Biochar addition regulates soil and earthworm gut microbiome and multifunctionality

Jin, Bing Jie; Liu, Xi-Peng; Roux, Xavier Le; Bi, Qing Fang; Li, Ke Jie; Wu, Chun Yan; Sun, Cheng Liang; Zhu, Yong Guan; Lin, Xian Yong

Published in:
Soil Biology and Biochemistry

DOI:
10.1016/j.soilbio.2022.108810

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2022

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 16-09-2023
Biochar addition regulates soil and earthworm gut microbiome and multifunctionality

Bing-Jie Jin\textsuperscript{a}, Xi-Peng Liu\textsuperscript{b}, Xavier Le Roux\textsuperscript{c}, Qing-Fang Bi\textsuperscript{d}, Ke-Jie Li\textsuperscript{a}, Chun-Yan Wu\textsuperscript{e}, Cheng-Liang Sun\textsuperscript{a}, Yong-Guan Zhu\textsuperscript{a,f}, Xian-Yong Lin\textsuperscript{a,*}

\textsuperscript{a} MOE Key Laboratory of Environment Remediation and Ecological Health, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou, 310058, PR China
\textsuperscript{b} Microbial Ecology Cluster, Genomics Research in Ecology and Evolution in Nature (GREEN), Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, 9747 AG, Groningen, the Netherlands
\textsuperscript{c} Laboratoire d’Ecologie Microbienne LEM, INRAE UMR 1418, CNRS UMR 5557, VetAgroSup, Université Lyon 1, Université de Lyon, 16 rue Raphaël Dubois, 69100, Villeurbanne, France
\textsuperscript{d} Max Planck Institute for Biogeochemistry, Jena, 07745, Germany
\textsuperscript{e} Zhejiang Academy of Agricultural Sciences, Hangzhou, 310021, PR China
\textsuperscript{f} Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, 361021, PR China

\textbf{ARTICLE INFO}

Keywords:
- Biochar type
- Earthworm gut bacterial community
- Enzymatic activities
- Structural equation model

\textbf{ABSTRACT}

The earthworm gut bacterial community and enzyme activities play a key role in many soil ecosystem functions, but how the addition of different types of biochar (used to improve soil quality) regulates soil and earthworm gut bacterial communities and associated enzyme activities is scarcely understood. An experiment was conducted using soil microcosms and seven treatments: no biochar application as control, and two types of biochar (maize straw and cow dung biochar, hereafter SB and CB treatments, respectively) with three biochar application rates (20, 50, and 100 g/kg soil). Eight enzymatic activities related to C-, N- and P-cycling (used to compute a “multifunctionality” index) and the abundance and composition of bacterial communities were assessed in both the soil and earthworm guts. Our results show that earthworm gut content, characterized by lower bacterial abundance but higher enzymatic activities than soil, was strongly influenced by biochar addition. Increasing biochar application rates significantly increased the nutrient content, bacterial abundance, and multifunctionality in the earthworm guts. Owing to the difference in physicochemical properties between SB and CB, the increased gut multifunctionality was linked to the significantly modified gut bacterial community composition in CB treatments but not in SB treatments. Structural equation modeling further revealed that the changed gut multifunctionalities in both biochar treatments were mostly related to the soil and gut physicochemical properties, especially in CB treatments, while the impacts of gut microbial variables on soil multifunctionality were higher in the SB than CB treatments. These results demonstrate that the influence of biochar amendment on earthworm gut microbiome and multifunctionality depends on the type of biochar, which also regulates the contributions of earthworm gut biochemical processes to soil ecosystem functions.

1. Introduction

Earthworms are often beneficial for soil nutrient cycling and crop growth since they regulate important soil (microbial) processes such as the cycling of carbon, C, nitrogen, N, and phosphorus, P (Blouin et al., 2013; Hoang et al., 2016; Medina-Sauza et al., 2019). Growing evidence suggests that many ecosystem services provided by earthworms, such as accelerated decomposition of organic matter and increased bioremediation of pollutants, are largely related to biochemical processes operated specifically or at particularly high levels in their gut (Wang et al., 2019, 2022; Sun et al., 2020; Zhu et al., 2021). The earthworm gut is indeed a neutral, anaerobic environment characterized by higher carbon and nutrient resource availability and higher enzymatic activities than soil (Drake and Horn, 2007). It acts as a microbial reactor with greater metabolic capacity than that of soil (Zhang et al., 2022). For example, many soil microorganisms contributing to soil...
Soil Biology and Biochemistry 173 (2022) 108810

organic matter decomposition and nutrient cycling can be stimulated in earthworm guts (Hu et al., 2020). These include N-fixing and P-solubilizing bacteria (e.g., *Serratia* and *Bacillus*) (Hussain et al., 2016), anammox bacteria (e.g., *Brocadia* and *Kuenenia*) (Li et al., 2021a), glucose fermenters (e.g., *Aeromonadaceae*, *Enterobacteriaceae* and *Clostridiae*) (Wüst et al., 2011; Meier et al., 2018), and denitrifiers (e.g., *Flavobacterium*, *Pseudomonas* and *Dechloromonas*) (Drake and Horn, 2007). In addition, it has been reported that the overall selection of the earthworm gut microbiome explains that some activities such as C-mineralization in the soil can be two to three-fold higher after transiting the earthworm gut (Aball et al., 2017).

