metabolite analysis. The variable importance (VIP) value of each variable in the PLS-DA model was calculated to indicate its contribution to the separation. Microbes and metabolites with VIP > 1.0 and $p < 0.05$ (one-way ANOVA) were regarded as the ones that were significantly influenced by CeO_2 NPs or by earthworms.

RESULTS AND DISCUSSION

Response of Enzyme Activities to CeO_2 NPs and Earthworms. In both the NP-spiked and nonspiked soils, the mortality of earthworms was less than 8% during the experiment (Table S2).

The microbial biomass C, activities of catalase, and keystone enzymes of C, N, and P cycles (i.e., β -glucosidase and peroxidase for C; acid protease for N; and acid phosphatase for P) were determined as the soil enzyme activity responses to CeO_2 NPs and earthworms.

Without earthworms in the soil, significant decreases of acid protease and acid phosphatase and an increase of catalase activities were observed in the NP-spiked soils compared with that in the control (Figure 1). The effects on catalase and acid phosphatase activities were dose-dependent ($p < 0.05$; Figure

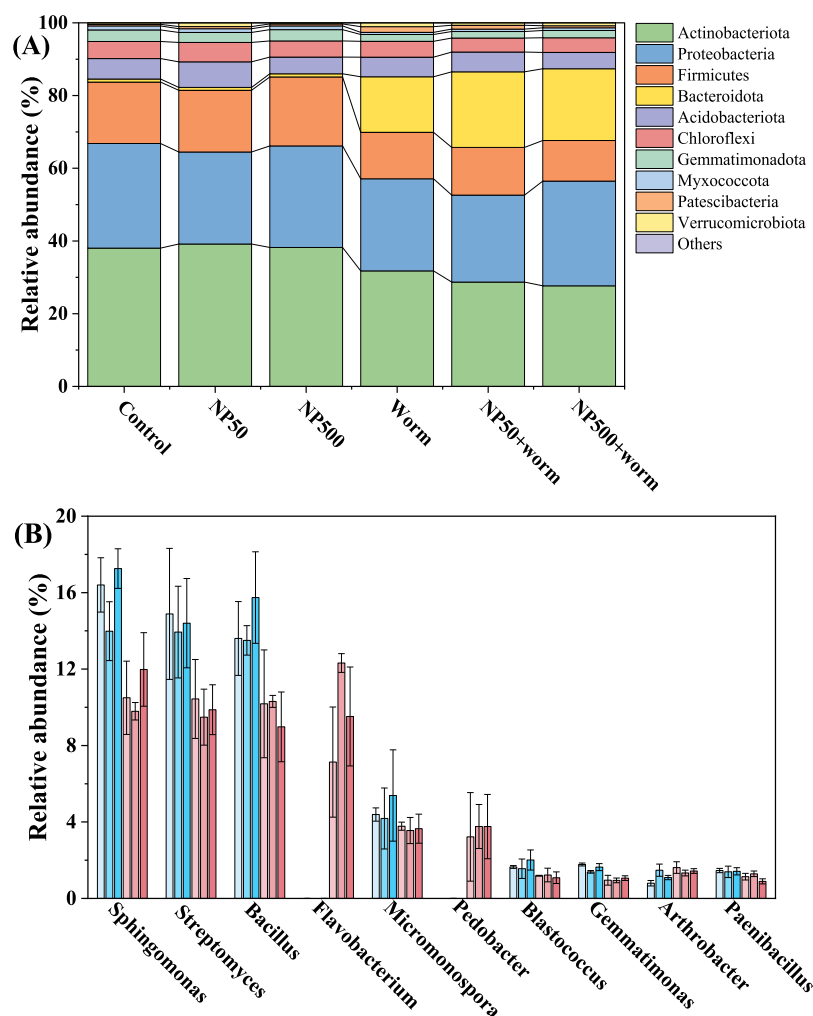


Figure 2. Relative abundance of soil bacteria at the level of phylum (A) and genus (top 10) (B) in soil spiked with CeO₂ NPs at 50 or 500 mg/kg dry soil and incubated for 28 days with or without earthworms (*E. fetida*). Values are mean $\pm$ standard deviation ($n = 3$).

