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Individual and combined effects of earthworms and *Sphingobacterium* sp. on soil organic C, N forms and enzyme activities in non-contaminated and Cd-contaminated soil

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Abstract:	<p>Earthworms and <i>Sphingobacterium</i> sp. are known for their strong organic compound decomposition ability and wide distribution in soil. However, interactions of soil organic matter decomposition with soil properties and whether microbial species such as <i>Sphingobacterium</i> sp. could assist earthworms in carbon and nitrogen transformation in soil remain poorly understood. Earthworms (<i>Eisenia fetida</i>, <i>Amyntas gracilis</i>) and <i>Sphingobacterium</i> sp. were introduced in non-contaminated and cadmium-contaminated soils under controlled laboratory conditions for 20 days. We examined their individual or combined effects on carbon and nitrogen forms and related enzyme activities to assess their influence on soil C and N cycling. Individual <i>Sphingobacterium</i> sp. inoculation led to significantly decreased organic carbon (SOC) contents, reducing it by 16.5% in non-contaminated soil and by 3.77%, in Cd-contaminated soil. It resulted in an increased microbial biomass carbon (MBC) contents, reaching 1685 ± 292 mg·kg⁻¹ in non-contaminated soil. Individual introductions of <i>E. fetida</i> and <i>A. gracilis</i> caused a decline in SOC content in non-contaminated soil, but increased significantly dissolved organic carbon (DOC) and alkali-hydrolysable nitrogen (AN) contents by 75.8%, 53.6% and 32.9%, 20.9%, respectively. In contrast, in Cd-contaminated soil, only the significant combined effects of earthworms and <i>Sphingobacterium</i> sp. were linked to significant increase in SOC contents, raising by 7.22% and 9.64% in <i>E. fetida</i> + <i>Sphingobacterium</i> sp. and <i>A. gracilis</i> + <i>Sphingobacterium</i> sp. treatments, respectively. In non-contaminated soil, the combined effects of earthworm and <i>Sphingobacterium</i> sp. further increased DOC and AN content by 212%, 134% and 31.3%, 25.4% in the treatments of <i>E. fetida</i> + <i>Sphingobacterium</i> sp. and <i>A. gracilis</i> + <i>Sphingobacterium</i> sp., respectively; the highest ratios of DOC to SOC and AN to total Nitrogen (TN) were found in the earthworm+<i>Sphingobacterium</i> sp. treatments as well. In non-contaminated soil, <i>Sphingobacterium</i> sp. and earthworms mainly influenced β-glucosidase (BG), urease (URE), N-acetyl-β-D-glucosaminidase (NAG) activities and</p>

	<p>fluorescein diacetate hydrolysis (FDA) hydrolysis, while in Cd-contaminated soil, they mainly influenced invertase (INV), NAG, URE, and protease (PRO) activities. Principal component analysis indicated that in non-contaminated soil, the earthworm activities dominated the mineralization processes of soil carbon and nitrogen, and <i>Sphingobacterium</i> sp. can intensify this process when it was inoculated in soil along with earthworms. Furthermore, both earthworm species increased C and N levels by elevated INV and PRO activities in combined inoculation. However, in contaminated soil, the impact of earthworm inoculation on soil C stabilization showed a species dependent pattern. <i>E. fetida</i> reduced C mineralization by decreasing URE activities, while <i>A. gracilis</i> enhanced C stabilization by increasing INV activities and decreasing PRO activities. In conclusion, earthworms played a key role in enhancing C and N mineralization in non-contaminated soil and promoting C stabilization in contaminated soil. Both earthworm species followed similar strategies in the former process but adopted different strategies in the latter. When introduced individually, <i>Sphingobacterium</i> sp. was able to promote mineralization in both soils, primarily assisting earthworms in improving carbon and nitrogen mineralization in non-contaminated soil but hindering these processes in Cd-contaminated soil. These findings provide insights into the combined effects of earthworms and microorganisms on carbon and nitrogen cycling.</p>
<p>Suggested Reviewers:</p>	<p>Brown George minhocassu@gmail.com</p> <p>xiaomin Li xiaomin.li@m.scnu.edu.cn</p> <p>Weixin Zhang weixinzhang@139.com</p> <p>Manuel Blouin manuel.blouin@agrosupdijon.fr</p>
<p>Response to Reviewers:</p>	<p>Title: Individual and combined effects of earthworms and <i>Sphingobacterium</i> sp. on soil organic C, N forms and enzyme activities in non-contaminated and Cd-contaminated soil</p> <p>Reviewer #1: The manuscript aims to examine the roles of one of dominant gut bacteria taxa (<i>Sphingobacterium</i> sp.) of earthworms and two earthworm species on soil organic matter transformation, as well as the associated changes in soil enzyme activities and other soil properties. Two types of soil, clean and Cd-contaminated, were used, and the results were interesting. There are some suggestions to be carefully considered in the revision.</p> <p>1. First, one or two scientific hypotheses could be given. In lines 91-92, the authors mentioned that <i>Sphingobacterium</i> sp. was usually one of dominant bacteria taxa in earthworm guts. This is very important base that a scientific hypothesis could be raised on. For example, given that <i>Sphingobacterium</i> sp. was dominant in worm gut, an inoculation of this bacteria may exert similar impacts on organic matter decomposition like earthworms, if it contributed great to the worm gut-mediated organic matter decomposition. or <i>Sphingobacterium</i> sp. inoculation did not affect organic matter transformation like earthworms, if its function relies on the special gut condition. or <i>Sphingobacterium</i> sp. may enhance earthworm impacts on carbon transformation by increasing the microbial activities in earthworm gut?</p> <p>Answer: Thanks for your comment and suggestion. Earthworm's preferential feeding and concentration processes have a substantial impact soil carbon and nitrogen cycling. Their bioturbation activities, including burrowing, feeding, and excretion (casts and surface mucus), affects the soil carbon and nitrogen distribution within soil profile. These effects result from complex mutualism interactions between earthworms and microorganisms. Previous studies have established connections between earthworms and rhizosphere growth-promoting bacteria or specific arbuscular mycorrhizal fungi to enhance nutrient availability or nitrogen storage in soil. Moreover, the collaboration of indigenous earthworms and gut bacteria has been employed to remediate metal contaminated soil and has shown superiority compared to chemical amendments. Likewise, combining earthworms with dominate gut bacteria taxa to enhance organic matter decomposition is also advantageous, because it is more readily accepted by earthworms. In our previous studies, <i>Sphingobacterium</i> sp. was identified as a</p>

dominant bacterial taxon in the earthworm gut. *Sphingobacterium* sp., a gram-negative bacterium, exhibits a wide range of nutrient sources, high adaptability, and a broad ecological distribution. In practice, it has been used to decompose agricultural waste and degrade organic pollutants (e.g. alkanes and polycyclic aromatic hydrocarbons) in soil. Therefore, this decomposition ability of earthworms probably derives from symbiotic microorganisms in their guts. Considering that earthworms and *Sphingobacterium* sp. impact organic matter decomposition and the latter was dominant bacterial taxon in earthworm gut, a synergistic relationship may exist, enhancing soil organic matter decomposition. However, under environment stress, such as metal contamination, the activities and complex interactions of them may be suppressed. Specifically, metal contamination can induce biomass loss, reduce survival rate, cause irreversible physiological damage, decrease the stability of gut bacterial network, and dampen its ecological functions. Similarly, the inhibition effects also impact soil microorganisms, leading to reduced soil enzyme functions critical for organic matter cycling. In addition, different earthworm species and ecological categories have varying effects on soil organic matter degradation and soil C and N cycles. The species of the experimental model earthworm *E. fetida* and local wild epigeic species may have different survival strategies that affect microbial biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in non-contaminated and Cd-contaminated soil.

Based on this, the same taxa were selected in this study to reveal that if the combined effects of this strain and earthworm on soil carbon and nitrogen turnover. Therefore, we added the hypothesis as:

Line 135-140: Given that *Sphingobacterium* sp. is the dominant bacterial taxon in the earthworm gut, we hypothesized that *Sphingobacterium* sp. and earthworm could individually promote soil organic matter decomposition and, when combined, exert reinforcing effects. These effects are negatively impacted by metal stress and influenced by the survival strategies of different earthworm species; Additionally, *Sphingobacterium* sp. and earthworms may play different roles in this combined process, with *Sphingobacterium* sp. stimulating microbial activities in the earthworm gut.

2. In addition, the possible interactions (and the associated consequences) between *Sphingobacterium* sp. and earthworms may differ in the clean and Cd-contaminated soils. Q: What

may be the difference and how did they happen? All these should be added in the introduction section and reflected in the hypotheses. For instance, in considering what..., earthworm and *Sphingobacterium* sp. will enhance soil carbon mineralization in clean soil, but enhance soil carbon sequestration in Cd-contaminated soil.; or, given that, the inoculated earthworm and bacteria may cooperate and exert an additive effect, or may compete or negatively interact with each other, and exert a negative effect?

Answer: Thanks for your comment. We rearrange the introduction part as:

Line 108-117: However, under environment stress, such as metal contamination, the activities and complex interactions of them may be suppressed. Specifically, metal contamination can induce biomass loss, reduce survival rate, cause irreversible physiological damage, decrease the stability of gut bacterial network, and dampen its ecological functions [27-29]. Similarly, the inhibition effects also impact soil microorganisms, leading to reduced soil enzyme functions critical for organic matter cycling [30]. In addition, different earthworm species and ecological categories have varying effects on soil organic matter degradation and soil C and N cycles [31]. The species of the experimental model earthworm *E. fetida* and local wild epigeic species may have different survival strategies that affect microbial biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in non-contaminated and Cd-contaminated soil.

In addition, we also added the hypothesis as:

Line 135-140: Given that *Sphingobacterium* sp. was dominant bacteria taxa in earthworm gut, we hypothesized that *Sphingobacterium* sp. and earthworm could promote soil organic matter decomposition in individual processes and exert reinforcement effects in combined processes, which effects are negatively impacted by metal stress and influenced by survival strategies of different earthworm species; Additionally, *Sphingobacterium* sp. and earthworms may play different roles in combined process in this system, with *Sphingobacterium* sp. making influence by stimulating microbial activities in earthworm gut.

3. Line 132, please check the biomass per ind. here, too big for a *E. fetida*.

Answer: Thanks for your comment. We added more detail about earthworm biomass after checking and confirming the data as “Each pot was inoculated with 3 g of earthworms, approximately 8 individual *E. fetida* (~ 0.37 g-ind⁻¹) or 5 individual *A. gracilis* (~ 0.60 g-ind⁻¹).”. In previous study, the average weight of *E. fetida* is around 0.4 g-ind⁻¹ (Yue et al., 2019). Therefore, we think it is in normal range of biomass of *E. fetida*.

Reference: S.Z. Yue, H.Q. Zhang, H.Y. Zhen, Z.Q. Lin, Y.H. Qiao, Selenium accumulation, speciation and bioaccessibility in selenium-enriched earthworm (*Eisenia fetida*), *Microchemical Journal* 145 (2019) 1-8, <https://doi.org/10.1016/j.microc.2018.10.015>.

4. Line 173, What is DTPA Cd, and how to measure it?

Answer: Thanks for your comment. We added more details about DTPA and measure method in lines 203-213 as:

2.4 Total and DTPA extractable Cadmium content

Total cadmium (Cd) in the soils was extracted by microwave digestion with aqua regia (HNO₃:HCl (v/v) = 1:3). The diethylenetriaminepentaacetic acid (DTPA) extractable form was regarded as bioavailable metals and toxic. DTPA-extractable Cd was extracted with DTPA extractant at a Soil:DTPA extractant ratio of 5:25 (g/mL), which included a solution of 5 Mm DTPA and 10 mM CaCl₂. The extracted suspensions were analyzed for total cadmium (Cd) and DTPA-extractable contents using inductively coupled plasma-mass spectrometry (ICP-MS, 7700ce, Agilent Technologies, Santa Clara, CA, USA) [43]. GBW07455 (GSS-26) was employed as certified reference material for soil analyses, and the measured concentrations were within 98% of the reported certified concentrations for Cd. Quality control measures included three repetitions for each soil and three blanks for each batch to ensure the quality of the analysis.

5. Lines 322-326, Not sure here, a lower standing MBC did not necessarily indicate a low growth of bacterial population, alternatively, this may also result from a strong feeding of earthworm on the bacterial biomass?

Answer: Thanks for your comment. We agree with you. The feeding of earthworm also strong impacts the microbial biomass and structure. We revised and improve this part as:

Line 402-411: Earthworm activities under the combined process dramatically decreased microbial biomass carbon compared to *Sphingobacterium* sp. activities alone. It is suggested that earthworms contribute to maintaining the ecosystem balance and stabilizing the microbial community structure through feeding [71] and regulating the allocation of active organic carbon and dissolved organic carbon pools [57,72]. Therefore, the rapid proliferation of *Sphingobacterium* sp. was prevented in CSE and CAS treatments. It was further confirmed by the significantly lower microbial quotient carbon in treatment CSE and CSA compared to treatment CS (Fig. 2E). It can be deduced that earthworms may reduce the conversion efficiency of soil microorganisms in utilizing carbon sources into microbial biomass carbon [52] or they can intensely feed and graze on microbes [10, 73-75].

6. Lines 355-356 and lines 414-415, so, how to understand such negative effects of earthworms? similar negative effect was also reported in literature, such as Lv et al., 2020 <https://doi.org/10.1016/j.scitotenv.2020.137227>;

Answer: Thanks for your comment. Likewise, in Lv et al. (2020), earthworm activities suppressed β -glucosidase activity during stimulating carbon mineralization processes. They speculated that when soil microorganisms could easily access the readily mineralized C released by earthworms, they would use a smaller portion of the carbon from enzyme-catalyzed decomposition of soil organic carbon. Additionally, the enzyme activity only represents the concomitant consequence of earthworm activities but does not serve as the major driver of organic matter decomposition. It is also controlled by multiple factors such as food type, pH, temperature, gut transit time, metal stress, substrates, et al., [65-67].

We revised the discussion of negative effects as:

Line 358-365 as: As for enzymes, the enzymes activity was influenced by earthworm species and metal stress. Likewise, in the previous study, earthworm activities suppressed β -glucosidase activity during the stimulation of C mineralization processes [65]. It was speculated that when soil microorganisms could easily access the readily mineralized C released by earthworms, they would use a smaller portion of the carbon from enzyme-catalyzed decomposition of soil organic carbon. Enzyme activity only represents the concomitant consequence of earthworm activities but does not serve as the major driver of organic matter decomposition. It is also controlled by multiple

factors such as food type, pH, temperature, gut transit time, metal stress, substrates, et al., [65-67].

7. lines 414-415 may suggest that soil microbiota did not play a key role in OC decomposition processes, but changed accordingly, worm dominated these processes.

Answer: Thanks for your comment. You are right. The soil enzymes activities shown inhibited which may suggest that the earthworm dominated the organic carbon decomposition processes and *Sphingobacterium* sp. play an assistance role. Another underlying factor is that *Sphingobacterium* sp. may assist earthworms in mineralizing soil organic carbon and nitrogen by stimulating the microbial activities in the earthworm gut. The subsequent studies need to focus on the earthworm gut microbial to reveal the internal mechanisms. We have added some discussion in the manuscript as:
Line 393-417: In these processes, earthworm activities dominated the soil carbon and nitrogen mineralization processes, while *Sphingobacterium* sp. played a supporting role in combined processes. When the effects of earthworm and *Sphingobacterium* sp. on carbon and nitrogen were consistent, they enhanced each other; otherwise they undermined each other. Hence, in non-contaminated soil, carbon mineralization was observed. While in Cd-contaminated soil, carbon storage was more prevalent due to the stronger effect of earthworms on stabilization than *Sphingobacterium* sp. on mineralization. This is further confirmed by PCA which demonstrated that earthworms play a critical role in soil organic carbon decomposition processes, following the same trend as inoculation with earthworm alone and in combination with *Sphingobacterium* sp. (Fig. 4A and C; Fig. 5A and C). Additionally, in this system, a strong interaction existed between earthworms and *Sphingobacterium* sp.. Earthworm activities under the combined process dramatically decreased microbial biomass carbon compared to *Sphingobacterium* sp. activities alone. It is suggested that earthworms contribute to maintaining the ecosystem balance and stabilizing the microbial community structure through feeding [71] and regulating the allocation of active organic carbon and dissolved organic carbon pools [57,72]. Therefore, the rapid proliferation of *Sphingobacterium* sp. was prevented in CSE and CAS treatments. It was further confirmed by the significantly lower microbial quotient carbon in treatment CSE and CSA compared to treatment CS (Fig. 2E). It can be deduced that earthworms may reduce the conversion efficiency of soil microorganisms in utilizing carbon sources into microbial biomass carbon [52] or they can intensely feed and graze on microbes [10, 73-75]. Additionally, the different impact of earthworm species may be caused by *E. fetida* being more conducive to the survival and growth of G- bacterial communities [75], thus promoting *Sphingobacterium* sp. to assist earthworms in carbon mineralization. Based on the above, another underlying factor was postulated that *Sphingobacterium* sp. may assist earthworms in mineralizing soil organic carbon and nitrogen by stimulating the microbial activities in the earthworm gut. The subsequent studies need to focus on the earthworm gut microbial to reveal the internal mechanisms.

8. lines 392-393, how could the assistance role of *Sphingobacterium* sp. on worm could be observed in Fig. 4a?

Answer: Thanks for your comment. Sorry for our mistakes. This conclusion can't be observed only in Fig. 4A, it should be analysis through combining Fig. 4A and Fig. 4C. The low PC1 values in Fig. 4A means high carbon and nitrogen mineralization such as DOC and AN. Inoculation earthworm treatments trend to have low PC1 values especially co-inoculation with *Sphingobacterium* sp. (Fig. 4C). It means that co-inoculation with *Sphingobacterium* sp. can enhance the earthworm effects on carbon and nitrogen transformation. We added the details of figure label as:

Line 399-401: This is further confirmed by PCA which demonstrated that earthworms play a critical role in soil organic carbon decomposition processes, following the same trend as inoculation with earthworm alone and in combination with *Sphingobacterium* sp. (Fig. 4A and C; Fig. 5A and C).

Reviewer #2:

Jia et al. investigated the effect of single and combined application of earthworms and *Sphingobacterium* sp. on the C and N cycles in a non-contaminated soil and a cadmium-contaminated soil. The C and N fractions in soil, ratio of C/N fractions, soil enzyme activities, relationships among the above parameters were determined. The results of this study can provide a better understanding of how different species of earthworms and their co-application with a key bacterium from the gut of earthworm regulate the carbon and nitrogen mineralization/fixation in the non-contaminated and Cd-contaminated acid soils. Yet, the current version of the manuscript still needs

further modification and improvement, particularly the "Discussion" part. More details need to be addressed clearly as well.

1. Abstract. The abstract should be revised and clearly introduce which kind of results comes to which kind of key finding, not just simply mention the results.

Answer: Thanks for your suggestion. We added the key finding and revised the "abstract" part as:

Line 35-75: Earthworms and *Sphingobacterium* sp. are known for their strong organic compound decomposition ability and wide distribution in soil. However, interactions of soil organic matter decomposition with soil properties and whether microbial species such as *Sphingobacterium* sp. could assist earthworms in carbon and nitrogen transformation in soil remain poorly understood. Earthworms (*Eisenia fetida*, *Amyntas gracilis*) and *Sphingobacterium* sp. were introduced in non-contaminated and cadmium-contaminated soils under controlled laboratory conditions for 20 days. We examined their individual or combined effects on carbon and nitrogen forms and related enzyme activities to assess their influence on soil C and N cycling. Individual *Sphingobacterium* sp. inoculation led to significantly decreased organic carbon (SOC) contents, reducing it by 16.5% in non-contaminated soil and by 3.77%, in Cd-contaminated soil. It resulted in an increased microbial biomass carbon (MBC) contents, reaching 1685 ± 292 mg·kg⁻¹ in non-contaminated soil. Individual introductions of *E. fetida* and *A. gracilis* caused a decline in SOC content in non-contaminated soil, but increased significantly dissolved organic carbon (DOC) and alkali-hydrolysable nitrogen (AN) contents by 75.8%, 53.6% and 32.9%, 20.9%, respectively. In contrast, in Cd-contaminated soil, only the significant combined effects of earthworms and *Sphingobacterium* sp. were linked to significant increase in SOC contents, raising by 7.22% and 9.64% in *E. fetida* + *Sphingobacterium* sp. and *A. gracilis* + *Sphingobacterium* sp. treatments, respectively. In non-contaminated soil, the combined effects of earthworm and *Sphingobacterium* sp. further increased DOC and AN content by 212%, 134% and 31.3%, 25.4% in the treatments of *E. fetida* + *Sphingobacterium* sp. and *A. gracilis* + *Sphingobacterium* sp., respectively; the highest ratios of DOC to SOC and AN to total Nitrogen (TN) were found in the earthworm+*Sphingobacterium* sp. treatments as well. In non-contaminated soil, *Sphingobacterium* sp. and earthworms mainly influenced β -glucosidase (BG), urease (URE), N-acetyl- β -D-glucosaminidase (NAG) activities and fluorescein diacetate hydrolysis (FDA) hydrolysis, while in Cd-contaminated soil, they mainly influenced invertase (INV), NAG, URE, and protease (PRO) activities. Principal component analysis indicated that in non-contaminated soil, the earthworm activities dominated the mineralization processes of soil carbon and nitrogen, and *Sphingobacterium* sp. can intensify this process when it was inoculated in soil along with earthworms. Furthermore, both earthworm species increased C and N levels by elevated INV and PRO activities in combined inoculation. However, in contaminated soil, the impact of earthworm inoculation on soil C stabilization showed a species dependent pattern. *E. fetida* reduced C mineralization by decreasing URE activities, while *A. gracilis* enhanced C stabilization by increasing INV activities and decreasing PRO activities. In conclusion, earthworms played a key role in enhancing C and N mineralization in non-contaminated soil and promoting C stabilization in contaminated soil. Both earthworm species followed similar strategies in the former process but adopted different strategies in the latter. When introduced individually, *Sphingobacterium* sp. was able to promote mineralization in both soils, primarily assisting earthworms in improving carbon and nitrogen mineralization in non-contaminated soil but hindering these processes in Cd-contaminated soil. These findings provide insights into the combined effects of earthworms and microorganisms on carbon and nitrogen cycling.

2. Highlights.

1) In the second bullet of highlights, the full names of the earthworms should be provided. Answer: Thanks for your reminding. We give the full names of the earthworms in the highlights.

2) The third bullet of highlights is very confusing. If the bacteria could not assist earthworm to improve C and N mineralization in the Cd-contaminated soil, please just delete "rather than contaminated soil".

Answer: Thanks for your comment and suggestion. We agree with you and delete "rather than contaminated soil". we revised it as "*Sphingobacterium* sp. could assist earthworms in improving C and N mineralization in non-contaminated soil."

3) How is the effect of *Sphingobacterium* sp. itself? Please provide one more bullet of highlight for this.

Answer: Thanks for your comment and suggestion. We added the effects of inoculation with *Sphingobacterium* sp. alone. Like below: "*Sphingobacterium* sp. could promote C mineralization in both soils."

3. Introduction.

1) What is the hypothesis for the potential effect of *Sphingobacterium* sp. on carbon and nitrogen cycling process in Cd-contaminated soil? The authors need to add more background information to support their hypothesis in the "Introduction" part.

Answer: Thanks for your comment. We rearrange the introduction part and supply the background information and the potential effect of invocation *Sphingobacterium* sp. alone as :

Line 108-112: However, under environment stress, such as metal contamination, the activities and complex interactions of them may be suppressed. Specifically, metal contamination can induce biomass loss, reduce survival rate, cause irreversible physiological damage, decrease the stability of gut bacterial network, and dampen its ecological functions [27-29]. Similarly, the inhibition effects also impact soil microorganisms, leading to reduced soil enzyme functions critical for organic matter cycling [30].

We also added the hypothesis in as:

Line 135-140: Given that *Sphingobacterium* sp. is the dominant bacterial taxon in the earthworm gut, we hypothesized that *Sphingobacterium* sp. and earthworm could individually promote soil organic matter decomposition and, when combined, exert reinforcing effects. These effects are negatively impacted by metal stress and influenced by the survival strategies of different earthworm species; Additionally, *Sphingobacterium* sp. and earthworms may play different roles in this combined process, with *Sphingobacterium* sp. stimulating microbial activities in the earthworm gut.

2) The specific objectives were not consistent with the "abstract" and "discussion".

Answer: Thanks for your comment. We revised the specific objectives as:

Line 143-148: The specific research objectives were (1) to analyze and compare the effects of *Sphingobacterium* sp. and the two earthworm species, when inoculated alone and in combination, on the carbon and nitrogen forms and related enzyme activities in non- and Cd-contaminated soils. (2) to elucidate the effects of Cd pollution stress on this process and the underline mechanisms. (3) to clarify the interaction and roles of *Sphingobacterium* sp. and earthworms in these processes.

4. Discussion.

1) There are seven subsections in this part, but the discussion seemed weak and not focused. In addition, since the authors present the "Discussion" part with the same order to that of the "Results" part, it is recommended that the authors combine them together and present them in the "Results and discussion" part. Otherwise, the authors need to make great effort to rewrite the "Discussion" part, in which the roles of earthworms, *Sphingobacterium* sp., and their combination should be discussed in a step-by-step and logical manner. 2) In the part of Effects of earthworms and *Sphingobacterium* sp. on soil carbon and nitrogen fractions, the authors should discuss the soil carbon and nitrogen cycling via experimental results and previous studies.

Answer: Thanks for your comment and suggestion. You are right. This part existed serious weaknesses in discussion and lack of focus. After discussion, we rewrite and improve the "Discussion" part and also discuss our result with previous studies as:
Lin 293-417:

4.1 The effects of *Sphingobacterium* sp. on C mineralization

Microorganisms are known to contribute to the mineralization and loss of SOC [44]. *Sphingobacterium* sp. is a type of gram-negative bacteria. Likewise, previous studies [45-46] used *Sphingobacterium* sp. to decompose the agricultural byproducts and to biodegrade organic pollutants. The performance of *Sphingobacterium* sp. in both non-contaminated and Cd contaminated soils indicates its ability to mineralize carbon and tolerate metal stress. These abilities align with a similar study [47], who demonstrated that the inoculation of *Sphingobacterium* sp. can enhance the degradation of organic pollutant residues in Cd (0.30 mg·kg⁻¹) contaminated soil. It also can be inferred from the aforementioned results that these functions of *Sphingobacterium* sp. were not dependent on the gut conditions of earthworms. Additionally, the highest microbial biomass carbon content and the microbial quotient carbon in the CS treatment (Fig. 1C and Fig.2E) were due to the inoculation of this strain, its adaption in the soil environment and substantial proliferation. However, this strong adaptive phenomenon was not observed in Cd-contaminated soil, where there is reduced microbial biomass carbon caused by metal stress. For instance, the adaptation process of

Sphingobacterium sp. was observed in the early stage (60 days) in Cd-contaminated soil (0.3 mg·kg⁻¹), with the strain population size gradually increasing after 90 days [47]. Nevertheless, this study only lasted for 20 days and more time is needed for Sphingobacterium sp. to complete the adaption processes under metal stress.