The assembly of the earthworm gut microbiome is actually determined by host selection processes in relation to the environmental conditions prescribed by the surrounding soil (Thakuria et al., 2010) and by organic inputs, such as agricultural wastes (such as dwarf shrubs, domestic sludge, and grass litter) (Knapp et al., 2009; Hu et al., 2020) and industrial wastes (such as fermented tannery waste) (Ravindran et al., 2015). These changes in the composition of the earthworm gut microbiome may in turn influence the ecological functions it ensures, particularly key enzymatic activities linked to soil nutrient cycling and important for soil fertility (Bastida et al., 2016; Loepmann et al., 2016). This role of earthworms might be particularly important in the context of practices such as biochar addition to the soil which is widely used to improve soil quality and fertility (Atkinson et al., 2010; Lehmann et al., 2011). For instance, Sanchez-Hernandez (2018) revealed that in soils with earthworms exposed to biochar addition, the hydrolytic activities on the biochar particles were nearly eight times higher compared with the biochar particles in earthworm-free soils. This suggests that the microbiome of the earthworm gut and the associated biochemical processes may have a great potential to optimize the beneficial effects of biochar for soil quality and bioremediation. However, previous studies on the responses of the microbial communities or enzymatic activities following biochar addition have focused on soils with or without earthworms. To what extent ingested biochar particles can influence the earthworm gut microbiome and the microbial functions it ensures is largely unknown. In addition, different types of biochar produced from different materials (e.g., maize straw, manure, and compost) usually possess different physicochemical properties, such as nutrient content, surface area, and pore size (Jin et al., 2016; Khadem and Raiesi, 2017; Zheng et al., 2019a). These parameters have been proved to be the crucial determinants of the biochar-induced effects on soil microbial communities and enzymatic activities (Ameloot et al., 2013; Gul et al., 2015; Lehmann et al., 2011; Pandey et al., 2020; Ji et al., 2022). However, we still need to understand how the addition of different types of biochar influences the earthworm gut microbiome as well as soil and gut multifunctionality. Multifunctionality refers here to a panel of enzymatic activities viewed as proxies of functions allowed by different kinds of microbial communities, i.e. an approach that has been used in many previous studies (Luo et al., 2018; Cui et al., 2020a; Li et al., 2021b).

In this study, we explored to what extent two types of biochar (maize straw and cow dung biochar, hereafter SB and CB, respectively) applied at three levels can affect the bacterial communities and multifunctionality of soil and earthworm gut content. Bacterial communities were characterized in terms of total abundance (qPCR targeting the 16S rRNA gene), composition (high-throughput sequencing of 16S rRNA), and network complexity (network analysis applied to bacterial OTUs). Multifunctionality was assessed based on biochemical assays for eight enzymatic activities related to C-, N- and P-cycling measured for both soil and earthworm guts, while the physicochemical characteristics of soil and gut content were also measured. The specific objectives of this study were:

1. To investigate to what extent different types of biochar addition influence the soil and earthworm gut microbial community and multifunctionality. We hypothesized that biochar addition would significantly influence the microbiome and increase multifunctionality in both soil and earthworm gut, and that such effects would be higher in the earthworm gut ‘bioreactor’ characterized by higher availability of carbon and nutrient sources (Jin et al., 2022; Zhang et al., 2022). However, different types of biochar might have different effects; and
2. To reveal –by using structural equation modeling– whether the changes in soil and earthworm gut multifunctionality induced by the biochar treatments were mainly related to modified soil/gut physicochemical characteristics or also modified bacterial diversities, total abundances and community compositions. Given the possible high level of functional redundancy in microbial communities (Wertz et al., 2007), we hypothesized that the biochar-induced changes in earthworm gut multifunctionality would mostly be due to modified gut biochemical characteristics, and possibly total bacterial abundances, while diversity/composition would be less important. We also assumed that soil multifunctionality could be indirectly related to gut multifunctionality since earthworms are known to influence soil functions due to the high volume of soil they process in their gut (Lavelle and Spain, 2001).

2. Materials and methods
2.1. Soil, biochar and earthworm materials used

The soil used in this study was sampled from the 0–20 cm soil layer in an orchard located in Fuyang (30° 04′ N, 119° 57′ E), Zhejiang Province, China. After removing stones and visible residues, the collected soils were air-dried, homogenized and sieved through a 2 mm mesh. Soil total C (TC) was 16.3 g/kg, total organic carbon (TOC) 2.58%, total N (TN) 1.64 g/kg, total P (TP) 560.40 mg kg⁻¹, available P (Psal) 70.28 mg kg⁻¹, pH (watersoil ratio of 5:1) 5.91, and electrical conductivity (EC) (watersoil of 5:1) 69.64 μS cm⁻¹.

The two different types of biochar used in this experiment were produced from maize straw (SB) and cow dung (CB), and pyrolyzed at 600 °C for 48h. The physicochemical properties of each biochar, including their elemental content, surface area, and pore size, are presented in Table S1. Compared with the SB, CB possesses relatively higher nutrient content (such as TC, TN, TP, and many other trace elements) but a lower C/N ratio, and is characterized by larger surface area but smaller pore size and particle size. Scanning electron microscopy images for each biochar are provided in Fig. S1, and SB possesses more clear vascular structures than CB.

The earthworms were purchased from an earthworm company in Huaiian City and were identified as *Lumbricida* sp. GL-2010 using cytochrome oxidase I barcode gene sequencing (Pass et al., 2015). To minimize the effect of the original earthworm gut microbiota, all earthworms were acclimated for two weeks in untreated soil under laboratory conditions before the experiment started. Cow dung was added as a food source (4–6 g dry cow dung kg⁻¹ dry weight soil) by thoroughly mixing the soil with dry cow dung powder.

2.2. Experimental design and recovery of gut and soil material

A microcosm experiment was designed to investigate the effects of the two types of biochar (SB and CB) on soil and earthworm gut bacterial communities and multifunctionality. Each soil microcosm was constructed using a polyethylene plastic box containing 1200 g of dry soil. Six biochar treatments were used, i.e. two types of biochar, each added at a dose of 20 g/kg soil (SB20 and CB20), 50 g/kg soil (SB50 and CB50) or 100 g/kg soil (SB100 and CB100). A seventh, control treatment without biochar addition (CK) was used. Five replicates were used for each treatment, resulting in a total of 35 microcosms.