1C,F). The alteration of acid phosphatase activities in our study is consistent with the results of Luo et al.,⁵ in which both 200 and 1000 mg/kg CeO₂ NP exposure had an inhibitory effect on the soil acid phosphatase activity. This is probably due to the negative effects of NPs on soil microbes such as the induction of lipid peroxidation, membrane damage, and reactive oxygen species (ROS) production within the microbial cells. Subsequently, the increased ROS can stimulate the catalase activity to defend against oxidative stress.³³ In the presence of earthworms, the activities of acid protease, acid phosphatase, and catalase, however, did not show a dose-related effect of NPs (Figure 1C,E,F). These results indicate a beneficial role of earthworms in mediating the negative effects of NPs on soil enzyme activities. The activities of worms such as burrowing, ingestion and egestion of soil particles, mucus secretion, and interactions between the gut microbiota and soil microbiota could not only change the physicochemical properties but also improve the microbial activities of soil.^{19,23} These changes might further mitigate the adverse effects of NPs on soil enzymatic activities.

Moreover, increased activities of catalase and peroxidase were observed in the presence of earthworms without or with NPs added ($p < 0.05$; Figure 1C,D). This could be due to the earthworm activities, such as decomposing, mineralizing, and burrowing, that subsequently may promote microbial activity

by altering the microhabitat and the nutrient status.^{19,23} Additionally, exposure to NPs caused oxidative stress of earthworms (unpublished data). The higher catalase activity could be partly due to the higher amounts of ROS produced by earthworms.³³ However, the presence of earthworms significantly decreased soil microbial biomass C (Figure 1A), probably due to the stronger competition of earthworms with microbes for organic materials³⁴ and the direct digestion of microorganisms.³⁵ Meanwhile, peroxidase and β -glucosidase activities did not show a clear pattern among treatments (Figure 1B,D). These results suggest that among the above soil enzymes, catalase, acid phosphatase, and acid protease could be the most sensitive enzymes in response to CeO₂ NP exposure in our test soil.

Alterations of Soil Bacterial Community Compositions. A higher alpha diversity was found in the groups in which earthworms were present (Worm, NP50 + worm, and NP500 + worm) (Table S4), indicating that the earthworms may promote bacterial community diversity. A further investigation of the responses of soil bacteria to CeO₂ NPs and earthworms at the phylum and genus levels was performed afterward. The dominant phyla (relative abundance >10%) across all soil samples were Actinobacteriota (27.3–38.7%), Proteobacteria (23.5–28.5%), and Firmicutes (11.0–18.8%), which accounted for more than 64.6% of the total abundance

