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:	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, Amynthas 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-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.
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Response to Reviewers:	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 dominate gut bacteria 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 an 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 cann'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, Amynthas 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-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:

Line135-140: 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 silimar 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 resistant Cd stress. Both species increases 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 ± 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 ± 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 ± 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:
Table2 Survival rate and mean biomass loss of earthworm at the 20th day
TreatmentsSurvival rate (%)Mean biomass loss (%)
CE95.2 ± 4.76a17.4 ± 13.3b
CA86.1 ± 7.35a7.68 ± 4.54b
CSE91.1 ± 4.49a19.4 ± 5.26b
CSA87.8 ± 6.19a13.7 ± 10.2b
SE77.8 ± 11.1a17.6 ± 5.22b
SA100 ± 0a18.9 ± 5.54b
SSE31.2 ± 6.10b77.2 ± 3.99a
SSA100 ± 0a18.9 ± 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 Amynthas 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 (HNO_3: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,

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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 dominate gut bacteria 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. *T*he 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 an 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]. *T*he 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 (HNO_3: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 cann'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*, *Amynthas 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 $mg \cdot kg^{-1}$ 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:

Line135-140: 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 $mg \cdot kg^{-1}$) 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 $mg \cdot kg^{-1}$), 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 silimar 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 resistant Cd stress. Both species increases 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:

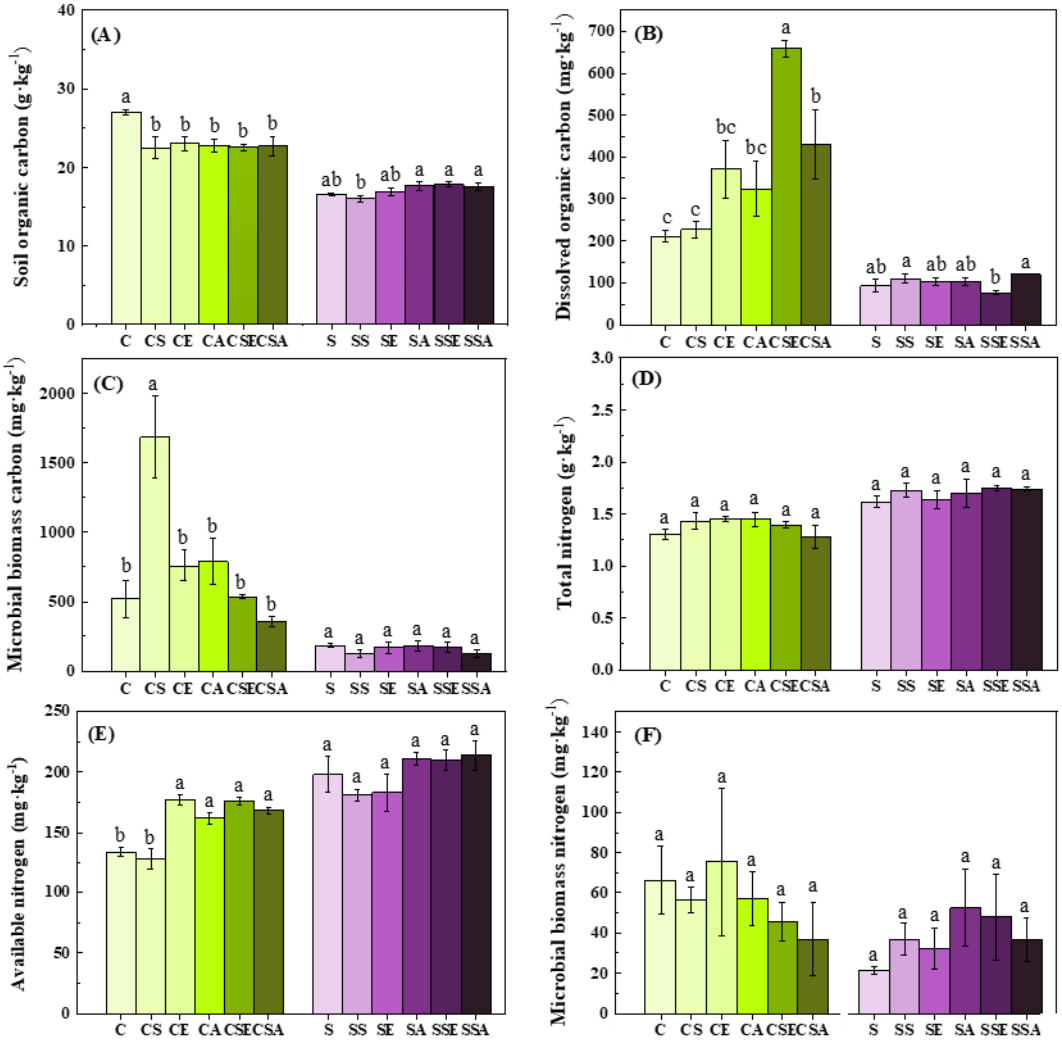

Fig. 1. Soil carbon and nitrogen form characteristics. Results are expressed as the mean value ± SE (n = 3). Different lowercase letters indicate significantly differences ($P < 0.05$) among the treatments.

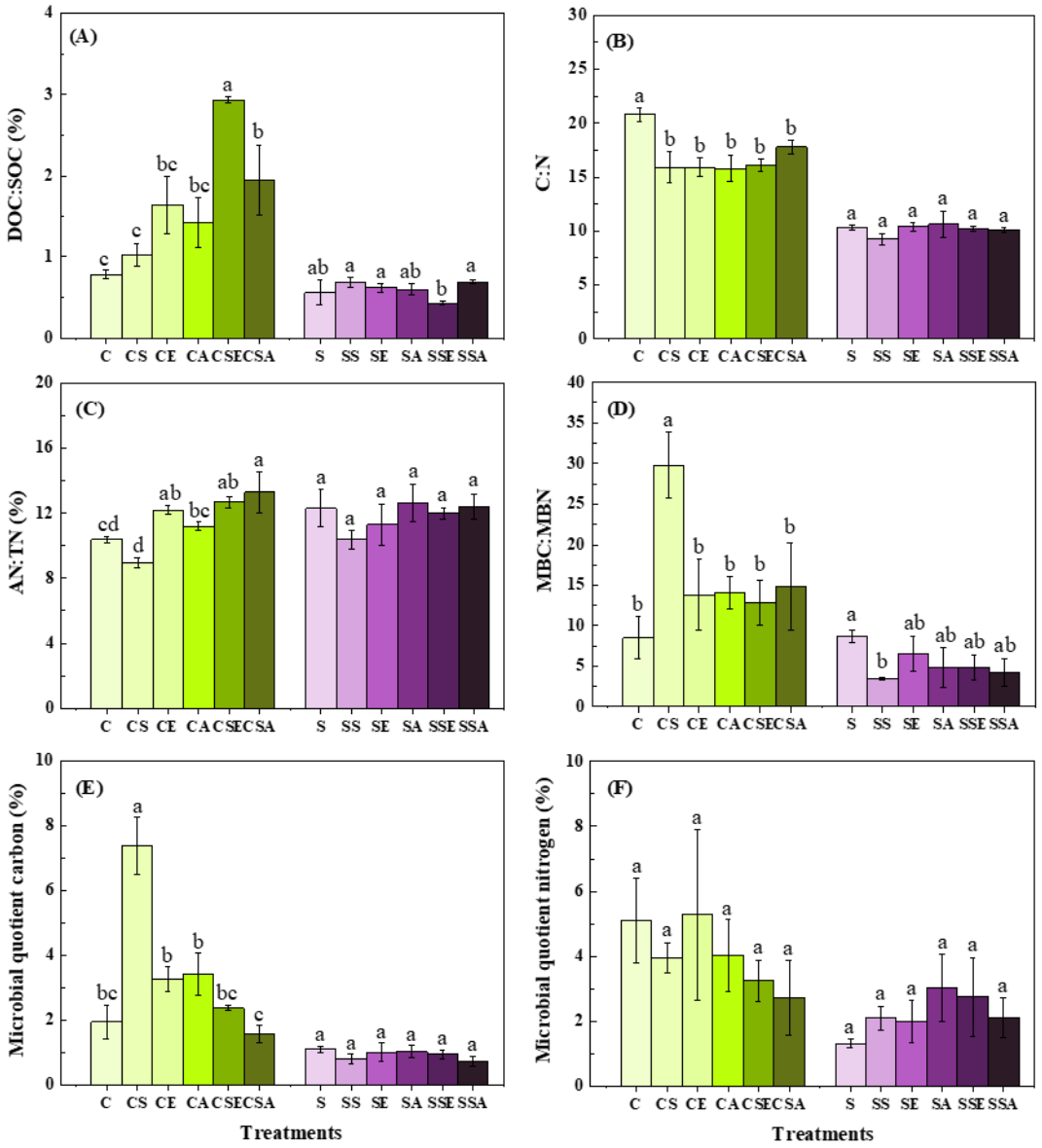

Fig. 2. The ratio of soil carbon and nitrogen forms. SOC, soil organic carbon; DOC, dissolved organic matter; TN, total nitrogen; AN, available nitrogen; C:N, soil organic carbon-to-total nitrogen ratio; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. Results are expressed as the mean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