An equal volume of sterilized water was added to the soil in each box to maintain the same soil humidity (60% of soil water holding capacity), and all microcosms were preincubated for two weeks to activate soil
microorganisms and stabilize the soil physicochemical properties before the experiment started. *Lumbricina* sp. individuals were rinsed with sterilized water to remove adhering soil. According to the Organization for Economic Cooperation and Development (OECD) (No. 222) protocols (OECD, 2016), ten adult earthworms of similar sizes and health conditions were introduced into each box (after acclimation). Each box was covered with a lid to prevent escape, and holes were drilled into the lid for aeration. All boxes were kept for 28 d in an incubator at 20 °C and relative humidity of 70%. During the entire incubation period, dry cow dung powder was added (addition of 5 g of powder every 3 days) to feed the earthworms, and soil humidity was maintained at 30% by regular addition of sterilized water to compensate for evaporation. During the experiment, 2 dead earthworms (i.e. one individual in 2 of the 35 boxes) were observed on the soil surface and removed immediately without any disturbance to the soil.

At the end of the incubation, all earthworms were collected immediately and euthanized using absolute ethyl alcohol. The adhering soil particles and mucus on the earthworm body were removed by gentle shaking and by rinsing five times in sterilized phosphate buffer solution. Earthworm individuals were dissected immediately with sterile scissors under sterile conditions. For each microcosm, the gut contents of 7 earthworm individuals were collected and mixed for quantification of physicochemical properties and multifunctionality, and the 3 other individuals were used for molecular biology assays. All earthworm gut content samples (composite samples among 7 or 3 indivudal) were freeze-dried immediately after dissection and then sieved through a 100-mesh sieve (0.15 mm) to remove earthworm body tissue as far as possible. To keep the enzymatic activities of soil and earthworm gut contents comparable, all the soil samples used for enzymatic activity assays were also freeze-dried and sieved under the same conditions.

2.3. Physicochemical properties of soil and gut content

The pH and EC values in the soil and earthworm gut were determined in a 1:5 (soil/water ratio, w:v) suspension using a pH- and conductivity meter, respectively. TC and TN contents in the soil and earthworm gut were measured by dry combustion using an elemental analyzer (Vario max CN, Elementar, Hanau, Germany). The C/N ratio was calculated by dividing TC by TN. TOC was determined using the external-heat potassium dichromate oxidation-colorimetric method. TP and PO4 were measured using the p-nitrophenol (PNP)-linked model substrate method (Sanchez-Hernandez, 2018), and the enzymatic activity was estimated by calculating the release of PNP. Urease activity was measured by using urea as a substrate (0.2 g⁻¹ soil or gut content) and was estimated by calculating the final concentration of NH4 after 24 h. Protease activity was measured by using gelatin as a substrate (0.05 g⁻¹ soil or gut content) and estimated by calculating the final concentration of glycine after 24 h. Although multifunctionality could also have included variables linked to available nutrients, we did not include these in the multifunctionality index here because the NH4 and NO3 contents in earthworm gut could hardly be assessed due to the possible influence of earthworm tissues as explained above. Hence, the multifunctionality index was only based on enzymatic activities, as in many other studies (Delgado-Baquerizo et al., 2017; Luo et al., 2018; Cui et al., 2020a; Li et al., 2021b).

All enzyme activities were standardized by transforming their values into Z-scores (Manning et al., 2018) before calculating the C-, N- and P-cycling and multifunctionality indices. We used an equation to normalize the activity of enzymes with functions involved in the same cycle, in order to characterize the strength of the soil and earthworm gut C-, N-, and P-cycling functions (García-Ruiz et al., 2006). For example, the N-cycling index was calculated as follows:

\[ N - \text{cycling index} = \sqrt{\text{NAG} \times \text{LAP} \times \text{UA} \times \text{PRO}} \]

All of the standardized enzymatic activities were averaged to obtain the multifunctionality index (Wagg et al., 2014).

2.5. DNA extraction from earthworm gut and soil

About 0.5 g of gut content (i.e. composite sample from the 3 earthworm individuals) was transferred into a 2 ml Eppendorf tube with 0.96 mL sterile phosphate buffer solution and stored at −20 °C before DNA extraction.

We used the FastDNA Spin Kit for Soil (MP Biomedical, Santa Ana, CA, USA) to extract soil and earthworm gut DNA according to the manufacturer’s instructions. A spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Waltham, MA) and a Qubit 4.0 fluorometer (Thermo Fisher, Singapore) were used to determine the quality and concentration of the extracted DNA. All DNA samples were stored at −20 °C until use.

2.6. qPCR and high-throughput sequencing of 16S rRNA gene

To calculate the total abundance of soil and earthworm gut bacteria, qPCR was performed on all DNA samples to determine the copy number of the 16S rRNA gene using the StepOnePlus™ Real-Time PCR system (Applied Biosystems, Singapore). The 20 μL qPCR reaction system contained 10 μL of 2 X SYBR premix Ex Taq, 2 μL of DNA, 0.4 μL of 515F primer, 0.4 μL of 907R primer, 0.8 μL of BSA, and 6.4 μL of sterilized water. Triplicates were used for each sample. The qPCR conditions were as follows: 95 °C for 15 min, followed by 40 cycles at 95 °C for 1 min, 53 °C for 30 s, and 72 °C for 1 min. The standard curve was obtained by using linearized plasmids with insertions of 16S rRNA gene fragments, with standards ranging from 10^3 to 10^9 gene copies μL⁻¹. Amplification efficiency ranged from 97% to 100%, with R² > 0.99.

DNA samples extracted from all soil and earthworm guts (i.e., 35 microcosms x 2 compartments = 70 samples) were used to amplify the hypervariable V4–V5 region of the 16S rDNA gene using 515F and 907R bacterial 16S rRNA primers (Zhou et al., 2011). The PCR protocol was as follows: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. All PCR products were purified using a Universal DNA Purification Kit (Tiangen, China) and quantified using the Quant-IT PicoGreen double-stranded DNA (dsDNA) assay kit (Invitrogen, USA). Seventy barcoded samples were pooled in equal concentrations and sequenced using the Illumina MiSeq2500 platform (Majorbio, Shanghai, China). The obtained sequences were dereplicated before clustering into OTUs at 97% identity using USEARCH that included an “on-the-fly” chimera detection algorithm (Edgar, 2013). All of the low-quality or ambiguous reads were trimmed to guarantee the quality of the
downstream analysis. All OTU sequences were filtered for chimeras once more by comparing with the GREENGEnES (DeSantis et al., 2006) databases using UCHIME (Edgar et al., 2011). The remaining sequences were tested for the presence of ribosomal signatures using MetaXa2 (Bengtsson-Palme et al., 2015) and subsequently mapped against all quality-filtered reads using USEARCH. A total of 2,480,402 high-quality sequences were obtained after assembling and quality filtering, and the sequencing number per sample ranged from 18,414 to 56,273. A Bayesian classifier (Wang et al., 2007) was used for taxonomic assignment of the OTUs, with a minimum bootstrap support of 60% implemented in MOTHUR (Schloss et al., 2009) by querying the bacterial reads against the GREENGEnES reference databases. The community dissimilarity was calculated by using the normalized percent frequency based on the rarified OTU table. Alpha diversity indices including Shannon diversity, Chao1 richness, and Observed OTU were also calculated using MOTHUR based on the rarified sequence data.