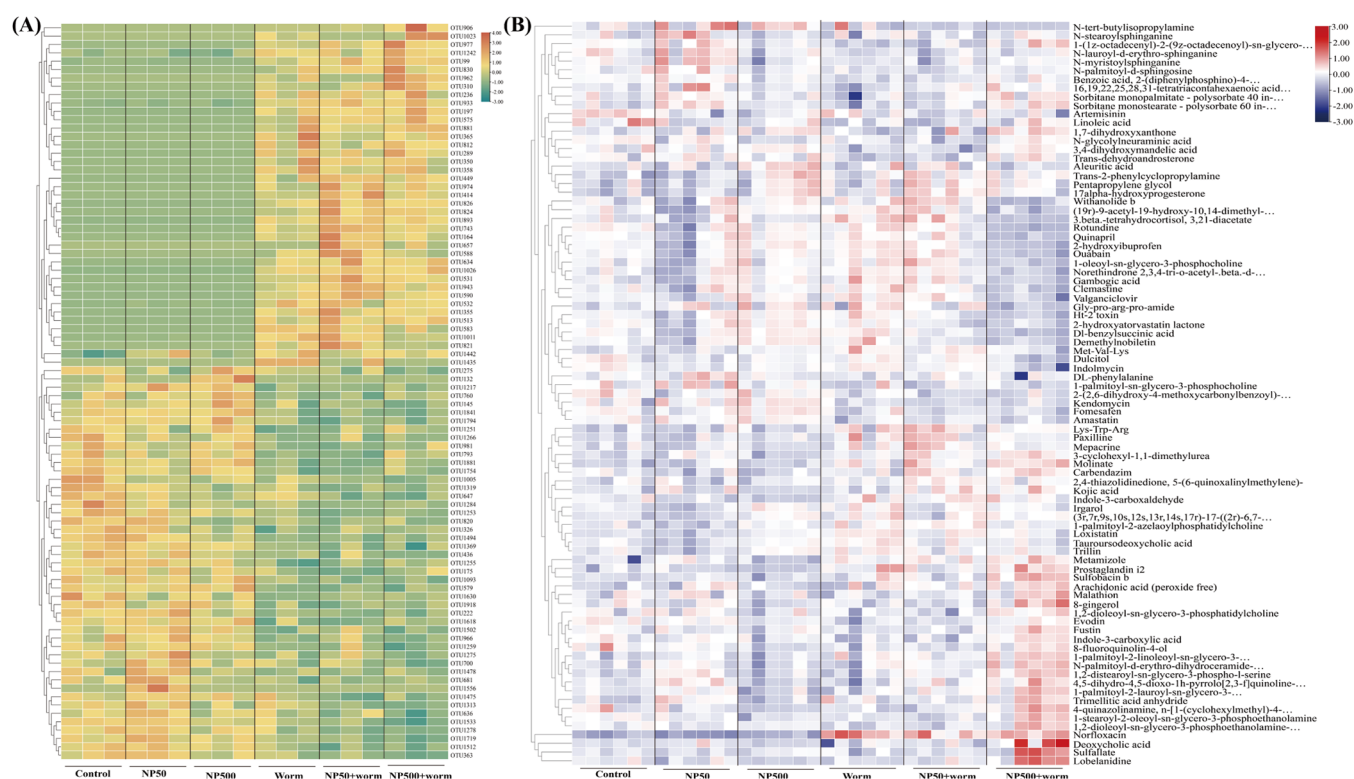


Figure 3. Hierarchical clustering of significantly changed microbes (A) and metabolites (B) ($p < 0.05$ and VIP > 1) in soil spiked with CeO₂ NPs at 50 or 500 mg/kg dry soil and incubated for 28 days with or without earthworms (*E. fetida*). Using the normalized relative abundance or expression data, 139 microbes and 85 metabolites (57 metabolites in the positive ion mode and 28 metabolites in the negative ion mode) were clustered.

