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Zheng, Y; Wang, S; Bonkowski, M; Chen, X; Griffiths, BS; Hu, F; Liu, M

*Published in:*
Soil Biology and Biochemistry

*DOI:*
10.1016/j.soilbio.2018.05.012

First published: 29/05/2018

*Document Version*
Peer reviewed version

Link to publication

*Citation for pulished version (APA):*
Zheng, Y., Wang, S., Bonkowski, M., Chen, X., Griffiths, BS., Hu, F., & Liu, M. (2018). Litter chemistry influences earthworm effects on soil carbon loss and microbial carbon acquisition. *Soil Biology and Biochemistry*, *123*, 105 - 114. https://doi.org/10.1016/j.soilbio.2018.05.012

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## Highlights

1. Earthworms reduce POC and SOC but have no effects on PON and TN under low lignin litters.

2. Earthworms decrease resource availability under low lignin litters therefore stimulate microbial competition for C.

3. Earthworms induce C loss mainly due to decreasing soil fungi abundance.

*Manuscript with continous line numbering
Click here to view linked References

Type of contribution: Original research paper

Date of resubmission: 10th May 2018

Text pages: 32; Tables: 3; Figures: 8

Lines: 704; Words: 7743

# Litter chemistry influences earthworm effects on soil carbon loss and microbial carbon acquisition

Yong Zheng [a,b], Shuai Wang [a,b], Michael Bonkowski [c], Xiaoyun Chen [a,b], Bryan Griffiths [d], Feng Hu [a,b], Manqiang Liu [a,b,*]

Affiliations of authors:

[a] Soil Ecology Lab, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, China

[b] Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization & Jiangsu Key Laboratory for Solid Organic Waste Utilization, China

[c] Center of Excellence in Plant Sciences (CEPLAS), Terrestrial Ecology, Institute of Zoology, University of Cologne, D-50674 Köln, Germany

[d] SRUC, Crop and Soil Systems Research Group, Edinburgh, EH9 3JG, UK

*Abbreviations*: NL, no litter; CL, clover; MA, maize stover; WH, wheat straw; RU, *Rumex*; BA, bagasse fiber; DOC, dissolved organic carbon; DON, dissolved organic nitrogen; POC, particulate organic carbon; PON, particulate organic nitrogen; SOC, soil organic carbon; TN, total nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen.

*Corresponding author: Manqiang Liu

Email address: liumq@njau.edu.cn and manqiang-liu@163.com

## Abstract

Earthworms could affect soil C and N cycling process to balance their energy and nutrients requirements, and they could also regulate soil microbial community structure and microbial acquisition for C and N. However, the connection between faunal and microbial stoichiometry in the coupling soil C and N cycling remains poorly understood. In a controlled laboratory experiment, we amended soil with five litters differing in litter chemistry (clover, maize stover, wheat straw, *Rumex* and bagasse fiber) including a no litter control and treated them without or with earthworms (*Metaphire guillelmi*). After 90 d incubation, we examined changes in earthworm tissue and microbial stoichiometry and different soil C and N fractions. Earthworm tissue C content was rather stable compared with the fluctuation in tissue N, implying that C is under stronger control and associated with higher demand than N. The presence of earthworm significantly enhanced $CO_2$ emissions and decreased particulate organic carbon (POC) and soil organic carbon (SOC) contents in the low lignin litter species clover, maize stover and wheat straw. Meanwhile, earthworm presence increased $N_2O$ cumulative emissions but exerted negligible effects on particulate organic nitrogen (PON) and soil total nitrogen (TN) contents irrespective of litter species. Correspondingly, earthworm regulated microbial C and N acquisition as C to N-degrading enzyme activity ratio were nearly doubled in the low lignin litter species clover, maize stover and wheat straw, while it was decreased in the high lignin litter species *Rumex* and bagasse fiber. However, the structural equation modeling indicated C loss induced by earthworms was mainly attributed to their effects on soil fungi and bacteria abundance, while much less related to C-degrading enzyme activities. In conclusion, litter species controlled earthworm effects on soil C and N loss and associated microbial acquisition for C and N, highlighting the pivotal role of