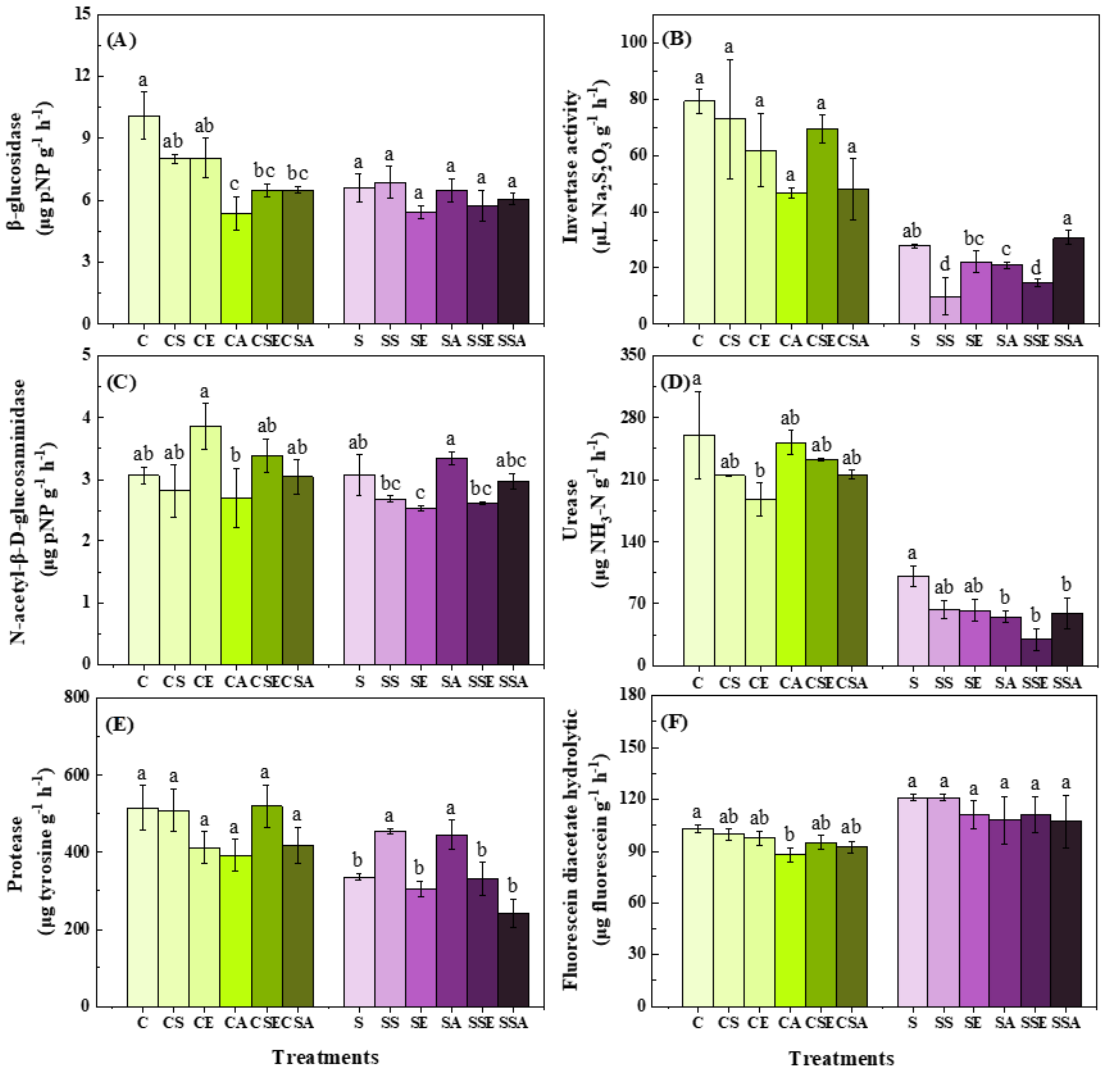

Fig. 3. Characterization of soil enzyme activity. Results are expressed as the mean value ± SE (n = 3). Different lowercase letters indicate significantly differences ($P < 0.05$) among the treatments.

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:

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.fetid*a 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. T*he 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]. T*he 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 Amynthas 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 (HNO_3: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.

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*, *Amynthas 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±292 $mg \cdot kg^{-1}$ 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 acivities). 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

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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Highlights

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

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*, *Amynthas 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±292 $mg \cdot kg^{-1}$ 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 acivities). 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. Introduction

Soil is an important source and sink of terrestrial carbon and nitrogen in the biosphere. There are a variety of soil organisms playing an essential role in soil carbon and nitrogen cycle[1,2]. The interactive processes of soil organisms, reconstituting soil carbon stocks and nitrogen cycling in sustainable soil management, have attracted broad attention[3,4]. However, soil metals pollution is becoming an more and more serious environmental and social problem, probably resulting in the changes in the interaction between soil organisms to affect soil carbon and nitrogen cycles[5]. It is of great ecological importance to understand the interactive relationship of soil organisms on the carbon and nitrogen cycle under metal contamination stress.

As "soil ecosystem engineers", earthworms could degrade soil organic matter and significantly influence soil carbon and nitrogen contents[13]. These processes is the result of complex interactions between soil organisms. Earthworm selective feeding activities have a significant impact on soil carbon and nitrogen cycling in gut[6]. Earthworm bioturbation (burrowing, feeding and excretion (casts and surface mucus)) affect directly or indirectly soil microbial communities and soil enzyme activities[7-9]. Nevertheless, currently, previous studies on earthworms in metal contaminated soil have mainly focused on earthworm effects on metal transformation and their ecotoxicological characteristics [10-12]. Earthworm effects on soil carbon and nitrogen cycling in metal contaminated soil have not been receiving sufficient attention.

Sphingobacterium sp., a kind of gram-negative bacterium, has a wide range of nutrient sources, high adaptability and wide ecological distribution [16-18]. In our previous studies, *Sphingobacterium* sp. was demonstrated as a kind of dominant microorganism in earthworm gut [25]. Although *Sphingobacterium* sp. has a strong capacity for organic matter degradation (alkanes and polycyclic aromatic hydrocarbons) to regulate soil carbon and nitrogen transformation in organic polluted soil[19-24], their effect in combination with earthworms on the soil carbon and nitrogen cycle have not yet been clarified, especially under metal pollution stress.

Soil C and N cycles invloved a series of biological and biochmical process. Soil carbon and nitrogen fractions, dissolved organic carbon(DOC), alkali-hydrolyzable nitrogen (AN), microbial biomass carbon and nitrogen(MBC and MBN) have been considered as the key indicators to reflect the state of the soil carbon and nitrogen cycles,

and their fraction ratios can further provide a more intuitive representation of the overall mineralization or sequestration of carbon and nitrogen in the system. Besides, soil enzymes are metabolic drivers of soil organisms, and their activity can, to some extent, reflect not only the changes in the soil microbial community [27], but also the intensity of soil organic matter decomposition and nutrient transformation [28]. Studies on the influence of enzymes on carbon and nitrogen transformation have mostly focused on β-glucosidases (BG) and invertase (INV), which were involved in the decomposition of labile cellulose, sucrose and carbohydrate polymers, and N-acetyl-glucosaminidase (NAG), urease (URE) and proteases (PRO) were involved in the hydrolysis of chitooligosaccharides, urea and protein, as these enzymes are mainly participating in terminal catalytic reactions or intra-systemic enzymatic reactions, which can reflect the level of carbon and nitrogen metabolism in soils [29]. Fluorescein diacetate hydrolysis (FDA) is an enzyme that can reflect the total microbial activity of soil [30]. Therefore, the effects of earthworm inoculation on soil carbon and nitrogen cycles can indirectly be reflected by studying the changes in soil enzyme activities related to carbon and nitrogen cycles.

The aim of this study was to investigate the effects of different species of earthworms (*Eisenia fetida* and *Amynthas gracilis*) and exogenous *Sphingobacterium* sp. on the transformation of soil carbon and nitrogen under clean and Cd-contaminated soil systems. The specific objectives were: (1) to compare and analyse the effects of exogenous *Sphingobacterium* sp. and two different species of earthworms, individually and simultaneously, on carbon and nitrogen fractions and related enzyme activities in

clean and Cd-contaminated soils. (2) to elucidate the role of *Sphingobacterium* sp. in the participation of earthworms in the carbon and nitrogen cycling process under Cd pollution stress. This study could provide a theoretical basis for an insight into soil carbon and nitrogen cycling processes and biologically driven mechanism, and also provide suggestions for adjusting soil remediation and their further sustainable management.