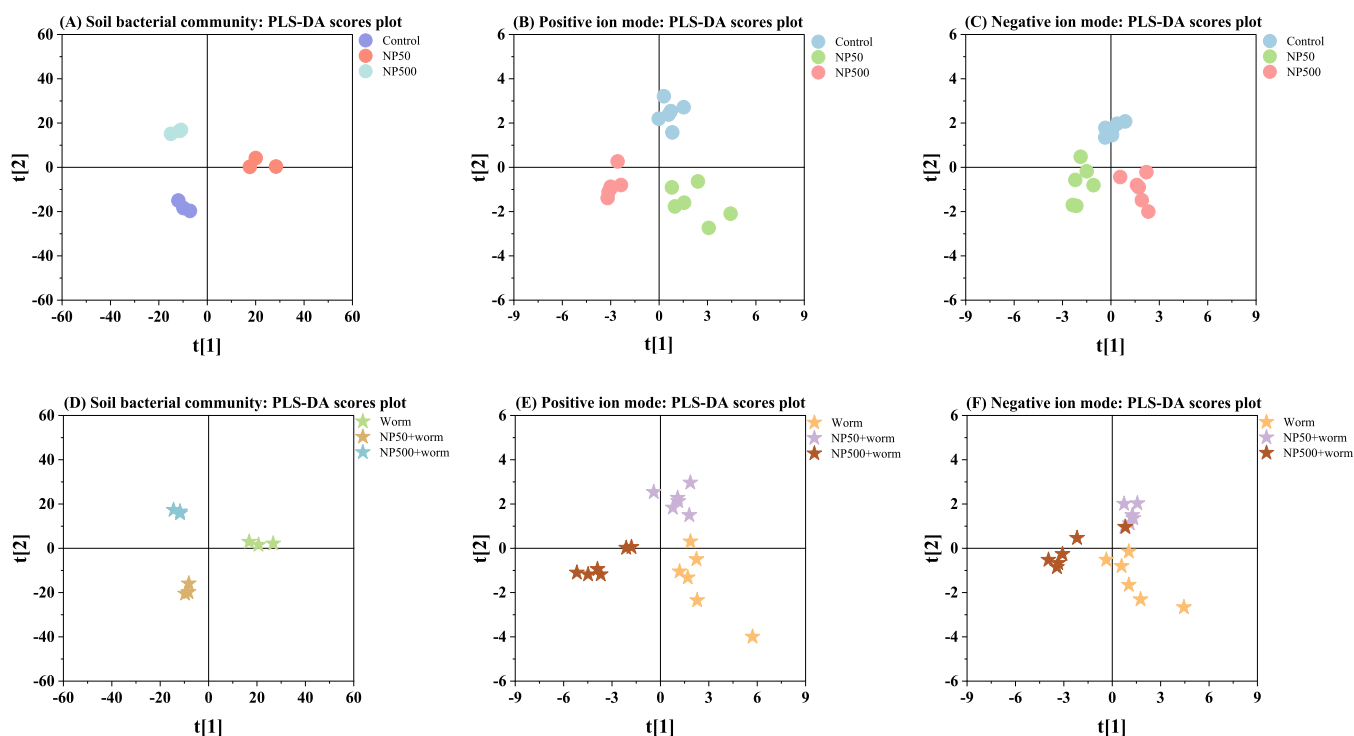


Figure 4. PLS-DA of the bacterial community (A,D) and metabolic profiles (B,C,E,F) in soil spiked with CeO₂ NPs at 50 or 500 mg/kg dry soil and incubated for 28 days with or without earthworms (*E. fetida*). The grouped comparisons include control vs NP50 vs NP500 (A–C) and worm vs NP50 + worm vs NP500 + worm (D–F).

in all groups (Figure 2A). These taxa are not only the main contributors to soil C, N, and P cycles but also the most abundant in soils.^{5,6,36}

The NMDS analysis showed that groups without earthworms (control, NP50, and NP500) were separated from earthworm-present groups along the first axis (Figure S3A).

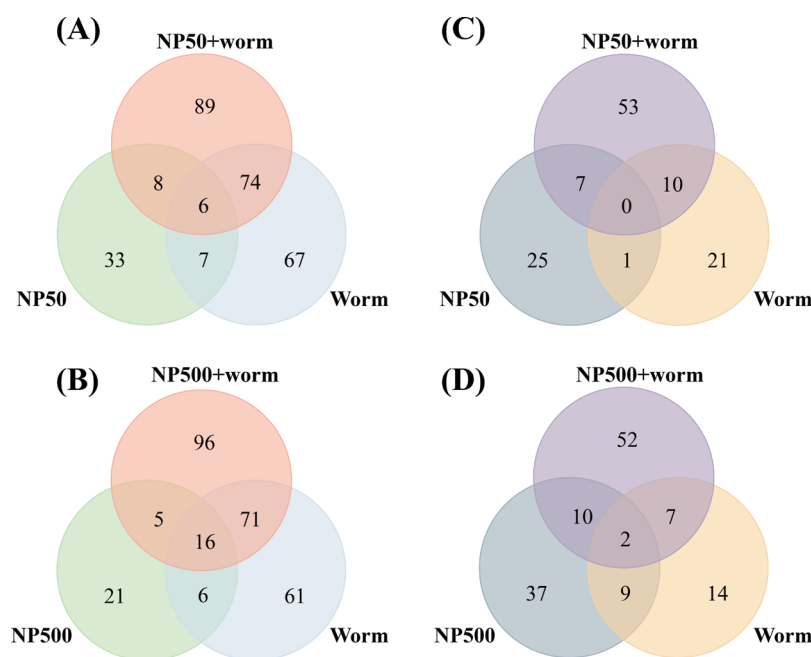


Figure 5. Edwards–Venn diagram of the significantly changed microbes (A,B) and metabolites (C,D) in soil spiked with CeO₂ NPs at 50 or 500 mg/kg dry soil and incubated for 28 days with or without earthworms (*E. fetida*). The significantly changed microbes and metabolites were separately obtained by performing PLS-DA for the NP50, NP500, worm, NP50 + worm, and NP500 + worm groups *vs* the control group.

This indicates that earthworms changed the composition of the soil bacterial communities. PERMANOVA further revealed that earthworms ($R^2 = 0.550$, $p = 0.002$) are the main factor explaining the variation of bacterial communities (Figure S3A). The presence of earthworms significantly decreased the relative abundance of *Firmicutes*, *Gemmatimonadota*, and *Myxococcota*, whereas they increased the relative abundance of *Bacteroidota* ($p < 0.05$), in particular the members of *Sphingomonas*, *Streptomyces*, and *Gemmatimonas* genera with a decreasing trend and the members of *Flavobacterium*, *Pedobacter*, and *Arthrobacter* genera with an increasing trend (Figures 2 and S2 and Table S5). Operational taxonomic units belonging to *Bacteroidota* increased significantly in our study with the presence of earthworms, possibly due to being typically found in the gut of earthworms.^{36,37} Earthworms are known to affect the soil microbial community through direct ingestion, which could result in the increasing dominance of several bacterial groups such as *Bacteroidota* by forming and altering the microbial habitats and nutrient status.^{26,37–39}