2.7. Bacterial co-occurrence network construction

We analyzed bacterial co-occurrence networks to get insight into the organization of the community and because highly connected nodes could be considered as the key taxa that maintain the stability of the network and occupy crucial ecological niches (Martín Gonzalez et al., 2010). Network computation should be made with a sufficiently high number of replicates, typically >8 (http://ieg2.ou.edu/MENA). To ensure the reliability of the correlation calculation for the assessment of networks of bacterial OTUs, OTU tables containing 10 replicates were created by integrating the two treatments with the highest application rates for each biochar type (i.e. SB50 and SB100; and CB50 and CB100), with the results for the CK treatment being based on only 5 replicates and hence having less robustness. Network analysis was performed using the pMENs analysis pipeline (http://ieg2.ou.edu/MENA). Only OTUs with relative abundance >0.01% and present in at least 8 of the 10 samples (or 4 of the 5) were selected for correlation calculation to minimize potential spurious correlations induced by the rare taxa. Random matrix theory was used to automatically determine the optimum threshold for network construction (Deng et al., 2012), and the co-occurrence networks were constructed with the same similarity threshold for the Spearman rank correlation matrix (0.90). The network graphs were constructed using the Gephi 0.9.2 software, and the topological properties of each network were calculated using the pMENs analysis pipeline and Gephi 0.9.2 software.

2.8. Statistical analyses

All one-way analysis of variance (ANOVA) were performed using Tukey tests with the SPSS (V20.0, IBM, USA) software. Permutational multivariate analysis of variance (ADONIS) and analysis of similarity (ANOSIM) tests based on Bray-Curtis distances were used to test the differences in the bacterial community structure between different treatments. Mantel tests were used to evaluate the effects of physicochemical properties on the bacterial communities in the soil or earthworm gut. All analyses were performed in R using the vegan package (Oksanen et al., 2016). Box plots and histograms were drawn using the OriginPro 2018 software. The relationships among physicochemical properties were evaluated by Pearson correlation analysis. All variables used in these analyses were standardized by transforming their values into Z-scores before the analysis. To visualize the results, plots were drawn using vegan, heatmap, psy, stringr, permute, lattice, dplyr, ggcorr, and ggplot2 packages in R.

Structural equation models (SEMs) were constructed using the maximum likelihood estimation method with the AMOS 21 software (SPSS Inc., Chicago, USA). The comparative fit index (CFI) (requirement: > 0.90), P-value (>0.05), and root mean square error of approximation (RMSEA) (<0.05) were used to test the overall goodness of the model fit.

3. Results

3.1. Physicochemical characteristics of soil and earthworm gut content

A majority of all the soil physicochemical properties were significantly influenced by the application of biochar, except for the soil TP and Posl in the SB treatments, and the application of CB had greater effects on the soil physicochemical characteristics than that of SB (Table S2). Most of the physicochemical characteristics of the earthworm gut content were significantly higher than those of the soil, except for the C/N ratio (Table 1; Table S2; Fig. S2).

Pearson correlation analysis demonstrated that for the CB treatments, all the soil and earthworm gut physicochemical properties were significantly correlated with the biochar application rates (Fig. 3). In contrast, only some of the soil and earthworm gut physicochemical properties were significantly correlated with the SB application rates (Fig. 3).

3.2. Enzymatic activities and multifunctionality of soil and earthworm gut

A majority of the individual enzymatic activities as well as the C-, N-, and P-cycling indices and the overall multifunctionality index, were significantly higher in earthworm guts than in soil (Fig. 2; Fig. S3). In addition, the higher application rates of CB had greater effects on gut enzymatic activities, which was not the case for SB treatments (Table 2; Fig. 2). However, the responses of enzymatic activities to biochar application differed between earthworm gut and soil (Table 2 and Table S3). BG, NAG, and LAP activities in the earthworm gut decreased when low amounts of biochar were added, but increased for the highest biochar application rates. In contrast, the activities of these enzymes in soil significantly decreased or remained unchanged in response to biochar addition. The C-, N- and P-cycling indices and the overall multifunctionality index showed a significant difference among the treatments.

### Table 1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>pH</th>
<th>C/N</th>
<th>EC (µS/cm)</th>
<th>TP (mg/kg)</th>
<th>Posl (mg/kg)</th>
<th>TC (%)</th>
<th>TN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>6.73</td>
<td>5.28</td>
<td>1801 ± 4102</td>
<td>2254 ± 59</td>
<td>670 ± 11</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>SB20</td>
<td>6.66</td>
<td>5.00</td>
<td>1701 ± 71</td>
<td>2102 ± 90d</td>
<td>634 ± 57</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>SB50</td>
<td>6.79</td>
<td>6.20</td>
<td>1626 ± 67c</td>
<td>2240 ± 77</td>
<td>632 ± 47</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>SB100</td>
<td>6.70</td>
<td>6.55</td>
<td>1954 ± 55c</td>
<td>2469 ± 145</td>
<td>668 ± 48</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>CR20</td>
<td>6.75</td>
<td>5.88</td>
<td>1602 ± 145</td>
<td>2178 ± 89</td>
<td>689 ± 69</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>CR50</td>
<td>6.64</td>
<td>6.62</td>
<td>1670 ± 337</td>
<td>2329 ± 129</td>
<td>743 ± 37</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>CR100</td>
<td>6.64</td>
<td>7.32</td>
<td>2037 ± 132</td>
<td>2642 ± 147</td>
<td>814 ± 41</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

Note: Values followed by the same letter within the same column are not significantly different at P < 0.05.
multifunctionality index in earthworm guts tended to decrease when lower amounts of biochar were applied but significantly increased for the highest biochar application rates, especially in the CB treatments (Fig. 2).