2. Materials and methods

2.1 Soil, earthworm and strain

Two representative soils from South China were selected, clean 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 soil are listed in Table 1.

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

Sphingobacterium sp. was purchased from BeNa Culture Collection (BNCC). Microbial CM0827 *Propionibacterium sp.* medium consisted of: tryptone 15 g, L-

cystine 0.5 g, yeast extract 5 g, sodium mercaptoethanolate 0.5 g, glucose 5 g, agar 0.7 g, sodium chloride 2.5 g, resazurin 0.001 g, distilled water 1 L. Incubate the strain at a constant temperature of 30°C for 2 days and set aside (pH 7.0, OD_{405} value 1.45).

2.2 Experimental designs

A two-factor (inoculation with *Sphingobacterium* sp. and different earthworm species (*E.fetida*, E, *A.gracilis*, A)) experimental design was used in clean (Soil I, C) and Cd- contaminated (Soil II, S) soils. Six treatments were set up for each of the two soils, 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).

Filled 200 g soil through 2 mm mesh into the pot (caliber x bottom diameter x height: 75 x 53 x 90 mm) with microporous bottom. Stabilized the soil moisture for 48 h by adjusting it to 60% of maximum filed water holding capacity and maintained at this moisture by weighting throughout. Inoculated with 3 g of earthworms per pot, approximately 8 *E.fetida* (3±0.3 g) or 5 *A.gracilis* (3±0.4 g). one milliliter of *Sphingobacterium* sp. solution (OD_{405} value 1.45) was added to the soil surface in CS, CSA, CSE, SS, SSA and SSE treatments, respectively. To prevent earthworms escape, the top and bottom of each pot were covered with a fine nylon mesh with 1 mm pore

diameter. The temperature in the laboratory was controlled at 25°C.

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

2.3 Soil properties, enzyme activity, content and DTPA of Cadmium

Soil pH was measured using a glass electrode in a 1:2.5 (*w*/*v*) soil/water suspension. Soil clay, and soil organic carbon (SOC), alkali-hydrolyzable nitrogen (AN) and total nitrogen (TN) was analyzed by the pipette [31], the dichromate oxidation [32], alkali N-proliferation [33] and Kjeldahl [32] methods, respectively；Microbial biomass carbon and nitrogen (MBC and MBN) were determined via the chloroform fumigation-extraction method [34,35]; Dissolved organic carbon (DOC) content was extracted with deionized water (1:5) and determined [15]. Total cadmium (Cd) content was prepared and determined by microwave digestion and ICP-MS [36], and the measured concentrations of referred samples within 98% of certified concentrations. Three repetitions for each soil were also implemented to ensure the quality of analysis.

β-glucosidase, protease and N-acetyl-β-D-glucosaminidase activities were all determined by colorimetric assay. Urease, invertase and fluorescein diacetate hydrolytic activities was determined by sodium phenolate colorimetric assay, titration and colourimetric using fluorescein methods [37], respectively.

2.4 Statistics analysis

All data was 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 was presented as the mean ± SE (standard error). Principal components analysis (PCA) was processed to analyze the relationships of soil carbon and nitrogen fractions and ratios (SOC, DOC, MBC, TN, AN, MBN, DOC:SOC, C:N, AN:TN, MBC:MBN, *q*MBC, *q*MBN) and enzymes (BG, INV, URE, NAG, PRO, FDA) in R version 3.4.3 using the "ade4" package. DOC:SOC=DOC/SOC, AN:TN=AN/TN, C:N= SOC/TN, MBC:MBN=MBC/MBN; Microbial quotient carbon (*q*MBC), *q*MBC=MBC/SOC; Microbial quotient nitrogen (*q*MBN), *q*MBN =MBN/TN.

3. Results

3.1 Earthworm survival rate and biomass

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

3.2 The content of carbon and nitrogen fractions

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

was reduced by 16.7%, 14.8%, 15.6%, 16.7%, and 15.9% in CS, CE, CA, CSE, CSA treatments, respectively. Furthermore, DOC increased significantly in the presence of earthworms ($p<0.05$), and was highest in the CSE and CSA treatments at 659±19.2 $mg \cdot kg^{-1}$and 430±82.8 $mg \cdot kg^{-1}$, respectively. Individual inoculation with *Sphingobacterium* sp. significantly increased MBC up to 1685±292 $mg \cdot kg^{-1}$ ($p<0.05$). Whereas, in all the treatments inoculated with earthworms, there was no significant change in MBC content compared to C treatment, but it was significantly decreased compared to CS treatment.

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

The content of nitrogen fractions can be found in Fig. 1d-f. In clean soil, the earthworm activity significantly increased AN content ($p<0.05$), which increased by 32.9%, 20.9%, 31.3% and 25.4% in CE, CA, CSE and CSA treatments respectively, while *Sphingobacterium* sp. did not significantly affect it ($p>0.05$). However, the mineralization indexes of nitrogen were not significantly changed in contaminated soil ($p>0.05$), but the trend of AN in all treatments was consistent with in clean soil. In addition, TN and MBN were relatively stable in both soils with no significant changes under the activities of *Sphingobacterium* sp. and earthworms.

3.3 The ratio characteristics of carbon and nitrogen fractions

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

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

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

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

inoculation of *A.gracilis* individually significantly decreased BG activities ($p<0.05$). In combination condition, both significantly inhibited BG activities ($p<0.05$), with no significant effect on other enzyme activities. In contaminated soil, *Sphingobacterium* sp. slight increased BG activities, but there was no significant difference among the treatments. PRO activity was significantly increased only in SS treatment, reaching 453±6.64 $\mu g \cdot g^{-1} \cdot h^{-1}$. As for the earthworm inoculation treatment, it was inhibited except for SSA, which increased INV, and SA, which increased NAG and PRO activities. There was no significant change in FDA activities ($p>0.05$).

3.5 Relationships among carbon, nitrogen and related enzymes activity with different treatments

In clean soils, two principal components (PCs) were identified in the PCA, representing 49.1% of the total variance (Fig. 4a-b). PC1 and PC2 explained 29.6% and 19.5% of the total variance, respectively. Significant differences were founded among the treatments, indicating that earthworms and *Sphingobacterium* sp. had significantly impacts on soil carbon and nitrogen fractions in clean soil ($p=0.001$). Treatments inoculated with earthworms and both earthworms and *Sphingobacterium* sp. tended to have low PC1 values, and treatments inoculated with *Sphingobacterium* sp. alone tended to have low PC2 values. The difference between the treatment C and CSA, CSE was reflected in the latter's significant enhancement of reactive carbon nitrogen such as DOC and AN, while the CE and CA treatments were intermediate between C and CSA, CSE. It can be clearly seen that the difference between C and CS was mainly in the second principal component of MBC, MBC:MBN and *q*MBC, while CE and CA, CSA

and CSE were in mid-way between them. In addition, inoculation with earthworms showed an inhibitory effect on enzyme activity. DOC was negatively correlated with soil enzyme activity.

In contaminated soils, two principal components (PCs) were identified in the PCA, representing 43.7% of the total variance (Fig. 4c-d). PC1 and PC2 explained 26.6% and 17.1% of the total variance, respectively. There were significant differences between the treatments, indicating that earthworms and *Sphingobacterium* sp. significantly influenced soil carbon and nitrogen composition in contaminated soils ($p=0.017$). Treatments inoculated with *Sphingobacterium* sp. tended to have high PC1 values and treatments inoculated with earthworms tended to have high PC2 values. The differences between the S treatment and the earthworm inoculation treatment were mainly reflected in the latter's enhanced effect on SOC, AN and MBN. While the S and SS treatments differed in the latter's enhanced effects on DOC, and the earthworm inoculated treatment was in between. Meanwhile, except for PRO enzyme, earthworm inoculation showed inhibitory effect on enzyme activity.

4. Discussion

4.1 Earthworm growth and survival

Earthworms abundance and biomass decreased after 20 days cultivation in both soils, with significant differences were observed especially in Cd-contaminated soil ($p < 0.05$), consistent with [38]. It might be due to soil nutrient limitation and toxic effects of high Cd concentration on earthworms [39]. In terms of earthworm species, *A.gracilis* showed higher resistant to environmental stresses than *E.fetida*, probably owing to the

adaptability of wild earthworm species in soil with limited organic matters.