Supervised multivariate analysis PLS-DA was further performed on all groups to maximize the difference among groups and to find the microbes contributing to this difference (Figure S3B). Similar to the results of NMDS and PERMANOVA analyses, the PLS-DA clearly separated the earthworm-present groups from the earthworm-absent groups along the first axis (Figure S3B). For all groups, a total of 139 significantly changed microbes (VIP > 1 and $p < 0.05$) were determined. Heatmap analysis of these significantly changed microbes indicated that their normalized relative abundance was discernibly different in earthworm-present and earthworm-absent soils and further grouped by the CeO₂ NP exposure level (Figure 3A). When we separately compared groups with the same level of CeO₂ NP exposure (i.e., 50 or 500 mg/kg CeO₂ NPs) with control and worm groups, the microbes in soils containing earthworms were clearly separated from those in the earthworm-absent soils along the first axis (Figure S4).

In line with these results, the bacterial composition of the earthworm-absent groups was separated from that of earthworm-present groups along CAP1, suggesting that earthworms had a significant impact on the alpha as well as the beta diversity.³⁷ This may be attributed to the soil structural, chemical, and biological changes due to the earthworm activity and mucus excretion⁴⁰ and to the presence of microbes in the gut of earthworms, which in turn modify the abundance, diversity, and activity of soil microbes.^{27,39,41}

To further disentangle the interactive effects of CeO₂ NPs and earthworms on soil microbes, we separately compared the microbial responses to CeO₂ NPs in the absence or presence of earthworms (Figure 4). CeO₂ NPs induced obvious microbial community changes as compared to the corresponding control groups, regardless of the presence or absence of earthworms (Figure 4A,D). Previous studies have found alterations of microbial communities in soil exposed to CeO₂ NPs at 25–1000 mg/kg dry soil.^{5,6,11} Tang et al.⁴² observed that more CeO₂ NPs were distributed in the cytomembrane of microbes than that inside the cell, which might cause membrane damage.^{5,43} NPs may also indirectly affect soil bacteria by changing the soil microhabitats due to their large surface area and high reactivity.^{44,45}

In order to determine how earthworms may affect the CeO₂ NP effects, we performed PLS-DA for control *versus* CeO₂ NP-exposed groups (i.e., NP50, NP500, NP50 + worm, and NP500 + worm) and worm groups, respectively. A total of 54, 48, 154, 177, and 188 significantly changed microbes were obtained in soil exposed to 50 mg/kg CeO₂ NPs, 500 mg/kg CeO₂ NPs, earthworms, 50 mg/kg CeO₂ NPs with earthworms, and 500 mg/kg CeO₂ NPs with earthworms, respectively (Tables S6–S10 and Figure 5). An Edwards–Venn diagram was obtained to reveal the overlapping and specific significantly changed microbes after CeO₂ NP exposure in the absence/presence of earthworms (Figure 5). There were 33 specific microbes in the NP50 group, 89 in the