52 resource chemistry in the regulation of soil fauna impact on soil functioning and
53 ecosystem services.
54
55 Keywords: Soil fauna; Litter chemistry; C and N fractions; Earthworm-microbe
56 competition; Enzyme activities
57
58

## 1. Introduction

Litter is an important resource providing one of the main sources of energy and nutrients for the soil food web (Wardle et al., 2004). Litter chemistry regulates growth and metabolism of soil biota and associated energy flows and nutrient cycling in terrestrial ecosystems (Scheu and Schaefer, 1998; Cornwell et al., 2008; Ott et al., 2014; Bradford et al., 2016; Cesarz et al., 2016). Litter species with high concentration of accessible organic compounds could significantly stimulate microbial activities and accelerate C and N mineralization (Hobbie, 2015). Therefore, soil fauna are likely to be most beneficial for the decomposition of litter species with high recalcitrant compounds. Nevertheless, earlier studies have indicated that higher resource availability litters could favor soil fauna utilization (Yatso and Lilleskov, 2016). So far, the interactions between soil fauna and litter chemistry and the consequences for C and N turnovers are not well understood.

It is well-known that burrowing, feeding and casting activities of earthworms affect C and N cycling by regulating soil microbial and biochemical process (Lavelle, 1988; Edwards, 2004; Blouin et al., 2013; van Groenigen et al., 2014; Bertrand et al., 2015). Earthworms can stimulate a small proportion of C and N gaseous loss by their respiration and gut-associated process (Scheu, 1991; Horn et al., 2003; Edwards, 2004). More importantly, earthworms facilitate microbial mineralization of labile organic substrates and greenhouse gas emissions by releasing C and N locked away in plant litter and soil organic matter (Bernard et al., 2012; Lubbers et al., 2013). Besides, earthworms showed stoichiometric invariability according to an investigative research conducted on different experimental plantations (Marichal et al., 2011). To balance their requirements for C and N, earthworms might have distinct strategies for C or N mining. Few studies applied stoichiometric principles when interpreting the combined

84 effects of earthworms and microorganisms on biogeochemical cycling (Tiunov and
85 Scheu 2004; Marichal et al. 2011; Fahey et al., 2013). Understanding the role of soil
86 faunal stoichiometry would improve our knowledge about the functional roles of
87 earthworm in soil C and N cycling.

88 Soil microbes produce extracellular enzymes to break down complex organic
89 matter compounds and acquire bioavailable C and N (Sinsabaugh et al., 2002; Waring
90 et al., 2013). The relative abundance of enzymes involved in C and N cycling reflects
91 the biogeochemical equilibrium between microbial biomass stoichiometry as well as
92 the quantity and quality of organic matter (Sinsabaugh and Follstad Shah, 2012).
93 Recently, Hoang et al. (2016) described distinct strategies of earthworms for
94 re-allocating C- and N-related enzyme activities in order to acquire the resource in the
95 shortest supply relative to their requirements. However, there are still two seemingly
96 contradictory mechanisms explaining how earthworms affect microbial enzyme
97 activities. As higher demand for the product can promote enzyme activities (Bell et al.,
98 2013; Manzoni et al., 2017), earthworm could utilize available C and/or N, therefore
99 increase microbial C- and/or N-mining activities to compensate for earthworm
100 competition. On the other hand, earthworms could enhance substrate availability,
101 hence stimulate microbial C-mining activities, as Allison et al. (2014) indicated low
102 substrate availability could suppress the production of an enzyme. So far, there is still
103 a lack of knowledge regarding how earthworms influence microbial stoichiometry and
104 the linkage with earthworm-induced C and N changes.

105 To explore whether faunal and microbial stoichiometry help to explain the
106 mechanisms of earthworm-driven soil C and N turnover, we performed a factorial
107 experiment with different litter species combined with or without earthworms.
108 Different C and N fractions as well as the $CO_2$ and $N_2O$ flux were measured.