### 3.3. Bacterial abundance, community diversity and structure in the soil and earthworm guts

Considering all treatments, the 16S rRNA gene copy number was significantly higher in soil than in earthworm guts (Fig. 1c). Soil bacterial abundance did not significantly respond to biochar addition (Fig. 1a), whereas the earthworm gut bacterial abundance was gradually

<table>
<thead>
<tr>
<th>Treatments</th>
<th>BG (mM(pNP)kg⁻¹h⁻¹)</th>
<th>NAG (mM(pNP)kg⁻¹h⁻¹)</th>
<th>LAP (mM(pNP)kg⁻¹h⁻¹)</th>
<th>ACP (mM(pNP)kg⁻¹h⁻¹)</th>
<th>ALP (mM(pNP)kg⁻¹h⁻¹)</th>
<th>PDE (mM(pNP)kg⁻¹h⁻¹)</th>
<th>UA (mg(Gly)kg⁻¹h⁻¹)</th>
<th>PRO (mg(Gly)kg⁻¹h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>17.61 ± 0.41 b</td>
<td>13.39 ± 0.25 b</td>
<td>7.20 ± 0.13 cd</td>
<td>16.37 ± 2.89 a</td>
<td>16.20 ± 3.29 b</td>
<td>10.58 ± 0.81 bc</td>
<td>0.092 ± 0.016 b</td>
<td>2.65 ± 0.13 b</td>
</tr>
<tr>
<td>SB20</td>
<td>15.50 ± 0.57 cd</td>
<td>10.75 ± 0.15 c</td>
<td>6.34 ± 0.43 de</td>
<td>12.43 ± 1.59 a</td>
<td>13.61 ± 0.56 b</td>
<td>8.16 ± 0.55 c</td>
<td>0.050 ± 0.007 b</td>
<td>2.62 ± 0.07 b</td>
</tr>
<tr>
<td>SB50</td>
<td>15.86 ± 1.14 bcd</td>
<td>10.91 ± 1.35 c</td>
<td>7.74 ± 0.25 cd</td>
<td>12.57 ± 1.84 a</td>
<td>13.65 ± 0.84 b</td>
<td>9.23 ± 0.35 bc</td>
<td>0.042 ± 0.008 c</td>
<td>2.58 ± 0.11 bc</td>
</tr>
<tr>
<td>SB100</td>
<td>18.06 ± 0.872 b</td>
<td>13.67 ± 0.99 b</td>
<td>8.54 ± 0.73 bc</td>
<td>16.79 ± 2.33 a</td>
<td>17.75 ± 0.41 b</td>
<td>11.60 ± 0.78 b</td>
<td>0.026 ± 0.001 c</td>
<td>2.56 ± 0.01 ab</td>
</tr>
<tr>
<td>CB20</td>
<td>14.53 ± 1.06 d</td>
<td>10.91 ± 0.68 c</td>
<td>5.42 ± 0.43 e</td>
<td>12.81 ± 0.43 a</td>
<td>14.37 ± 1.08 b</td>
<td>10.10 ± 0.33 bc</td>
<td>0.074 ± 0.019 ab</td>
<td>2.43 ± 0.08 bc</td>
</tr>
<tr>
<td>CB50</td>
<td>21.06 ± 1.27 a</td>
<td>13.99 ± 0.73 ab</td>
<td>9.75 ± 1.09 ab</td>
<td>15.50 ± 2.08 a</td>
<td>18.03 ± 2.61 b</td>
<td>11.29 ± 0.60 ab</td>
<td>0.073 ± 0.001 ab</td>
<td>2.67 ± 0.05 ab</td>
</tr>
<tr>
<td>CB100</td>
<td>22.76 ± 0.16 a</td>
<td>16.05 ± 0.47 a</td>
<td>10.93 ± 0.63 a</td>
<td>13.93 ± 4.13 a</td>
<td>24.17 ± 2.60 a</td>
<td>15.68 ± 2.53 a</td>
<td>0.084 ± 0.011 a</td>
<td>2.33 ± 0.01 c</td>
</tr>
</tbody>
</table>

Table 2: Enzyme activities of earthworm gut contents under the different treatments. For each activity, different letters indicate a significant difference between the treatments at P < 0.05. BG: β-1,4-glucosidase; NAG: β-N acetyl-D-glucosaminidase; LAP: leucine-aminopeptidase; UA: urease; PRO: protease; ACP: acid phosphomonoesterase; ALP: alkaline phosphomonoesterase; PDE: phosphodiesterase.
increased with increasing SB or CB biochar application rates (Fig. 1b).

Compared with the biochar-free soil, the application of both types of biochar significantly increased the Shannon index, Chao1 index, and OTU number of soil bacterial communities ($P < 0.05$) (Figs. S5a, b, c). In contrast, the application of SB had no significant effect on earthworm gut bacterial diversity, whereas the application of CB significantly decreased earthworm gut bacterial diversity ($P < 0.01$) (Figs. S5d, e, f).

Based on the calculation of the compositional dissimilarity (Bray-Curtis index) between each biochar treatment and the control, the biochar-induced compositional variations in bacterial communities were significantly higher for earthworm gut than soil (Fig. 1e).

Furthermore, according to the results of ADONIS and ANOSIM analyses (Table 3), both soil and earthworm gut bacterial community structures were significantly influenced by biochar application. Increasing the application rates of CB significantly affected earthworm gut bacterial communities (ADONIS test: $R = 0.213$, $P = 0.009$), whereas no significant impact of biochar amount was detected for the SB treatments (ADONIS: $R = 0.127$, $P = 0.054$). In addition, according to the R-values of ADONIS analyses, the dissimilarities between soil and earthworm gut bacterial communities were higher in treatments with biochar than without, especially for the CB treatments (R = 0.44 in the CK treatments, R = 0.50 in the SB treatments, and R = 0.74 in the CB treatments).