4.2 Effects of earthworms and Sphingobacterium sp. *on soil carbon and nitrogen fractions*

In clean soils with high organic carbon, both earthworm and *Sphingobacterium* sp. alone significantly facilitated the mineralization of organic carbon (Fig. 1b, Fig. 2b) and is consistent with the trend of carbon and nitrogen changes in vermicomposting by [40]. Both species of earthworms significantly increased nitrogen mineralization ($p < 0.05$) (Fig. 1e), presumably through digestion in the earthworm gut, which promotes the mineralization of organic nitrogen. A study investigated the changes in the gut microbiota and functional characteristics of earthworms with the chronological sequence of cultivated soils, revealing that tillage disturbance increases the formation of functional genes that promote nutrient cycling in the earthworm gut microbiota [41]. The combination of the two species of earthworm with *Sphingobacterium* sp. also further significantly increased the reactive carbon and nitrogen content, demonstrating that earthworms and *Sphingobacterium* sp. were able to stimulate soil carbon and nitrogen cycling, and that there was an intensified effect of the combination. The high microbial biomass carbon content in CS treatment inoculated with *Sphingobacterium* sp. alone may be due to its adaption to this environment, rapid multiplication and becoming the dominant strain. In contrast, earthworm activities decreased microbial biomass as compared to *Sphingobacterium* sp. activities alone. It is suggested that earthworms have a role in maintain the ecosystem balance and stabilizing the microbial community structure through feeding [42] and regulating the allocation of active

organic carbon in the microbial proliferation and dissolved organic carbon pools [43,44]. Therefore, in the treatments of *Sphingobacterium* sp. in combination with earthworm, the rapid proliferation of *Sphingobacterium* sp. was prevented. This was further confirmed by the significantly lower *q*MBC of CSE and CSA compared to CS in Fig. 2e. That is, earthworms reduced the efficiency of soil microorganisms in utilizing carbon sources for conversion into microbial biomass carbon. Additionally, since *Sphingobacterium* sp. is the dominant gut bacterium of *E.fetida* and genera of *Amythas*, the gut can provide a shelter for it to not die completely under Cd-contaminated stress [25].

In contaminated soil, *Sphingobacterium* sp. still showed a facilitation of carbon mineralization (Fig. 1a, b; Fig. 2a), although the ability to do so was diminished, indicating its ability to mineralize carbon and to tolerate metal contamination stress. Earthworms, on the other hand, showed carbon sequestration in Cd-contaminated soils with low soil organic carbon content, in contrast to their behavior in clean soils. This is probably due to the biased feeding of organic matter by earthworms to resist metal toxicity, and then, through gut peristalsis and body surface mucus, promote the formation of organic matter-rich aggregate and the protective effect of mucus substances, which reduces carbon loss [6, 44-47]. It's also probably explained by contaminated soils containing carbon that is stable and not easily mineralized. In PCA analysis, positive correlation between β-glucosidase and N-acetyl-β-D-glucosaminidase activity also indicated that soil microorganisms tend to use the active carbon pool generated by the decomposition of humus formed by litter (Fig. 4c; Fig.

S1), i.e., favor the accumulation of recalcitrant soil carbon pools. In addition, both dissolved organic carbon and the dissolved organic carbon to soil organic carbon ratio were significantly lower in the SSE treatment than in the other treatments, which may be influenced by the lower survival rate of earthworms and their reduced biological effect capacity in this treatment. For the other carbon and nitrogen fractions, earthworm and *Sphingobacterium* sp. activities had non-significant effect, presumably due to nutrient and metal pollution stress [48], which in turn reduces its ecological role.

4.3 Effect of earthworms and Sphingobacterium sp. *on soil enzyme activity*

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

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

the activities of invertase, N-acetyl-β-D glucosaminidase and urease, but there was no obvious regularity among treatments (Fig. 3b-d). It is possibly due to that soil exhibited carbon storage and the corresponding mineralizing enzyme activities were reduced under the activities of earthworms. Or these enzyme activities were intimately related to soil water-soluble organic carbon and microbials, i.e., metal stress and low soil fertility, and soil fauna activities in turn reduced the soil microbiota [52,53]. In addition, metal can inhibite soil urease and fluorescein diacetate hydrolytic activity, and postulated that the mechanism may be that Cd^{2+} exchanges with Mg^{2+}, resulting in blockage of protein synthesis, which in turn inhibits the growth and proliferation and decreases enzyme synthesis and secretion in microorganisms, and ultimately leads to decreased soil enzyme activities [54].

In terms of earthworm species, soil enzyme activities in clean soil with *E.fetida* participating were generally higher than those of *A.gracilis*. In contaminated soils, *A.gracilis* increased N-acetyl-β-D glucosaminidase, protease, and urease activities individual and combined, which may be related to that *A.gracilis* is wild species with strong resistance to environment stress. Moreover, *A.gracilis* influenced fluorescein diacetate hydrolytic to a greater extent, both individually and combinatorially in two soils, presumably as a result of differences in feeding habits of different species [55,10,56]. Earthworms preferred to feed on microorganisms as secondary food source in soils where organic matter is difficult to degrade [8]. The body fluids of earthworms may contain bacteriostatic substances [57,58], resulting in a lower bacterial content in their gut and excreta than in original soil.

4.4 *Effect of earthworms and Sphingobacterium* sp. *on the balance of soil carbon and nitrogen stabilization and mineralization*

Organisms have strong interaction among themselves in soil [3], earthworm and microorganisms contributed together in this study on the balance of soil carbon and nitrogen stabilization and mineralization. In our study, *Sphingobacterium* sp. can promote soil carbon and nitrogen mineralization, especially in clean soils (Fig.1-2). It also assist earthworm to further promote carbon and nitrogen mineralization(Fig.4a), and *E.fetida* showed more efficiencies than *A.gracilis*. In contrast, the combined effect of them in metal contaminated soils was not significant and exhibited carbon storage(Fig. 4b), probably due to the biased feeding of organic matter by earthworms to resist metal toxicity, and the nutrient and metal contaminated stress in the soil not only affects the survival status of earthworms, but also makes it difficult for microorganisms to play its part. Ultimately, the contaminated soil exhibited carbon storage because of the stronger stabilizing effect of earthworms than mineralizing effect of *Sphingobacterium* sp.. It's also probably owing to mineralizable carbon decreases with time of mine contaminated soils and residue more mineral associated organic matter that is stable and not easily mineralized by organisms [59]. Among earthworm species, *E.fetida* had a stronger effect on soil carbon and nitrogen mineralization than *A.gracilis* (Fig. 1b), which may be related that *E.fetida* favors ingestion of easily available organic matter, accelerating the mineralization and decomposition of organic matter [60,61]. By contrast, *A.gracilis* mainly fed on soil mineral particles and organic matter, facilitating the mixing and bind of organic matter with soil minerals. In addition,

the soil of this study (Table 1), *E. fetida* was easier to obtain food in clean soil with relatively high organic matter content and the high carbon to nitrogen ratio was favored for earthworm growth [62], so this species performed better. Unfortunately, their ecological effects were limited by difficulties in access to food in Cd-contaminated soils with low organic matter content(Table 1). Additionally, only parts of treatment increased soil enzyme activities. Mostly treatment enzymes had no significant different compared with control treatment and showed weak inhibited by earthworm activities. This phenomenon also can be founded in PCA analysis (Fig. 4). That is, soil carbon and nitrogen index did not show a strong correlation. To reveal the under mechanisms, we need to analyze them in the context of multiple biological and environmental factors.

5. Conclusion

This study on soil carbon and nitrogen fractions and enzyme activities to investigate whether *Sphingobacterium* sp. can assist different species of earthworm in influencing soil carbon stabilization and nitrogen cycling under clean and Cd-contaminated soil. Overall, earthworm inoculation alone exhibited facilitating carbon and nitrogen mineralization in clean soil, while *Sphingobacterium* sp. inoculation alone facilitated carbon and nitrogen mineralization in both soils. In clean soil, earthworm effect showed species-dependent and *E.fetida* was more effective than that of *A.gracilis*. *Sphingobacterium* sp. could enhance earthworm effects on carbon mineralization in clean soil. While in cadmium contaminated soil, earthworm and *Sphingobacterium* sp. combined inoculation improved soil carbon storage rather than carbon mineralization in treatments with earthworm, but no significant effects between earthworm species.

However, the effect of earthworm and *Sphingobacterium* sp. on soil nitrogen was no significant in contaminated soil. This study provides a clear understanding on the different effects of earthworm and microorganisms on soil carbon and nitrogen cycling in clean and contaminated soil. In further study, the complex interaction mechanisms of earthworm and microorganisms can be designed and tested by some methods about molecular biology or metabolomics. Additionally, the interaction of earthworm and microorganisms in soil remediation technology could be explored to adjust and rebuild a sustainable soil management practice.

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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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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.

ABSTRACT

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*, *Amynthas 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 $mg \cdot kg^{-1}$ 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.