4.2 Earthworm survival, growth and species-specific effects on C and N transformation

Earthworm abundance and biomass decreased after 20 days of cultivation in both soils, especially in Cd-contaminated soil, consistent with findings from a previous study [29]. This decline may be attributed to soil nutrient limitation and the toxic effects of high Cd concentration on earthworms [48]. Regarding earthworm species, *A. gracilis* showed higher resistance to environmental stresses than *E. fetida*, probably owing to the different strategies employed by earthworms in metal handling and resistance. In a previous study, a toxicokinetic model was employed, which found that wild earthworm species were more specialized than *E. fetida* in balancing the trade-off between metal uptake and excretion processes based on their surrounding environment [49].

There are different effects of earthworms on soil C and N transformation in non-contaminated and Cd-contaminated soil. In non-contaminated soil with a high organic carbon content, earthworms alone facilitated soil organic carbon and nitrogen mineralization. This is consistent with the trend of carbon and nitrogen changes observed in vermicomposting [50], presumably through the decomposition of organic matter by specific microorganisms under the unique conditions within the earthworm gut [51]. In addition, it can be found that earthworm activities increased the soil microbial quotient carbon compared to the control, indicating improved efficiency of soil organic matter utilization by microorganisms [52]. However, soil carbon stabilization in Cd-contaminated soil was probably due to earthworms consuming more organic matter to resist metal toxicity [53]. Moreover, it has been proved that gut peristalsis associates partly degraded organic matter with minerals [54] and leads to formation of organic matter-rich aggregates [55-56], which are conducive to SOC sequestration [7,57-60]. It was concluded that earthworms can slow down SOC mineralization in the presence of more minerals [52]. Therefore, earthworm activities reduced carbon mineralization in mineral-rich Cd-contaminated soil. Furthermore, this effect may also be explained by the fact that contaminated soils contain stable carbon that is not easily mineralized.

The positive correlation between β -glucosidase and N-acetyl- β -D-glucosaminidase activity, demonstrated with PCA, also supports the notion that soil microorganisms tend to use the active carbon pool generated by the decomposition of humus formed by litter (Fig. 5A; Fig. S1), and this favors the accumulation of recalcitrant soil carbon pools.

The earthworm species have great impact on biogeochemical cycling through their feeding habits and cast properties [31]. In non-contaminated and Cd-contaminated soil, both *E. fetida* and *A. gracilis* perform similar ecological functions; however, their performance is also influenced by earthworm survival strategies. In environments without metal stress, both species can enhance C and N mineralization. It is worth noting that *E. fetida* has a more pronounced effect on soil carbon and nitrogen mineralization than *A. gracilis* does (Fig. 1B and E; Fig. 2A and C). This could be attributed to the preference of *E. fetida* for ingesting high-quality organic matter. Their castings have a high level of particulate organic matter content and porosity that provides plentiful food and spaces for soil microorganisms [31], thereby accelerating the decomposition of organic matter [61-63]. On the contrary, the wild earthworm *A. gracilis* not only fed on soil organic matter, but also on more mineral particles. In addition, *E. fetida* found it easier to obtain food in non-contaminated soil with a relatively high organic matter content (Table 1). A high carbon to nitrogen ratio was favored for earthworm growth [64], so this species performed better. In contrast, in Cd-contaminated soil, the mineralization processes of soil organic matter slow down. The wild earthworm species *A. gracilis* demonstrated better survival and had more significant effects on C stabilization than *E. fetida* (Table 2, Fig.1 and Fig. 2). The low organic matter content in Cd-contaminated soils limited the decomposition functions of *E. fetida*. Furthermore, earthworms need to consume more food to resist Cd stress. Both species increase their food consumption to feed on more organic matter. In this process, earthworms were compelled to ingest more mineral particles in soils characterized by low organic matter (Table 1) and high soil mineral content. The gut-grazing process facilitated the thorough mixing and binding of organic matter with soil minerals [54]. As for enzymes, the enzymes activity was influenced by earthworm species and metal stress. Likewise, in the previous study, earthworm activities suppressed β -glucosidase activity during the stimulation of C mineralization processes [65]. It was speculated that when soil microorganisms could easily access the readily

mineralized C released by earthworms, they would use a smaller portion of the carbon from enzyme-catalyzed decomposition of soil organic carbon. Enzyme activity only represents the concomitant consequence of earthworm activities but does not serve as the major driver of organic matter decomposition. It is also controlled by multiple factors such as food type, pH, temperature, gut transit time, metal stress, substrates, et al., [65-67].

4.3 The combined effects of earthworm and *Sphingobacterium* sp. on the balance between C and N stabilization and mineralization

The combination processes showed that *Sphingobacterium* sp. assisted earthworms in further promoting carbon and nitrogen mineralization in non-contaminated soils (Fig. 4A and C) and carbon stabilization in Cd-contaminated soils (Fig. 5A and C), as reflected by the increased contents of mineralization (DOC, AN) and the stabilization indicators (SOC), respectively. It was also found that inoculated earthworm can process organically polluted water and excess sludge, and *Sphingobacterium*, known for its high ability to degrade organic matter, is one of the most abundant microbes in earthworm castings [68]. Nevertheless, our study demonstrated that external addition of *Sphingobacterium* sp. can help earthworms adapt more easily to environment and exert their ecological functions. Despite having similar effects when used in combination or when being inoculated alone, the strategies of the two species differed in non-contaminated and Cd-contaminated soil (Fig. 4D). Compared with treatments that inoculate earthworms alone, CSA and CSE treatments further activated INV and PRO activities in non-contaminated soil (Fig. 3 and Fig. 4D); On the other hand, SSA further activated INV activities and inhibited PRO activities, while SSE further inhibited URE activities (Fig. 3 and Fig. 5D). It is well known that soil enzyme activity is mainly derived from microorganisms and can reflect microbial activity [69]. Inoculation of *Sphingobacterium* sp. may stimulate microbial activities in the soil and the earthworm gut in non-contaminated soil [16]. In Cd-contaminated soil, it may represent the natural response of earthworms to the inoculation of *Sphingobacterium* sp. and metal stress. Compared to non-contaminated soil, metal stress induced inhibition of PRO activities in the treatment with *A. gracilis* inoculation and URE activities in the treatment with *E. fetida* inoculation, indirectly demonstrating distinct coping strategies for metal stress among earthworm species. Given that researchers have [70] found that the vermiwash and mucus extracted from earthworms can greatly inhibit the growth of pathogenic fungi, it is also possible that the mucus compounds changed due to metal pollutant stress in this study, such as secretion of specific organic substances to protect itself from physiological damage, while inhibiting the microbial activities. The underlying mechanisms are still not clear and require further research.

In these processes, earthworm activities dominated the soil carbon and nitrogen mineralization processes, while *Sphingobacterium* sp. played a supporting role in combined processes. When the effects of earthworm and *Sphingobacterium* sp. on carbon and nitrogen were consistent, they enhanced carbon mineralization; otherwise they undermined this process. Hence, in non-contaminated soil, carbon mineralization was observed. While in Cd-contaminated soil, carbon storage was more prevalent due to the stronger effect of earthworms on stabilization than *Sphingobacterium* sp. on mineralization. This is further confirmed by PCA which demonstrated that earthworms play a critical role in soil organic carbon decomposition processes, following the same trend as inoculation with earthworm alone and in combination with *Sphingobacterium* sp. (Fig. 4A and C; Fig. 5A and C). Additionally, in this system, a strong interaction existed between earthworms and *Sphingobacterium* sp.. Earthworm activities under the combined process dramatically decreased microbial biomass carbon compared to *Sphingobacterium* sp. activities alone. It is suggested that earthworms contribute to maintaining the ecosystem balance and stabilizing the microbial community structure through feeding [71] and regulating the allocation of active organic carbon and dissolved organic carbon pools [57,72]. Therefore, the rapid proliferation of *Sphingobacterium* sp. was prevented in CSE and CAS treatments. It was further confirmed by the significantly lower microbial quotient carbon in treatment CSE and CSA compared to treatment CS (Fig. 2E). It can be deduced that earthworms may reduce the conversion efficiency of soil microorganisms in utilizing carbon sources into microbial biomass carbon [52] or they can intensely feed and graze on microbes [10, 73-75]. Additionally, the different impact of earthworm species may be caused by *E. fetida* being more conducive to the survival and growth of G- bacterial communities [75], thus promoting *Sphingobacterium* sp. to assist earthworms in carbon mineralization. Based on the above, another underlying factor was postulated that

Sphingobacterium sp. may assist earthworms in mineralizing soil organic carbon and nitrogen by stimulating the microbial activities in the earthworm gut. The subsequent studies need to focus on the earthworm gut microbial to reveal the internal mechanisms.

5. Writing and grammar. There are a number of sentences that need improvement. For example,

1) The clean soil in this study means non-Cd-contaminated soil. Therefore, it should be better to use the Cd-contaminated and non-contaminated soils to describe these two soils.

Answer: Thanks for your comment and suggestion. We agree with you and have been replaced "clean soil and contaminated soil" to "non-contaminated soil and Cd-contaminated soil" to describe these two soils.

2) INV, inverse activity (in the figure caption of Fig. 3) has a spelling error.

Answer: Thanks for your comment. This word in the full text has been revised.

3) The name of earthworm in this study should be "E. fetida" and "A. gracilis", not "E.fetida" and "A.gracilis".

Answer: Thanks for your comment. Sorry about this mistake. The abbreviation format of earthworm species in the full-text has been revised as "E. fetida" and "A. gracilis".

4) There should be a space before the parentheses and brackets. Many similar typing mistakes can be found through the manuscript. Please carefully check and revise them.

Answer: Thanks for your reminding and suggestion. The full-text has been checked and revised.

6. Please find a professional editing service or a native speaker of English to check the language. Answer: Thanks for your suggestion. This manuscript of new version has been checked and polished by the professional institute.

7. Data presentation.

1) The text in the figures was too small to match the text in the manuscript. Try not to abbreviate the titles of vertical coordinates.

Answer: Thanks for your comment and suggestion. The text of all the figures was enlarged. We replaced the abbreviation by the full name in the titles of vertical coordinates. In fig. 2, there do not have more space to put the full name of the ratio such as DOC:DON, C:N, AN:TN, and MBC:MBN. Therefore, we keep same vertical coordinates with before and noted it under the figure. Thanks for your understanding. Like below:

Fig. 1. Soil carbon and nitrogen form characteristics. Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significantly differences (P < 0.05) among the treatments.

Fig. 2. The ratio of soil carbon and nitrogen forms. SOC, soil organic carbon; DOC, dissolved organic matter; TN, total nitrogen; AN, available nitrogen; C:N, soil organic carbon-to-total nitrogen ratio; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significantly differences (P < 0.05) among the treatments.

Fig. 3. Characterization of soil enzyme activity. Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significantly differences (P < 0.05) among the treatments.

2) Table 2. The significance difference in biomass on the 20th day (a, b) was mislabeled. Answer: Thanks for your suggestion. We revised the data format and check the significance between the treatments, like below:

Table 2 Survival rate and mean biomass loss of earthworm at the 20th day

Treatments	Survival rate (%)	Mean biomass loss (%)
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CE	95.2 \pm 4.76a	17.4 \pm 13.3b
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CA	86.1 \pm 7.35a	7.68 \pm 4.54b
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CSE	91.1 \pm 4.49a	19.4 \pm 5.26b
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CSA	87.8 \pm 6.19a	13.7 \pm 10.2b
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SE	77.8 \pm 11.1a	17.6 \pm 5.22b
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SA	100 \pm 0a	18.9 \pm 5.54b
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SSE	31.2 \pm 6.10b	77.2 \pm 3.99a
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SSA	100 \pm 0a	18.9 \pm 3.27b
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Note: Results are expressed as the mean value \pm SE (n = 3). The initial letters C and S represents the soil I and soil II. CE, CA, CSE and CSA represents the treatments with E. fetida, A. gracilis, Sphingobacterium sp. + E.fetida and Sphingobacterium sp. + A.gracilis in soil I, while SE, SA, SSE and SSA represents the treatments with E. fetida,

A. gracilis, Sphingobacterium sp.+ E. fetida and Sphingobacterium sp. + A. gracilis in soil II, respectively. Different lowercase letters indicate significant differences ($P < 0.05$) between different treatments.

8. Line 81-82. "Earthworm selective feeding activities have a significant impact on soil carbon and nitrogen cycling in gut [6]." What do you mean "soil carbon and nitrogen cycling in gut"? Please clarify.

Answer: Thanks for your comment. Sorry for our omission. It should be earthworm selective feeding activities have a significant impact on soil carbon and nitrogen cycling. We have revised as:

Line 89-90: Earthworm's preferential feeding and concentration processes have a substantial impact soil carbon and nitrogen cycling [7].

9. Line 138-139. Why did the authors select two epigeic species of earthworms in this study? Please clarify.

Answer: Thanks for your comment. The different species and ecological categories have different impacts on soil organic matter decomposition and soil C and N cycles. The species of the experimental model earthworm E. fetida and local wild epigeic species may have different survival strategies that affect microbial biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in non-contaminated and Cd-contaminated soil. Importantly, it is worth to be noticed that the different effects of them and trying to convert laboratory tests into field experiments. We clarified in the "Introduction" part as:

Line 112-117: In addition, different earthworm species and ecological categories have varying effects on soil organic matter degradation and soil C and N cycles [31]. The species of the experimental model earthworm E. fetida and local wild epigeic species may have different survival strategies that affect microbial biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in non-contaminated and Cd-contaminated soil.

10. Line 139. The abbreviation of Amynthus gracilis is in a wrong format. A space should be added between the genus name and the species name. Many similar typing mistakes can be found through the manuscript. Please carefully check and revise them.

Answer: Thanks for your comment. The abbreviation format of earthworm species has been revised as "E. fetida" and "A. gracilis". In addition, the full-text has been checked and revised.

11. Line 144-146. What is microbial CM0827 Propionibacterium sp. medium? Please clarify why use this kind of medium. In addition, there should be no commas followed by consist of.

Answer: Thanks for your comment and suggestion. The CM0827 Propionibacterium sp. medium means medium with the number CM0827 and the name Propionibacterium sp. medium, which can support the growth of gram-negative bacteria. Thanks for your kind reminding. We have deleted the punctuation followed by consist of.

12. Line 147. The pH and OD405 value 1.45 should be placed after the recipe of medium and before the incubation of bacteria in the tested soils, respectively. By the way, what do you mean "set aside"? Please clarify.

Answer: Thanks for your comment and suggestion. Sorry to describe not clear. We already modified it as:

Line 165-168: CM0827 Propionibacterium sp. medium, which consisted of tryptone 15 g, L-cystine 0.5 g, yeast extract 5 g, sodium thioglycolate 0.5 g, glucose 5 g, agar 0.7 g, sodium chloride 2.5 g, resazurin 0.001 g, distilled water 1 L, was employed to cultural Sphingobacterium sp. and was set at pH 7.0.

In addition, the means of "set aside" is "prepared the experiment materials waiting to be used in the subsequent experiments". We already revised it as:

Line 168-169: The strain was incubated at a constant temperature of 30°C for 2 days before inoculation into the soil.

13. Line 180-182. This sentence needs revision. For example, "Total cadmium (Cd) content" of whom? What was the certified standard material? Please provide.

Answer: Thanks for your comment. We added the explanation of DTPA, the determination methods of DTPA and total Cd content of soil, and the certified standard materials. This part be revised as:

Line 203-213: 2.4 Total and DTPA extractable Cadmium content

Total cadmium (Cd) in the soils was extracted by microwave digestion with aqua regia ($\text{HNO}_3:\text{HCl}$ (v/v) = 1:3). The diethylenetriaminepentaacetic acid (DTPA) extractable form was regarded as bioavailable metals and toxic. DTPA-extractable Cd was extracted with DTPA extractant at a Soil:DTPA extractant ratio of 5:25 (g/mL), which

included a solution of 5 Mm DTPA and 10 mM CaCl₂. The extracted suspensions were analyzed for total cadmium (Cd) and DTPA-extractable contents using inductively coupled plasma-mass spectrometry (ICP-MS, 7700ce, Agilent Technologies, Santa Clara, CA, USA) [43]. GBW07455 (GSS-26) was employed as certified reference material for soil analyses, and the measured concentrations were within 98% of the reported certified concentrations for Cd. Quality control measures included three repetitions for each soil and three blanks for each batch to ensure the quality of the analysis.

14. Line 205-207. This sentence is very confusing. Please rewrite it.

Answer: Thanks for your comment. We revised the data format order to show the contents of Line 205-207 in Table 2, and deleted Line 205-207. It will be clearer and easier to understand than before.

15. Line 251-253. Technically, INV activity is one of the enzymes activity. Please revise this sentence.

Answer: Thanks for your comment. This sentence really induced the misunderstanding. We deleted “, but greater variation in the effect on INV activities.” in Line 252-253 after reconsidering.

16. Line 394-399. This sentence is too long and hard to digest. Please rewrite.

Answer: Thanks for your comment. Because we reorganized the discussion part, the relevant contents were revised as:

Line 327-328: However, soil carbon stabilization in Cd-contaminated soil was probably due to earthworms consuming more organic matter to resist metal toxicity [53].

Moreover, it has been proved that gut peristalsis associates partly degraded organic matter with minerals [54] and leads to formation of organic matter-rich aggregates [55-56], which are conducive to SOC sequestration [7,57-60]. It was concluded that earthworms can slow down SOC mineralization in the presence of more minerals [52]. Therefore, earthworm activities reduced carbon mineralization in mineral-rich Cd-contaminated soil.

Dear Editor,

We would like to submit a manuscript entitled "**Different combined effects of earthworm and *Sphingobacterium* sp. on soil C, N forms and enzyme activities in clean and Cd-contaminated soil**" for possible publication in European Journal of Soil Biology.

Soil carbon and nitrogen dynamics is efficiency for climate change and environment resource. As "soil ecosystem engineers", earthworm have a significant impact on soil carbon and nitrogen cycling. In our previous studies, *Sphingobacterium* sp., a kind of gram-negative bacterium, was demonstrated as a kind of core microorganism in earthworm gut. To date, some previous studies evidenced that *Sphingobacterium* sp. has a strong degrading capacity for organic pollutants. Nevertheless, the relationship between *Sphingobacterium* sp. and the processes of earthworms affecting on soil organic matter degradation has not been studied. Moreover, the effect of *Sphingobacterium* sp. in combination with earthworms on the soil carbon and nitrogen cycle have not yet been investigated as well. The effect of metal contamination, which severely affects the environmental quality and organisms, on this process are not clear.

In our study, two epigeic species of earthworm (*E.fetida* and *A.gracilis*) and *Sphingobacterium* sp. were inoculated in clean and cadmium-contaminated soil. We investigated their single or combined effects on carbon and nitrogen forms, enzyme activities and evaluated their interaction on soil C and N cycles, investigating how *Sphingobacterium* sp. assist earthworm in influencing soil carbon stabilization and nitrogen cycling under clean and metal contaminated soil. The significance and findings are as follows: Earthworm promoted mineralization of C and N in clean soil and C storage in contaminated soil. Inoculation *Sphingobacterium* sp. individually facilitated soil carbon and nitrogen mineralization in both soils, and this effect was enhanced by combined with earthworms in clean soil. In addition, *E.fetida* was more effective than that of *A.gracilis*. While in cadmium (Cd) contaminated environment, soil exhibited carbon storage under the combination inoculated *Sphingobacterium* sp. with earthworm and no significant effects of earthworm species.

This study provides a clear understanding on the different effects of earthworm and *Sphingobacterium* sp. on soil carbon and nitrogen cycling in clean and contaminated soil.

We hereby certify that this paper consists of original, unpublished work which is not under consideration for publication elsewhere and all authors in agreement with the content of the manuscript have contributed significantly.

Thank you very much for your considering our manuscript for potential publication. We are looking forward to hearing from you soon.

Sincerely,

Dr. Chi Zhang

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E-mail: zhangchi2012@scau.edu.cn

Article Type: Full Length Article

Types of contribution: Regular paper

Date of preparation: July, 2023

The manuscript has 40 pages including:

34 pages of text,

5 pages of tables and figures

1 page of supplementary figure

Responses to reviewers

Dear Editors and Reviewers,

Thank you very much for your careful review of our paper. The feedback is very valuable. We have revised the manuscript in response to your suggestions and questions. All the modifications made according to reviewer's comments are highlighted in yellow in our manuscript. Our responses are also outlined below following your comments. I hope the revised manuscript is acceptable for publication in our journal.

Sincerely,

Chi Zhang, Ph. D.

Corresponding author for the manuscript

Manuscript Number: EJSOBI-D-23-00255

Code: #AU_EJSOBI#

Title: Individual and combined effects of earthworms and *Sphingobacterium* sp. on soil organic C, N forms and enzyme activities in non-contaminated and Cd-contaminated soil

Reviewer #1:

The manuscript aims to examine the roles of one of dominant gut bacteria taxa (*Sphingobacterium* sp.) of earthworms and two earthworm species on soil organic matter transformation, as well as the associated changes in soil enzyme activities and other soil properties. Two types of soil, clean and Cd-contaminated, were used, and the results were interesting. There are some suggestions to be carefully considered in the revision.

1. First, one or two scientific hypotheses could be given. In lines 91-92, the authors mentioned that *Sphingobacterium* sp. was usually one of dominant bacteria taxa in earthworm guts. This is very important base that a scientific hypothesis could be raised on. For example, given that *Sphingobacterium* sp. was dominant in worm gut, an inoculation of this bacteria may exert similar impacts on organic matter decomposition like earthworms, if it contributed great to the worm gut-mediated organic matter decomposition. or *Sphingobacterium* sp. inoculation did not affect organic matter transformation like earthworms, if its function relies on the special gut condition. or *Sphingobacterium* sp. may enhance earthworm impacts on carbon transformation by increasing the microbial activities in earthworm gut?

Answer: Thanks for your comment and suggestion. Earthworm's preferential feeding and concentration processes have a substantial impact soil carbon and nitrogen cycling. Their bioturbation activities, including burrowing, feeding, and excretion (casts and surface mucus), affects the soil carbon and nitrogen distribution within soil profile. These effects result from complex mutualism interactions between earthworms and microorganisms. Previous studies have established connections between earthworms and rhizosphere growth-promoting bacteria or

specific arbuscular mycorrhizal fungi to enhance nutrient availability or nitrogen storage in soil. Moreover, the collaboration of indigenous earthworms and gut bacteria has been employed to remediate metal contaminated soil and has shown superiority compared to chemical amendments. Likewise, combining earthworms with dominant gut bacterial taxa to enhance organic matter decomposition is also advantageous, because it is more readily accepted by earthworms. In our previous studies, *Sphingobacterium* sp. was identified as a dominant bacterial taxon in the earthworm gut. *Sphingobacterium* sp., a gram-negative bacterium, exhibits a wide range of nutrient sources, high adaptability, and a broad ecological distribution. In practice, it has been used to decompose agricultural waste and degrade organic pollutants (e.g. alkanes and polycyclic aromatic hydrocarbons) in soil. Therefore, this decomposition ability of earthworms probably derives from symbiotic microorganisms in their guts. Considering that earthworms and *Sphingobacterium* sp. impact organic matter decomposition and the latter was dominant bacterial taxon in earthworm gut, a synergistic relationship may exist, enhancing soil organic matter decomposition. However, under environment stress, such as metal contamination, the activities and complex interactions of them may be suppressed. Specifically, metal contamination can induce biomass loss, reduce survival rate, cause irreversible physiological damage, decrease the stability of gut bacterial network, and dampen its ecological functions. Similarly, the inhibition effects also impact soil microorganisms, leading to reduced soil enzyme functions critical for organic matter cycling. In addition, different earthworm species and ecological categories have varying effects on soil organic matter degradation and soil C and N cycles. The species of the experimental model earthworm *E. fetida* and local wild epigeic species may have different survival strategies that affect microbial biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in non-contaminated and Cd-contaminated soil.

Based on this, the same taxa were selected in this study to reveal that if the combined effects of this strain and earthworm on soil carbon and nitrogen turnover. Therefore, we added the hypothesis as:

Line 135-140: Given that *Sphingobacterium* sp. is the dominant bacterial taxon in the earthworm gut, we hypothesized that *Sphingobacterium* sp. and earthworm could individually promote soil organic matter decomposition and, when combined, exert reinforcing effects. These effects are negatively impacted by metal stress and influenced by the survival strategies of different

earthworm species; Additionally, *Sphingobacterium* sp. and earthworms may play different roles in this combined process, with *Sphingobacterium* sp. stimulating microbial activities in the earthworm gut.

2. In addition, the possible interactions (and the associated consequences) between *Sphingobacterium* sp. and earthworms may differ in the clean and Cd-contaminated soils. Q: What may be the difference and how did they happen? All these should be added in the introduction section and reflected in the hypotheses. For instance, in considering what..., earthworm and *Sphingobacterium* sp. will enhance soil carbon mineralization in clean soil, but enhance soil carbon sequestration in Cd-contaminated soil.; or, given that, the inoculated earthworm and bacteria may cooperate and exert an additive effect, or may compete or negatively interact with each other, and exert a negative effect?

Answer: Thanks for your comment. We rearrange the introduction part as:

Line 108-117: However, under environment stress, such as metal contamination, the activities and complex interactions of them may be suppressed. Specifically, metal contamination can induce biomass loss, reduce survival rate, cause irreversible physiological damage, decrease the stability of gut bacterial network, and dampen its ecological functions [27-29]. Similarly, the inhibition effects also impact soil microorganisms, leading to reduced soil enzyme functions critical for organic matter cycling [30]. In addition, different earthworm species and ecological categories have varying effects on soil organic matter degradation and soil C and N cycles [31]. The species of the experimental model earthworm *E. fetida* and local wild epigeic species may have different survival strategies that affect microbial biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in non-contaminated and Cd-contaminated soil.

In addition, we also added the hypothesis as:

Line 135-140: Given that *Sphingobacterium* sp. was dominant bacteria taxa in earthworm gut, we hypothesized that *Sphingobacterium* sp. and earthworm could promote soil organic matter decomposition in individual processes and exert reinforcement effects in combined processes, which effects are negatively impacted by metal stress and influenced by survival strategies of different earthworm species; Additionally, *Sphingobacterium* sp. and earthworms may play different roles in combined process in this system, with *Sphingobacterium* sp. making influence

by stimulating microbial activities in earthworm gut.

3. Line 132, please check the biomass per ind. here, too big for a *E. fetida*.

Answer: Thanks for your comment. We added more detail about earthworm biomass after checking and confirming the data as “Each pot was inoculated with 3 g of earthworms, approximately 8 individual *E. fetida* (~ 0.37 g·ind⁻¹) or 5 individual *A. gracilis* (~ 0.60 g·ind⁻¹).”. In previous study, the average weight of *E. fetida* is around 0.4 g·ind⁻¹ (Yue et al., 2019). Therefore, we think it is in normal range of biomass of *E. fetida*.