The taxonomic composition of soil and earthworm gut bacterial communities at the family and at the phylum levels are shown in Fig. 1d and Fig. S4, respectively. The application of biochar had a greater impact on bacterial community structure for the earthworm gut than the soil, especially when analyzed at the family level (Fig. 1d). For example, the relative abundance of Bacillaceae in the earthworm gut strongly increased when biochar was applied, from 3.7% in the CK treatment to 40.6% in the CB100 treatment. However, such drastic changes were not detected in the soil. Moreover, the relative abundances of many bacterial families in the earthworm gut were influenced by biochar application rates. The relative abundances of Xanthomonadaceae, Aeromonadaceae and Flavobacteriaceae significantly decreased with increasing SB application rates, while the relative abundances of Burkholderiaceae, Enterobacteriaceae, Planococcaceae and Paenibacillaceae gradually increased. Increased CB application rates resulted in a significant decrease in the relative abundance of Burkholderiaceae and Thermoactinomycetaceae, and a significant increase for Planococcaceae.

3.4. Bacterial co-occurrence networks

In both SB and CB treatments, the earthworm gut bacterial networks contained a higher number of edges among nodes and had higher proportions of negative links, and were more complex with higher connectedness than the soil networks (Fig. 4). In addition, many topological properties of the earthworm gut networks such as the proportions of negative links, average degree, average clustering coefficient, density, and transitivity, were higher in the SB than CB treatments (Table S4).

The taxonomic affiliation of highly connected nodes within the networks significantly differed between soil and earthworm gut. In earthworm gut networks, most of the highly connected nodes belonged to Alphaproteobacteria, Bacilli and Actinobacteria, while the application of SB significantly increased the degrees of the nodes belonging to Actinobacteria. In contrast, the application of CB increased the degrees of the nodes belonging to Alphaproteobacteria (Fig. 4b). In addition, in the SB treatments and for the earthworm gut, the biochar-induced changes in the overall gut bacterial community structures were strongly correlated with the changes in the relative abundance of Actinobacteria (Fig. S7).

3.5. Abiotic and biotic determinants of biochar-induced changes in multifunctionality

According to Pearson’s correlation analysis applied to gut samples across all treatments, the gut multifunctionality index was significantly
and positively correlated with a majority of the earthworm gut physicochemical properties, except pH (Fig. S6). When focusing on the CB treatments, bacterial community multifunctionality was significantly correlated with a high number of physicochemical properties, especially for the earthworm gut bacterial communities (Fig. 3a and b). For both biochar treatments, the changes in soil multifunctionality were significantly correlated with the compositional shifts in the soil bacterial community represented by the Bray-Curtis dissimilarity index (P = 0.033 in the SB treatments, P < 0.001 in the CB treatments) (Fig. 3c and e). In contrast, the changes in gut multifunctionality were significantly correlated to the compositional shifts in gut bacterial communities only for the CB treatments (P = 0.046) (Fig. 3f) but not for the SB treatments (P = 0.54) (Fig. 3d).

Structural equation models (SEM) were employed to evaluate the relationships between the different physicochemical characteristics of the soil and earthworm gut content (represented by the first axis of NMDS run for samples based on these characteristics), total bacterial

![Fig. 3](image-url)

**Fig. 3.** Analysis of the correlations between the physicochemical properties of soil and earthworm guts with their bacterial community structure and multifunctionality, for the maize straw biochar, SB (a) and cow dung biochar, CB (b) treatments. The relationships between the biochar-induced changes in multifunctionality and the bacterial compositional shifts (Bray-Curtis dissimilarity compared with the control) are presented for soil (c, e) and earthworm guts (d, f) for the SB (c, d) and CB treatments (e, f).

**Table 3**

The differences in bacterial community structures between different treatment groups. Values at P < 0.05 are shown in bold.

<table>
<thead>
<tr>
<th>Comparisons Sample considered</th>
<th>Adonis Test</th>
<th>Anosim Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil vs. earthworm All samples</td>
<td>0.427 (&lt;0.001)</td>
<td>0.752 (&lt;0.001)</td>
</tr>
<tr>
<td>CK samples</td>
<td>0.441 (0.011)</td>
<td>0.624 (0.008)</td>
</tr>
<tr>
<td>SB samples</td>
<td>0.504 (&lt;0.001)</td>
<td>0.444 (&lt;0.001)</td>
</tr>
<tr>
<td>CB samples</td>
<td>0.736 (&lt;0.001)</td>
<td>0.678 (&lt;0.001)</td>
</tr>
<tr>
<td>Biochar type Soil samples</td>
<td>0.255 (&lt;0.001)</td>
<td>0.444 (&lt;0.001)</td>
</tr>
<tr>
<td>Earthworm samples</td>
<td>0.308 (&lt;0.001)</td>
<td>0.377 (&lt;0.001)</td>
</tr>
<tr>
<td>Soil samples</td>
<td>0.233 (0.003)</td>
<td>0.301 (0.006)</td>
</tr>
<tr>
<td>Earthworm samples</td>
<td>0.127 (0.054)</td>
<td>0.099 (0.128)</td>
</tr>
<tr>
<td>CB biochar amount Soil samples</td>
<td>0.207 (0.006)</td>
<td>0.470 (&lt;0.001)</td>
</tr>
<tr>
<td>Earthworm samples</td>
<td>0.213 (0.009)</td>
<td>0.541 (&lt;0.001)</td>
</tr>
</tbody>
</table>
abundance, bacterial community structure (represented by the first axis of NMDS run for samples based on the OTU matrices), bacterial diversity (Shannon index), and multifunctionality. The number of significant effects selected by the SEM was higher for the CB than SB treatments, and the total variance of gut multifunctionality explained by the SEM was higher in the CB treatments ($R^2 = 0.77$) than in the SB treatments ($R^2 = 0.59$). For the gut multifunctionality, the gut physicochemical properties were the most influential gut variables in both biochar treatments, with a less important role of the gut bacterial abundance and gut bacterial community composition, the effects of gut bacterial diversity being the lowest, especially in CB treatments (Fig. 5b and Fig. 5e). The gut multifunctionality was significantly related to the soil multifunctionality in SB treatments (Fig. 5a and d). The soil multifunctionality was mostly related to soil physicochemical properties and to soil bacterial community structure and/or diversity (Fig. 5c and f). In both biochar treatments, the earthworm gut bacterial diversities (Shannon index) had a much lower contribution to multifunctionality than other variables (Fig. 5b; Fig. 5c; Fig. 5e; Fig. 5f).