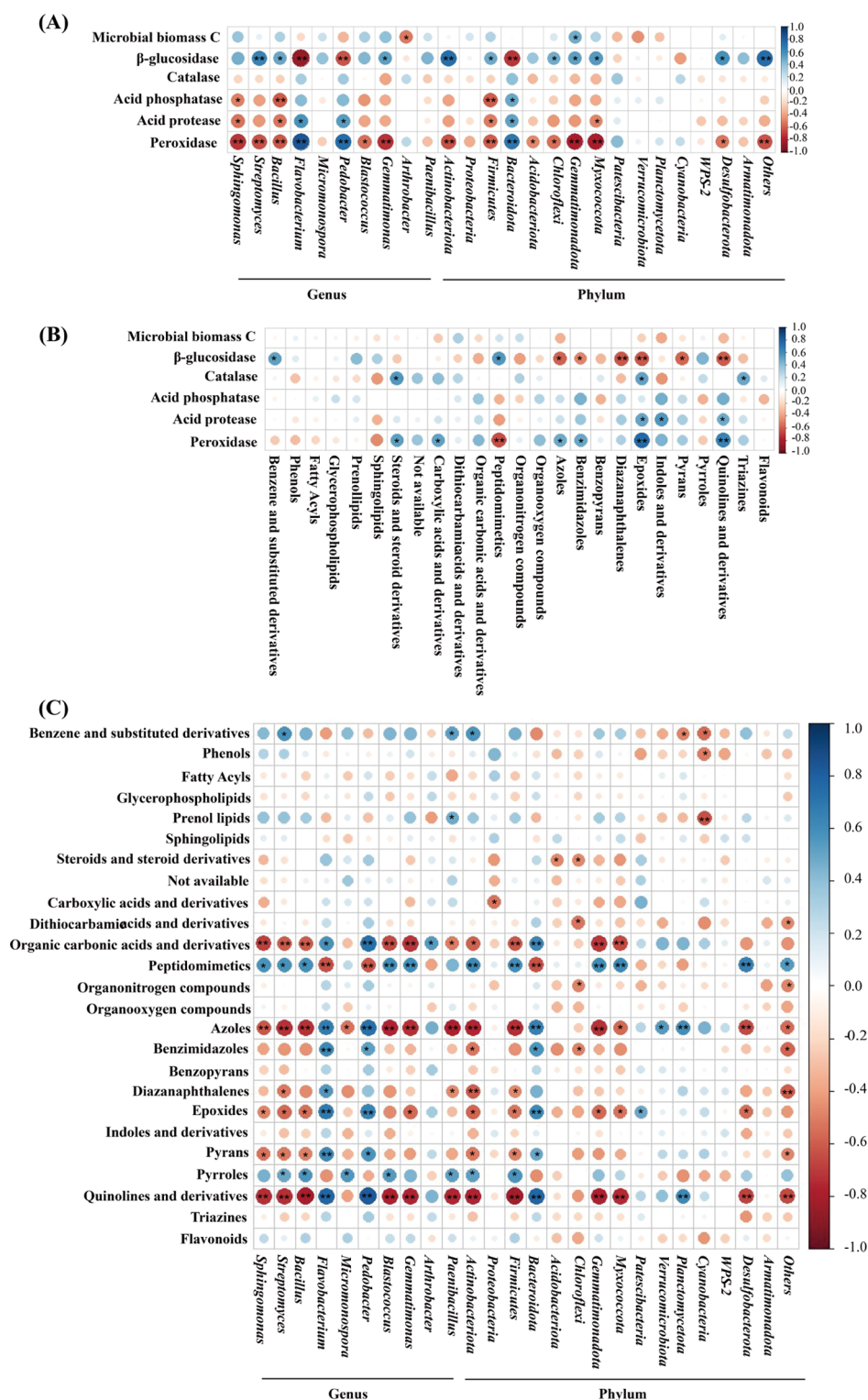


Figure 6. Correlation analyses between soil enzyme activities and soil bacterial communities at the phylum/genus level (A), soil metabolites at the class level (B), soil bacterial communities at the phylum/genus level, and soil metabolites at the class level (C) in soil spiked with CeO₂ NPs at 50 or 500 mg/kg dry soil and incubated for 28 days with or without earthworms (*E. fetida*).

NP50 + worm group, 21 in the NP500 group, and 96 in the NP500 + worm group. Regarding the effects of earthworms in the presence of CeO₂ NPs, the NP50 + worm group shared 14 of the 54 microbes that were significantly changed in the NP50 group. The NP500 + worm treatment shared 21 of the 48 microbes that were significantly changed by the NP500 treatment. For the CeO₂ NP effects in the presence of

earthworms, the NP50 + worm treatment and NP500 + worm treatment shared 80 and 87 microbes, respectively, of the 154 microbes that were significantly changed from the worm treatment. Our results thus suggest a promoting effect on the soil bacteria of CeO₂ NPs in the presence of earthworms. This not only may be the result of the effect of earthworm activity (i.e., burrowing and mucus production) on the mobility and

bioavailability of the CeO₂ NPs^{46,47} but might also be attributed to interactions between the soil microbiota and earthworm gut microbiota.⁴¹