Reference: S.Z. Yue, H.Q. Zhang, H.Y. Zhen, Z.Q. Lin, Y.H. Qiao, Selenium accumulation, speciation and bioaccessibility in selenium-enriched earthworm (*Eisenia fetida*), *Microchemical Journal* 145 (2019) 1-8, <https://doi.org/10.1016/j.microc.2018.10.015>.

4. Line 173, What is DTPA Cd, and how to measure it?

Answer: Thanks for your comment. We added more details about DTPA and measure method in lines 203-213 as:

2.4 Total and DTPA extractable Cadmium content

Total cadmium (Cd) in the soils was extracted by microwave digestion with aqua regia (HNO₃:HCl (v/v) = 1:3). The diethylenetriaminepentaacetic acid (DTPA) extractable form was regarded as bioavailable metals and toxic. DTPA-extractable Cd was extracted with DTPA extractant at a Soil:DTPA extractant ratio of 5:25 (g/mL), which included a solution of 5 Mm DTPA and 10 mM CaCl₂. The extracted suspensions were analyzed for total cadmium (Cd) and DTPA-extractable contents using inductively coupled plasma-mass spectrometry (ICP-MS, 7700ce, Agilent Technologies, Santa Clara, CA, USA) [43]. GBW07455 (GSS-26) was employed as certified reference material for soil analyses, and the measured concentrations were within 98% of the reported certified concentrations for Cd. Quality control measures included three repetitions for each soil and three blanks for each batch to ensure the quality of the analysis.

5. Lines 322-326, Not sure here, a lower standing MBC did not necessarily indicate a low growth of bacterial population, alternatively, this may also result from a strong feeding of earthworm on the bacterial biomass?

Answer: Thanks for your comment. We agree with you. The feeding of earthworm also strong impacts the microbial biomass and structure. We revised and improve this part as:

Line 402-411: Earthworm activities under the combined process dramatically decreased microbial biomass carbon compared to *Sphingobacterium* sp. activities alone. It is suggested that earthworms contribute to maintaining the ecosystem balance and stabilizing the microbial community structure through feeding [71] and regulating the allocation of active organic carbon and dissolved organic carbon pools [57,72]. Therefore, the rapid proliferation of *Sphingobacterium* sp. was prevented in CSE and CAS treatments. It was further confirmed by the significantly lower microbial quotient carbon in treatment CSE and CSA compared to treatment CS (Fig. 2E). It can be deduced that earthworms may reduce the conversion efficiency of soil microorganisms in utilizing carbon sources into microbial biomass carbon [52] or they can intensely feed and graze on microbes [10, 73-75].

6. Lines 355-356 and lines 414-415, so, how to understand such negative effects of earthworms? similar negative effect was also reported in literature, such as Lv et al., 2020 <https://doi.org/10.1016/j.scitotenv.2020.137227>;

Answer: Thanks for your comment. Likewise, in Lv et al. (2020), earthworm activities suppressed β -glucosidase activity during stimulating carbon mineralization processes. They speculated that when soil microorganisms could easily access the readily mineralized C released by earthworms, they would use a smaller portion of the carbon from enzyme-catalyzed decomposition of soil organic carbon. Additionally, the enzyme activity only represents the concomitant consequence of earthworm activities but does not serve as the major driver of organic matter decomposition. It is also controlled by multiple factors such as food type, pH, temperature, gut transit time, metal stress, substrates, et al., [65-67].

We revised the discussion of negative effects as:

Line 358-365 as: As for enzymes, the enzymes activity was influenced by earthworm species and metal stress. Likewise, in the previous study, earthworm activities suppressed β -glucosidase activity during the stimulation of C mineralization processes [65]. It was speculated that when soil microorganisms could easily access the readily mineralized C released by earthworms, they would use a smaller portion of the carbon from enzyme-catalyzed decomposition of soil organic carbon. Enzyme activity only represents the concomitant consequence of earthworm activities but does not serve as the major driver of organic matter decomposition. It is also controlled by multiple factors such as food type, pH, temperature, gut transit time, metal stress, substrates, et al., [65-67].

7. lines 414-415 may suggest that soil microbiota did not play a key role in OC decomposition processes, but changed accordingly, worm dominated these processes.

Answer: Thanks for your comment. You are right. The soil enzymes activities shown inhibited which may suggest that the earthworm dominated the organic carbon decomposition processes and *Sphingobacterium* sp. play an assistance role. Another underlying factor is that *Sphingobacterium* sp. may assist earthworms in mineralizing soil organic carbon and nitrogen by stimulating the microbial activities in the earthworm gut. The subsequent studies need to focus on the earthworm gut microbial to reveal the internal mechanisms. We have added some discussion in the manuscript as:

Line 393-417: In these processes, earthworm activities dominated the soil carbon and nitrogen mineralization processes, while *Sphingobacterium* sp. played a supporting role in combined processes. When the effects of earthworm and *Sphingobacterium* sp. on carbon and nitrogen were consistent, they enhanced each other; otherwise they undermined each other. Hence, in non-contaminated soil, carbon mineralization was observed. While in Cd-contaminated soil, carbon storage was more prevalent due to the stronger effect of earthworms on stabilization than *Sphingobacterium* sp. on mineralization. This is further confirmed by PCA which demonstrated that earthworms play a critical role in soil organic carbon decomposition processes, following the same trend as inoculation with earthworm alone and in combination with *Sphingobacterium* sp. (Fig. 4A and C; Fig. 5A and C). Additionally, in this system, a strong interaction existed between earthworms and *Sphingobacterium* sp.. Earthworm activities under the combined process dramatically decreased microbial biomass carbon compared to *Sphingobacterium* sp. activities alone. It is suggested that earthworms contribute to maintaining the ecosystem balance and stabilizing the microbial community structure through feeding [71] and regulating the allocation of active organic carbon and dissolved organic carbon pools [57,72]. Therefore, the rapid proliferation of *Sphingobacterium* sp. was prevented in CSE and CAS treatments. It was further confirmed by the significantly lower microbial quotient carbon in treatment CSE and CSA compared to treatment CS (Fig. 2E). It can be deduced that earthworms may reduce the conversion efficiency of soil microorganisms in utilizing carbon sources into microbial biomass carbon [52] or they can intensely feed and graze on microbes [10, 73-75]. Additionally, the different impact of earthworm species may be caused by *E. fetida* being more conducive to the

survival and growth of G⁻ bacterial communities [75], thus promoting *Sphingobacterium* sp. to assist earthworms in carbon mineralization. Based on the above, another underlying factor was postulated that *Sphingobacterium* sp. may assist earthworms in mineralizing soil organic carbon and nitrogen by stimulating the microbial activities in the earthworm gut. The subsequent studies need to focus on the earthworm gut microbial to reveal the internal mechanisms.

8. lines 392-393, how could the assistance role of *Sphingobacterium* sp. on worm could be observed in Fig. 4a?

Answer: Thanks for your comment. Sorry for our mistakes. This conclusion can't be observed only in Fig. 4A, it should be analysis through combining Fig. 4A and Fig. 4C. The low PC1 values in Fig. 4A means high carbon and nitrogen mineralization such as DOC and AN. Inoculation earthworm treatments trend to have low PC1 values especially co-inoculation with *Sphingobacterium* sp. (Fig. 4C). It means that co-inoculation with *Sphingobacterium* sp. can enhance the earthworm effects on carbon and nitrogen transformation. We added the details of figure label as:

Line 399-401: This is further confirmed by PCA which demonstrated that earthworms play a critical role in soil organic carbon decomposition processes, following the same trend as inoculation with earthworm alone and in combination with *Sphingobacterium* sp. (Fig. 4A and C; Fig. 5A and C).

Reviewer #2:

Jia et al. investigated the effect of single and combined application of earthworms and *Sphingobacterium* sp. on the C and N cycles in a non-contaminated soil and a cadmium-contaminated soil. The C and N fractions in soil, ratio of C/N fractions, soil enzyme activities, relationships among the above parameters were determined. The results of this study can provide a better understanding of how different species of earthworms and their co-application with a key bacterium from the gut of earthworm regulate the carbon and nitrogen mineralization/fixation in the non-contaminated and Cd-contaminated acid soils. Yet, the current version of the manuscript still needs further modification and improvement, particularly the "Discussion" part. More details

need to be addressed clearly as well.

1. Abstract. The abstract should be revised and clearly introduce which kind of results comes to which kind of key finding, not just simply mention the results.

Answer: Thanks for your suggestion. We added the key finding and revised the “abstract” part as:

Line 35-75: Earthworms and *Sphingobacterium* sp. are known for their strong organic compound decomposition ability and wide distribution in soil. However, interactions of soil organic matter decomposition with soil properties and whether microbial species such as *Sphingobacterium* sp. could assist earthworms in carbon and nitrogen transformation in soil remain poorly understood. Earthworms (*Eisenia fetida*, *Amyntas gracilis*) and *Sphingobacterium* sp. were introduced in non-contaminated and cadmium-contaminated soils under controlled laboratory conditions for 20 days. We examined their individual or combined effects on carbon and nitrogen forms and related enzyme activities to assess their influence on soil C and N cycling. Individual *Sphingobacterium* sp. inoculation led to significantly decreased organic carbon (SOC) contents, reducing it by 16.5% in non-contaminated soil and by 3.77%, in Cd-contaminated soil. It resulted in an increased microbial biomass carbon (MBC) contents, reaching $1685 \pm 292 \text{ mg}\cdot\text{kg}^{-1}$ in non-contaminated soil. Individual introductions of *E. fetida* and *A. gracilis* caused a decline in SOC content in non-contaminated soil, but increased significantly dissolved organic carbon (DOC) and alkali-hydrolysable nitrogen (AN) contents by 75.8%, 53.6% and 32.9%, 20.9%, respectively. In contrast, in Cd-contaminated soil, only the significant combined effects of earthworms and *Sphingobacterium* sp. were linked to significant increase in SOC contents, raising by 7.22% and 9.64% in *E. fetida* + *Sphingobacterium* sp. and *A. gracilis* + *Sphingobacterium* sp. treatments, respectively. In non-contaminate soil, the combined effects of earthworm and *Sphingobacterium* sp. further increased DOC and AN content by 212%, 134% and 31.3%, 25.4% in the treatments of *E. fetida* + *Sphingobacterium* sp. and *A. gracilis* + *Sphingobacterium* sp., respectively; the highest ratios of DOC to SOC and AN to total Nitrogen (TN) were found in the earthworm+*Sphingobacterium* sp. treatments as well. In non-contaminated soil, *Sphingobacterium* sp. and earthworms mainly influenced β -glucosidase (BG), urease (URE), N-acetyl- β -D-glucosaminidase (NAG) activities and fluorescein diacetate hydrolysis (FDA)

hydrolysis, while in Cd-contaminated soil, they mainly influenced invertase (INV), NAG, URE, and protease (PRO) activities. Principal component analysis indicated that in non-contaminated soil, the earthworm activities dominated the mineralization processes of soil carbon and nitrogen, and *Sphingobacterium* sp. can intensify this process when it was inoculated in soil along with earthworms. Furthermore, both earthworm species increased C and N levels by elevated INV and PRO activities in combined inoculation. However, in contaminated soil, the impact of earthworm inoculation on soil C stabilization showed a species dependent pattern. *E. fetida* reduced C mineralization by decreasing URE activities, while *A. gracilis* enhanced C stabilization by increasing INV activities and decreasing PRO activities. In conclusion, earthworms played a key role in enhancing C and N mineralization in non-contaminated soil and promoting C stabilization in contaminated soil. Both earthworm species followed similar strategies in the former process but adopted different strategies in the latter. When introduced individually, *Sphingobacterium* sp. was able to promote mineralization in both soils, primarily assisting earthworms in improving carbon and nitrogen mineralization in non-contaminated soil but hindering these processes in Cd-contaminated soil. These findings provide insights into the combined effects of earthworms and microorganisms on carbon and nitrogen cycling.

2. Highlights.

1) In the second bullet of highlights, the full names of the earthworms should be provided.

Answer: Thanks for your reminding. We give the full names of the earthworms in the highlights.

2) The third bullet of highlights is very confusing. If the bacteria could not assist earthworm to improve C and N mineralization in the Cd-contaminated soil, please just delete "rather than contaminated soil".

Answer: Thanks for your comment and suggestion. We agree with you and delete "rather than contaminated soil". we revised it as "*Sphingobacterium* sp. could assist earthworms in improving C and N mineralization in non-contaminated soil."

3) How is the effect of *Sphingobacterium* sp. itself? Please provide one more bullet of highlight for this.

Answer: Thanks for your comment and suggestion. We added the effects of inoculation with *Sphingobacterium* sp. alone. Like below: "*Sphingobacterium* sp. could promote C mineralization

in both soils.”.

3. Introduction.

1) What is the hypothesis for the potential effect of *Sphingobacterium* sp. on carbon and nitrogen cycling process in Cd-contaminated soil? The authors need to add more background information to support their hypothesis in the "Introduction" part.

Answer: Thanks for your comment. We rearrange the introduction part and supply the background information and the potential effect of invocation *Sphingobacterium* sp. alone as:

Line 108-112: However, under environment stress, such as metal contamination, the activities and complex interactions of them may be suppressed. Specifically, metal contamination can induce biomass loss, reduce survival rate, cause irreversible physiological damage, decrease the stability of gut bacterial network, and dampen its ecological functions [27-29]. Similarly, the inhibition effects also impact soil microorganisms, leading to reduced soil enzyme functions critical for organic matter cycling [30].

We also added the hypothesis in as:

Line 135-140: Given that *Sphingobacterium* sp. is the dominant bacterial taxon in the earthworm gut, we hypothesized that *Sphingobacterium* sp. and earthworm could individually promote soil organic matter decomposition and, when combined, exert reinforcing effects. These effects are negatively impacted by metal stress and influenced by the survival strategies of different earthworm species; Additionally, *Sphingobacterium* sp. and earthworms may play different roles in this combined process, with *Sphingobacterium* sp. stimulating microbial activities in the earthworm gut.

2) The specific objectives were not consistent with the "abstract" and "discussion".

Answer: Thanks for your comment. We revised the specific objectives as:

Line 143-148: The specific research objectives were (1) to analyze and compare the effects of *Sphingobacterium* sp. and the two earthworm species, when inoculated alone and in combination, on the carbon and nitrogen forms and related enzyme activities in non- and Cd-contaminated soils. (2) to elucidate the effects of Cd pollution stress on this process and the underline mechanisms. (3) to clarify the interaction and roles of *Sphingobacterium* sp. and earthworms in these processes.

4. Discussion.

1) There are seven subsections in this part, but the discussion seemed weak and not focused. In addition, since the authors present the "Discussion" part with the same order to that of the "Results" part, it is recommended that the authors combine them together and present them in the "Results and discussion" part. Otherwise, the authors need to make great effort to rewrite the "Discussion" part, in which the roles of earthworms, *Sphingobacterium* sp., and their combination should be discussed in a step-by-step and logical manner. 2) In the part of Effects of earthworms and *Sphingobacterium* sp. on soil carbon and nitrogen fractions, the authors should discuss the soil carbon and nitrogen cycling via experimental results and previous studies.

Answer: Thanks for your comment and suggestion. You are right. This part existed serious weaknesses in discussion and lack of focus. After discussion, we rewrite and improve the "Discussion" part and also discuss our result with previous studies as:

Lin 293-417:

*4.1 The effects of *Sphingobacterium* sp. on C mineralization*

Microorganisms are known to contribute to the mineralization and loss of SOC [44]. *Sphingobacterium* sp. is a type of gram-negative bacteria. Likewise, previous studies [45-46] used *Sphingobacterium* sp. to decompose the agricultural byproducts and to biodegrade organic pollutants. The performance of *Sphingobacterium* sp. in both non-contaminated and Cd contaminated soils indicates its ability to mineralize carbon and tolerate metal stress. These abilities align with a similar study [47], who demonstrated that the inoculation of *Sphingobacterium* sp. can enhance the degradation of organic pollutant residues in Cd (0.30 mg·kg⁻¹) contaminated soil. It also can be inferred from the aforementioned results that these functions of *Sphingobacterium* sp. were not dependent on the gut conditions of earthworms. Additionally, the highest microbial biomass carbon content and the microbial quotient carbon in the CS treatment (Fig. 1C and Fig.2E) were due to the inoculation of this strain, its adaption in the soil environment and substantial proliferation. However, this strong adaptive phenomenon was not observed in Cd-contaminated soil, where there is reduced microbial biomass carbon caused by metal stress. For instance, the adaptation process of *Sphingobacterium* sp. was observed in the early stage (60 days) in Cd-contaminated soil (0.3 mg·kg⁻¹), with the strain population size gradually increasing after 90 days [47]. Nevertheless, this study only lasted for 20 days and more time is needed for *Sphingobacterium* sp. to complete the adaption processes under metal stress.

4.2 Earthworm survival, growth and species-specific effects on C and N transformation

Earthworm abundance and biomass decreased after 20 days of cultivation in both soils, especially in Cd-contaminated soil, consistent with findings from a previous study [29]. This decline may be attributed to soil nutrient limitation and the toxic effects of high Cd concentration on earthworms [48]. Regarding earthworm species, *A. gracilis* showed higher resistance to environmental stresses than *E. fetida*, probably owing to the different strategies employed by earthworms in metal handling and resistance. In a previous study, a toxicokinetic model was employed, which found that wild earthworm species were more specialized than *E. fetida* in balancing the trade-off between metal uptake and excretion processes based on their surrounding environment [49].

There are different effects of earthworms on soil C and N transformation in non-contaminated and Cd-contaminated soil. In non-contaminated soil with a high organic carbon content, earthworms alone facilitated soil organic carbon and nitrogen mineralization. This is consistent with the trend of carbon and nitrogen changes observed in vermicomposting [50], presumably through the decomposition of organic matter by specific microorganisms under the unique conditions within the earthworm gut [51]. In addition, it can be found that earthworm activities increased the soil microbial quotient carbon compared to the control, indicating improved efficiency of soil organic matter utilization by microorganisms [52]. However, soil carbon stabilization in Cd-contaminated soil was probably due to earthworms consuming more organic matter to resist metal toxicity [53]. Moreover, it has been proved that gut peristalsis associates partly degraded organic matter with minerals [54] and leads to formation of organic matter-rich aggregates [55-56], which are conducive to SOC sequestration [7,57-60]. It was concluded that earthworms can slow down SOC mineralization in the presence of more minerals [52]. Therefore, earthworm activities reduced carbon mineralization in mineral-rich Cd-contaminated soil. Furthermore, this effect may also be explained by the fact that contaminated soils contain stable carbon that is not easily mineralized. The positive correlation between β -glucosidase and N-acetyl- β -D-glucosaminidase activity, demonstrated with PCA, also supports the notion that soil microorganisms tend to use the active carbon pool generated by the decomposition of humus formed by litter (Fig. 5A; Fig. S1), and this favors the accumulation of recalcitrant soil carbon pools.

The earthworm species have great impact on biogeochemical cycling through their feeding habits and cast properties [31]. In non-contaminated and Cd-contaminated soil, both *E. fetida* and *A. gracilis* perform similar ecological functions; however, their performance is also influenced by earthworm survival strategies. In environments without metal stress, both species can enhance C and N mineralization. It is worth noting that *E. fetida* has a more pronounced effect on soil carbon and nitrogen mineralization than *A. gracilis* does (Fig. 1B and E; Fig. 2A and C). This could be attributed to the preference of *E. fetida* for ingesting high-quality organic matter. Their castings have a high level of particulate organic matter content and porosity that provides plentiful food and spaces for soil microorganisms [31], thereby accelerating the decomposition of organic matter [61-63]. On the contrary, the wild earthworm *A. gracilis* not only fed on soil organic matter, but also on more mineral particles. In addition, *E. fetida* found it easier to obtain food in non-contaminated soil with a relatively high organic matter content (Table 1). A high carbon to nitrogen ratio was favored for earthworm growth [64], so this species performed better. In contrast, in Cd-contaminated soil, the mineralization processes of soil organic matter slow down. The wild earthworm species *A. gracilis* demonstrated better survival and had more significant effects on C stabilization than *E. fetida* (Table 2, Fig.1 and Fig. 2). The low organic matter content in Cd-contaminated soils limited the decomposition functions of *E. fetida*. Furthermore, earthworms need to consume more food to resist Cd stress. Both species increase their food consumption to feed on more organic matter. In this process, earthworms were compelled to ingest more mineral particles in soils characterized by low organic matter (Table 1) and high soil mineral content. The gut-grazing process facilitated the thorough mixing and binding of organic matter with soil minerals [54]. As for enzymes, the enzyme activity was influenced by earthworm species and metal stress. Likewise, in the previous study, earthworm activities suppressed β -glucosidase activity during the stimulation of C mineralization processes [65]. It was speculated that when soil microorganisms could easily access the readily mineralized C released by earthworms, they would use a smaller portion of the carbon from enzyme-catalyzed decomposition of soil organic carbon. Enzyme activity only represents the concomitant consequence of earthworm activities but does not serve as the major driver of organic matter decomposition. It is also controlled by multiple factors such as food type, pH, temperature, gut transit time, metal stress, substrates, et al., [65-67].

4.3 The combined effects of earthworm and *Sphingobacterium* sp. on the balance between C and N stabilization and mineralization

The combination processes showed that *Sphingobacterium* sp. assisted earthworms in further promoting carbon and nitrogen mineralization in non-contaminated soils (Fig. 4A and C) and carbon stabilization in Cd-contaminated soils (Fig. 5A and C), as reflected by the increased contents of mineralization (DOC, AN) and the stabilization indicators (SOC), respectively. It was also found that inoculated earthworm can process organically polluted water and excess sludge, and *Sphingobacterium*, known for its high ability to degrade organic matter, is one of the most abundant microbes in earthworm castings [68]. Nevertheless, our study demonstrated that external addition of *Sphingobacterium* sp. can help earthworms adapt more easily to environment and exert their ecological functions. Despite having similar effects when used in combination or when being inoculated alone, the strategies of the two species differed in non-contaminated and Cd-contaminated soil (Fig. 4D). Compared with treatments that inoculate earthworms alone, CSA and CSE treatments further activated INV and PRO activities in non-contaminated soil (Fig. 3 and Fig. 4D); On the other hand, SSA further activated INV activities and inhibited PRO activities, while SSE further inhibited URE activities (Fig. 3 and Fig. 5D). It is well known that soil enzyme activity is mainly derived from microorganisms and can reflect microbial activity [69]. Inoculation of *Sphingobacterium* sp. may stimulate microbial activities in the soil and the earthworm gut in non-contaminated soil [16]. In Cd-contaminated soil, it may represent the natural response of earthworms to the inoculation of *Sphingobacterium* sp. and metal stress. Compared to non-contaminated soil, metal stress induced inhibition of PRO activities in the treatment with *A. gracilis* inoculation and URE activities in the treatment with *E. fetida* inoculation, indirectly demonstrating distinct coping strategies for metal stress among earthworm species. Given that researchers have [70] found that the vermiwash and mucus extracted from earthworms can greatly inhibit the growth of pathogenic fungi, it is also possible that the mucus compounds changed due to metal pollutant stress in this study, such as secretion of specific organic substances to protect itself from physiological damage, while inhibiting the microbial activities. The underlying mechanisms are still not clear and require further research.

In these processes, earthworm activities dominated the soil carbon and nitrogen mineralization processes, while *Sphingobacterium* sp. played a supporting role in combined

processes. When the effects of earthworm and *Sphingobacterium* sp. on carbon and nitrogen were consistent, they enhanced carbon mineralization; otherwise they undermined this process. Hence, in non-contaminated soil, carbon mineralization was observed. While in Cd-contaminated soil, carbon storage was more prevalent due to the stronger effect of earthworms on stabilization than *Sphingobacterium* sp. on mineralization. This is further confirmed by PCA which demonstrated that earthworms play a critical role in soil organic carbon decomposition processes, following the same trend as inoculation with earthworm alone and in combination with *Sphingobacterium* sp. (Fig. 4A and C; Fig. 5A and C). Additionally, in this system, a strong interaction existed between earthworms and *Sphingobacterium* sp.. Earthworm activities under the combined process dramatically decreased microbial biomass carbon compared to *Sphingobacterium* sp. activities alone. It is suggested that earthworms contribute to maintaining the ecosystem balance and stabilizing the microbial community structure through feeding [71] and regulating the allocation of active organic carbon and dissolved organic carbon pools [57,72]. Therefore, the rapid proliferation of *Sphingobacterium* sp. was prevented in CSE and CAS treatments. It was further confirmed by the significantly lower microbial quotient carbon in treatment CSE and CSA compared to treatment CS (Fig. 2E). It can be deduced that earthworms may reduce the conversion efficiency of soil microorganisms in utilizing carbon sources into microbial biomass carbon [52] or they can intensely feed and graze on microbes [10, 73-75]. Additionally, the different impact of earthworm species may be caused by *E. fetida* being more conducive to the survival and growth of G⁻ bacterial communities [75], thus promoting *Sphingobacterium* sp. to assist earthworms in carbon mineralization. Based on the above, another underlying factor was postulated that *Sphingobacterium* sp. may assist earthworms in mineralizing soil organic carbon and nitrogen by stimulating the microbial activities in the earthworm gut. The subsequent studies need to focus on the earthworm gut microbial to reveal the internal mechanisms.

5. Writing and grammar. There are a number of sentences that need improvement. For example,

1) The clean soil in this study means non-Cd-contaminated soil. Therefore, it should be better to use the Cd-contaminated and non-contaminated soils to describe these two soils.

Answer: Thanks for your comment and suggestion. We agree with you and have been replaced “clean soil and contaminated soil” to “non-contaminated soil and Cd-contaminated soil” to describe these two soils.

2) INV, inverse activity (in the figure caption of Fig. 3) has a spelling error.

Answer: Thanks for your comment. This word in the full text has been revised.

3) The name of earthworm in this study should be "*E. fetida*" and "*A. gracilis*", not "*E.fetida*" and "*A.gracilis*".

Answer: Thanks for your comment. Sorry about this mistake. The abbreviation format of earthworm species in the full-text has been revised as "*E. fetida*" and "*A. gracilis*".

4) There should be a space before the parentheses and brackets. Many similar typing mistakes can be found through the manuscript. Please carefully check and revise them.

Answer: Thanks for your reminding and suggestion. The full-text has been checked and revised.

6. Please find a professional editing service or a native speaker of English to check the language.

Answer: Thanks for your suggestion. This manuscript of new version has been checked and polished by the professional institute.

7. Data presentation.

1) The text in the figures was too small to match the text in the manuscript. Try not to abbreviate the titles of vertical coordinates.