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

1. Introduction

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

As "soil ecosystem engineers", earthworm can degrade soil organic matter and significantly influence the forms and contents of soil carbon and nitrogen [6]. Earthworm's preferential feeding and concentration processes have a substantial impact soil carbon and nitrogen cycling [7]. Their bioturbation activities, including burrowing, feeding, and excretion (casts and surface mucus), affects the soil carbon and nitrogen distribution within soil profile [8-11]. These effects result from complex mutualism interactions between earthworms and microorganisms [7,12-13]. 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 [14-15]. 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 [16]. 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, *Sphingobacterium* sp. was identified as a dominant bacterial taxon in the earthworm gut [17]. *Sphingobacterium* sp., a gram-negative bacterium, exhibits a wide range of nutrient sources, high adaptability, and a broad ecological distribution [18-20]. In practice, it has been used to decompose agricultural waste and degrade organic pollutants (e.g. alkanes and polycyclic aromatic

hydrocarbons) in soil [21-26]. 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 [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.

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

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.

This study aimed to investigate the effects of different earthworm species (*Eisenia fetida* and *Amynthas gracilis*) and exogenous *Sphingobacterium* sp. on the transformation of soil carbon and nitrogen under non-contaminated and Cd-contaminated stresses. 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. This study can provide a theoretical basis for insight into the combined effects of earthworms and microorganisms on soil carbon and nitrogen cycling processes, the biologically driven mechanisms, and offer suggestions for adjusting soil remediation practices and their further sustainable management.

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 *Amynthas 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 $g \cdot ind^{-1}$) or 5 individual *A. gracilis* (~ 0.60 $g \cdot ind^{-1}$). One milliliter of *Sphingobacterium* sp. solution (OD_{405}

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 (HNO_3: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.

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 ± SE (standard error). DOC:SOC = DOC/SOC, AN:TN = AN/TN, C:N = SOC/TN, MBC:MBN=MBC/MBN; Microbial quotient carbon (*q*MBC), *q*MBC = MBC/SOC; Microbial

quotient nitrogen (*q*MBN), *q*MBN = MBN/TN.

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

3. Results

3.1 The contents of carbon and nitrogen forms

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

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

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

3.2 The ratios of carbon and nitrogen forms

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

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

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

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

3.4 Earthworm survival rate and mean biomass loss

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

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

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

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

4. Discussion

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 $mg \cdot kg^{-1}$) 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 $mg \cdot kg^{-1}$), 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 silimar 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 resistant Cd stress. Both species increases 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. Conclusion

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

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Table list:

Table1 Properties and Cd concentrations of soil

	pH	Clay (%)	SOC ($g \cdot kg^{-1}$)	TN ($g \cdot kg^{-1}$)	C:N	Cd ($mg \cdot kg^{-1}$)	DTPA-Cd ($mg \cdot kg^{-1}$)
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).

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.fetid*a 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.

Figure captions:

Fig. 1. Soil carbon and nitrogen form characteristics.

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

Fig. 3. Characterization of soil enzyme activity.

Results are expressed as the mean value ± SE (n = 3). Different lowercase letters indicate sicanificantly 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.

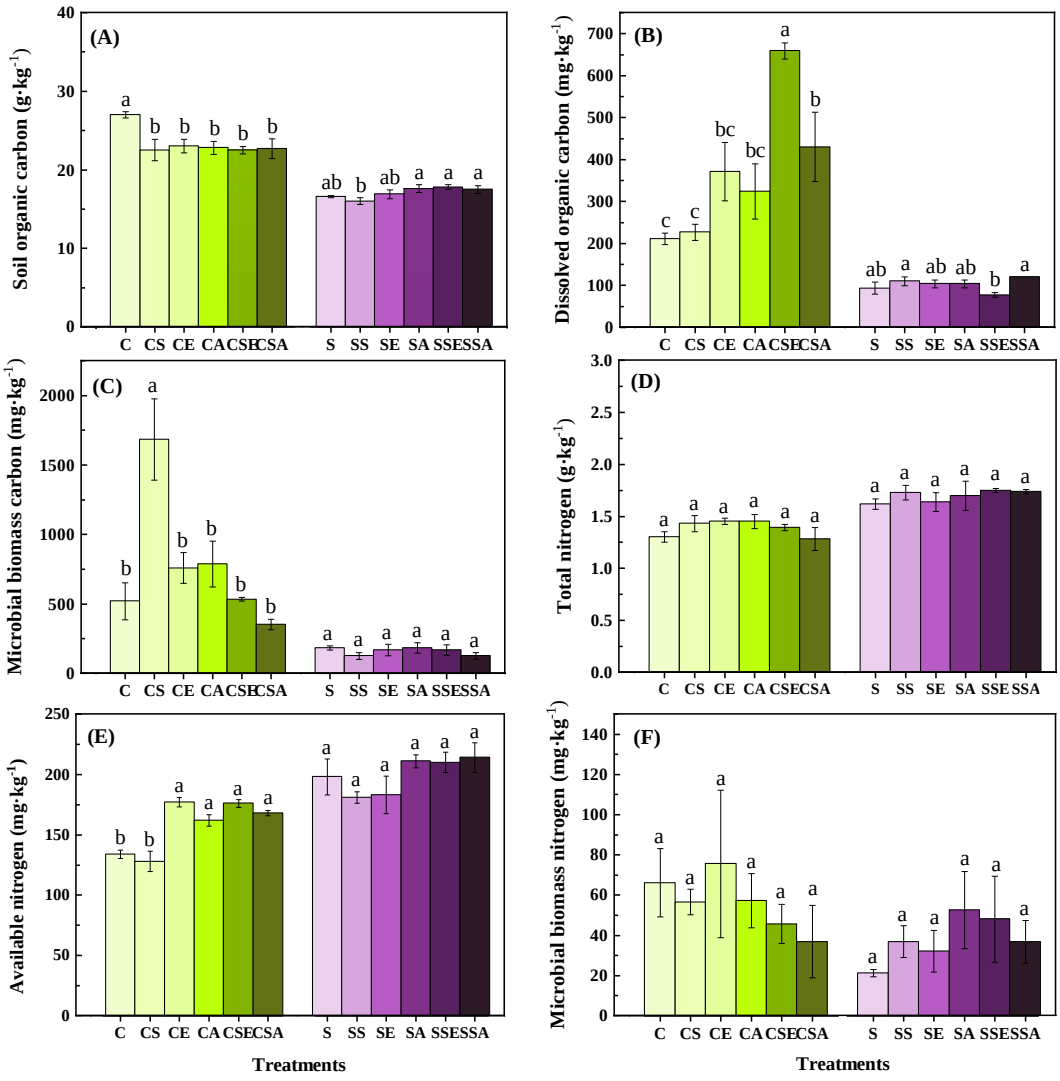


Fig. 1. Soil carbon and nitrogen form characteristics. Results are expressed as the mean value ± SE (n = 3). Different lowercase letters indicate sicanificantly differences ($P < 0.05$) among the treatments.