Alterations of Soil Metabolite Profiles. Using untargeted UPLC–Q-TOF–MS analysis, a total of 243 and 142 compounds under the positive and negative modes, respectively, were detected in soil. Initially, unsupervised PCA clearly separated the earthworm-present groups from the corresponding earthworm-absent groups (Figure S3C,E). Supervised PLS-DA further maximized these separations and helped identify the responsible metabolites leading to these separations (Figure S3D,F). For all test groups, a total of 57 and 28 differential metabolites (VIP >1.0 and $p < 0.05$) under the positive and negative modes, respectively, were subjected to hierarchical clustering (Figure 3B). It is noteworthy that a number of metabolites such as Lys–Trp–Arg, prostaglandin i2, and 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) and organoheterocyclic compounds including indolmycin, indole-3-carboxaldehyde, indole-3-carboxylic acid, epoxides, and norfloxacin decreased with CeO₂ NPs (50 and 500 mg/kg) alone. Additionally, sphingolipids including *N*-palmitoyl-*d*-sphingosine, *N*-lauroyl-*d*-erythro-sphinganine, *N*-myristoylsphinganine, and *N*-stearoylsphinganine increased for 50 mg/kg CeO₂ NPs alone, while decreased for 500 mg/kg CeO₂ NPs alone (Figure 3B). Sphingolipids have been found to have a number of biological functions, for instance, to maintain the membrane function and integrity and preserve the lipoprotein structure and functions. The addition of earthworms changed these metabolite expression patterns, possibly due to earthworm activities such as mucus secretion and gut digestion.⁴⁰ Upon increasing the NP concentration, the differences in the expression patterns of metabolites between the control and NP-spiked groups became more apparent. This is likely due to the antibacterial properties of NPs, which result in changes in the microbial metabolic activity and the release of extracellular metabolites.⁴⁸

We further focused on the separate effects from the NP exposure and earthworm presence on the differences in the expression of soil metabolites (Figure 4). CeO₂ NPs were responsible for soil metabolic reprogramming even though the earthworms were present (Figure 4B,C,E,F), which echoed the microbial response to CeO₂ NPs. At the same NP concentration, the metabolic alteration trends were consistent with that of soil bacterial changes (Figure S4). To illustrate how earthworms modified the responses of soil metabolite profiles to NPs, we separately performed PLS-DA for the control versus NP and worm samples and obtained 33, 58, 70, and 71 differential metabolites in soil after exposure to NP50, NP500, NP50 + worm, and NP500 + worm treatments, respectively (Tables S11–S15). Generally, these significantly changed metabolites (SCMs) could be classified into the following categories: (1) benzene and substituted derivatives, (2) carboxylic acids and derivatives, (3) fatty acyls, (4) organonitrogen compounds, (5) organooxygen compounds, and (6) prenol lipids (Figure S7). With earthworms, the CeO₂ NPs induced more differential metabolites in terms of the number of metabolites. Some of these differential metabolites participate in a series of important microbial metabolic pathways. The perturbed biological pathways were identified using MetaboAnalyst 5.0. The results of this analysis revealed that there were two and six altered pathways in soil exposed to CeO₂ NPs at 50 mg/kg and 500 mg/kg without earthworms and eight and five in soil exposed to CeO₂ NPs at 50 mg/kg

and 500 mg/kg with earthworms, respectively (Table S16). It is noteworthy that several perturbed pathways were related to the microbial nitrogen and phosphorus metabolism such as amino acid, glycerophospholipid, purine, and pyrimidine metabolisms. We constructed an Edwards–Venn diagram to reveal the overlapping and specific differential metabolites after exposure to CeO₂ NPs in the absence/presence of earthworms (Figure 5). There were 25 specific metabolites in the NP50 treatment (mainly naphthalenes, linear 1,3-diarylpropanoids, glycerolipids, and benzofurans), 53 in the NP50 + worm treatment (mainly diazanaphthalenes, epoxides, imidazopyrimidines, isoflavonoids, purine nucleotides, pyrans, and pyrimidine nucleotides), 37 in the NP500 treatment (mainly isoquinolines and pyridines), and 52 in the NP500 + worm treatment (mainly peptidomimetics, dithiocarbamic acids, cinnamic acids, benzimidazoles, and anthracenes) (Figure S7). For effects of earthworms in the presence of CeO₂ NPs, the NP50 + worm treatment shared 7 out of 33 metabolites that were significantly changed from the NP50 exposure only. The NP500 + worm treatment shared 12 of the 58 metabolites that were significantly changed from the NP500 exposure only. For the CeO₂ NP effects in the presence of earthworms, NP50 + worm and NP500 + worm treatment separately shared 10 and 9 of the 32 metabolites, respectively, that were significantly changed in the worm-only exposure. The modification of the effects of CeO₂ NPs on soil metabolites by the presence of earthworms was in parallel with the response of the soil bacterial community to NPs, that is, a promoting effect by earthworms under CeO₂ NP exposure (Figure 5C,D).