Answer: Thanks for your comment and suggestion. The text of all the figures was enlarged. We replaced the abbreviation by the full name in the titles of vertical coordinates. In fig. 2, there do not have more space to put the full name of the ratio such as DOC:DON, C:N, AN:TN, and MBC:MBN. Therefore, we keep same vertical coordinates with before and noted it under the figure. Thanks for your understanding. Like below:

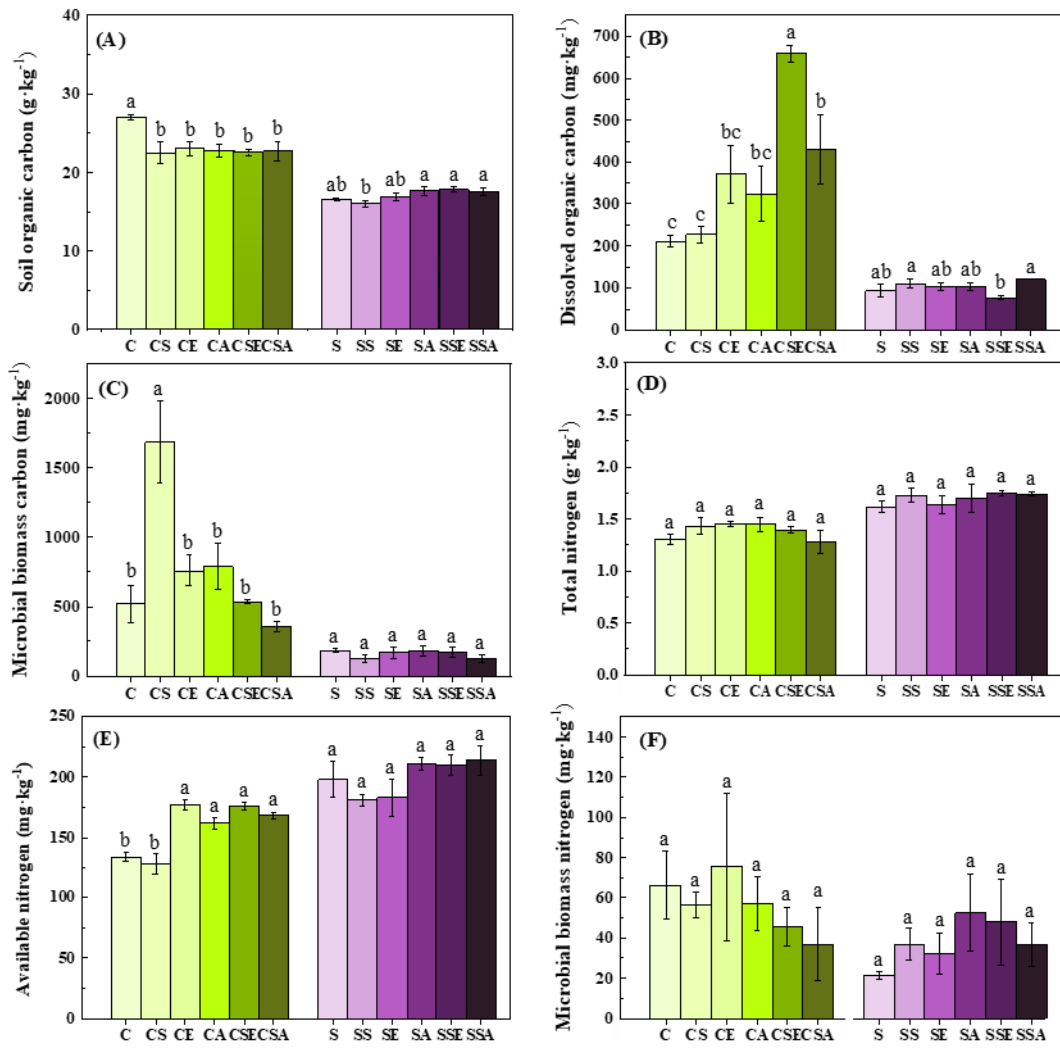


Fig. 1. Soil carbon and nitrogen form characteristics. Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significantly differences ($P < 0.05$) among the treatments.

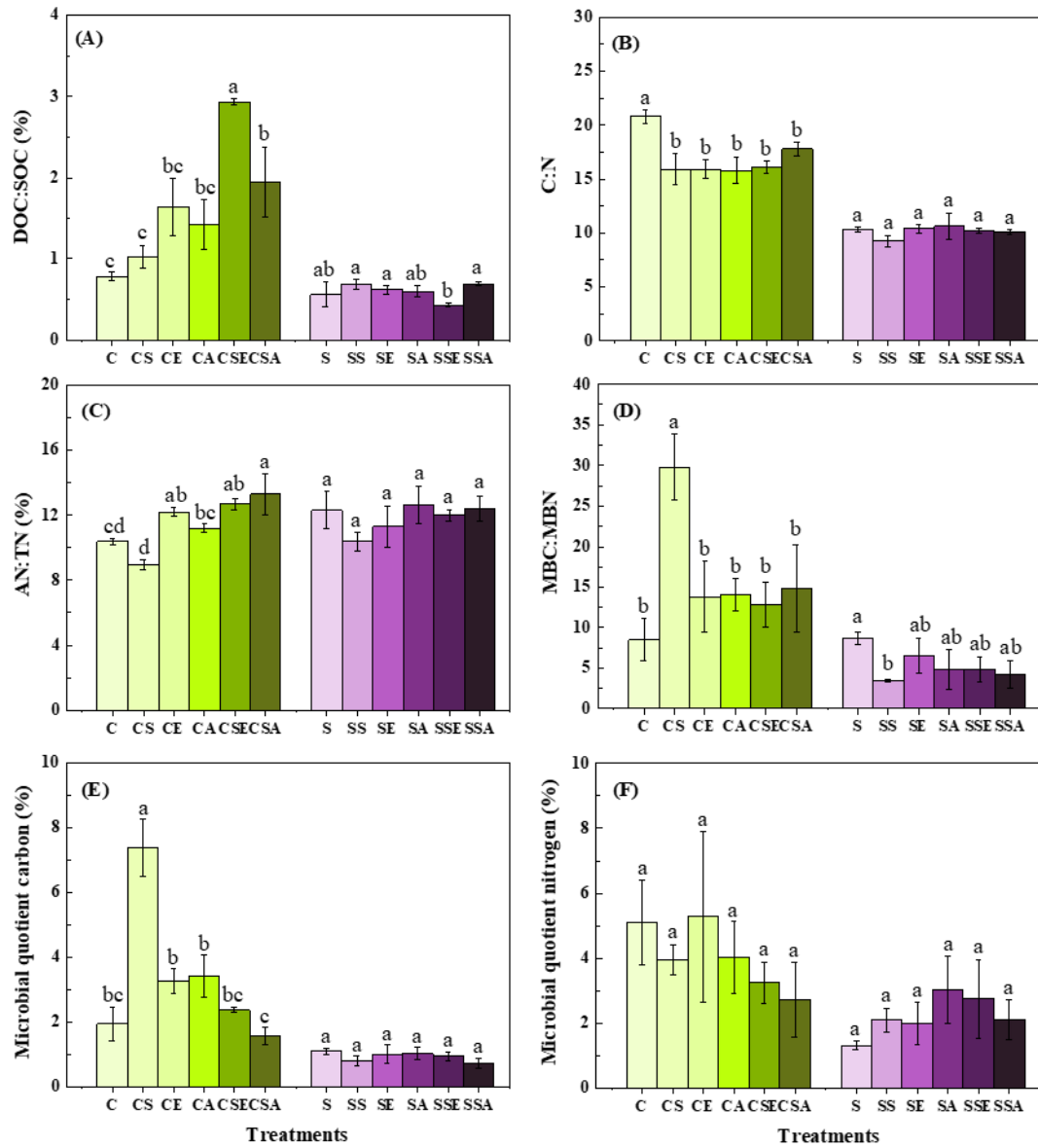


Fig. 2. The ratio of soil carbon and nitrogen forms. SOC, soil organic carbon; DOC, dissolved organic matter; TN, total nitrogen; AN, available nitrogen; C:N, soil organic carbon-to-total nitrogen ratio; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. Results are expressed as the mean value \pm SE ($n = 3$). Different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

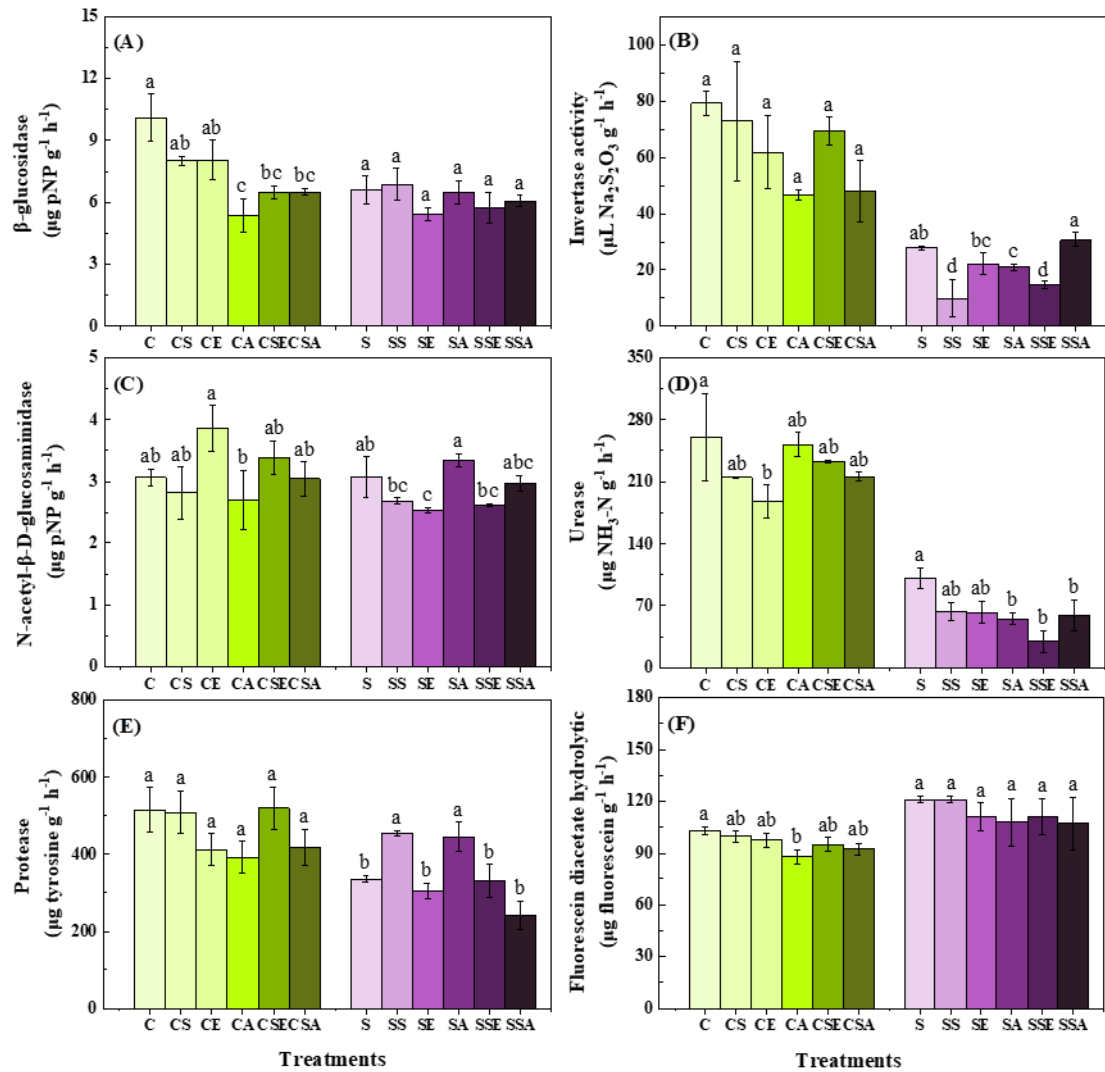


Fig. 3. Characterization of soil enzyme activity. Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significantly differences ($P < 0.05$) among the treatments.

2) Table 2. The significance difference in biomass on the 20th day (a, b) was mislabeled.

Answer: Thanks for your suggestion. We revised the data format and check the significance between the treatments, like below:

Table 2 Survival rate and mean biomass loss of earthworm at the 20th day

Treatments	Survival rate (%)	Mean biomass loss (%)
CE	95.2 \pm 4.76a	17.4 \pm 13.3b
CA	86.1 \pm 7.35a	7.68 \pm 4.54b
CSE	91.1 \pm 4.49a	19.4 \pm 5.26b
CSA	87.8 \pm 6.19a	13.7 \pm 10.2b
SE	77.8 \pm 11.1a	17.6 \pm 5.22b

SA	100 ± 0a	18.9 ± 5.54b
SSE	31.2 ± 6.10b	77.2 ± 3.99a
SSA	100 ± 0a	18.9 ± 3.27b

Note: Results are expressed as the mean value ± SE (n = 3). The initial letters C and S represents the soil I and soil II. CE, CA, CSE and CSA represents the treatments with *E. fetida*, *A. gracilis*, *Sphingobacterium* sp. + *E.fetida* and *Sphingobacterium* sp. + *A.gracilis* in soil I, while SE, SA, SSE and SSA represents the treatments with *E. fetida*, *A. gracilis*, *Sphingobacterium* sp.+ *E. fetida* and *Sphingobacterium* sp. + *A. gracilis* in soil II, respectively. Different lowercase letters indicate significant differences ($P < 0.05$) between different treatments.

8. Line 81-82. "Earthworm selective feeding activities have a significant impact on soil carbon and nitrogen cycling in gut [6]." What do you mean "soil carbon and nitrogen cycling in gut"? Please clarify.

Answer: Thanks for your comment. Sorry for our omission. It should be earthworm selective feeding activities have a significant impact on soil carbon and nitrogen cycling. We have revised as:

Line 89-90: Earthworm's preferential feeding and concentration processes have a substantial impact soil carbon and nitrogen cycling [7].

9. Line 138-139. Why did the authors select two epigeic species of earthworms in this study? Please clarify.

Answer: Thanks for your comment. The different species and ecological categories have different impacts on soil organic matter decomposition and soil C and N cycles. *The species of the experimental model earthworm E. fetida and local wild epigeic species* may have different survival strategies that affect microbial biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in non-contaminated and Cd-contaminated soil. Importantly, it is worth to be noticed that the different effects of them and trying to convert laboratory tests into field experiments. We clarified in the "Introduction" part as:

Line 112-117: In addition, different earthworm species and ecological categories have varying effects on soil organic matter degradation and soil C and N cycles [31]. *The species of the experimental model earthworm E. fetida and local wild epigeic species* may have different survival strategies that affect microbial biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in non-contaminated and Cd-contaminated soil.

10. Line 139. The abbreviation of *Amyntas gracilis* is in a wrong format. A space should be added between the genus name and the species name. Many similar typing mistakes can be found through the manuscript. Please carefully check and revise them.

Answer: Thanks for your comment. The abbreviation format of earthworm species has been revised as "*E. fetida*" and "*A. gracilis*". In addition, the full-text has been checked and revised.

11. Line 144-146. What is microbial CM0827 *Propionibacterium* sp. medium? Please clarify why use this kind of medium. In addition, there should be no commas followed by consist of.

Answer: Thanks for your comment and suggestion. The CM0827 *Propionibacterium* sp. medium means medium with the number CM0827 and the name *Propionibacterium* sp. medium, which can support the growth of gram-negative bacteria. Thanks for your kind reminding. We have deleted the punctuation followed by consist of.

12. Line 147. The pH and OD405 value 1.45 should be placed after the recipe of medium and before the incubation of bacteria in the tested soils, respectively. By the way, what do you mean "set aside"? Please clarify.

Answer: Thanks for your comment and suggestion. Sorry to describe not clear. We already modified it as:

Line 165-168: CM0827 *Propionibacterium* sp. medium, which consisted of tryptone 15 g, L-cystine 0.5 g, yeast extract 5 g, sodium thioglycolate 0.5 g, glucose 5 g, agar 0.7 g, sodium chloride 2.5 g, resazurin 0.001 g, distilled water 1 L, was employed to cultural *Spingobacterium* sp. and was set at pH 7.0.

In addition, the means of "set aside" is "prepared the experiment materials waiting to be used in the subsequent experiments". We already revised it as:

Line 168-169: The strain was incubated at a constant temperature of 30°C for 2 days before inoculation into the soil.

13. Line 180-182. This sentence needs revision. For example, "Total cadmium (Cd) content" of whom? What was the certified standard material? Please provide.

Answer: Thanks for your comment. We added the explanation of DTPA, the determination methods of DTPA and total Cd content of soil, and the certified standard materials. This part be revised as:

Line 203-213: 2.4 Total and DTPA extractable Cadmium content

Total cadmium (Cd) in the soils was extracted by microwave digestion with aqua regia ($\text{HNO}_3\text{:HCl}$ (v/v) = 1:3). The diethylenetriaminepentaacetic acid (DTPA) extractable form was regarded as bioavailable metals and toxic. DTPA-extractable Cd was extracted with DTPA extractant at a Soil:DTPA extractant ratio of 5:25 (g/mL), which included a solution of 5 Mm DTPA and 10 mM CaCl_2 . The extracted suspensions were analyzed for total cadmium (Cd) and DTPA-extractable contents using inductively coupled plasma-mass spectrometry (ICP-MS, 7700ce, Agilent Technologies, Santa Clara, CA, USA) [43]. GBW07455 (GSS-26) was employed as certified reference material for soil analyses, and the measured concentrations were within 98% of the reported certified concentrations for Cd. Quality control measures included three repetitions for each soil and three blanks for each batch to ensure the quality of the analysis.

14. Line 205-207. This sentence is very confusing. Please rewrite it.

Answer: Thanks for your comment. We revised the data format order to show the contents of Line 205-207 in Table 2, and deleted Line 205-207. It will be clearer and easier to understand than before.

15. Line 251-253. Technically, INV activity is one of the enzymes activity. Please revise this sentence.

Answer: Thanks for your comment. This sentence really induced the misunderstanding. We deleted “, but greater variation in the effect on INV activities.” in Line 252-253 after reconsidering.

16. Line 394-399. This sentence is too long and hard to digest. Please rewrite.

Answer: Thanks for your comment. Because we reorganized the discussion part, the relevant contents were revised as:

Line 327-328: However, soil carbon stabilization in Cd-contaminated soil was probably due to earthworms consuming more organic matter to resist metal toxicity [53]. Moreover, it has been proved that gut peristalsis associates partly degraded organic matter with minerals [54] and leads

to formation of organic matter-rich aggregates [55-56], which are conducive to SOC sequestration [7,57-60]. It was concluded that earthworms can slow down SOC mineralization in the presence of more minerals [52]. Therefore, earthworm activities reduced carbon mineralization in mineral-rich Cd-contaminated soil.

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Different combined effects of earthworm and *Sphingobacterium* sp. on soil C, N forms and enzyme activities in clean and Cd-contaminated soil

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ABSTRACT

Earthworm and *Sphingobacterium* sp. are both characterized by their strong decomposition ability of organic compound and broad distribution in soil. Little is known about their interaction on soil organic matter decomposition and whether *Sphingobacterium* sp. could assist earthworms with carbon and nitrogen cycles. In this study, earthworms (*Eisenia fetida*, *Amyntas gracilis*) and *Sphingobacterium* sp. were inoculated in clean and contaminated soils under laboratory condition for 20 days. We investigated their single or combined effects on carbon and nitrogen forms (organic carbon, total Nitrogen, dissolved organic carbon (DOC), alkali-hydrolyzable nitrogen (AN), microbial biomass carbon and nitrogen) and C- and N- related enzyme activities (β -glucosidase(BG), invertase (INV), urease(URE), N-acetyl- β -D-glucosaminidase(NAG), protease(PRO) and fluorescein diacetate hydrolysis (FDA)), evaluating their interaction on soil C and N cycles. *E. fetida* and *A. gracilis* inoculation alone increased DOC contents significantly by 75.8 % and 53.6 % comparing with no earthworm control in clean soil; while they increased AN contents significantly by 32.9% and 20.9% as well, respectively. Inoculation of *Sphingobacterium* sp. alone promoted microbial biomass carbon reaching $1685 \pm 292 \text{ mg} \cdot \text{kg}^{-1}$ in clean soil tripled of control. Moreover, the combination of earthworms and *Sphingobacterium* sp. increased DOC contents by 212% and 134%, and AN contents by 31.3% and 25.4% in treatments of *E. fetida*+*Sphingobacterium* sp. and *A. gracilis*+*Sphingobacterium* sp. in clean soil, respectively. In these two soils, most of enzyme activities were inhibited in treatments with earthworm alone and earthworm+*Sphingobacterium* sp.. However, N-related enzyme activities were increased in treatments with *E. fetida* in clean soil and treatments with *A. gracilis* in contaminated soil(NAG activities), protease activities in treatments with *Sphingobacterium* sp. and treatments with *A. gracilis* in contaminated soil(PRO activities). Our results concluded that earthworm inoculation

alone significantly promoted mineralization of carbon and nitrogen in clean soil rather than contaminated soil; *Sphingobacterium* sp. was able to assist earthworms with improving carbon and nitrogen mineralization in clean soil. In contaminated soil, earthworm alone and earthworm combined with *Sphingobacterium* sp. both facilitated carbon storage. These findings provided insights into the combined effects of earthworms and microbes on carbon and nitrogen cycling during soil remediation.

Keywords: Earthworms; *Sphingobacterium* sp.; Cd-contaminated soil; carbon and nitrogen fractions; enzyme activities

1 **Different combined effects of earthworm and**
2 ***Sphingobacterium* sp. on soil C, N forms and enzyme**
3 **activities in clean and Cd-contaminated soil**

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28 **Highlights**

- 29 • Earthworm improved mineralization of C and N in clean soil and C storage in
30 contaminated soil.
- 31 • *E. fetida* was more effective on C mineralization than *A. gracilis*.
- 32 • *Sphingobacterium* sp. assisted earthworm with improving C and N
33 mineralization in clean soil rather than contaminated soil.

34

1 35 **ABSTRACT**

2
3 36 Earthworm and *Sphingobacterium* sp. are both characterized by their strong
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6 37 decomposition ability of organic compound and broad distribution in soil. Little is
7
8
9 38 known about their interaction on soil organic matter decomposition and whether
10
11 39 *Sphingobacterium* sp. could assist earthworms with carbon and nitrogen cycles. In this
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13
14 40 study, earthworms (*Eisenia fetida*, *Amyntas gracilis*) and *Sphingobacterium* sp. were
15
16
17 41 inoculated in clean and contaminated soils under laboratory condition for 20 days. We
18
19
20 42 investigated their single or combined effects on carbon and nitrogen forms (organic
21
22 43 carbon, total Nitrogen, dissolved organic carbon (DOC), alkali-hydrolyzable nitrogen
23
24
25 44 (AN), microbial biomass carbon and nitrogen) and C- and N- related enzyme activities
26
27
28 45 (β -glucosidase(BG), invertase (INV), urease(URE), N-acetyl- β -D-
29
30
31 46 glucosaminidase(NAG), protease(PRO) and fluorescein diacetate hydrolysis (FDA)),
32
33
34 47 evaluating their interaction on soil C and N cycles. *E. fetida* and *A. gracilis* inoculation
35
36 48 alone increased DOC contents significantly by 75.8 % and 53.6 % comparing with no
37
38
39 49 earthworm control in clean soil; while they increased AN contents significantly by 32.9%
40
41
42 50 and 20.9% as well, respectively. Inoculation of *Sphingobacterium* sp. alone promoted
43
44
45 51 microbial biomass carbon reaching 1685 ± 292 mg·kg⁻¹ in clean soil tripled of control.
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48 52 Moreover, the combination of earthworms and *Sphingobacterium* sp. increased DOC
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50
51 53 contents by 212% and 134%, and AN contents by 31.3% and 25.4% in treatments of *E.*
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53 54 *fetida*+*Sphingobacterium* sp. and *A. gracilis*+*Sphingobacterium* sp. in clean soil,
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56 55 respectively. In these two soils, most of enzyme activities were inhibited in treatments
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58
59 56 with earthworm alone and earthworm+*Sphingobacterium* sp.. However, N-related
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1 57 enzyme activities were increased in treatments with *E. fetida* in clean soil and
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3 58 treatments with *A. gracilis* in contaminated soil(NAG activities), protease activities in
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6 59 treatments with *Sphingobacterium* sp. and treatments with *A. gracilis* in contaminated
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9 60 soil(PRO activities). Our results concluded that earthworm inoculation alone
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11 61 significantly promoted mineralization of carbon and nitrogen in clean soil rather than
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14 62 contaminated soil; *Sphingobacterium* sp. was able to assist earthworms with improving
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17 63 carbon and nitrogen mineralization in clean soil. In contaminated soil, earthworm alone
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19
20 64 and earthworm combined with *Sphingobacterium* sp. both facilitated carbon storage.
21
22 65 These findings provided insights into the combined effects of earthworms and microbes
23
24
25 66 on carbon and nitrogen cycling during soil remediation.
26
27

28 67 **Keywords :** Earthworms; *Sphingobacterium* sp.; Cd-contaminated soil; carbon and nitrogen
29
30
31 68 fractions; enzyme activities
32

33 69 **1. Introduction**

34
35
36 70 Soil is an important source and sink of terrestrial carbon and nitrogen in the
37
38
39 71 biosphere. There are a variety of soil organisms playing an essential role in soil carbon
40
41
42 72 and nitrogen cycle[1,2]. The interactive processes of soil organisms, reconstituting soil
43
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45 73 carbon stocks and nitrogen cycling in sustainable soil management, have attracted
46
47
48 74 broad attention[3,4]. However, soil metals pollution is becoming an more and more
49
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51 75 serious environmental and social problem, probably resulting in the changes in the
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54 76 interaction between soil organisms to affect soil carbon and nitrogen cycles[5]. It is of
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57 77 great ecological importance to understand the interactive relationship of soil organisms
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60 78 on the carbon and nitrogen cycle under metal contamination stress.
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1 79 As "soil ecosystem engineers", earthworms could degrade soil organic matter and
2
3 80 significantly influence soil carbon and nitrogen contents[13]. These processes is the
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6 81 result of complex interactions between soil organisms. Earthworm selective feeding
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8
9 82 activities have a significant impact on soil carbon and nitrogen cycling in gut[6].
10
11 83 Earthworm bioturbation (burrowing, feeding and excretion (casts and surface mucus))
12
13
14 84 affect directly or indirectly soil microbial communities and soil enzyme activities[7-9].
15
16
17 85 Nevertheless, currently, previous studies on earthworms in metal contaminated soil
18
19
20 86 have mainly focused on earthworm effects on metal transformation and their
21
22
23 87 ecotoxicological characteristics [10-12]. Earthworm effects on soil carbon and nitrogen
24
25
26 88 cycling in metal contaminated soil have not been receiving sufficient attention.

27
28 89 *Sphingobacterium* sp., a kind of gram-negative bacterium, has a wide range of
29
30
31 90 nutrient sources, high adaptability and wide ecological distribution [16-18]. In our
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33
34 91 previous studies, *Sphingobacterium* sp. was demonstrated as a kind of dominant
35
36
37 92 microorganism in earthworm gut [25]. Although *Sphingobacterium* sp. has a strong
38
39
40 93 capacity for organic matter degradation (alkanes and polycyclic aromatic hydrocarbons)
41
42
43 94 to regulate soil carbon and nitrogen transformation in organic polluted soil[19-24], their
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45
46 95 effect in combination with earthworms on the soil carbon and nitrogen cycle have not
47
48
49 96 yet been clarified, especially under metal pollution stress.