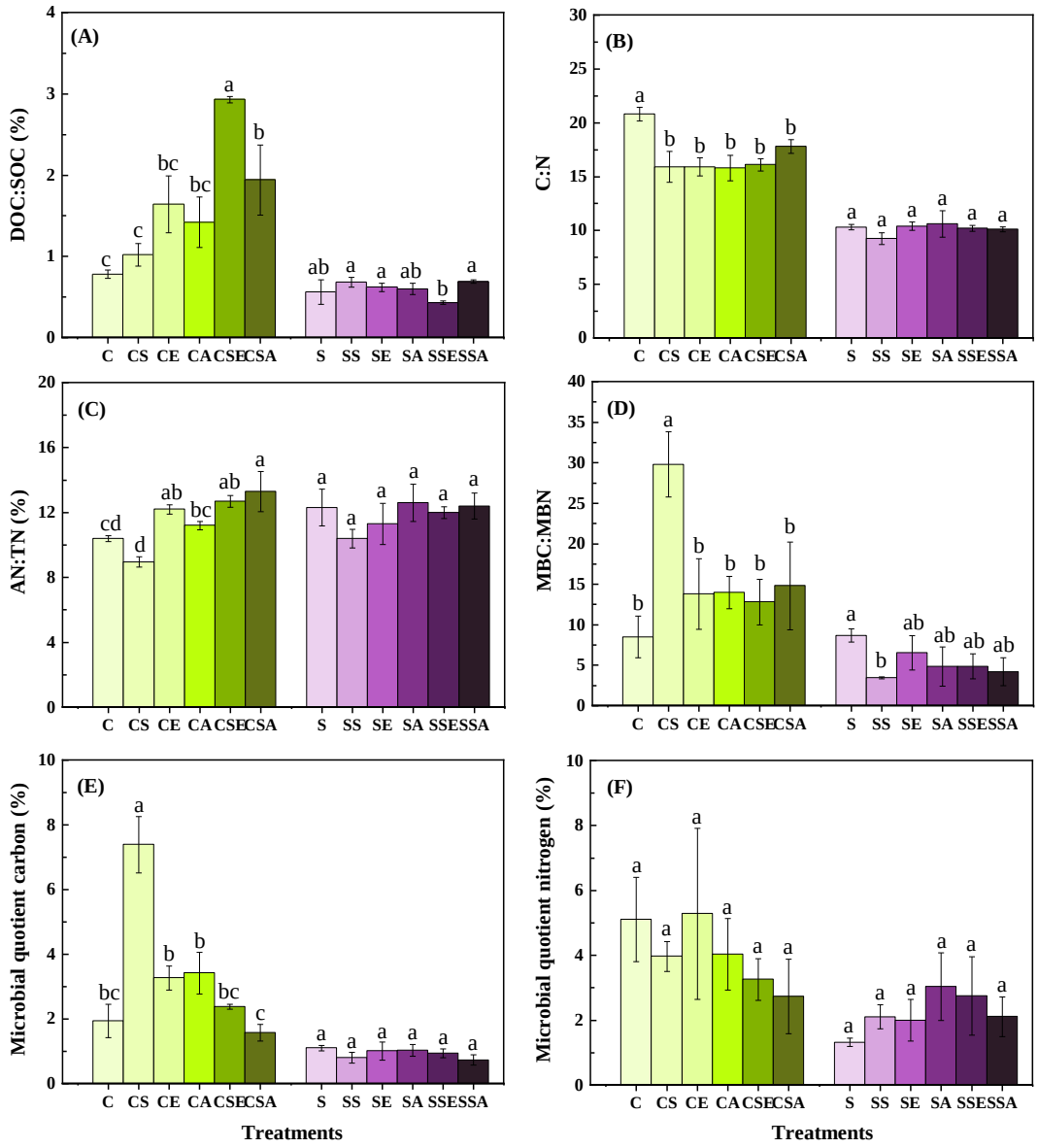


Fig. 2. The ratio of soil carbon and nitrogen forms. SOC, soil organic carbon; DOC, dissolved organic matter; TN, total nitrogen; AN, available nitrogen; C:N, soil organic carbon-to-total nitrogen ratio; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. Results are expressed as the mean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

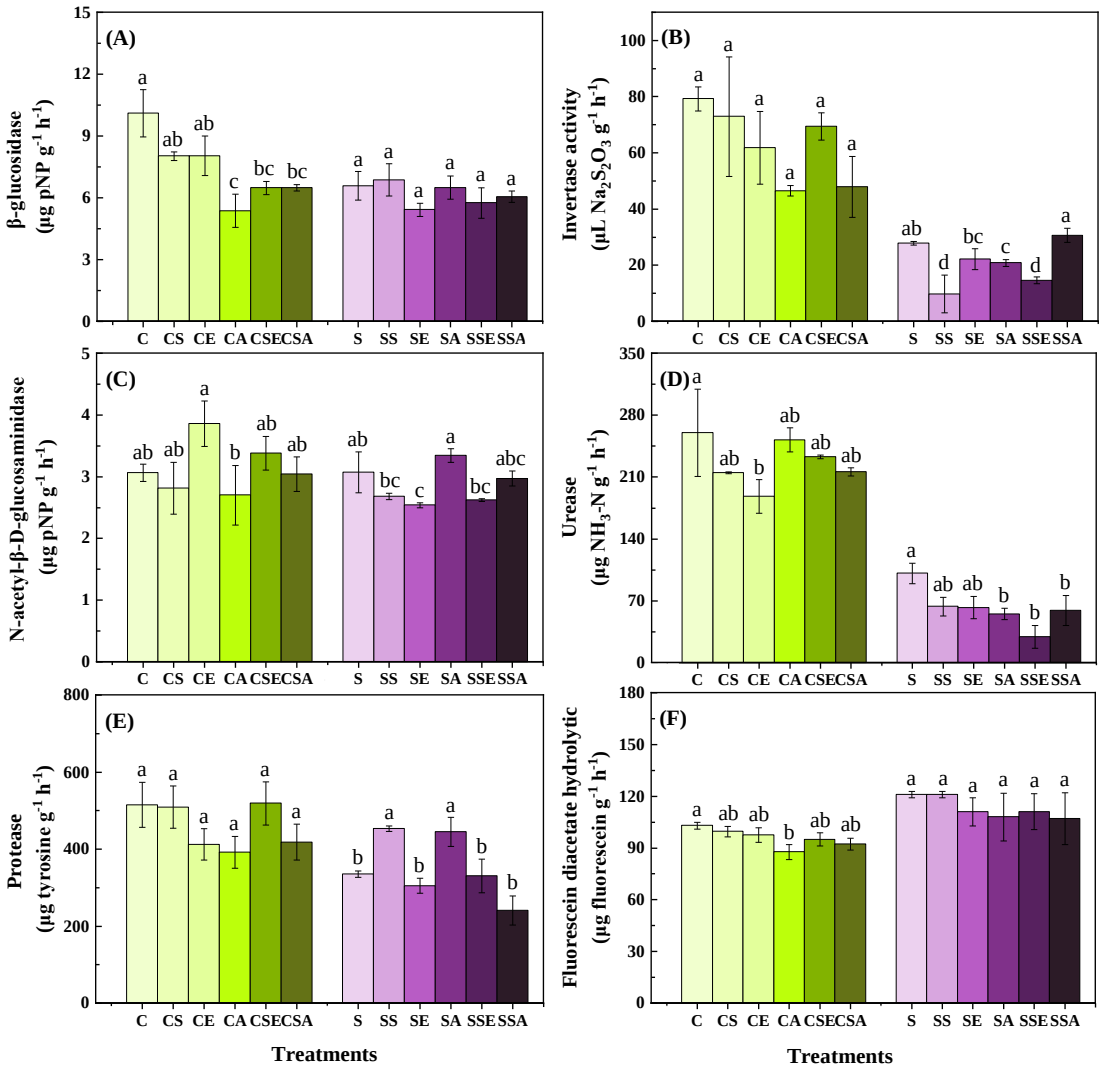


Fig. 3. Characterization of soil enzyme activity. Results are expressed as the mean value ± SE (n = 3). Different lowercase letters indicate significantly differences ($P < 0.05$) among the treatments.

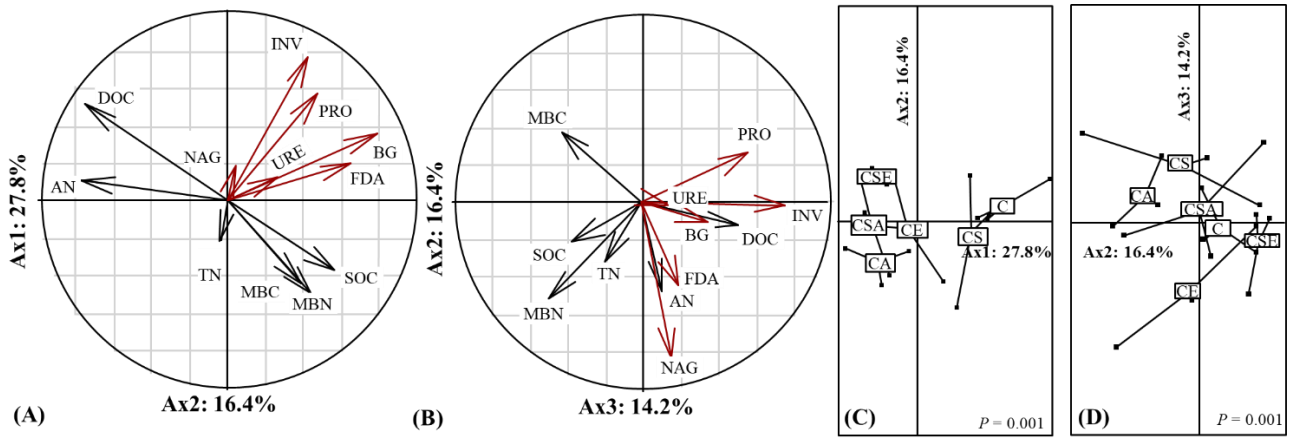


Fig. 4. Principal component analysis (PCA) of the effect of earthworms and *Sphingobacterium* sp. on soil carbon and nitrogen fractions and enzyme activities in non-contaminated soil in fractal F1, F2 and F3 planes.

(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.