Correlations among Soil Enzyme Activities, Microbes, and Metabolites. Soil enzymes, metabolites, and microbes are known to have close relationships^{6,49} and are sensitive to environmental changes.^{3,4,31} Spearman correlation analyses were performed of soil enzymes with metabolites and soil bacterial communities. The presence of earthworm significantly increased the relative abundances of *Flavobacterium*, *Pedobacter*, and *Bacteroidota* (Figure 2). This contributed to increased acid protease and acid phosphatase activities (Figures 1 and 6A). Moreover, acid phosphatase and acid protease activities were negatively correlated with the relative abundances of *Sphingomonas*, *Bacillus*, and *Firmicutes* (Figure 6A). Therefore, in the presence of earthworms, the decrease of the relative abundances of *Sphingomonas*, *Bacillus*, and *Firmicutes* (Table S5 and Figure S2) contributed to ameliorate the inhibition of acid protease and acid phosphatase by CeO₂ NPs alone (Figure 1). The catalase and peroxidase activities had a significant positive correlation with steroids (Figure 6B), which are the main products of ROS in cells.⁵⁰ Some metabolites from organoheterocyclic compounds including indoles and derivatives, epoxides, and quinolines and derivatives were negatively correlated with β -glucosidase or positively correlated with peroxidase, catalase, and acid protease activities ($p < 0.05$ or $p < 0.01$) (Figure 6B). The inclusion of earthworms increased the expression of organoheterocyclic compounds (Figure 3B) and contributed to mitigating the adverse responses of soil enzymatic activities to CeO₂ NPs. Besides, these negative and positive correlations might be due to the specific enzymatic consumption or degradation and production, respectively.⁶ Meanwhile, the dissimilarity of the bacterial community was significantly related to that of metabolites, indicating that the bacterial community is an important driver of the distribution of metabolite profiles (Figure S7). The interconnections between metabolites and

microbes were further explored using Spearman correlation analysis (Figure 6C). The results revealed the strong associations between specific soil metabolites (i.e., organic carbon acids, peptidomimetics, azoles, epoxides, and quinolines) and soil microbes. Specifically, the earthworms remarkably increased the relative abundances of *Flavobacterium*, *Pedobacter*, and *Bacteroidota* (Figure 2), which contributed to the increased expressions of quinolines and epoxides (Figure 3B). The changes in soil microbes and metabolites with the addition of worms might be attributed to the activities of worms, that is, ingestion and epidermal mucus secretion under CeO₂ NP stress. Additionally, the unpublished data have shown that CeO₂ NPs caused perturbation in the metabolite profiles and gut microbiota of worms, which further affected the microbial community and metabolite profiles in soil. Overall, the changes of these bacterial relative abundances and metabolite expressions contributed to alleviate the inhibitory effect of CeO₂ NPs alone on soil acid protease and acid phosphatase activities in the presence of earthworms (Figure 6).

Although soil microbes can be under direct exposure of contaminants such as NPs, estimating their toxicity to microbes alone can be unilateral given that ecological interactions (e.g., predation or mutualistic facilitation) within the highly complex soil ecosystem are diverse.⁵¹ This can modify the toxicity of NPs on the soil microbial community structure and ultimately affect their functions. Here, in our study, the inclusion of one more component (i.e., earthworms) in the NP–soil microbe system can drastically reverse the negative impact induced by NPs through promoting microbes associated with soil N and P cycles and expressed metabolites associated with microbial N and P metabolisms. In short, the top-down control of earthworms on the soil microbes alone, possibly due to their grazing or facilitation, diminished the adverse effect of the NPs. Hence, a comprehensive understanding of the toxic effect of NPs to soil health requires the integrative investigations of the interplays among the focused components in the soil as they may act as one entity to respond to the same disturbance.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.1c06592>.

Additional experimental details, materials, methods, and bioinformatics analysis of sequencing and metabolomics data (PDF)

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Notes

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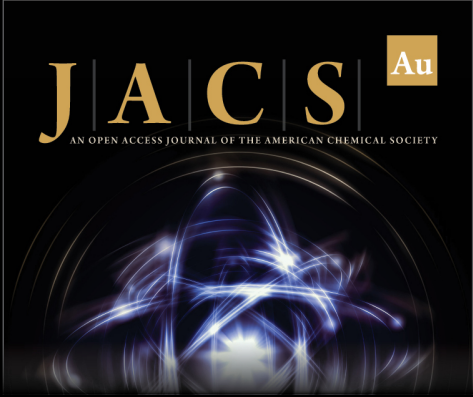
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