Particulate organic C and N which are characteristic of intermediately decomposed plant litter, were used to express earthworm-induced litter C and N losses as they are much more sensitive than total soil organic C and N (Cambardella and Elliott, 1992; Benbi et al., 2014). Microbial biomass, microbial community structure and enzyme activities were also determined to explore the stoichiometric mechanisms underlying the effects of earthworms on soil C and N changes. The C-degrading enzyme activities including α-1,4-glucosidase (AG), β-1,4-glucosidase (BG), and β-D-cellobiohydrolase (CB) and N-degrading enzyme activities including β-1,4-N-acetylglucosaminidase (NAG) and leucine aminopeptidase (LAP) were measured. The ratio of C- to N-degrading enzyme activities was used as indicators of microbial resources demand between C and N (Waring et al., 2013). We hypothesized that (i) earthworm effects on C and N pools vary with plant chemistry, for example high resource availability litters (low C:N ratio, low lignin content and high soluble compounds) could favor earthworms utilization compared to the high recalcitrant compounds litters (high C:N ratio, high lignin content and low soluble compounds), therefore reinforce earthworm effects on C and N cycling process; (ii) increased microbial mining for C (or N) will be reflected by shifts in the relevant enzyme activities as well as shifts in microbial community structure to favor bacteria over fungi (or vice versa).

## 2. Materials and methods

### *2.1. Experimental set-up*

The endogeic earthworm *Metaphire guillelmi* was collected from an arable field rotated with soybean, maize and different kinds of vegetables each year in Rudong county (32°33′N, 121°15′E), Jiangsu, China. To avoid the earthworm cast from

134 diminishing the effects of earthworms in the following experiment, soil was collected
135 from the top 5-20 cm layer. The background soil properties were soil pH (water:soil
136 2.5:1) 6.5, 30.0% sand, 63.5% silt, 6.5% clay, 13.9 g of organic C $kg^{-1}$ and 0.7 g of
137 total N $kg^{-1}$. The soil was sieved (< 2 mm) and all visible debris and fauna were
138 removed before the incubation experiment.

139 This experiment was set up with a two-way factorial design (earthworm × litters),
140 with five litters including residues of clover (*Trifolium repens* L.), maize stover (*Zea*
141 *mays* L.), wheat straw (*Triticum aestivum* L.), *Rumex* (*Rumex japonicus* Houtt.),
142 bagasse fiber (*Saccharum officinarum*) and a control (no litter input) and across
143 earthworm treatments (with or without earthworms). Litters are abbreviated as
144 following: clover (CL), maize stover (MA), wheat straw (WH), *Rumex* (RU) and
145 bagasse fiber (BA). Each treatment had five replicates leading to 60 experimental
146 units in total. The selected litters spanned a gradient of litter chemistry (Table 1).
147 Litters were collected from the same location as earthworms and subsequently dried at
148 60 °C for 24 h and milled and sieved (1 mm mesh). Each litter was added at a rate
149 equivalent to 10.0 g litter C $kg^{-1}$ dry soil. Litters were homogeneously mixed with soil
150 to separate the litter-mixing effect of earthworms from the stoichiometric effects.
151 After 10 d of pre-incubation, three adult earthworms with a total fresh weight of 7.5g
152 were added to each microcosm. Litter C and N concentrations were determined by
153 potassium dichromate oxidation-ferrous sulphate titration and the Kjeldahl digestion
154 with sulfuric acid and hydrogen peroxide, respectively (Sparks et al, 1996). Litter
155 soluble C and N were obtained by extracting 3.0 g air-dried litter in 30 mL distilled
156 water (20℃, 30 min), then centrifuged (3500 rpm, 20 min) and filtered through
157 0.45-μm cellulose nitrate membrane filter (Ghani et al., 2003), then determined using
158 a TOC analyser (Elementar, Germany) and a continuous flow analyzer (Skalar, Breda,

159 The Netherlands), respectively. Cellulose and lignin were determined using a Fibertec
160 System 2021 FiberCap (Foss Tecator, Höganäs, Sweden) following the procedures
161 described by Soest et al. (1967).