50 97 Soil C and N cycles invloved a series of biological and biochemical process. Soil
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52
53 98 carbon and nitrogen fractions, dissolved organic carbon(DOC), alkali-hydrolyzable
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56 99 nitrogen (AN), microbial biomass carbon and nitrogen(MBC and MBN) have been
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58
59 100 considered as the key indicators to reflect the state of the soil carbon and nitrogen cycles,

1 101 and their fraction ratios can further provide a more intuitive representation of the overall
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3 102 mineralization or sequestration of carbon and nitrogen in the system. Besides, soil
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6 103 enzymes are metabolic drivers of soil organisms, and their activity can, to some extent,
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9 104 reflect not only the changes in the soil microbial community [27], but also the intensity
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11 105 of soil organic matter decomposition and nutrient transformation [28]. Studies on the
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14 106 influence of enzymes on carbon and nitrogen transformation have mostly focused on
15
16
17 107 β -glucosidases (BG) and invertase (INV), which were involved in the decomposition
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20 108 of labile cellulose, sucrose and carbohydrate polymers, and N-acetyl-glucosaminidase
21
22 109 (NAG), urease (URE) and proteases (PRO) were involved in the hydrolysis of chitooli-
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25 110 gosaccharides, urea and protein, as these enzymes are mainly participating in terminal
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28 111 catalytic reactions or intra-systemic enzymatic reactions, which can reflect the level of
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31 112 carbon and nitrogen metabolism in soils [29]. Fluorescein diacetate hydrolysis (FDA)
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33
34 113 is an enzyme that can reflect the total microbial activity of soil [30]. Therefore, the
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37 114 effects of earthworm inoculation on soil carbon and nitrogen cycles can indirectly be
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39
40 115 reflected by studying the changes in soil enzyme activities related to carbon and
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42 116 nitrogen cycles.

43
44 117 The aim of this study was to investigate the effects of different species of
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47 118 earthworms (*Eisenia fetida* and *Amyntas gracilis*) and exogenous *Sphingobacterium*
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49
50 119 sp. on the transformation of soil carbon and nitrogen under clean and Cd-contaminated
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52
53 120 soil systems. The specific objectives were: (1) to compare and analyse the effects of
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56 121 exogenous *Sphingobacterium* sp. and two different species of earthworms, individually
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59 122 and simultaneously, on carbon and nitrogen fractions and related enzyme activities in
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1 123 clean and Cd-contaminated soils. (2) to elucidate the role of *Sphingobacterium* sp. in
2
3 124 the participation of earthworms in the carbon and nitrogen cycling process under Cd
4
5
6 125 pollution stress. This study could provide a theoretical basis for an insight into soil
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9 126 carbon and nitrogen cycling processes and biologically driven mechanism, and also
10
11 127 provide suggestions for adjusting soil remediation and their further sustainable
12
13
14 128 management.

17 129 **2. Materials and methods**

20 130 *2.1 Soil, earthworm and strain*

22 131 Two representative soils from South China were selected, clean soil (Soil I) and
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24
25 132 soil contaminated with metals from mining areas (Soil II). Soil I was collected from the
26
27
28 133 tree garden of South China Agricultural University (23°9'N, 113°21'E); Soil II was
29
30
31 134 collected from a paddy field (24°30'N, 113°45'E) about 6 km downstream of the
32
33
34 135 opencast mining area of the copper-sulfur ore, limonite and lead-zinc mine in Dabao
35
36 136 Mountain, northern Guangdong Province. The basic physicochemical properties of soil
37
38
39 137 are listed in Table 1.

41 138 Earthworms *Eisenia fetida* (*E. fetida*, epigeic specie) and *Amyntas gracilis*
42
43
44 139 (*A. gracilis*, epigeic species) were selected. *A. gracilis* was collected from South China
45
46
47 140 Agricultural University, *E. fetida* were laboratory-raised earthworms. After two weeks
48
49
50 141 of acclimation in the laboratory, earthworms with similar individual weight and a
51
52
53 142 mature clitellum were selected for experiment.

55 143 *Sphingobacterium* sp. was purchased from BeNa Culture Collection (BNCC).

58 144 Microbial CM0827 *Propionibacterium* sp. medium consisted of: tryptone 15 g, L-

1 145 cystine 0.5 g, yeast extract 5 g, sodium mercaptoethanolate 0.5 g, glucose 5 g, agar 0.7
2
3
4 146 g, sodium chloride 2.5 g, resazurin 0.001 g, distilled water 1 L. Incubate the strain at a
5
6 147 constant temperature of 30°C for 2 days and set aside (pH 7.0, OD₄₀₅ value 1.45).
7
8

9 148 2.2 *Experimental designs*

10
11 149 A two-factor (inoculation with *Sphingobacterium* sp. and different earthworm
12
13 150 species (*E.fetida*, E, *A.gracilis*, A)) experimental design was used in clean (Soil I, C)
14
15 151 and Cd- contaminated (Soil II, S) soils. Six treatments were set up for each of the two
16
17 152 soils, with three replicates for each treatment, and 36 pots in total.
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20
21
22 153 Soil I: (1) Soil I (C); (2) Soil I + *Sphingobacterium* sp. (CS); (3) Soil I + *E. fetida* (CE);
23
24
25 154 (4) Soil I + *A. gracilis* (CA); (5) Soil I + *Sphingobacterium* sp. + *E. fetida* (CSE); (6)
26
27 155 Soil I + *Sphingobacterium* sp. + *A.gracilis* (CSA).
28
29

30
31 156 Soil II: (1) Soil II (S); (2) Soil II + *Sphingobacterium* sp. (SS); (3) Soil II + *E. fetida*
32
33 157 (*SE*); (4) Soil II + *A.gracilis* (SA); (5) Soil II + *Sphingobacterium* sp. + *E. fetida* (SSE);
34
35 158 (6) Soil II + *Sphingobacterium* sp. + *A. gracilis* (SSA).
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37
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39 159 Filled 200 g soil through 2 mm mesh into the pot (caliber x bottom diameter x
40
41 160 height: 75 x 53 x 90 mm) with microporous bottom. Stabilized the soil moisture for 48
42
43 161 h by adjusting it to 60% of maximum field water holding capacity and maintained at
44
45 162 this moisture by weighting throughout. Inoculated with 3 g of earthworms per pot,
46
47 163 approximately 8 *E.fetida* (3±0.3 g) or 5 *A.gracilis* (3±0.4 g). one milliliter of
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49 164 *Sphingobacterium* sp. solution (OD₄₀₅ value 1.45) was added to the soil surface in CS,
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51 165 CSA, CSE, SS, SSA and SSE treatments, respectively. To prevent earthworms escape,
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53 166 the top and bottom of each pot were covered with a fine nylon mesh with 1 mm pore
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1 167 diameter. The temperature in the laboratory was controlled at 25°C.

2
3 168 After 20 days, the earthworms were hand-picked and soil samples were collected
4
5
6 169 as well. A portion of the fresh soil was refrigerated at 4°C for analyzing microbial
7
8
9 170 characteristics and microbial biomass carbon and nitrogen. The remainder was fully
10
11
12 171 air-dried, ground and sieved for analyzing soil physical and chemical properties.
13
14 172 Earthworms were counted and weighted during sampling.

17 173 *2.3 Soil properties, enzyme activity, content and DTPA of Cadmium*

20 174 Soil pH was measured using a glass electrode in a 1:2.5 (w/v) soil/water
21
22 175 suspension. Soil clay, and soil organic carbon (SOC), alkali-hydrolyzable nitrogen (AN)
23
24
25 176 and total nitrogen (TN) was analyzed by the pipette [31], the dichromate oxidation [32],
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27
28 177 alkali N-proliferation [33] and Kjeldahl [32] methods, respectively; Microbial biomass
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31 178 carbon and nitrogen (MBC and MBN) were determined via the chloroform fumigation-
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33
34 179 extraction method [34,35]; Dissolved organic carbon (DOC) content was extracted with
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36
37 180 deionized water (1:5) and determined [15]. Total cadmium (Cd) content was prepared
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39
40 181 and determined by microwave digestion and ICP-MS [36], and the measured
41
42
43 182 concentrations of referred samples within 98% of certified concentrations. Three
44
45 183 repetitions for each soil were also implemented to ensure the quality of analysis.

47 184 β -glucosidase, protease and N-acetyl- β -D-glucosaminidase activities were all
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50 185 determined by colorimetric assay. Urease, invertase and fluorescein diacetate hydrolytic
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52
53 186 activities was determined by sodium phenolate colorimetric assay, titration and
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56 187 colourimetric using fluorescein methods [37], respectively.

58 188 *2.4 Statistics analysis*

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189 All data was analyzed and plotted with SPSS statistical software (Version 24.0),
190 Origin 9.0, and R (Version 3.4.3). T test and variance (ANOVA) analysis were
191 performed. Data was presented as the mean \pm SE (standard error). Principal components
192 analysis (PCA) was processed to analyze the relationships of soil carbon and nitrogen
193 fractions and ratios (SOC, DOC, MBC, TN, AN, MBN, DOC:SOC, C:N, AN:TN,
194 MBC:MBN, q MBC, q MBN) and enzymes (BG, INV, URE, NAG, PRO, FDA) in R
195 version 3.4.3 using the “ade4” package. DOC:SOC=DOC/SOC, AN:TN=AN/TN,
196 C:N= SOC/TN, MBC:MBN=MBC/MBN; Microbial quotient carbon (q MBC),
197 q MBC=MBC/SOC; Microbial quotient nitrogen (q MBN), q MBN =MBN/TN.

198 **3. Results**

199 *3.1 Earthworm survival rate and biomass*

200 After 20 d, the survival rate and biomass of both earthworm species decreased
201 (Table 2). In terms of survival rate, only the SSE treatment showed a significant
202 decrease of 68.7% ($p < 0.05$), while the other treatments showed no significant variation.
203 While earthworm biomass decreased by 7.68%-77.2% ($p < 0.05$), the decrease was
204 greater in Cd-contaminated soil than in clean soil, and more in *E.fetida* than *A.gracilis*.
205 Specifically, CE and CSE decreased by 17.4% and 19.4%, respectively, CA and CSA
206 by 7.68% and 13.7%, respectively; SE and SSE decreased by 17.6% and 77.2%,
207 respectively; SA and SSA both by 18.9%.

208 *3.2 The content of carbon and nitrogen fractions*

209 The soil carbon fraction contents were shown in Fig. 1a-c. In clean soil,
210 *Sphingobacterium* sp. and earthworms significantly reduced SOC content ($p < 0.05$). It

1 211 was reduced by 16.7%, 14.8%, 15.6%, 16.7%, and 15.9% in CS, CE, CA, CSE, CSA
2
3 212 treatments, respectively. Furthermore, DOC increased significantly in the presence of
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5
6 213 earthworms ($p < 0.05$), and was highest in the CSE and CSA treatments at 659 ± 19.2
7
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9 214 $\text{mg} \cdot \text{kg}^{-1}$ and $430 \pm 82.8 \text{ mg} \cdot \text{kg}^{-1}$, respectively. Individual inoculation with
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11 215 *Sphingobacterium* sp. significantly increased MBC up to $1685 \pm 292 \text{ mg} \cdot \text{kg}^{-1}$ ($p < 0.05$).
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14 216 Whereas, in all the treatments inoculated with earthworms, there was no significant
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16
17 217 change in MBC content compared to C treatment, but it was significantly decreased
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20 218 compared to CS treatment.

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22 219 In Cd-contaminated soil, SS treatment reduced the SOC content, decreasing to
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25 220 $16.0 \pm 0.44 \text{ g} \cdot \text{kg}^{-1}$. while SOC increased in all treatments involving earthworms and was
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28 221 significantly higher than S treatment ($16.0 \pm 0.44 \text{ g} \cdot \text{kg}^{-1}$) under combined effect of SSE
29
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31 222 ($17.8 \pm 0.28 \text{ g} \cdot \text{kg}^{-1}$) and SSA ($17.5 \pm 0.50 \text{ g} \cdot \text{kg}^{-1}$) ($p < 0.05$). Compared to control, MBC
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34 223 was slightly decreased in SS treatment and the variation was smaller than in CS
35
36
37 224 treatment.

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39 225 The content of nitrogen fractions can be found in Fig. 1d-f. In clean soil, the
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42 226 earthworm activity significantly increased AN content ($p < 0.05$), which increased by
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44
45 227 32.9%, 20.9%, 31.3% and 25.4% in CE, CA, CSE and CSA treatments respectively,
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47
48 228 while *Sphingobacterium* sp. did not significantly affect it ($p > 0.05$). However, the
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50
51 229 mineralization indexes of nitrogen were not significantly changed in contaminated soil
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53
54 230 ($p > 0.05$), but the trend of AN in all treatments was consistent with in clean soil. In
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57 231 addition, TN and MBN were relatively stable in both soils with no significant changes
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60 232 under the activities of *Sphingobacterium* sp. and earthworms.
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233 3.3 *The ratio characteristics of carbon and nitrogen fractions*

234 Soil carbon and nitrogen fraction ratios are shown in Fig. 2. In both soils, the
235 ratios showed consistency with the changes in the corresponding carbon and nitrogen
236 fractions.

237 The ratio of DOC:SOC followed the same trend as DOC, increasing to 2.93%
238 and 1.94% in the CSE and CSA treatments, respectively (Fig. 2a). The ratio of C:N
239 significantly decreased by 23.6%, 23.6%, 24.0%, 22.6% and 14.4% in the CS, CE, CA,
240 CSE and CSA treatments. In clean soil, the ratio of AN:TN increased significantly in
241 the treatments involving earthworms and further increased in combination with
242 *Sphingobacterium* sp. ($p < 0.05$). MBC:MBN was significantly increased in CS
243 treatment in clean soil and significantly decreased in SS treatment in Cd-contaminated
244 soil ($p < 0.05$). In turn, the ratio significantly decreased in clean soil and increased in
245 contaminated soil when combined *Sphingobacterium* sp. with earthworms. In clean soil,
246 inoculation with *Sphingobacterium* sp. and earthworms individually increased $qMBC$
247 and was highest in CS treatment to control, but decreased in the combined condition.
248 $qMBN$ did not change significantly ($p > 0.05$) in all treatment for both soils.

249 3.4 *Response of soil enzymes activity to inoculated earthworm and* 250 *Sphingobacterium* sp.

251 The soil enzyme activities were shown in Fig. 3. In clean soil, inoculation of
252 *Sphingobacterium* sp. alone had no significant effect on enzymes activity, but greater
253 variation in the effect on INV activities. Inoculation of *E.fetida* individually increased
254 NAG ($3.86 \pm 0.37 \mu\text{g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) activities but decreased URE activities. Moreover,

1 255 inoculation of *A.gracilis* individually significantly decreased BG activities ($p < 0.05$).
2
3 256 In combination condition, both significantly inhibited BG activities ($p < 0.05$), with no
4
5
6 257 significant effect on other enzyme activities. In contaminated soil, *Sphingobacterium*
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8
9 258 sp. slight increased BG activities, but there was no significant difference among the
10
11 259 treatments. PRO activity was significantly increased only in SS treatment, reaching
12
13 260 $453 \pm 6.64 \mu\text{g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$. As for the earthworm inoculation treatment, it was inhibited except
14
15 261 for SSA, which increased INV, and SA, which increased NAG and PRO activities.
16
17 262 There was no significant change in FDA activities ($p > 0.05$).
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21

22 263 *3.5 Relationships among carbon, nitrogen and related enzymes activity* 23 24 25 264 *with different treatments* 26 27

28 265 In clean soils, two principal components (PCs) were identified in the PCA,
29
30 266 representing 49.1% of the total variance (Fig. 4a-b). PC1 and PC2 explained 29.6% and
31
32 267 19.5% of the total variance, respectively. Significant differences were founded among
33
34 268 the treatments, indicating that earthworms and *Sphingobacterium* sp. had significantly
35
36 269 impacts on soil carbon and nitrogen fractions in clean soil ($p = 0.001$). Treatments
37
38 270 inoculated with earthworms and both earthworms and *Sphingobacterium* sp. tended to
39
40 271 have low PC1 values, and treatments inoculated with *Sphingobacterium* sp. alone
41
42 272 tended to have low PC2 values. The difference between the treatment C and CSA, CSE
43
44 273 was reflected in the latter's significant enhancement of reactive carbon nitrogen such as
45
46 274 DOC and AN, while the CE and CA treatments were intermediate between C and CSA,
47
48 275 CSE. It can be clearly seen that the difference between C and CS was mainly in the
49
50 276 second principal component of MBC, MBC:MBN and $q\text{MBC}$, while CE and CA, CSA
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1 277 and CSE were in mid-way between them. In addition, inoculation with earthworms
2
3 278 showed an inhibitory effect on enzyme activity. DOC was negatively correlated with
4
5
6 279 soil enzyme activity.
7

8
9 280 In contaminated soils, two principal components (PCs) were identified in the
10
11 281 PCA, representing 43.7% of the total variance (Fig. 4c-d). PC1 and PC2 explained 26.6%
12
13
14 282 and 17.1% of the total variance, respectively. There were significant differences
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16
17 283 between the treatments, indicating that earthworms and *Sphingobacterium* sp.
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19
20 284 significantly influenced soil carbon and nitrogen composition in contaminated soils
21
22
23 285 ($p=0.017$). Treatments inoculated with *Sphingobacterium* sp. tended to have high PC1
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25
26 286 values and treatments inoculated with earthworms tended to have high PC2 values. The
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29 287 differences between the S treatment and the earthworm inoculation treatment were
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31
32 288 mainly reflected in the latter's enhanced effect on SOC, AN and MBN. While the S and
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34
35 289 SS treatments differed in the latter's enhanced effects on DOC, and the earthworm
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38 290 inoculated treatment was in between. Meanwhile, except for PRO enzyme, earthworm
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41 291 inoculation showed inhibitory effect on enzyme activity.

42 292 **4. Discussion**

43 44 45 293 *4.1 Earthworm growth and survival*

46
47 294 Earthworms abundance and biomass decreased after 20 days cultivation in both
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49
50 295 soils, with significant differences were observed especially in Cd-contaminated soil (p
51
52
53 296 <0.05), consistent with [38]. It might be due to soil nutrient limitation and toxic effects
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55
56 297 of high Cd concentration on earthworms [39]. In terms of earthworm species, *A.gracilis*
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59 298 showed higher resistant to environmental stresses than *E.fetida*, probably owing to the
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1 299 adaptability of wild earthworm species in soil with limited organic matters.

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3 300 *4.2 Effects of earthworms and Sphingobacterium sp. on soil carbon and*
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5
6 301 *nitrogen fractions*

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9 302 In clean soils with high organic carbon, both earthworm and *Sphingobacterium*
10
11 303 sp. alone significantly facilitated the mineralization of organic carbon (Fig. 1b, Fig. 2b)
12
13
14 304 and is consistent with the trend of carbon and nitrogen changes in vermicomposting by
15
16
17 305 [40]. Both species of earthworms significantly increased nitrogen mineralization ($p <$
18
19
20 306 0.05) (Fig. 1e), presumably through digestion in the earthworm gut, which promotes
21
22
23 307 the mineralization of organic nitrogen. A study investigated the changes in the gut
24
25
26 308 microbiota and functional characteristics of earthworms with the chronological
27
28
29 309 sequence of cultivated soils, revealing that tillage disturbance increases the formation
30
31
32 310 of functional genes that promote nutrient cycling in the earthworm gut microbiota [41].
33
34
35 311 The combination of the two species of earthworm with *Sphingobacterium* sp. also
36
37
38 312 further significantly increased the reactive carbon and nitrogen content, demonstrating
39
40
41 313 that earthworms and *Sphingobacterium* sp. were able to stimulate soil carbon and
42
43
44 314 nitrogen cycling, and that there was an intensified effect of the combination. The high
45
46
47 315 microbial biomass carbon content in CS treatment inoculated with *Sphingobacterium*
48
49
50 316 sp. alone may be due to its adaption to this environment, rapid multiplication and
51
52
53 317 becoming the dominant strain. In contrast, earthworm activities decreased microbial
54
55
56 318 biomass as compared to *Sphingobacterium* sp. activities alone. It is suggested that
57
58
59 319 earthworms have a role in maintain the ecosystem balance and stabilizing the microbial
60
61
62 320 community structure through feeding [42] and regulating the allocation of active
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1 321 organic carbon in the microbial proliferation and dissolved organic carbon pools [43,44].
2
3 322 Therefore, in the treatments of *Sphingobacterium* sp. in combination with earthworm,
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5
6 323 the rapid proliferation of *Sphingobacterium* sp. was prevented. This was further
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8
9 324 confirmed by the significantly lower q MBC of CSE and CSA compared to CS in Fig.
10
11
12 325 2e. That is, earthworms reduced the efficiency of soil microorganisms in utilizing
13
14
15 326 carbon sources for conversion into microbial biomass carbon. Additionally, since
16
17 327 *Sphingobacterium* sp. is the dominant gut bacterium of *E.fetida* and genera of *Amythas*,
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19
20 328 the gut can provide a shelter for it to not die completely under Cd-contaminated stress
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22
23 329 [25].

25 330 In contaminated soil, *Sphingobacterium* sp. still showed a facilitation of carbon
26
27
28 331 mineralization (Fig. 1a, b; Fig. 2a), although the ability to do so was diminished,
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30
31 332 indicating its ability to mineralize carbon and to tolerate metal contamination stress.
32
33
34 333 Earthworms, on the other hand, showed carbon sequestration in Cd-contaminated soils
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36
37 334 with low soil organic carbon content, in contrast to their behavior in clean soils. This is
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39
40 335 probably due to the biased feeding of organic matter by earthworms to resist metal
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42
43 336 toxicity, and then, through gut peristalsis and body surface mucus, promote the
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46 337 formation of organic matter-rich aggregate and the protective effect of mucus
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48
49 338 substances, which reduces carbon loss [6, 44-47]. It's also probably explained by
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51
52 339 contaminated soils containing carbon that is stable and not easily mineralized. In PCA
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54
55 340 analysis, positive correlation between β -glucosidase and N-acetyl- β -D-
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57
58 341 glucosaminidase activity also indicated that soil microorganisms tend to use the active
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61 342 carbon pool generated by the decomposition of humus formed by litter (Fig. 4c; Fig.

1 343 S1), i.e., favor the accumulation of recalcitrant soil carbon pools. In addition, both
2
3 344 dissolved organic carbon and the dissolved organic carbon to soil organic carbon ratio
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6 345 were significantly lower in the SSE treatment than in the other treatments, which may
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8
9 346 be influenced by the lower survival rate of earthworms and their reduced biological
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11
12 347 effect capacity in this treatment. For the other carbon and nitrogen fractions, earthworm
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14 348 and *Sphingobacterium* sp. activities had non-significant effect, presumably due to
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16
17 349 nutrient and metal pollution stress [48], which in turn reduces its ecological role.

20 350 *4.3 Effect of earthworms and Sphingobacterium* sp. on soil enzyme 21 22 351 *activity*

25 352 The soil enzyme activity in both clean and Cd-contaminated soils showed in Fig.
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27
28 353 3. In clean soil, inoculation of earthworms and *Sphingobacterium* sp. had no significant
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31 354 effect on soil enzyme activities on the whole, but activated enzyme activities in some
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33
34 355 treatment. In terms of trends, inoculation with earthworms showed weak inhibition of
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36 356 β -glucosidase, urease, and fluorescein diacetate hydrolytic activities, opposite to the
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39 357 results of most studies [49,50]. However, it did not affect the soil carbon and nitrogen
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42 358 mineralization. Similarly, a study found that acid and alkaline phosphatase activities
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44
45 359 were decreased in earthworm casts but inorganic phosphorus in the soil was increased
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47
48 360 [8]. This also suggests that enzyme activity is affected by a variety of factors such as
49
50
51 361 food type, pH, temperature, gut transit time, etc., [51]. Besides, enzymes only play a
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54 362 catalytic role and do not completely determine soil matter mineralization, which needs
55
56
57 363 to be analyzed in the context of multiple biological and environmental factors.

58 364 In contaminated soil, earthworms and *Sphingobacterium* sp. activities decreased

1 365 the activities of invertase, N-acetyl- β -D glucosaminidase and urease, but there was no
2
3 366 obvious regularity among treatments (Fig. 3b-d). It is possibly due to that soil exhibited
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5
6 367 carbon storage and the corresponding mineralizing enzyme activities were reduced
7
8
9 368 under the activities of earthworms. Or these enzyme activities were intimately related
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11
12 369 to soil water-soluble organic carbon and microbials, i.e., metal stress and low soil
13
14 370 fertility, and soil fauna activities in turn reduced the soil microbiota [52,53]. In addition,
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17 371 metal can inhibit soil urease and fluorescein diacetate hydrolytic activity, and
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19
20 372 postulated that the mechanism may be that Cd^{2+} exchanges with Mg^{2+} , resulting in
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23 373 blockage of protein synthesis, which in turn inhibits the growth and proliferation and
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26 374 decreases enzyme synthesis and secretion in microorganisms, and ultimately leads to
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29 375 decreased soil enzyme activities [54].

30
31 376 In terms of earthworm species, soil enzyme activities in clean soil with *E.fetida*
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33
34 377 participating were generally higher than those of *A.gracilis*. In contaminated soils,
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37 378 *A.gracilis* increased N-acetyl- β -D glucosaminidase, protease, and urease activities
38
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40 379 individual and combined, which may be related to that *A.gracilis* is wild species with
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43 380 strong resistance to environment stress. Moreover, *A.gracilis* influenced fluorescein
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46 381 diacetate hydrolytic to a greater extent, both individually and combinatorially in two
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48
49 382 soils, presumably as a result of differences in feeding habits of different species
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51
52 383 [55,10,56]. Earthworms preferred to feed on microorganisms as secondary food source
53
54
55 384 in soils where organic matter is difficult to degrade [8]. The body fluids of earthworms
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58 385 may contain bacteriostatic substances [57,58], resulting in a lower bacterial content in
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61 386 their gut and excreta than in original soil.