4. Discussion

It has been reported that the environmental conditions and bacterial communities in the earthworm gut differ from those in the surrounding soil (Bi et al., 2021; Jin et al., 2022). Consistently, our results confirmed the significantly higher contents of nutrients (e.g., TC, TN, TP, and Posl) and the specificities of bacterial community compositions in the earthworm gut compared to soil. These differences are likely due to the selectively feeding of earthworms on biochar and the strong selection pressure of the gut environment (Thakuria et al., 2010). Specifically, many well-known copiotrophic taxa such as Bacilli, Clostridia and Actinobacteria were dominant in earthworm guts, while the abundance of Acidobacteria, one of the typical oligotrophic taxa, was significantly reduced when passing through earthworm gut (consistent with results from Fierer et al., 2007). Our results also confirmed the significantly higher enzymatic activities in earthworm gut than in soil, highlighting the higher capabilities of earthworm gut bacterial communities regarding C and nutrient turnovers. In this discussion, we focus on the novel aspects of our work which unfold the way biochar addition, including biochar type and amount, modifies bacterial abundance,
diversity and community composition as well as multifunctionality in soil and earthworm guts.

4.1. Biochar addition modifies bacterial communities more in gut than in soil

Our results showed that biochar addition modifies bacterial community structure more in earthworm gut than in soil. Whereas the addition of organic materials such as compost can contribute to changes in community compositions through the microorganisms they bear (Saison et al., 2006), biochar does not bear many microorganisms because it is a material obtained by pyrolysis. Hence the influence of biochar addition on native microbial communities was likely mainly due to modified soil physicochemical characteristics rather than to the introduction of biochar-borne microorganisms. We therefore assume that the profound alteration of the gut bacterial community observed following biochar (in particular CB) addition could be attributed to strong modifications in the abundances (absolute and relative values) of initially present taxa, likely in response to the biochar-induced increase in the gut nutrient content. Specifically, as a typical copiotrophic taxon with a high ability to decompose various organic substrates (Mendes et al., 2013), Bacillaceae experienced the most drastic changes when biochar application rates increased. Copiotrophic taxa actually dominate in resource-rich environments such as rhizosphere and earthworm gut, and they respond more rapidly to nutrient inputs than oligotrophic taxa (Blagodatskaya et al., 2007; Nemergut et al., 2016; Cui et al., 2020b; Ling et al., 2022). Consistently to this interpretation, Dai et al. (2016) found that the bacterial communities in the rhizosphere were more sensitive to biochar addition than in bulk soil. Overall, our results demonstrate that the bacterial communities in the earthworm gut responded more actively to biochar addition than soil, suggesting the possibility of adding biochar to manipulate the earthworm gut microbiome and thereby soil ecosystem functions.

4.2. Biochar physicochemical properties impact earthworm gut enzymatic activities, with an influence of biochar type and application rates

Our results showed that the biochar-induced changes in earthworm gut physicochemical properties play a crucial role in regulating gut multifunctionality. Generally, a greater effect of CB than of SB was observed on gut enzymatic activity. This was very likely due to the higher contents of nutrients and trace elements in CB than in SB (Table S1), since it has been reported that biochar can stimulate soil enzymatic activities directly by providing trace elements that act as cofactors of many enzymes and/or indirectly by providing organic substrates to bacterial communities (Gorovtsov et al., 2020). However, our findings also revealed that a low biochar application rate (CB20 and moreover SB20) resulted in a decrease in earthworm gut enzymatic activities. This might be explained by economic theories of bacterial metabolism stating that microbes may decrease their production of enzymes that degrade complex substrates when assimilable resources are available (Allison and Vitousek, 2005). Therefore, it is possible that the limited resources provided by smaller amounts of biochar could be directly consumed by the gut microorganisms, and subsequently induced a reduction of their enzyme production but were not sufficient for stimulating the proliferation of gut microorganisms.
We also observed that both the soil 16S rRNA copy number and C-cycling related enzymes were significantly decreased or remained unchanged following biochar application rate, whereas biochar addition boosted bacterial abundance and multifunctionality in earthworm gut. We speculate that the lower C/N ratio measured in earthworm gut than in soil could explain these different responses to biochar addition between the soil and guts. Indeed, it has been reported that biochar addition could increase the C turnovers only in soil with a low C/N ratio, likely by providing sufficient resources and alleviating N-limitations on soil microorganism growth (Guo et al., 2020). Therefore, our findings suggest that whereas biochar addition can have a negative (or lack of) effect on several soil enzymatic activities, a positive effect could be obtained by stimulating specific biochemical processes in the earthworm gut. Considering the unique environmental conditions and high enzymatic activity in the earthworm gut, more information is urgently needed to decipher the mechanisms explaining the biochar-induced changes in earthworm gut enzymatic activities and their impacts on soil nutrient cycling.