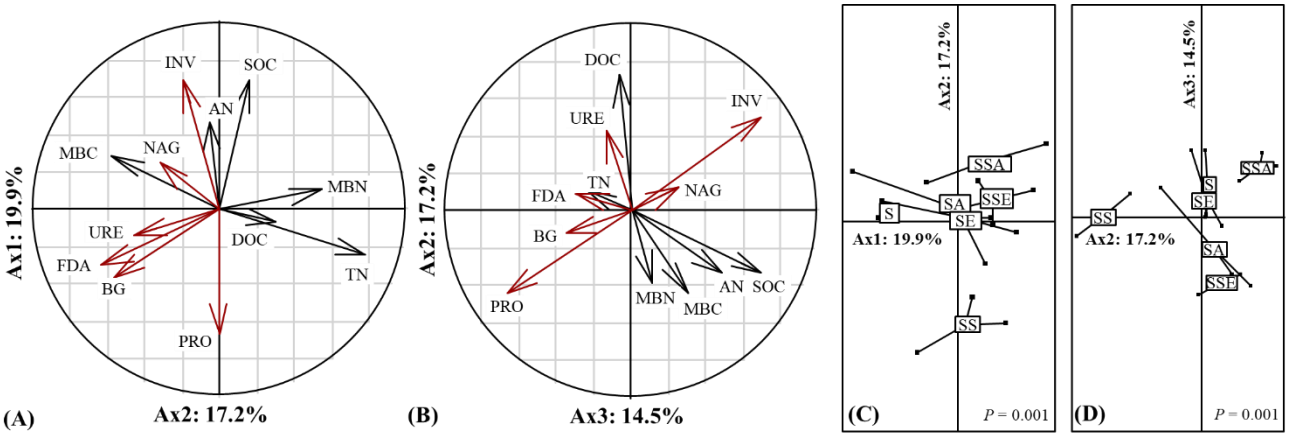


Fig. 5. Principal component analysis (PCA) of the effect of earthworms and *Sphingobacterium* sp. on soil carbon and nitrogen fractions and enzyme activities in Cd-contaminated soil in fractal F1, F2 and F3 planes.

(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.

Figure captions:

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 ± SE (n=3). Different letters indicate significant differences ($p < 0.05$) among the treatments.

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

*q*MC, microbial quotient carbon, microbial quotient nitrogen. Results are expressed as the mean value ± 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 ± SE (n=3).

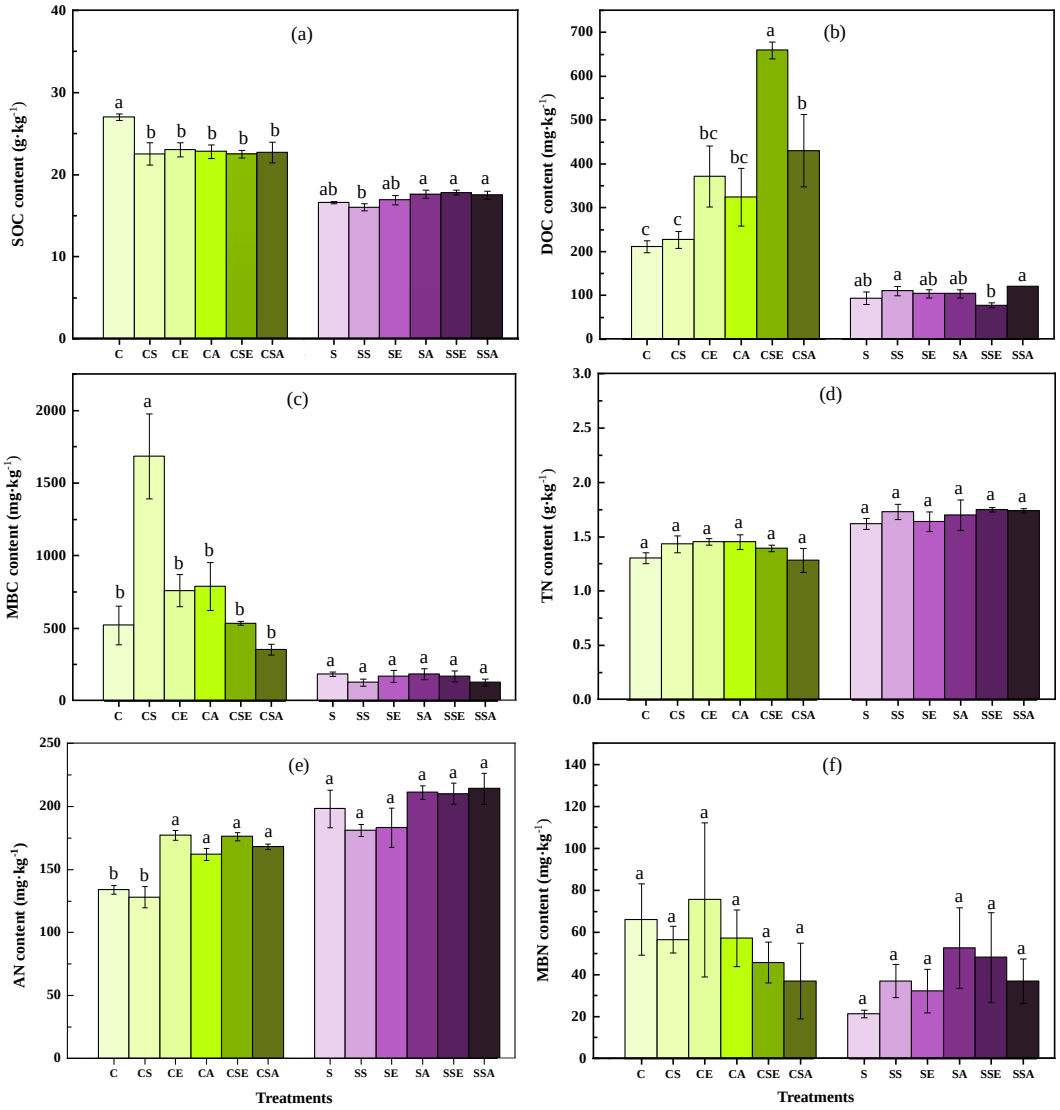
Fig. 4. Principal component analysis (PCA) of the effect of earthworms and Sphingobacterium sp. on soil carbon and nitrogen fractions and enzyme activities in clean and contaminated soil.

(a) correlation circles ofcarbon 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.



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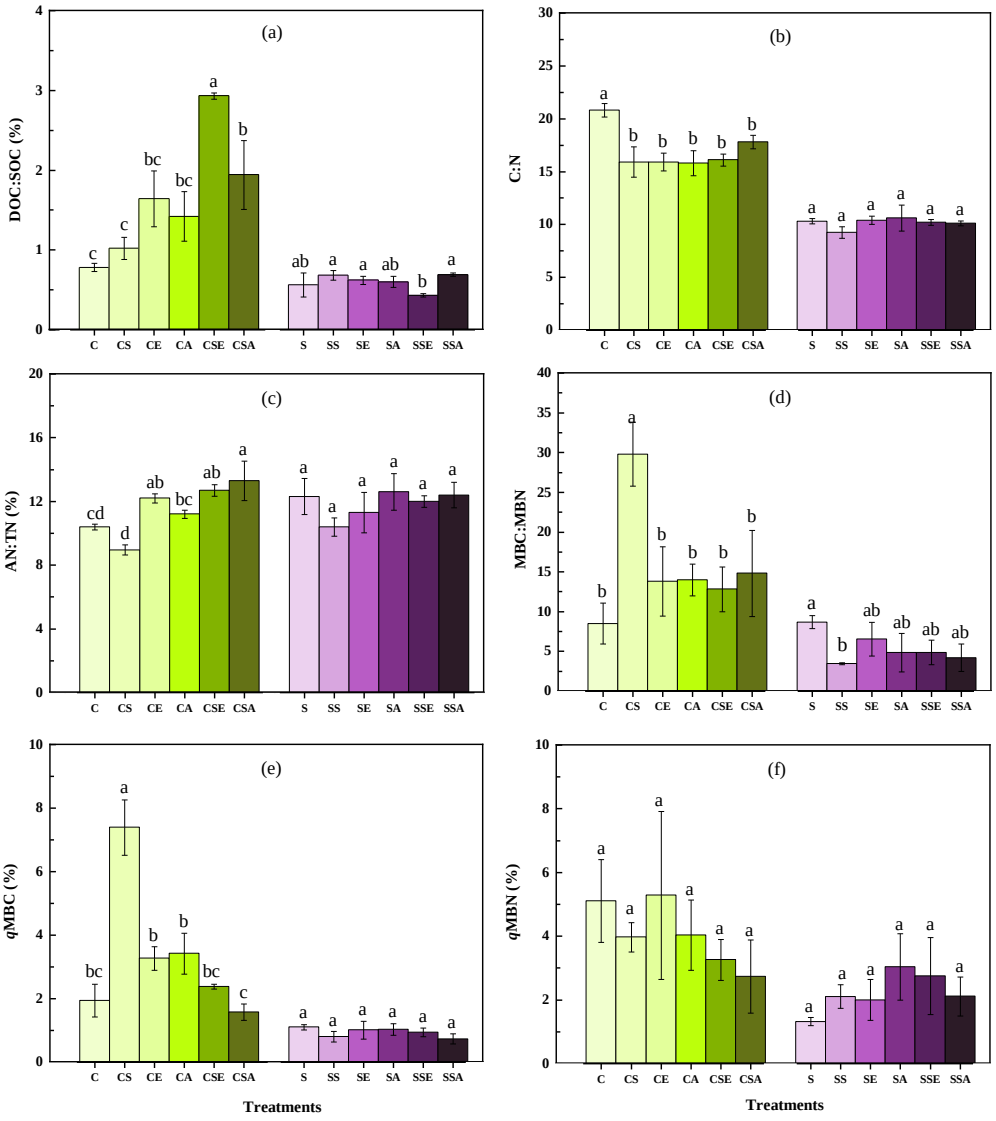