1 387 4.4 *Effect of earthworms and Sphingobacterium sp. on the balance of soil*
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3 388 *carbon and nitrogen stabilization and mineralization*
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6 389 Organisms have strong interaction among themselves in soil [3], earthworm and
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8 390 microorganisms contributed together in this study on the balance of soil carbon and
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10 391 nitrogen stabilization and mineralization. In our study, *Sphingobacterium sp.* can
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12 392 promote soil carbon and nitrogen mineralization, especially in clean soils (Fig.1-2). It
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14 393 also assist earthworm to further promote carbon and nitrogen mineralization(Fig.4a),
15
16 394 and *E.fetida* showed more efficiencies than *A.gracilis*. In contrast, the combined effect
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18 395 of them in metal contaminated soils was not significant and exhibited carbon
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20 396 storage(Fig. 4b), probably due to the biased feeding of organic matter by earthworms
21
22 397 to resist metal toxicity, and the nutrient and metal contaminated stress in the soil not
23
24 398 only affects the survival status of earthworms, but also makes it difficult for
25
26 399 microorganisms to play its part. Ultimately, the contaminated soil exhibited carbon
27
28 400 storage because of the stronger stabilizing effect of earthworms than mineralizing effect
29
30 401 of *Sphingobacterium sp.*. It's also probably owing to mineralizable carbon decreases
31
32 402 with time of mine contaminated soils and residue more mineral associated organic
33
34 403 matter that is stable and not easily mineralized by organisms [59]. Among earthworm
35
36 404 species, *E.fetida* had a stronger effect on soil carbon and nitrogen mineralization than
37
38 405 *A.gracilis* (Fig. 1b), which may be related that *E.fetida* favors ingestion of easily
39
40 406 available organic matter, accelerating the mineralization and decomposition of organic
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42 407 matter [60,61]. By contrast, *A.gracilis* mainly fed on soil mineral particles and organic
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44 408 matter, facilitating the mixing and bind of organic matter with soil minerals. In addition,
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1 409 the soil of this study (Table 1), *E. fetida* was easier to obtain food in clean soil with
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3 410 relatively high organic matter content and the high carbon to nitrogen ratio was favored
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6 411 for earthworm growth [62], so this species performed better. Unfortunately, their
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8
9 412 ecological effects were limited by difficulties in access to food in Cd-contaminated soils
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11
12 413 with low organic matter content (Table 1). Additionally, only parts of treatment
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15 414 increased soil enzyme activities. Mostly treatment enzymes had no significant different
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18 415 compared with control treatment and showed weak inhibited by earthworm activities.
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20 416 This phenomenon also can be founded in PCA analysis (Fig. 4). That is, soil carbon and
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23 417 nitrogen index did not show a strong correlation. To reveal the under mechanisms, we
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25
26 418 need to analyze them in the context of multiple biological and environmental factors.

27 28 419 **5. Conclusion**

29
30 420 This study on soil carbon and nitrogen fractions and enzyme activities to
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33 421 investigate whether *Sphingobacterium* sp. can assist different species of earthworm in
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35
36 422 influencing soil carbon stabilization and nitrogen cycling under clean and Cd-
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39 423 contaminated soil. Overall, earthworm inoculation alone exhibited facilitating carbon
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41
42 424 and nitrogen mineralization in clean soil, while *Sphingobacterium* sp. inoculation alone
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45 425 facilitated carbon and nitrogen mineralization in both soils. In clean soil, earthworm
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48 426 effect showed species-dependent and *E. fetida* was more effective than that of *A. gracilis*.
49
50 427 *Sphingobacterium* sp. could enhance earthworm effects on carbon mineralization in
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52
53 428 clean soil. While in cadmium contaminated soil, earthworm and *Sphingobacterium* sp.
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56 429 combined inoculation improved soil carbon storage rather than carbon mineralization
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59 430 in treatments with earthworm, but no significant effects between earthworm species.
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1 431 However, the effect of earthworm and *Sphingobacterium* sp. on soil nitrogen was no
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3 432 significant in contaminated soil. This study provides a clear understanding on the
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6 433 different effects of earthworm and microorganisms on soil carbon and nitrogen cycling
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9 434 in clean and contaminated soil. In further study, the complex interaction mechanisms
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12 435 of earthworm and microorganisms can be designed and tested by some methods about
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14
15 436 molecular biology or metabolomics. Additionally, the interaction of earthworm and
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17
18 437 microorganisms in soil remediation technology could be explored to adjust and rebuild
19
20
21 438 a sustainable soil management practice.

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1 **Individual and combined effects of earthworms and**
2
3 ***Sphingobacterium* sp. on soil organic C, N forms and enzyme activities**
4
5 **in non-contaminated and Cd-contaminated soil**

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Highlights

- *Sphingobacterium* sp. could promote C mineralization in both soils.
- Earthworms facilitated C and N mineralization in non-contaminated soil.
- Earthworms enhanced C stabilization in Cd-contaminated soil.
- *Sphingobacterium* sp. could assist earthworms in improving C and N mineralization in non-contaminated soil.
- Earthworm activities dominated the C and N turnover in this strain-earthworm-soil system.

34 ABSTRACT

35 Earthworms and *Sphingobacterium* sp. are known for their strong organic compound
36 decomposition ability and wide distribution in soil. However, interactions of soil organic matter
37 decomposition with soil properties and whether microbial species such as *Sphingobacterium* sp. could
38 assist earthworms in carbon and nitrogen transformation in soil remain poorly understood. Earthworms
39 (*Eisenia fetida*, *Amyntas gracilis*) and *Sphingobacterium* sp. were introduced in non-contaminated
40 and cadmium-contaminated soils under controlled laboratory conditions for 20 days. We examined
41 their individual or combined effects on carbon and nitrogen forms and related enzyme activities to
42 assess their influence on soil C and N cycling. Individual *Sphingobacterium* sp. inoculation led to
43 significantly decreased organic carbon (SOC) contents, reducing it by 16.5% in non-contaminated soil
44 and by 3.77%, in Cd-contaminated soil. It resulted in an increased microbial biomass carbon (MBC)
45 contents, reaching $1685 \pm 292 \text{ mg} \cdot \text{kg}^{-1}$ in non-contaminated soil. Individual introductions of *E. fetida*
46 and *A. gracilis* caused a decline in SOC content in non-contaminated soil, but increased significantly
47 dissolved organic carbon (DOC) and alkali-hydrolysable nitrogen (AN) contents by 75.8%, 53.6% and
48 32.9%, 20.9%, respectively. In contrast, in Cd-contaminated soil, only the significant combined effects
49 of earthworms and *Sphingobacterium* sp. were linked to significant increase in SOC contents, raising
50 by 7.22% and 9.64% in *E. fetida* + *Sphingobacterium* sp. and *A. gracilis* + *Sphingobacterium* sp.
51 treatments, respectively. In non-contaminate soil, the combined effects of earthworm and
52 *Sphingobacterium* sp. further increased DOC and AN content by 212%, 134% and 31.3%, 25.4% in
53 the treatments of *E. fetida* + *Sphingobacterium* sp. and *A. gracilis* + *Sphingobacterium* sp.,
54 respectively; the highest ratios of DOC to SOC and AN to total Nitrogen (TN) were found in the
55 earthworm+*Sphingobacterium* sp. treatments as well. In non-contaminated soil, *Sphingobacterium* sp.
56 and earthworms mainly influenced β -glucosidase (BG), urease (URE), N-acetyl- β -D-glucosaminidase
57 (NAG) activities and fluorescein diacetate hydrolysis (FDA) hydrolysis, while in Cd-contaminated
58 soil, they mainly influenced invertase (INV), NAG, URE, and protease (PRO) activities. Principal
59 component analysis indicated that in non-contaminated soil, the earthworm activities dominated the
60 mineralization processes of soil carbon and nitrogen, and *Sphingobacterium* sp. can intensify this
61 process when it was inoculated in soil along with earthworms. Furthermore, both earthworm species
62 increased C and N levels by elevated INV and PRO activities in combined inoculation. However, in
63 contaminated soil, the impact of earthworm inoculation on soil C stabilization showed a species
64 dependent pattern. *E. fetida* reduced C mineralization by decreasing URE activities, while *A. gracilis*
65 enhanced C stabilization by increasing INV activities and decreasing PRO activities. In conclusion,
66 earthworms played a key role in enhancing C and N mineralization in non-contaminated soil and
67 promoting C stabilization in contaminated soil. Both earthworm species followed similar strategies in

68 the former process but adopted different strategies in the latter. When introduced individually,
69 *Sphingobacterium* sp. was able to promote mineralization in both soils, primarily assisting earthworms
70 in improving carbon and nitrogen mineralization in non-contaminated soil but hindering these
71 processes in Cd-contaminated soil. These findings provide insights into the combined effects of
72 earthworms and microorganisms on carbon and nitrogen cycling.

73 **Keywords** : Earthworm; *Sphingobacterium* sp.; Cd-contaminated soil; carbon and nitrogen forms;
74 enzyme activities

75 1. Introduction

76 Soil serves as both an important source and sink for terrestrial carbon and nitrogen in the
77 biosphere. Various soil organisms play essential roles in soil carbon and nitrogen cycles [1,2]. The
78 interactive processes involving soil organisms and restoration of soil carbon and nitrogen stocks in
79 sustainable soil management have garnered widespread attention [3,4]. However, soil metal pollution
80 is increasingly emerging as a serious environmental and social concern, potentially resulting in
81 changes in the interactions among soil organisms and affecting soil carbon and nitrogen cycles [5]. It
82 is of great ecological importance to understand the interactive relationships among soil organisms in
83 the context of the carbon and nitrogen cycle, particularly under non-contaminated and metal-
84 contamination stress.

85 As "soil ecosystem engineers", earthworm can degrade soil organic matter and significantly
86 influence the forms and contents of soil carbon and nitrogen [6]. Earthworm's preferential feeding and
87 concentration processes have a substantial impact soil carbon and nitrogen cycling [7]. Their
88 bioturbation activities, including burrowing, feeding, and excretion (casts and surface mucus), affects
89 the soil carbon and nitrogen distribution within soil profile [8-11]. These effects result from complex
90 mutualism interactions between earthworms and microorganisms [7,12-13]. Previous studies have
91 established connections between earthworms and rhizosphere growth-promoting bacteria or specific
92 arbuscular mycorrhizal fungi to enhance nutrient availability or nitrogen storage in soil [14-15].
93 Moreover, the collaboration of indigenous earthworms and gut bacteria has been employed to
94 remediate metal contaminated soil and has shown superiority compared to chemical amendments [16].
95 Likewise, combining earthworms with dominate gut bacteria taxa to enhance organic matter
96 decomposition is also advantageous, because it is more readily accepted by earthworms. In our
97 previous studies, *Sphingobacterium* sp. was identified as a dominant bacterial taxon in the earthworm
98 gut [17]. *Sphingobacterium* sp., a gram-negative bacterium, exhibits a wide range of nutrient sources,
99 high adaptability, and a broad ecological distribution [18-20]. In practice, it has been used to
100 decompose agricultural waste and degrade organic pollutants (e.g. alkanes and polycyclic aromatic

101 hydrocarbons) in soil [21-26]. Therefore, this decomposition ability of earthworms probably derives
102 from symbiotic microorganisms in their guts. Considering that earthworms and *Sphingobacterium* sp.
103 impact organic matter decomposition and the latter was dominant bacterial taxon in earthworm gut, a
104 synergistic relationship may exist, enhancing soil organic matter decomposition.

105 However, under environment stress, such as metal contamination, the activities and complex
106 interactions of them may be suppressed. Specifically, metal contamination can induce biomass loss,
107 reduce survival rate, cause irreversible physiological damage, decrease the stability of gut bacterial
108 network, and dampen its ecological functions [27-29]. Similarly, the inhibition effects also impact soil
109 microorganisms, leading to reduced soil enzyme functions critical for organic matter cycling [30]. In
110 addition, different earthworm species and ecological categories have varying effects on soil organic
111 matter degradation and soil C and N cycles [31]. The species of the experimental model earthworm *E.*
112 *fetida* and local wild epigeic species may have different survival strategies that affect microbial
113 biomass or activities, resulting in their different performances in soil carbon and nitrogen cycles in
114 non-contaminated and Cd-contaminated soil.

115 Soil C and N cycles involve a series of biological and biochemical processes. Soil carbon and
116 nitrogen forms, dissolved organic carbon (DOC), alkali-hydrolysable nitrogen (AN), microbial
117 biomass carbon and nitrogen (MBC and MBN) have been considered as the key indicators for
118 assessing the status of the soil carbon and nitrogen cycles. The ratios of these forms can further provide
119 a more intuitive representation of the overall mineralization or sequestration of carbon and nitrogen.
120 Besides, soil enzymes are metabolic drivers for soil organisms, and their activity can, to some extent,
121 reflect the changes in the soil microbial community [32], and the intensity of soil organic matter
122 decomposition and nutrient transformation [33]. Studies on the influence of enzymes on carbon and
123 nitrogen transformation have mainly focused on β -glucosidases (BG) and invertase (INV), which are
124 involved in the decomposition of labile cellulose, sucrose, and carbohydrate polymers. N-acetyl-
125 glucosaminidase (NAG), urease (URE), and proteases (PRO) are involved in the hydrolysis of
126 chitooligosaccharide, urea and protein. These enzymes mainly participate in terminal catalytic
127 reactions or intra-systemic enzymatic reactions, which can reflect the level of carbon and nitrogen
128 metabolism in soils [34]. Furthermore, fluorescein diacetate hydrolysis (FDA) is an enzyme that can
129 indicate the total microbial activity in the soil [35]. Therefore, the effects of earthworm inoculation on
130 soil carbon and nitrogen cycles can indirectly be assessed by studying the changes in soil enzyme
131 activities related to carbon and nitrogen cycles.

132 Given that *Sphingobacterium* sp. is the dominant bacterial taxon in the earthworm gut, we
133 hypothesized that *Sphingobacterium* sp. and earthworm could individually promote soil organic matter
134 decomposition and, when combined, exert reinforcing effects. These effects are negatively impacted

135 by metal stress and influenced by the survival strategies of different earthworm species; Additionally,
136 *Sphingobacterium* sp. and earthworms may play different roles in this combined process, with
137 *Sphingobacterium* sp. stimulating microbial activities in the earthworm gut.

138 This study aimed to investigate the effects of different earthworm species (*Eisenia fetida* and
139 *Amyntas gracilis*) and exogenous *Sphingobacterium* sp. on the transformation of soil carbon and
140 nitrogen under non-contaminated and Cd-contaminated stresses. The specific research objectives were
141 (1) to analyze and compare the effects of *Sphingobacterium* sp. and the two earthworm species, when
142 inoculated alone and in combination, on the carbon and nitrogen forms and related enzyme activities
143 in non- and Cd-contaminated soils. (2) to elucidate the effects of Cd pollution stress on this process
144 and the underline mechanisms. (3) to clarify the interaction and roles of *Sphingobacterium* sp. and
145 earthworms in these processes. This study can provide a theoretical basis for insight into the combined
146 effects of earthworms and microorganisms on soil carbon and nitrogen cycling processes, the
147 biologically driven mechanisms, and offer suggestions for adjusting soil remediation practices and
148 their further sustainable management.

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2. Materials and methods

2.1 Soil, earthworms, and strain

Two representative soils from South China were selected: non-contaminated soil (Soil I) and soil contaminated with metals from mining areas (Soil II). Soil I was collected from the tree garden of South China Agricultural University (23°9'N, 113°21'E); Soil II was collected from a paddy field (24°30'N, 113°45'E) about 6 km downstream of the opencast mining area of the copper-sulfur ore, limonite and lead-zinc mine in Dabao Mountain, northern Guangdong Province. The basic physicochemical properties of soils are listed in Table 1.

Earthworm species *Eisenia fetida* (*E. fetida*, epigeic species) and *Amyntas gracilis* (*A. gracilis*, epigeic species) were selected. *A. gracilis* was collected from South China Agricultural University, while *E. fetida* were laboratory-raised earthworms. After two weeks of acclimation in the laboratory, earthworms with similar individual weights and a mature clitellum were chosen for the experiment.

Sphingobacterium sp. was purchased from BeNa Culture Collection (BNCC). CM0827 *Propionibacterium* sp. medium, which consisted of tryptone 15 g, L-cystine 0.5 g, yeast extract 5 g, sodium thioglycolate 0.5 g, glucose 5 g, agar 0.7 g, sodium chloride 2.5 g, resazurin 0.001 g, distilled water 1 L, was employed to cultural *Sphingobacterium* sp. and was set at pH 7.0. The strain was incubated at a constant temperature of 30°C for 2 days before inoculation into the soil.

2.2 Experimental design

A two-factor (inoculation with *Sphingobacterium* sp. and different earthworm species (*E. fetida*, *E. A. gracilis*, *A*)) experimental design was employed in non-contaminated (Soil I, C) and Cd-contaminated (Soil II, S) soils. Six treatments were set up for each soil, with three replicates for each treatment and 36 pots in total.

Soil I: (1) Soil I (C); (2) Soil I + *Sphingobacterium* sp. (CS); (3) Soil I + *E. fetida* (CE); (4) Soil I + *A. gracilis* (CA); (5) Soil I + *Sphingobacterium* sp. + *E. fetida* (CSE); (6) Soil I + *Sphingobacterium* sp. + *A. gracilis* (CSA).

Soil II: (1) Soil II (S); (2) Soil II + *Sphingobacterium* sp. (SS); (3) Soil II + *E. fetida* (SE); (4) Soil II + *A. gracilis* (SA); (5) Soil II + *Sphingobacterium* sp. + *E. fetida* (SSE); (6) Soil II + *Sphingobacterium* sp. + *A. gracilis* (SSA).

Each pot was filled with 200 g of soil, which was passed through a 2 mm mesh. The pots have dimensions of caliber × bottom diameter × height of 75 × 53 × 90 mm and a microporous bottom. Soil moisture was adjusted to 60% of the maximum field water holding capacity and stabilized for 48 h. Each pot was inoculated with 3 g of earthworms, approximately 8 individual *E. fetida* (~ 0.37 g·ind⁻¹) or 5 individual *A. gracilis* (~ 0.60 g·ind⁻¹). One milliliter of *Sphingobacterium* sp. solution (OD₄₀₅

value of 1.45) was added to the soil surface in CS, CSA, CSE, SS, SSA, and SSE treatments. To prevent earthworms from escaping, the top and bottom of each pot were covered with a fine nylon mesh with a 1 mm pore diameter. The temperature in the laboratory was maintained at 25°C.

After 20 days, the earthworms were hand-picked, and soil samples were collected. A portion of the fresh soil was refrigerated at 4°C for the analysis of microbial characteristics and microbial biomass carbon and nitrogen. The remainder was thoroughly air-dried, ground, and sieved for the analysis of soil physical and chemical properties. Earthworms were counted and weighed during sampling.

2.3 Soil properties, carbon and nitrogen forms, enzyme activities

Soil pH was measured using a glass electrode in a 1:2.5 (w/v) soil/water suspension. Soil clay, soil organic carbon (SOC), alkali-hydrolysable nitrogen (AN), and total nitrogen (TN) were analyzed by the pipette [36], dichromate oxidation [37], alkali N-proliferation [38] and Kjeldahl [39] methods, respectively. Microbial biomass carbon and nitrogen (MBC and MBN) were determined via the chloroform fumigation-extraction method [39-40]. Dissolved organic carbon (DOC) content was extracted with deionized water (1:5) and determined [41]. β -glucosidase, protease, and N-acetyl- β -D-glucosaminidase activities were all determined by colorimetric assay. Urease, invertase, and fluorescein diacetate hydrolytic activities were determined by sodium phenolate colorimetric assay, titration, and colorimetric using fluorescein methods [42], respectively.

2.4 Total and DTPA extractable Cadmium content

Total cadmium (Cd) in the soils was extracted by microwave digestion with aqua regia ($\text{HNO}_3:\text{HCl}$ (v/v) = 1:3). The diethylenetriaminepentaacetic acid (DTPA) extractable form was regarded as bioavailable metals and toxic. DTPA-extractable Cd was extracted with DTPA extractant at a Soil:DTPA extractant ratio of 5:25 (g/mL), which included a solution of 5 Mm DTPA and 10 mM CaCl_2 . The extracted suspensions were analyzed for total cadmium (Cd) and DTPA-extractable contents using inductively coupled plasma-mass spectrometry (ICP-MS, 7700ce, Agilent Technologies, Santa Clara, CA, USA) [43]. GBW07455 (GSS-26) was employed as certified reference material for soil analyses, and the measured concentrations were within 98% of the reported certified concentrations for Cd. Quality control measures included three repetitions for each soil and three blanks for each batch to ensure the quality of the analysis.

2.5 Statistics analysis

All data were analyzed and plotted with SPSS statistical software (Version 24.0), Origin 9.0, and R (Version 3.4.3). T-test and variance (ANOVA) analysis were performed. Data were presented as the mean \pm SE (standard error). $\text{DOC}:\text{SOC} = \text{DOC}/\text{SOC}$, $\text{AN}:\text{TN} = \text{AN}/\text{TN}$, $\text{C}:\text{N} = \text{SOC}/\text{TN}$, $\text{MBC}:\text{MBN} = \text{MBC}/\text{MBN}$; Microbial quotient carbon ($q\text{MBC}$), $q\text{MBC} = \text{MBC}/\text{SOC}$; Microbial

216 quotient nitrogen ($q\text{MBN}$), $q\text{MBN} = \text{MBN}/\text{TN}$.

217 Principal components analysis (PCA) and Person's correlation analysis were conducted to
218 analyze the relationships of soil carbon and nitrogen forms and ratios (SOC, DOC, MBC, TN, AN,
219 MBN, DOC:SOC, C:N, AN:TN, MBC:MBN, $q\text{MBC}$, $q\text{MBN}$), as well as enzyme activities (BG, INV,
220 URE, NAG, PRO, FDA). The analysis was performed in R version 3.4.3 using the "ade4" and "corrplot"
221 packages.

222 223 3. Results

224 3.1 The contents of carbon and nitrogen forms

225 The soil carbon form contents are shown in Fig. 1A, B, and C. In non-contaminated soil, both
226 *Sphingobacterium* sp. and earthworms significantly reduced SOC content ($P < 0.05$). Reductions
227 reached 16.7%, 14.8%, 15.6%, 16.7%, and 15.9% in CS, CE, CA, CSE, and CSA treatments,
228 respectively. Furthermore, DOC increased significantly in the presence of earthworms ($P < 0.05$) and
229 was highest in the CSE and CSA treatments at $659 \pm 19.2 \text{ mg} \cdot \text{kg}^{-1}$ and $430 \pm 82.8 \text{ mg} \cdot \text{kg}^{-1}$, respectively.
230 Individual inoculation with *Sphingobacterium* sp. significantly increased MBC to $1685 \pm 292 \text{ mg} \cdot \text{kg}^{-1}$
231 ($P < 0.05$). However, in all the treatments inoculated with earthworms, there was no significant
232 change in MBC content compared to C treatment, but it was significantly decreased compared to the
233 CS treatment.

234 SS treatment reduced the SOC content in Cd-contaminated soil, decreasing to $16.0 \pm 0.44 \text{ g} \cdot \text{kg}^{-1}$
235 ¹. In contrast, SOC increased in all treatments involving earthworms and was significantly higher than
236 that in the S treatment ($16.6 \pm 0.21 \text{ g} \cdot \text{kg}^{-1}$) under the combined effect of SSE ($17.8 \pm 0.28 \text{ g} \cdot \text{kg}^{-1}$) and
237 SSA ($17.5 \pm 0.50 \text{ g} \cdot \text{kg}^{-1}$) ($P < 0.05$). Compared to the control, MBC was slightly decreased in the SS
238 treatment, and the variation was smaller than that in the CS treatment.

239 The contents of nitrogen forms are displayed in Fig. 1D, E, and F. In non-contaminated soil,
240 earthworm activity significantly increased AN content ($P < 0.05$) by 32.9%, 20.9%, 31.3%, and 25.4%
241 in CE, CA, CSE, and CSA treatments, respectively, while no significant change in the CS treatment
242 was observed. The indexes of nitrogen were not significantly changed in Cd-contaminated soil ($P >$
243 0.05), but the trend of AN in all treatments was consistent with that in non-contaminated soil. In
244 addition, TN and MBN in both soils did not show significant changes under the influence of
245 *Sphingobacterium* sp. and earthworms.

246 3.2 The ratios of carbon and nitrogen forms

247 Soil carbon and nitrogen form ratios are shown in Fig. 2. In general, the trend of ratios in both
248 soils was consistent with those of the corresponding carbon and nitrogen forms. Specifically, the ratio

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249 of DOC:SOC followed the same trend as DOC, increasing to 2.93% and 1.94% in the CSE and CSA
250 treatments, respectively. The C:N ratio significantly decreased by 23.6%, 23.6%, 24.0%, 22.6%, and
251 14.4% in the CS, CE, CA, CSE, and CSA treatments, respectively. The AN:TN ratio increased
252 significantly in the CE and CA treatments ($P < 0.05$) and further increased in combination treatments.
253 The MBC:MBN ratio was significantly increased in CS treatment and significantly decreased in the
254 SS treatment ($P < 0.05$). In contrast, this ratio significantly decreased in non-contaminated soil ($P <$
255 0.05) and increased in Cd-contaminated soil when combining *Sphingobacterium* sp. with earthworms.
256 In non-contaminated soil, compared with C treatment, inoculation with *Sphingobacterium* sp. and
257 earthworms individually increased q MBC compared to the C treatment and q MBC was highest in CS
258 treatment. However, q MBC was decreased in the combined treatment. q MBN did not change
259 significantly ($P > 0.05$) in all treatments for both soils.

260 3.3 Response of soil enzyme activity to inoculated earthworms and *Sphingobacterium* 261 sp.

262 The soil enzyme activities are illustrated in Fig. 3. In non-contaminated soil, inoculation of
263 *Sphingobacterium* sp., *E. fetida*, and *A. gracili*, both individually and in combination significantly
264 decreased BG, URE activities and FDA hydrolysis ($P < 0.05$). Additionally, *E. fetida* alone
265 significantly increased NAG activities ($3.86 \pm 0.37 \mu\text{g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) while *A. gracilis* had the opposite effects
266 ($P < 0.05$). In Cd-contaminated soil, inoculation of *Sphingobacterium* sp. and *E. fetida* alone and in
267 combination significantly decreased INV, NAG, and URE activities, with *Sphingobacterium* sp. alone
268 significantly increasing PRO activities ($P < 0.05$). Inoculation of *A. gracilis* alone significantly
269 decreased INV, URE activities and increased NAG, PRO activities while increasing INV, NAG, and
270 URE activities when combined with *Sphingobacterium* sp.

271 3.4 Earthworm survival rate and mean biomass loss

272 After 20 days, the survival rate and biomass of both earthworm species decreased (Table 2). In
273 terms of survival rate, only the SSE treatment exhibited a significant decrease of 68.7% ($P < 0.05$).
274 Earthworm biomass decreased by 7.68% - 77.2%, with a particularly significant decreasing in SSE
275 treatment ($P < 0.05$). The biomass decreases were higher in Cd-contaminated soil than in non-
276 contaminated soil and was more pronounced in *E. fetida* than *A. gracilis* treatment.