4.3. Biochar type alters gut bacterial communities and their relationships with gut multifunctionality

Our results further revealed that biochar type largely regulated the relationship between multifunctionality and the earthworm gut bacterial community. It has been reported that organic substrates with a lower C/N ratio commonly possess a higher proportion of labile C, which could favor SOC decomposition by selectively stimulating soil copiotrophic taxa (Fontaine and Barot, 2005; Baldiaviseo-Freitas et al., 2018). Likely due to the low C/N ratio and particle size of CB, as well as the high nutrient content and surface area, increasing CB application rates induced more drastic changes in the soil and earthworm gut physicochemical properties than that of SB, which induced significant compositional changes of earthworm gut bacterial communities in CB treatments. This is highlighted by the strong correlations observed between the physicochemical properties and bacterial community structures or multifunctionality in both soil and guts for CB treatments, whereas such significant correlations were not observed under SB treatments. In addition, the SB induced a higher relative abundance of Actinobacteria in the earthworm gut, which is often associated with the degradation of recalcitrant carbon resources (Kirby 2006; Ventura et al., 2007; Gorovtsov et al., 2020). This tends to confirm the lower bioavailability of SB than CB. Noticeably, the application of CB also resulted in particularly high earthworm gut multifunctionality but low gut bacterial diversity, which is not consistent with some previous studies reporting positive correlations between soil multifunctionality and the alpha-diversity of soil bacterial communities (Wagg et al., 2014; Delgado-Baquerizo et al., 2016; Luo et al., 2018; Zheng et al., 2019b). Given the initially high proportions of copiotrophic taxa in earthworm gut and higher bioavailability of CB, this apparently counter-intuitive result is likely explained by the changed total abundances of bacteria with the proliferation of a few copiotrophic taxa rather than the changed bacterial diversities. This interpretation is supported by the SEM results, which proved that the effects of gut bacterial abundance on gut multifunctionality were higher than those of gut bacterial diversities, especially for CB treatments. Consistently, a recent study showed how high application rates of organic manure could cause rapid proliferation of copiotrophic species in the earthworm gut and a concurrent decrease in bacterial diversity (Jin et al., 2022).

Our results also showed that with higher biochar application rates, the complexity of earthworm gut bacterial networks and the proportions of the negative links were higher in the SB treatments compared with the CB treatments (Fig. 4a). In complement to the labile C resources naturally present in the earthworm gut, the biochar-derived recalcitrant C resources (Bruun et al., 2011) likely provided additional niches for some gut microorganisms that tend to interact with each other, especially for SB treatments. For example, the vascular structures and low bioavailability of SB could facilitate the thriving of Actinobacteria in the earthworm gut due to their filamentous growth patterns (Aksenov--Gribanov et al., 2016; Gorovtsov et al., 2020) and great degradation abilities on recalcitrant C resources (Kirby 2006; Gorovtsov et al., 2020). This consequently boosted the formation of a complex gut bacterial network with Actinobacteria as the main keystone species. Additionally, considering the well-known antibacterial activities of Actinobacteria (Ventura et al., 2007; Hussain et al., 2017), the dominance of Actinobacteria may also be responsible for the relatively stable earthworm gut bacterial community structures in the SB treatments. Besides, the more condensed gut bacterial networks and higher proportions of negative links in SB treatments than that in CB treatments could also explain the relatively stable earthworm gut bacterial diversity and community structures in this study (Stouffer and Bascombe, 2011; Coyte et al., 2015; Morrien et al., 2017; de Vries et al., 2018). Altogether, these results suggest that different types of biochar have different regulatory effects on earthworm gut bacterial communities and their relationships with multifunctionality, and the direction and magnitude of these effects highly depend on the physicochemical properties of biochar.

4.4. Biochar alters the gut multifunctionality mainly through the changed gut and/or physicochemical properties rather than community structures

Our results from SEM analysis suggested that across SB treatments gut multifunctionality was mainly related to gut physicochemical properties, whereas across CB treatments, it was mostly driven by both soil and gut physicochemical properties as well as application rates. Consistently, previous studies reported that changes in soil enzymatic activities can be mostly related to changes in environmental parameters rather than community diversity and composition (Attard et al., 2011; Bowles et al., 2014; Huang et al., 2019), probably owing to the level of functional redundancy within soil microbial communities. Our results hence proved that the biochar-induced changes in (gut and soil) physicochemical properties were the primary determinants of changes in gut multifunctionality.

In addition, the gut microbial multifunctionality significantly affected soil multifunctionality in the SB treatments but not in the CB treatments, and the total variance of soil multifunctionality explained by SEM was also higher in SB treatments. Moreover, the effects of earthworm gut biochemical variables on soil multifunctionality were also higher in the SB than in CB treatments. Altogether, these findings indicate greater contributions of gut biochemical variables on soil multifunctionality in SB treatments. A possible explanation is that the bacterial community excreted by earthworms into the soil was more stable owing to their higher bacterial diversities and stronger gut bacterial interactions observed in SB treatments. In addition, the higher pore size of SB might also facilitate the colonization of gut microbes and then their maintenance in the excreted casts for a relatively long time (Atkinson et al., 2010), which would favor the maintenance of soil enzymatic activities influenced by earthworm activity. This is also consistent with the observation that biochar can allow the immobilization of microbial inoculants, often improving the maintenance of their beneficial activity and efficiency in soil (Lehmann et al., 2011; Palamooriya et al., 2019). In summary, our findings provided insights into how biochar addition (including biochar type and amount) influences earthworm gut bacterial communities and expanded the understanding of the relationships between earthworm gut bacterial communities and nutrient cycling. This study hence sheds light on the potential of using biochar to regulate earthworm gut bacterial communities and improve soil ecosystem function.

5. Conclusions

Our study revealed that earthworm gut bacterial communities had higher metabolic capacities and were more responsive to biochar application than soil. Besides, the biochar-induced responses in
earthworm gut multifunctionality differed between the two types of biochar applied and biochar application rates. Increasing the application rates of CB significantly increased earthworm gut multifunctionality by modifying the soil physicochemical properties in the earthworm gut and, to a lesser extent, by altering gut bacterial abundance and community structure. In contrast, high application rates of SB lead to more earthworm gut microbiome and multifunctionality through the strategic application of biochar in the soil by carefully selecting biochar type and amount.

Declaration of competing interest

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.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (42077088). Xi-Peng Liu was supported by a scholarship from the China Scholarship Council. Xavier Le Roux was funded by the National Research Institute for Agriculture, Food and Environment INRAE (ECODIV Department). We thank Dr. Liang Ni (AES of Zhejiang University) for his help in field experiment management and soil sample collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108810.

References


OECD, 2016. Test No. 222: Earthworm Reproduction Test