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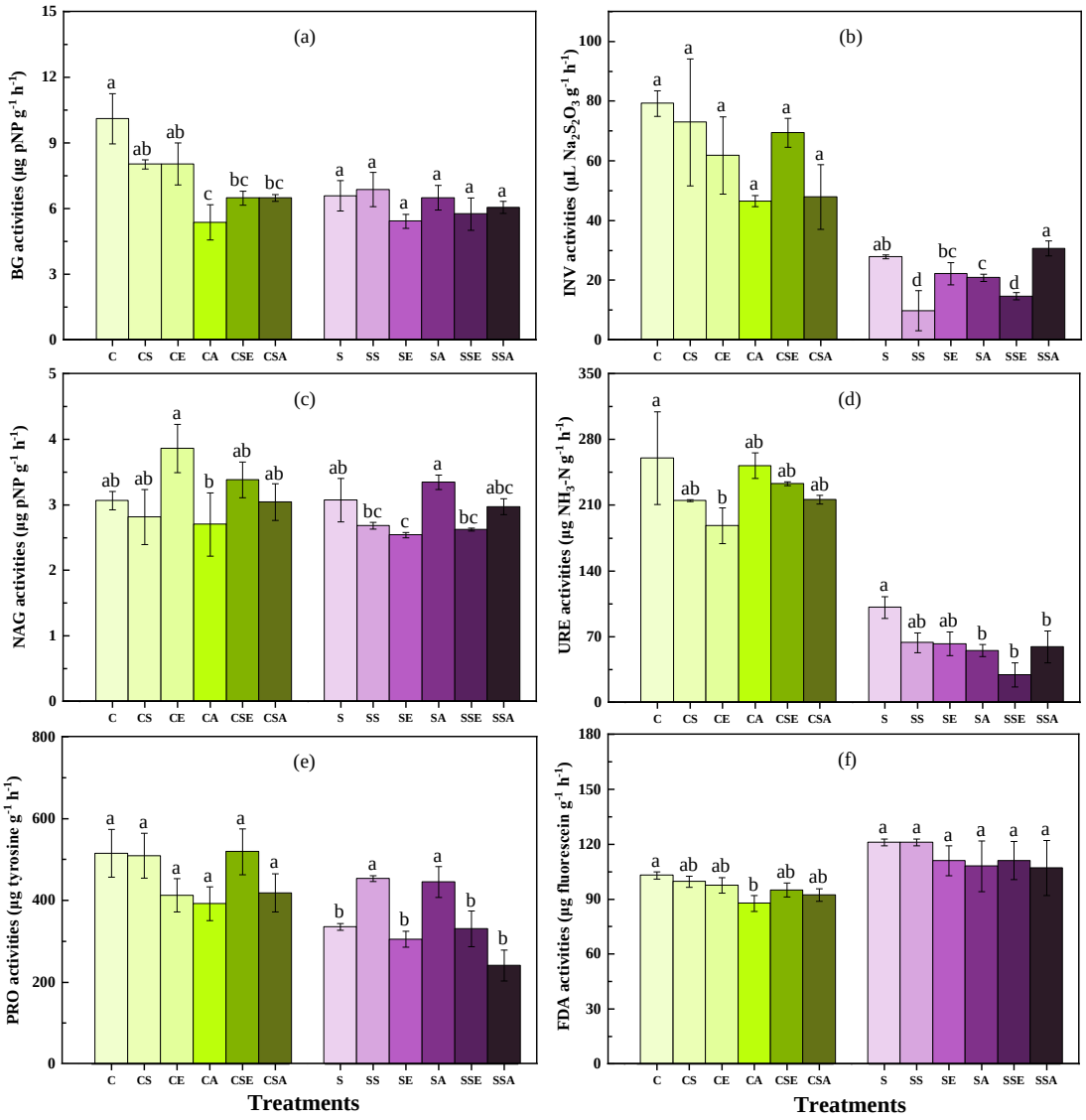

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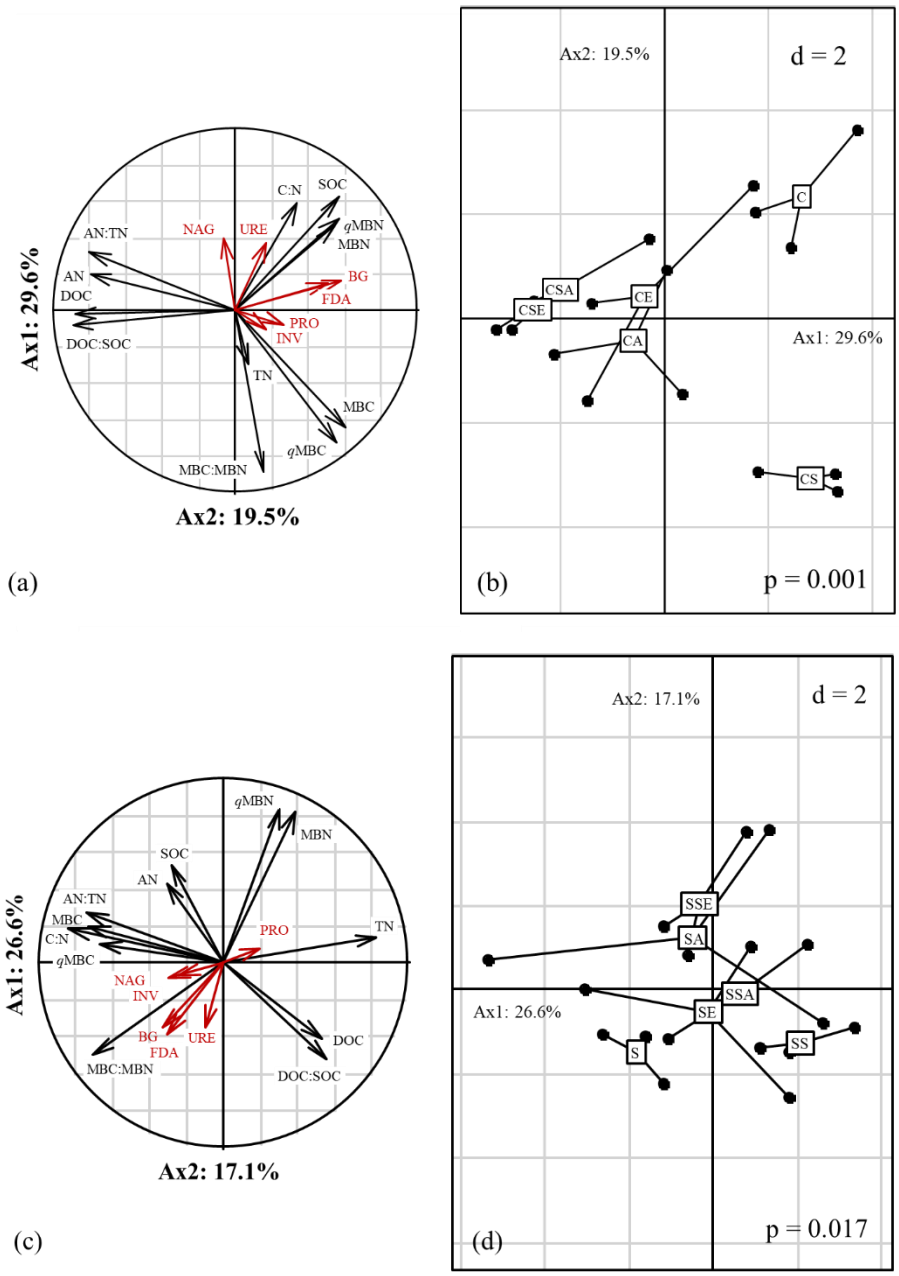

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(a) correlation circles ofcarbon and nitrogen fractions with enzymes in clean soil .

(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.

Table list:

Table1 Properties and Cd concentrations of soil.

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

Table1 Properties and Cd concentrations of soil

	pH	Clay (%)	SOC ($g \cdot kg^{-1}$)	TN ($g \cdot kg^{-1}$)	C:N	Cd ($mg \cdot kg^{-1}$)	DTPA-Cd ($mg \cdot kg^{-1}$)
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.fetid*a 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:

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