277 3.5 General effects of the treatments on soil C, N, and related enzyme activity

278 Principal Component Analysis (PCA) was carried out to identify the overall trends in soil C, N,
279 and enzyme activity in response to *Sphingobacterium* sp. and earthworm activities in non-
280 contaminated and Cd-contaminated soils (Fig. 4 and Fig. 5). In non-contaminated soils (Fig. 4 A and
281 B), Axis 1 (27.8% variance explained) was primarily associated with DOC, AN, BG, FDA, SOC, MBC,

282 and MBN. Axis 2 (16.4% variance explained) was mainly linked to INV, PRO, and URE. Axis 3 (14.2%
283 variance explained) was mainly related to TN and NAG. Soils with added organisms were significantly
284 differentiated from the control soils (Fig. 4C and D) ($P < 0.001$). In Cd-contaminated soils (Fig. 5 A
285 and B), Axis 1 (19.9% variance explained) was mainly related to TN, MBN, MBC, FDA, BG, URE,
286 and NAG. Axis 2 (17.2% variance explained) was mainly related to SOC, AN, PRO, INV. Axis 3 (14.5%
287 variance explained) was mainly related to DOC. There were significant differences among the
288 treatments (Fig. 5C and D) ($P < 0.001$).

289 4. Discussion

290 4.1 The effects of *Sphingobacterium* sp. on C mineralization

291 Microorganisms are known to contribute to the mineralization and loss of SOC [44].
292 *Sphingobacterium* sp. is a type of gram-negative bacteria. Likewise, previous studies [45-46] used
293 *Sphingobacterium* sp. to decompose the agricultural byproducts and to biodegrade organic pollutants.
294 The performance of *Sphingobacterium* sp. in both non-contaminated and Cd contaminated soils
295 indicates its ability to mineralize carbon and tolerate metal stress. These abilities align with a similar
296 study [47], who demonstrated that the inoculation of *Sphingobacterium* sp. can enhance the
297 degradation of organic pollutant residues in Cd ($0.30 \text{ mg} \cdot \text{kg}^{-1}$) contaminated soil. It also can be inferred
298 from the aforementioned results that these functions of *Sphingobacterium* sp. were not dependent on
299 the gut conditions of earthworms. Additionally, the highest microbial biomass carbon content and the
300 microbial quotient carbon in the CS treatment (Fig. 1C and Fig.2E) were due to the inoculation of this
301 strain, its adaption in the soil environment and substantial proliferation. However, this strong adaptive
302 phenomenon was not observed in Cd-contaminated soil, where there is reduced microbial biomass
303 carbon caused by metal stress. For instance, the adaptation process of *Sphingobacterium* sp. was
304 observed in the early stage (60 days) in Cd-contaminated soil ($0.3 \text{ mg} \cdot \text{kg}^{-1}$), with the strain population
305 size gradually increasing after 90 days [47]. Nevertheless, this study only lasted for 20 days and more
306 time is needed for *Sphingobacterium* sp. to complete the adaption processes under metal stress.

307 4.2 Earthworm survival, growth and species-specific effects on C and N 308 transformation

309 Earthworm abundance and biomass decreased after 20 days of cultivation in both soils,
310 especially in Cd-contaminated soil, consistent with findings from a previous study [29]. This decline
311 may be attributed to soil nutrient limitation and the toxic effects of high Cd concentration on
312 earthworms [48]. Regarding earthworm species, *A. gracilis* showed higher resistance to environmental
313 stresses than *E. fetida*, probably owing to the different strategies employed by earthworms in metal
314 handling and resistance. In a previous study, a toxicokinetic model was employed, which found that

315 wild earthworm species were more specialized than *E. fetida* in balancing the trade-off between metal
316 uptake and excretion processes based on their surrounding environment [49].

317 There are different effects of earthworms on soil C and N transformation in non-contaminated
318 and Cd-contaminated soil. In non-contaminated soil with a high organic carbon content, earthworms
319 alone facilitated soil organic carbon and nitrogen mineralization. This is consistent with the trend of
320 carbon and nitrogen changes observed in vermicomposting [50], presumably through the
321 decomposition of organic matter by specific microorganisms under the unique conditions within the
322 earthworm gut [51]. In addition, it can be found that earthworm activities increased the soil microbial
323 quotient carbon compared to the control, indicating improved efficiency of soil organic matter
324 utilization by microorganisms [52]. However, soil carbon stabilization in Cd-contaminated soil was
325 probably due to earthworms consuming more organic matter to resist metal toxicity [53]. Moreover, it
326 has been proved that gut peristalsis associates partly degraded organic matter with minerals [54] and
327 leads to formation of organic matter-rich aggregates [55-56], which are conducive to SOC
328 sequestration [7,57-60]. It was concluded that earthworms can slow down SOC mineralization in the
329 presence of more minerals [52]. Therefore, earthworm activities reduced carbon mineralization in
330 mineral-rich Cd-contaminated soil. Furthermore, this effect may also be explained by the fact that
331 contaminated soils contain stable carbon that is not easily mineralized. The positive correlation
332 between β -glucosidase and N-acetyl- β -D-glucosaminidase activity, demonstrated with PCA, also
333 supports the notion that soil microorganisms tend to use the active carbon pool generated by the
334 decomposition of humus formed by litter (Fig. 5A; Fig. S1), and this favors the accumulation of
335 recalcitrant soil carbon pools.

336 The earthworm species have great impact on biogeochemical cycling through their feeding
337 habits and cast properties [31]. In non-contaminated and Cd-contaminated soil, both *E. fetida* and *A.*
338 *gracilis* perform similar ecological functions; however, their performance is also influenced by
339 earthworm survival strategies. In environments without metal stress, both species can enhance C and
340 N mineralization. It is worth noting that *E. fetida* has a more pronounced effect on soil carbon and
341 nitrogen mineralization than *A. gracilis* does (Fig. 1B and E; Fig. 2A and C). This could be attributed
342 to the preference of *E. fetida* for ingesting high-quality organic matter. Their castings have a high level
343 of particulate organic matter content and porosity that provides plentiful food and spaces for soil
344 microorganisms [31], thereby accelerating the decomposition of organic matter [61-63]. On the
345 contrary, the wild earthworm *A. gracilis* not only fed on soil organic matter, but also on more mineral
346 particles. In addition, *E. fetida* found it easier to obtain food in non-contaminated soil with a relatively
347 high organic matter content (Table 1). A high carbon to nitrogen ratio was favored for earthworm

348 growth [64], so this species performed better. In contrast, in Cd-contaminated soil, the mineralization
349 processes of soil organic matter slow down. The wild earthworm species *A. gracilis* demonstrated
350 better survival and had more significant effects on C stabilization than *E. fetida* (Table 2, Fig.1 and
351 Fig. 2). The low organic matter content in Cd-contaminated soils limited the decomposition functions
352 of *E. fetida*. Furthermore, earthworms need to consume more food to resistant Cd stress. Both species
353 increases their food consumption to feed on more organic matter. In this process, earthworms were
354 compelled to ingest more mineral particles in soils characterized by low organic matter (Table 1) and
355 high soil mineral content. The gut-grazing process facilitated the thorough mixing and binding of
356 organic matter with soil minerals [54]. As for enzymes, the enzymes activity was influenced by
357 earthworm species and metal stress. Likewise, in the previous study, earthworm activities suppressed
358 β -glucosidase activity during the stimulation of C mineralization processes [65]. It was speculated that
359 when soil microorganisms could easily access the readily mineralized C released by earthworms, they
360 would use a smaller portion of the carbon from enzyme-catalyzed decomposition of soil organic carbon.
361 Enzyme activity only represents the concomitant consequence of earthworm activities but does not
362 serve as the major driver of organic matter decomposition. It is also controlled by multiple factors such
363 as food type, pH, temperature, gut transit time, metal stress, substrates, et al., [65-67].

364 *4.3 The combined effects of earthworm and Sphingobacterium sp. on the balance* 365 *between C and N stabilization and mineralization*

366 The combination processes showed that *Sphingobacterium sp.* assisted earthworms in further
367 promoting carbon and nitrogen mineralization in non-contaminated soils (Fig. 4A and C) and carbon
368 stabilization in Cd-contaminated soils (Fig. 5A and C), as reflected by the increased contents of
369 mineralization (DOC, AN) and the stabilization indicators (SOC), respectively. It was also found that
370 inoculated earthworm can process organically polluted water and excess sludge, and
371 *Sphingobacterium*, known for its high ability to degrade organic matter, is one of the most abundant
372 microbes in earthworm castings [68]. Nevertheless, our study demonstrated that external addition of
373 *Sphingobacterium sp.* can help earthworms adapt more easily to environment and exert their ecological
374 functions. Despite having similar effects when used in combination or when being inoculated alone,
375 the strategies of the two species differed in non-contaminated and Cd-contaminated soil (Fig. 4D).
376 Compared with treatments that inoculate earthworms alone, CSA and CSE treatments further activated
377 INV and PRO activities in non-contaminated soil (Fig. 3 and Fig. 4D); On the other hand, SSA further
378 activated INV activities and inhibited PRO activities, while SSE further inhibited URE activities (Fig.
379 3 and Fig. 5D). It is well known that soil enzyme activity is mainly derived from microorganisms and
380 can reflect microbial activity [69]. Inoculation of *Sphingobacterium sp.* may stimulate microbial

381 activities in the soil and the earthworm gut in non-contaminated soil [16]. In Cd-contaminated soil, it
382 may represent the natural response of earthworms to the inoculation of *Sphingobacterium* sp. and metal
383 stress. Compared to non-contaminated soil, metal stress induced inhibition of PRO activities in the
384 treatment with *A. gracilis* inoculation and URE activities in the treatment with *E. fetida* inoculation,
385 indirectly demonstrating distinct coping strategies for metal stress among earthworm species. Given
386 that researchers have [70] found that the vermiwash and mucus extracted from earthworms can greatly
387 inhibit the growth of pathogenic fungi, it is also possible that the mucus compounds changed due to
388 metal pollutant stress in this study, such as secretion of specific organic substances to protect itself
389 from physiological damage, while inhibiting the microbial activities. The underlying mechanisms are
390 still not clear and require further research.

391 In these processes, earthworm activities dominated the soil carbon and nitrogen mineralization
392 processes, while *Sphingobacterium* sp. played a supporting role in combined processes. When the
393 effects of earthworm and *Sphingobacterium* sp. on carbon and nitrogen were consistent, they enhanced
394 carbon mineralization; otherwise they undermined this process. Hence, in non-contaminated soil,
395 carbon mineralization was observed. While in Cd-contaminated soil, carbon storage was more
396 prevalent due to the stronger effect of earthworms on stabilization than *Sphingobacterium* sp. on
397 mineralization. This is further confirmed by PCA which demonstrated that earthworms play a critical
398 role in soil organic carbon decomposition processes, following the same trend as inoculation with
399 earthworm alone and in combination with *Sphingobacterium* sp. (Fig. 4A and C; Fig. 5A and C).
400 Additionally, in this system, a strong interaction existed between earthworms and *Sphingobacterium*
401 sp.. Earthworm activities under the combined process dramatically decreased microbial biomass
402 carbon compared to *Sphingobacterium* sp. activities alone. It is suggested that earthworms contribute
403 to maintaining the ecosystem balance and stabilizing the microbial community structure through
404 feeding [71] and regulating the allocation of active organic carbon and dissolved organic carbon pools
405 [57,72]. Therefore, the rapid proliferation of *Sphingobacterium* sp. was prevented in CSE and CAS
406 treatments. It was further confirmed by the significantly lower microbial quotient carbon in treatment
407 CSE and CSA compared to treatment CS (Fig. 2E). It can be deduced that earthworms may reduce the
408 conversion efficiency of soil microorganisms in utilizing carbon sources into microbial biomass carbon
409 [52] or they can intensely feed and graze on microbes [10, 73-75]. Additionally, the different impact
410 of earthworm species may be caused by *E. fetida* being more conducive to the survival and growth of
411 G⁻ bacterial communities [75], thus promoting *Sphingobacterium* sp. to assist earthworms in carbon
412 mineralization. Based on the above, another underlying factor was postulated that *Sphingobacterium*
413 sp. may assist earthworms in mineralizing soil organic carbon and nitrogen by stimulating the

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414 microbial activities in the earthworm gut. The subsequent studies need to focus on the earthworm gut
415 microbial to reveal the internal mechanisms.

416 5. Conclusion

417 This study focuses on soil carbon and nitrogen forms and enzyme activities to investigate whether
418 *Sphingobacterium* sp. can assist different earthworm species in influencing soil carbon stabilization
419 and nitrogen cycling under non-contaminated and Cd-contaminated soil. Generally, *Sphingobacterium*
420 sp. inoculation individually facilitated carbon mineralization in both soils, and this function did not
421 depend on the gut conditions of earthworms. In terms of species of earthworms, a species-specific
422 effect was observed with *E. fetida* being more effective in C mineralization than *A. gracilis*. Earthworm
423 activities may have a weak inhibitory effect on enzyme activity during this process. Additionally, Cd
424 pollution stress impacts C and N cycles and results in opposite effects. Earthworms facilitated the
425 mineralization of C and N in non-contaminated soil while promoted C storage in Cd-contaminated soil.
426 Similarly, *Sphingobacterium* sp. assisted earthworms in improving carbon and nitrogen mineralization
427 in non-contaminated soil, with *E. fetida* being more effective than *A. gracilis*. However, they failed to
428 do so in Cd-contaminated soil and promoted carbon storage instead. Therefore, earthworm activity
429 dominates the processes of carbon and nitrogen stabilization and mineralization, with
430 *Sphingobacterium* sp. playing a supporting role. This study provides a clear understanding of the
431 different effects of earthworms and microorganisms on soil carbon and nitrogen cycling in non-
432 contaminated and Cd-contaminated soil. In further studies, the complex interaction mechanisms of
433 earthworms and microorganisms can be designed and tested by some methods of molecular biology
434 or metabolomics methods. Additionally, exploring the interaction of earthworms and microorganisms
435 in soil remediation technology could lead to the adjustment and reconstruction of sustainable soil
436 management practices.

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Table list:

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Table1 Properties and Cd concentrations of soil.

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Table2 Survival rate and mean biomass loss of earthworm at the 20th day.

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Table1 Properties and Cd concentrations of soil

	pH	Clay (%)	SOC (g·kg ⁻¹)	TN (g·kg ⁻¹)	C:N	Cd (mg·kg ⁻¹)	DTPA-Cd (mg·kg ⁻¹)
Soil I	4.73 ± 0.05	18.60 ± 3.85	27.0 ± 0.37	1.30 ± 0.05	20.8 ± 0.64	0.13 ± 0.15	0.007 ± 0.001
Soil II	4.40 ± 0.02	19.42 ± 0.49	16.6 ± 0.12	1.62 ± 0.05	10.3 ± 0.25	0.64 ± 0.20	0.164 ± 0.000

Note: Soil I and Soil II represents non-contaminated soil and contaminated soil, respectively. SOC, soil organic carbon; TN, total nitrogen; Cd, cadmium. Results are expressed as the mean ± SE (n = 3).

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Table2 Survival rate and mean biomass loss of earthworm at the 20th day

Treatments	Survival rate (%)	Mean biomass loss (%)
CE	95.2 ± 4.76a	17.4 ± 13.3b
CA	86.1 ± 7.35a	7.68 ± 4.54b
CSE	91.1 ± 4.49a	19.4 ± 5.26b
CSA	87.8 ± 6.19a	13.7 ± 10.2b
SE	77.8 ± 11.1a	17.6 ± 5.22b
SA	100 ± 0a	18.9 ± 5.54b
SSE	31.2 ± 6.10b	77.2 ± 3.99a
SSA	100 ± 0a	18.9 ± 3.27b

Note: Results are expressed as the mean value ± SE (n = 3). The initial letters C and S represents the soil I and soil II. CE, CA, CSE and CSA represents the treatments with *E. fetida*, *A. gracilis*, *Sphingobacterium* sp. + *E.fetida* and *Sphingobacterium* sp. + *A.gracilis* in soil I, while SE, SA, SSE and SSA represents the treatments with *E. fetida*, *A. gracilis*, *Sphingobacterium* sp.+ *E. fetida* and *Sphingobacterium* sp. + *A. gracilis* in soil II, respectively. Different lowercase letters indicate sicanificantly differences ($P < 0.05$) between different treatments.

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Figure captions:

Fig. 1. Soil carbon and nitrogen form characteristics.

Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

Fig. 2. The ratio of soil carbon and nitrogen forms.

SOC, soil organic carbon; DOC, dissolved organic matter; TN, total nitrogen; AN, available nitrogen; C:N, soil organic carbon-to-total nitrogen ratio; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

Fig. 3. Characterization of soil enzyme activity.

Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

Fig. 4. Principal component analysis (PCA) of the effect of earthworms and *Sphingobacterium* sp. on soil carbon and nitrogen fractions and enzyme activities in non- and Cd-contaminated soil.

(A) Correlation circles of carbon and nitrogen fractions with enzymes in non-contaminated soil.

(B) Projection of experimental points according to treatments with or without earthworm and *Sphingobacterium* sp. in non-contaminated soil.

(C) Correlation circles of carbon and nitrogen fractions with enzymes activities in Cd-contaminated soil.

(D) Projection of experimental points according to treatments with or without earthworm and *Sphingobacterium* sp. in Cd-contaminated soil.

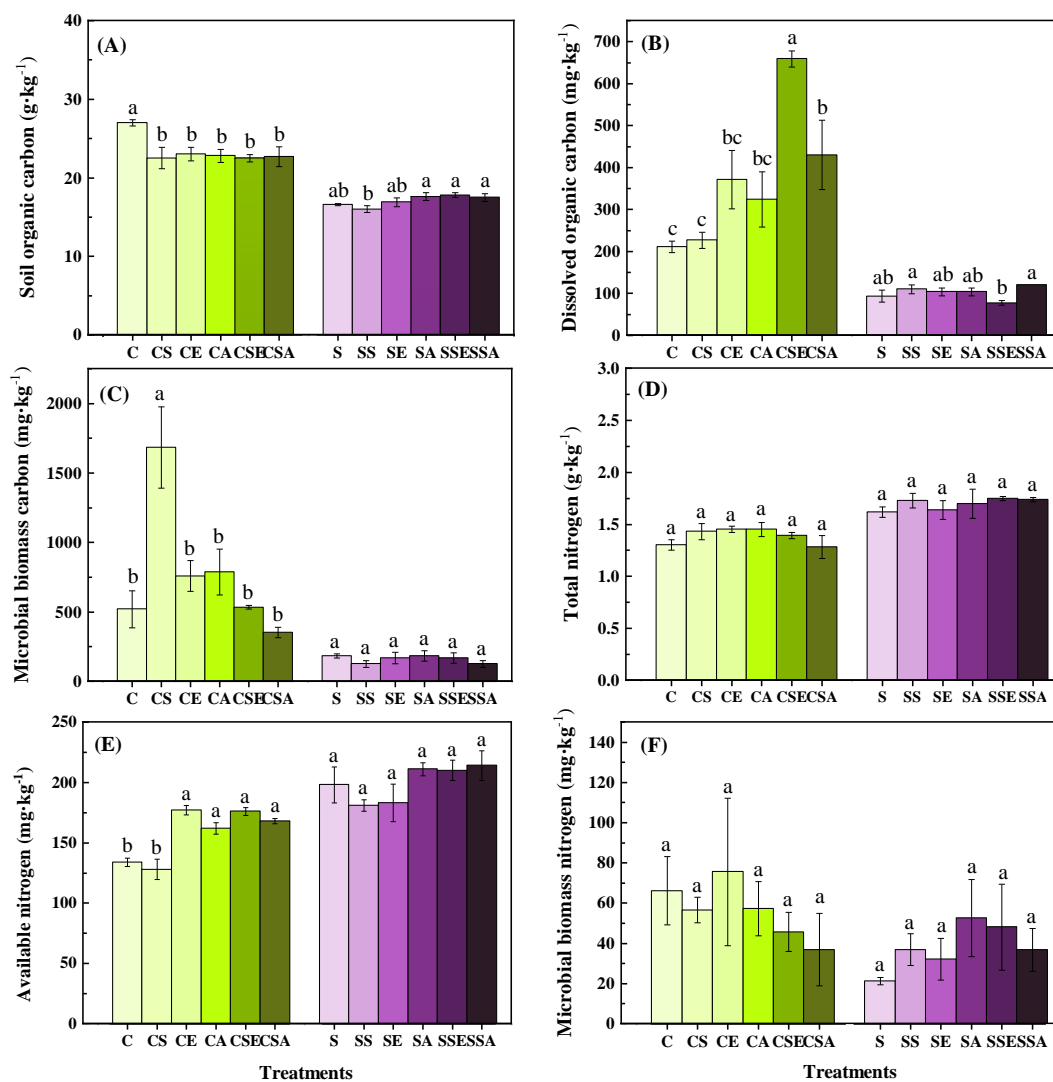


Fig. 1. Soil carbon and nitrogen form characteristics. Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

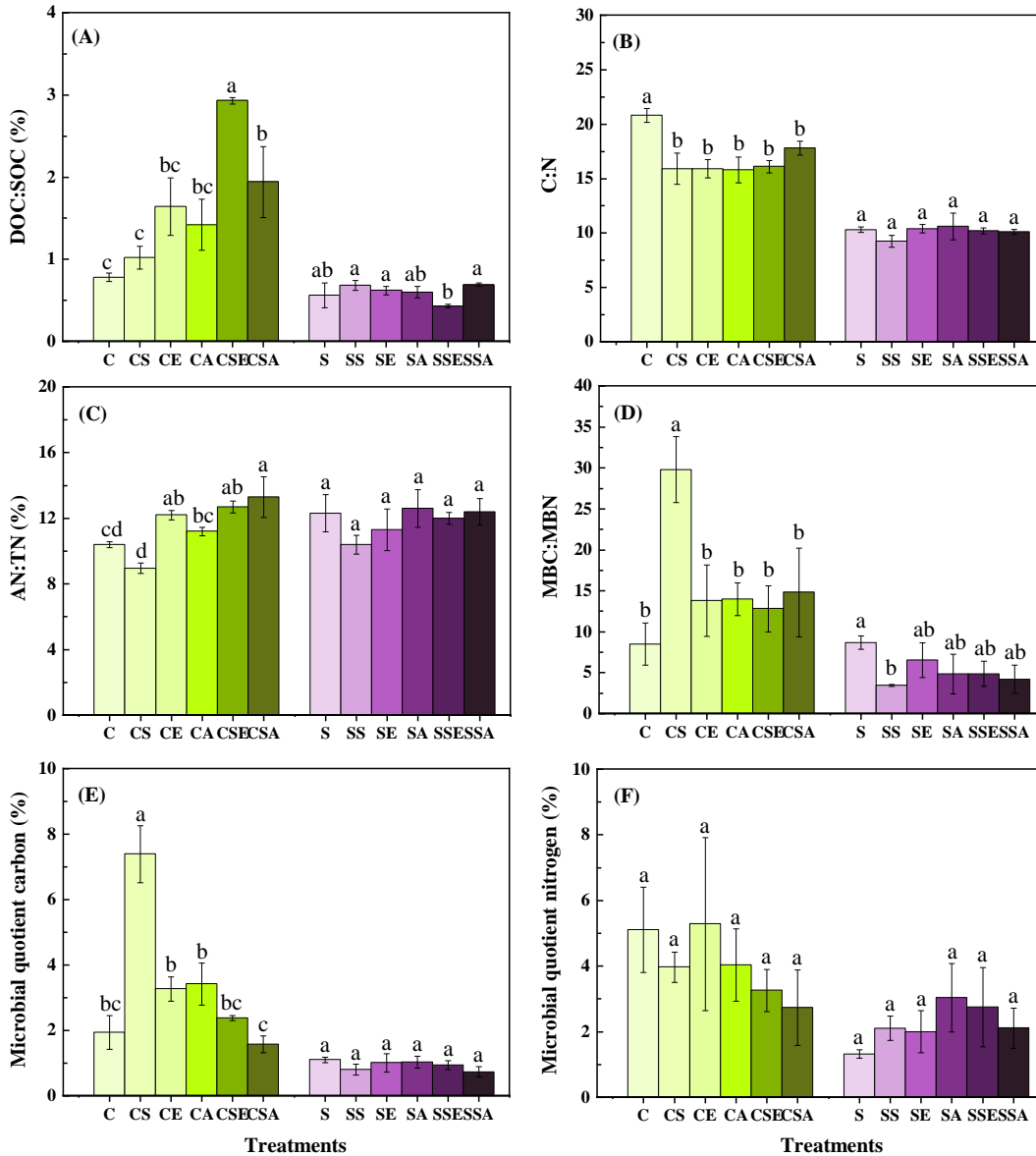


Fig. 2. The ratio of soil carbon and nitrogen forms. SOC, soil organic carbon; DOC, dissolved organic matter; TN, total nitrogen; AN, available nitrogen; C:N, soil organic carbon-to-total nitrogen ratio; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. Results are expressed as the mean value \pm SE (n = 3). Different lowercase letters indicate significant differences ($P < 0.05$) among the treatments.

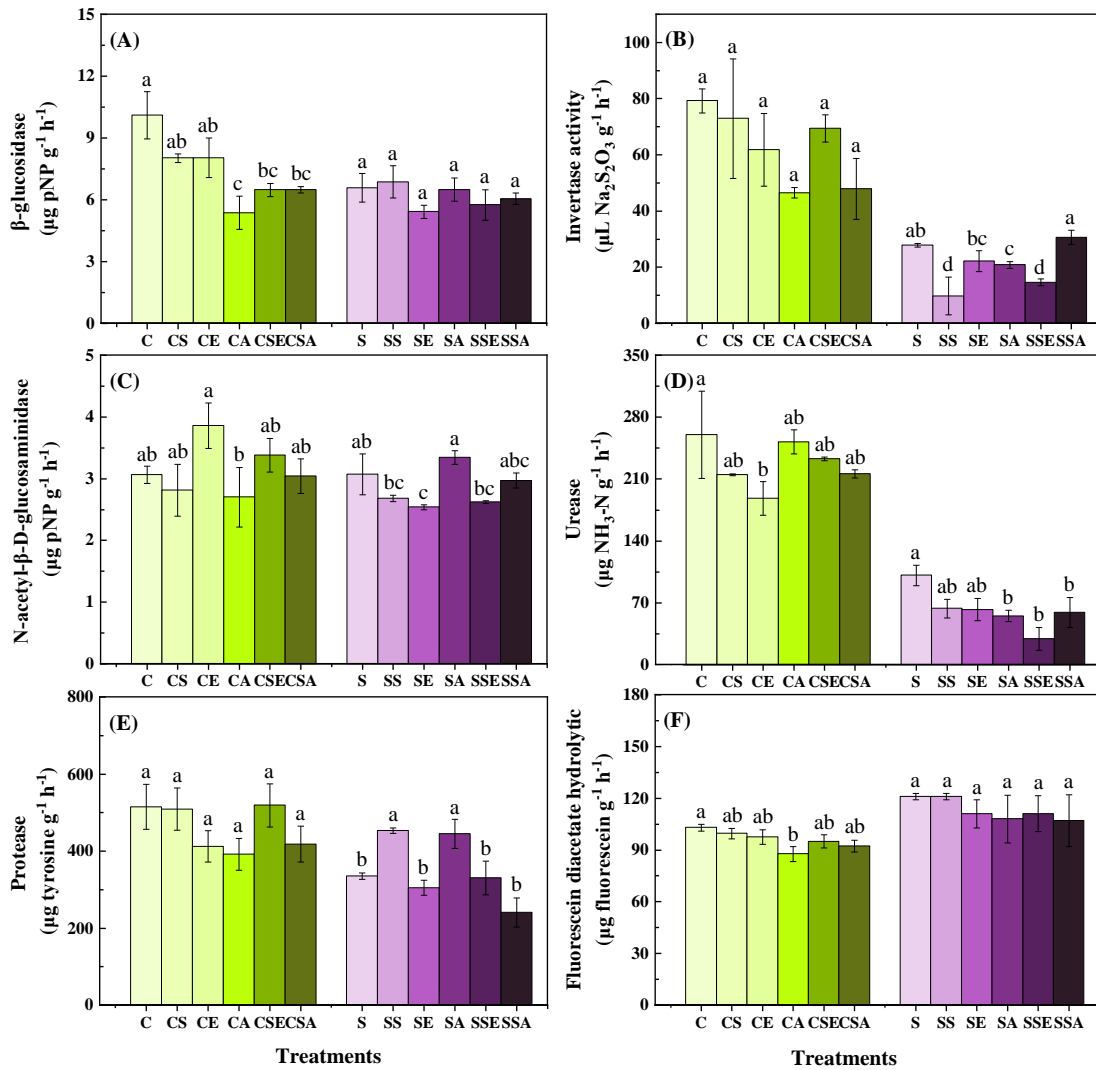


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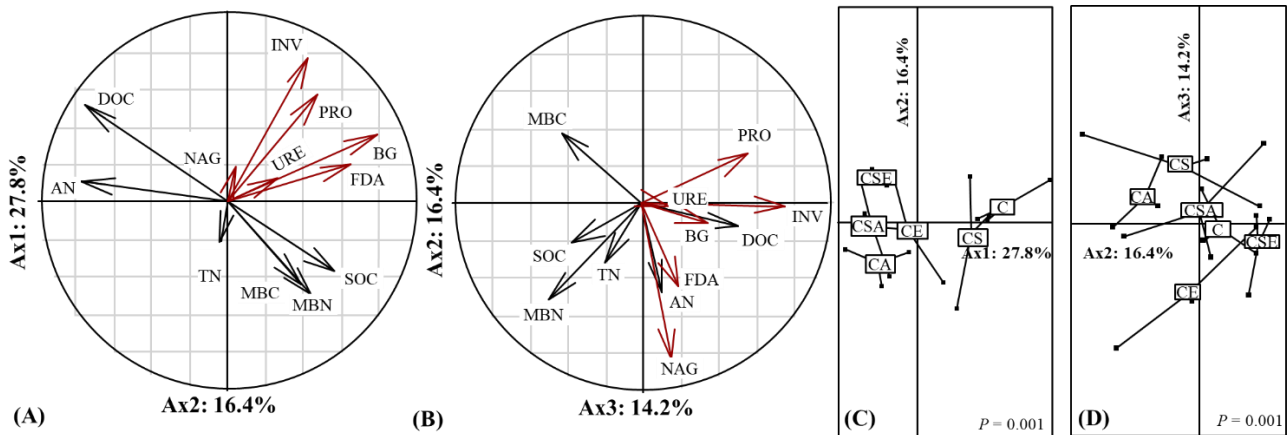


Fig. 4. Principal component analysis (PCA) of the effect of earthworms and *Sphingobacterium* sp. on soil carbon and nitrogen fractions and enzyme activities in non-contaminated soil in fractal F1, F2 and F3 planes.

(A) Correlation circles of carbon and nitrogen fractions with enzymes activities in non-contaminated soil in fractal F1 and

F2 planes.

(B) Correlation circles of carbon and nitrogen fractions with enzymes activities in non-contaminated soil in fractal F2 and F3 planes.

(C) Projection of experimental points according to treatments with or without earthworm and *Sphingobacterium sp.* in non-contaminated soil in fractal F1 and F2 planes.

(D) Projection of experimental points according to classification with or without strain and worm in non-contaminated soil in fractal F2 and F3 planes.

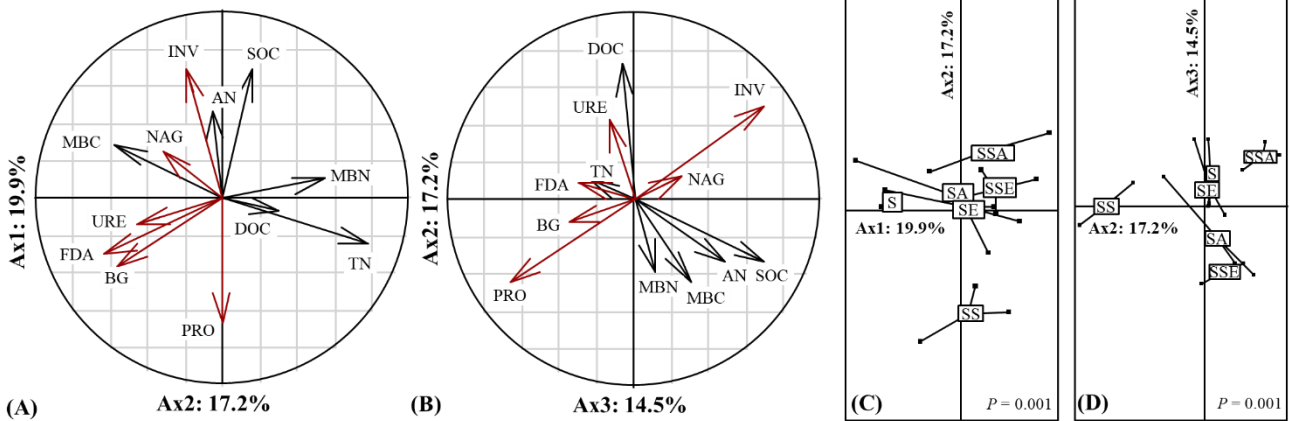


Fig. 5. Principal component analysis (PCA) of the effect of earthworms and *Sphingobacterium sp.* on soil carbon and nitrogen fractions and enzyme activities in Cd-contaminated soil in fractal F1, F2 and F3 planes.

(A) Correlation circles of carbon and nitrogen fractions with enzymes activities in Cd-contaminated soil in fractal F1 and F2 planes.

(B) Correlation circles of carbon and nitrogen fractions with enzymes activities in Cd-contaminated soil in fractal F2 and F3 planes.

(C) Projection of experimental points according to treatments with or without earthworm and *Sphingobacterium sp.* in Cd-contaminated soil in fractal F1 and F2 planes.

(D) Projection of experimental points according to classification with or without strain and worm in Cd-contaminated soil in fractal F2 and F3 planes.

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1 **Figure captions:**

2 **Fig. 1. Soil carbon and nitrogen fraction characteristics.**

3 SOC, soil organic carbon; DOC, dissolved organic matter; MBC, microbial biomass carbon; TN, total
4 nitrogen; AN, available nitrogen; MBN, microbial biomass nitrogen. Results are expressed as the mean
5 value \pm SE (n=3). Different letters indicate significant differences ($p < 0.05$) among the treatments.

6

7 **Fig. 2. The ratio of Soil carbon and nitrogen fractions.**

8 qMC , microbial quotient carbon, microbial quotient nitrogen. Results are expressed as the mean value
9 \pm SE (n=3). Different letters indicate significant differences ($p < 0.05$) among the treatments.

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12 **Fig. 3. Characterization of soil enzyme activity.**

13 BG, β -glucosidase activity; INV, inverse activity; NAG, N-acetyl- β -D-glucosaminidase activity; URE,
14 urease activity; PRO, protease activity; FDA, fluorescein diacetate hydrolytic activity. Results are
15 expressed as the mean value \pm SE (n=3).

16

17 **Fig. 4. Principal component analysis (PCA) of the effect of earthworms and *Sphingobacterium* sp.
18 on soil carbon and nitrogen fractions and enzyme activities in clean and contaminated soil.**

19 (a) correlation circles of carbon and nitrogen fractions with enzymes in clean soil .

20 (b) Projection of experimental points according to treatments with or without earthworm and
21 *Sphingobacterium* sp. in clean soil.

22 (c) Correlation circles of carbon and nitrogen fractions with enzymes activities in contaminated soil.

23 (d)Projection of experimental points according to treatments with or without earthworm and

24 *Sphingobacterium* sp. in contaminated soil.

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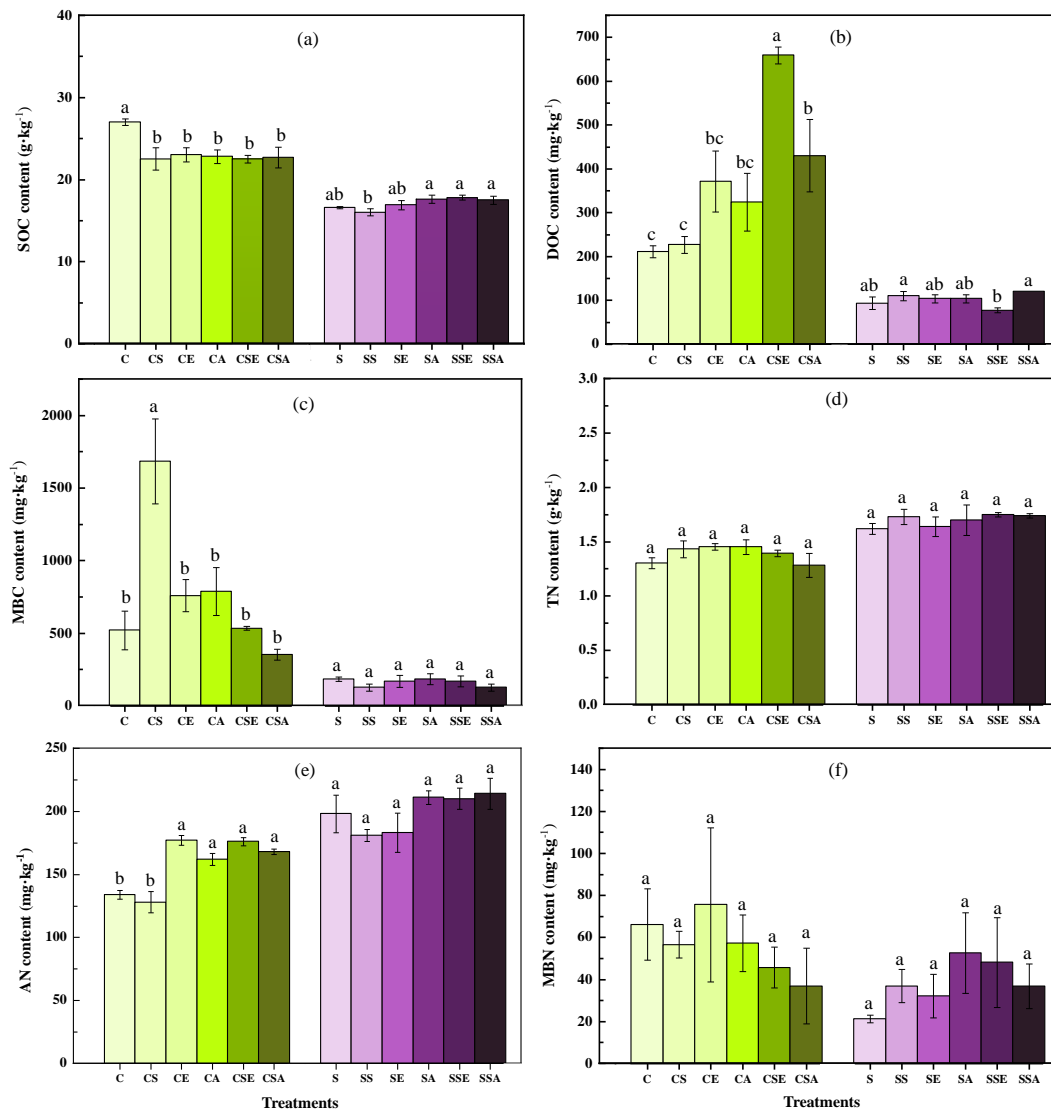


Fig. 1. Soil carbon and nitrogen fraction characteristics. SOC, soil organic carbon; DOC, dissolved organic matter; MBC, microbial biomass carbon; TN, total nitrogen; AN, available nitrogen; MBN, microbial biomass nitrogen. Results are expressed as the mean value \pm SE ($n=3$). Different letters indicate significant differences ($p < 0.05$) among the treatments.

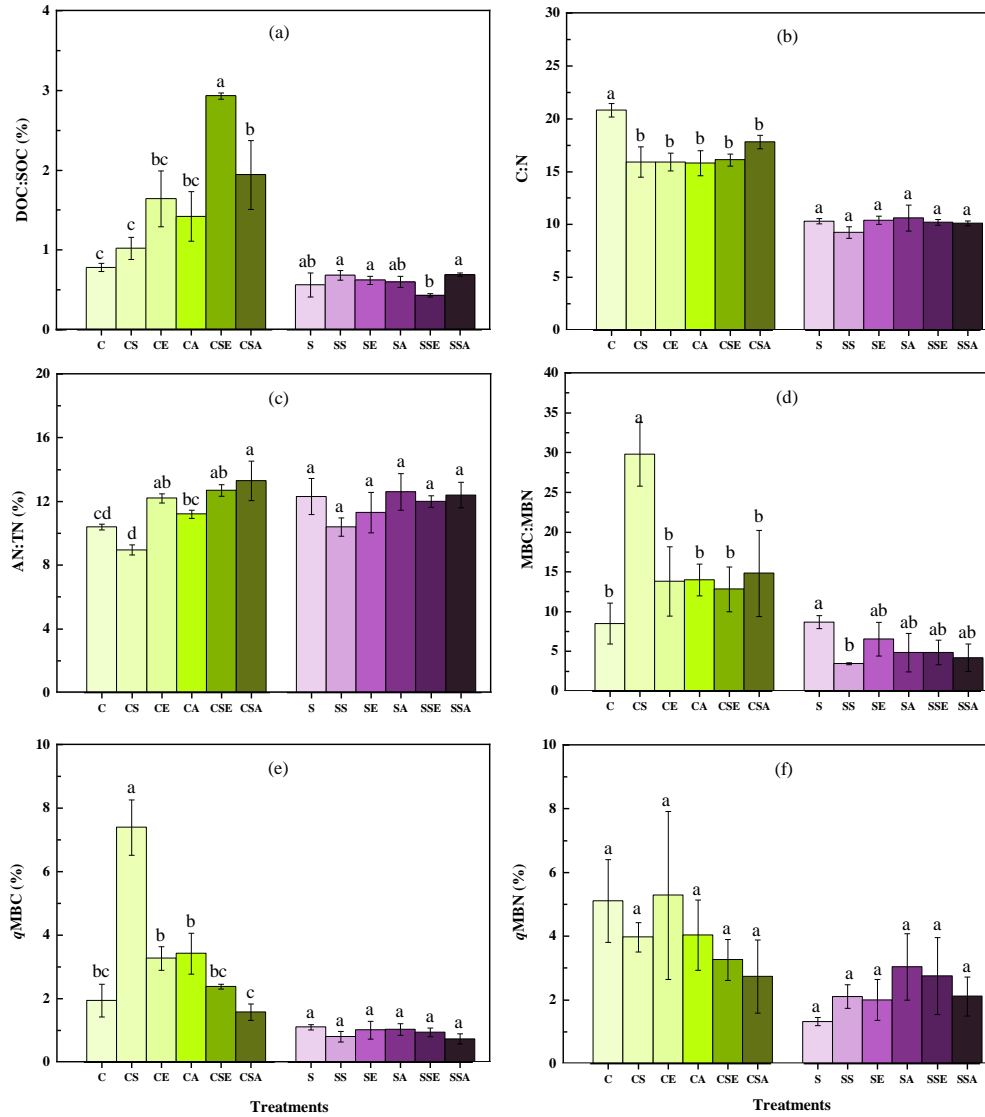


Fig. 2. The ratio of Soil carbon and nitrogen fractions. qMC , microbial quotient carbon, microbial quotient nitrogen. Results are expressed as the mean value \pm SE (n=3). Different letters indicate significant differences ($p < 0.05$) among the treatments.

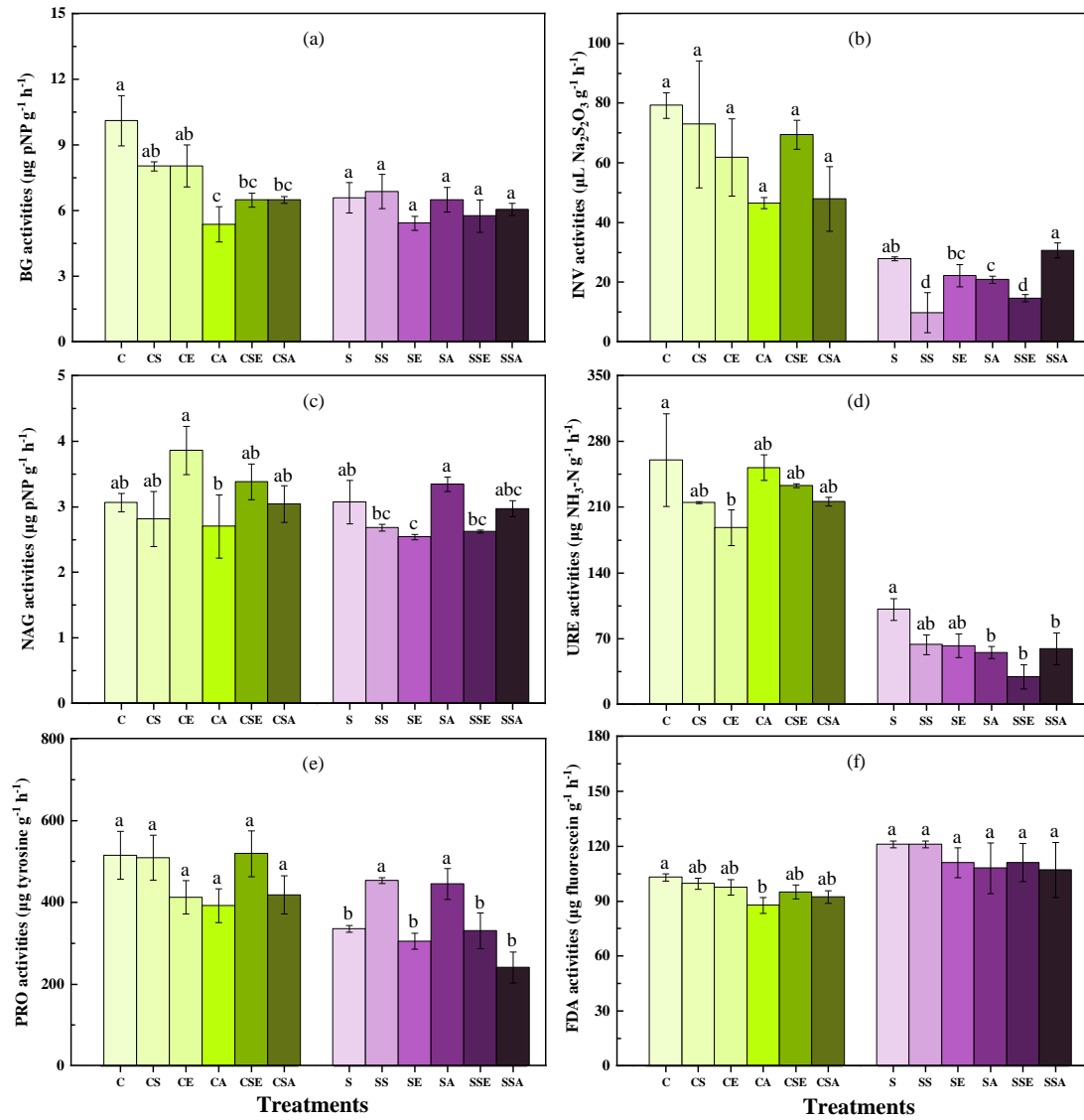


Fig. 3. Characterization of soil enzyme activity. BG, β -glucosidase activity; INV, inverse activity; NAG, N-acetyl- β -D-glucosaminidase activity; URE, urease activity; PRO, protease activity; FDA, fluorescein diacetate hydrolytic activity. Results are expressed as the mean value \pm SE (n=3). Different letters indicate significant differences ($p < 0.05$) among the treatments.

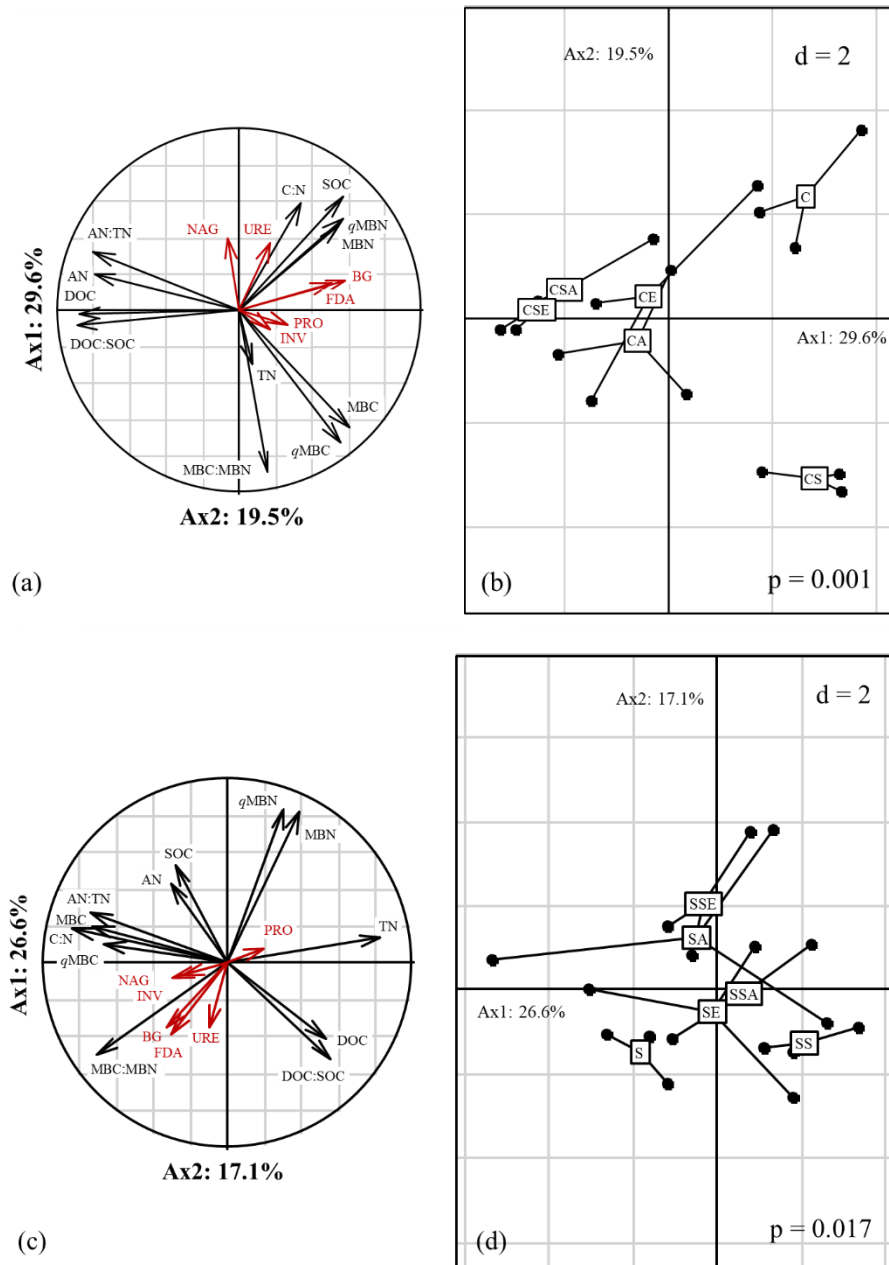


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(a) correlation circles of carbon and nitrogen fractions with enzymes in clean soil .

(b) Projection of experimental points according to treatments with or without earthworm and *Sphingobacterium* sp. in clean soil.

(c) Correlation circles of carbon and nitrogen fractions with enzymes activities in contaminated soil.

(d) Projection of experimental points according to treatments with or without earthworm and *Sphingobacterium* sp. in contaminated soil.

1 **Table list:**

2 **Table1** Properties and Cd concentrations of soil.

3 **Table2** The survival rate and biomass of earthworm at the 20th day.

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Table1 Properties and Cd concentrations of soil

	pH	Clay (%)	SOC (g·kg ⁻¹)	TN (g·kg ⁻¹)	C:N	Cd (mg·kg ⁻¹)	DTPA-Cd (mg·kg ⁻¹)
Soil I	4.73±0.05a	18.60±3.85a	27.0±0.37a	1.30±0.05b	20.8±0.64a	0.13±0.15b	0.007±0.001b
Soil II	4.40±0.02b	19.42±0.49a	16.6±0.12b	1.62±0.05a	10.3±0.25b	0.64±0.20a	0.164±0.000a

Note: Soil I and Soil II represents clean soil and contaminated soil, respectively. SOC, soil organic carbon; TN, total nitrogen; Cd, cadmium. Results are expressed as the mean ± SE (n=3). Different lower lowercase letters represent significant differences ($p < 0.05$) among the soils. The same below.

Table2 The survival rate and biomass of earthworm at the 20th day

Treatments	Survival rate/%		Biomass /g·pot ⁻¹	
	Before incubation	The 20th day	Before incubation	The 20th day
CE	100±0a	95.2±4.76a	3.02±0.05a	2.49±0.19a
CA	100±0a	86.1±7.35a	3.05±0.05a	2.81±0.03b
CSE	100±0a	91.1±4.49a	3.00±0.11a	2.41±0.07b
CSA	100±0a	87.8±6.19a	3.22±0.06a	2.79±0.24a
SE	100±0a	77.8 ±11.1a	2.94±0.04a	2.43±0.12b
SA	100±0a	100±0a	3.10±0.11a	2.52±0.19a
SSE	100±0a	31.2±6.10b	3.12±0.13a	0.71±0.05b
SSA	100±0a	100±0a	3.07±0.04a	2.49±0.05b

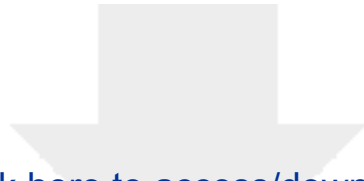
Note: Results are expressed as the mean value ± SE (n=3). Initial C and S represents the soil I and soil II. CE, CA, CSE and CSA represents the treatments with *E.fetida*, *A.gracilis*, *Sphingobacterium* sp.+ *E.fetida* and *Sphingobacterium* sp.+*A.gracilis* in soil I, while SE, SA, SSE and SSA represents the treatments with *E.fetida*, *A.gracilis*, *Sphingobacterium* sp.+ *E.fetida* and *Sphingobacterium* sp.+*A.gracilis* in soil II, respectively. The same below. Different letters indicate significant differences ($p < 0.05$) between different days.

Declaration of interests

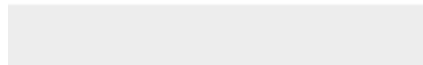
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Chi ZHANG reports financial support was provided by Guangdong Provincial Natural Science Foundation.
Chi ZHANG reports financial support was provided by National Natural Science Foundation of China.



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