162 The microcosms were incubated in a climate chamber at 25/15 °C day/nighttime
163 and 12/12 h light/dark periods. The microcosms were composed of a polyvinyl
164 chloride (PVC) pot (15 cm height, 15 cm diameter), holding 2.0 kg dry equivalent soil
165 which was adjusted to 60% water-holding capacity, and watered weekly to constant
166 weight with distilled water to compensate for evaporation losses. The pots were
167 covered with nylon mesh (1 mm) to keep the earthworms in the microcosms (Fig. S1).
168 Before introducing earthworms to the corresponding microcosms, earthworms were
169 placed in a plastic container spread with wet filter paper for 48 hours to evacuate their
170 guts (Dalby et al., 1996).

171 *2.2. Element content of earthworm tissue*

172 To calculate the survival rates and biomass changes relative to initial values,
173 earthworms were washed in distilled water and again kept on wet filter paper for 48 h
174 to void their guts. Earthworms of each microcosm were freeze-dried and ground into
175 powder (Marichal et al., 2011). Earthworm body tissue C and N concentrations were
176 determined at the end of the experiment, following the methods similarly to litter C
177 and N.

178 *2.3. Determination of C and N fractions*

179 Nitrate ($NO_3^-$-N) was extracted from soil with 50 mL of 2 M KCl after shaking (30
180 min) and filtering and determined using a continuous flow analyzer (Skalar, Breda,
181 The Netherlands). Dissolved organic carbon (DOC) and nitrogen (DON) were
182 extracted from fresh soil with ultrapure water (at 2:1 w/w water:soil) and centrifuged
183 at 3000 rpm for 5 min, then determined similar to litter soluble C and N. Particulate

184 organic matter was separated by dispersing 10 g air-dried soil in 30 mL of 5 g $L^{-1}$
185 sodium hexametaphosphate solution, shaking for 15 h on a reciprocal shaker, then
186 collected on a 53 μm sieve after thorough washing with distilled water. After drying
187 (60 °C) and grinding by mortar and pestle, the powder was analysed for particulate
188 organic C (POC) and N (PON) (Cambardella and Elliott, 1992). SOC and POC were
189 measured using the Walkley and Black method, while TN and PON were measured
190 by Kjeldahl digestion (Sparks et al, 1996).

191 $CO_2$ and $N_2O$ samples were collected after earthworms were introduced to the
192 microcosm on average every four days during the 90 d incubation by capping the
193 microcosm by a lid with a septum, and taking gas samples from the chamber
194 headspace 0 min and 45 min after closure (Fig. S1). The 20 ml collected gas samples
195 were analyzed using a gas chromatograph (Agilent 7890A, USA) equipped with a
196 63Ni electron capture detector. The gas chromatograph setup and configuration were
197 described in detail by Wu et al. (2015).

198 *2.4. Microbial community indices*

199 Soil microbial biomass carbon (MBC) and nitrogen (MBN) were determined by the
200 fumigation-extraction method. Briefly, soil samples were divided into two subsamples,
201 of which one subsample was extracted with 0.5M $K_2SO_4$ directly and another
202 subsample was extracted after 24 hours chloroform fumigation. MBC and MBN were
203 calculated from the extracted organic C and N by multiplication factors of 0.38 and
204 0.45, respectively (Brookes et al., 1985; Vance et al., 1987).

205 Microbial community structure was determined by analysis of phospholipid fatty
206 acid (PLFA) based on the method described by Bossio and Scow (1998). Lipids were
207 extracted from 10.0 g freeze-dried soil with a chloroform-methanol-citrated buffer
208 mixture (25 mL at a 1:2:0.8 by volume). The lipid extract was separated into neutral

lipids, glycolipids and phospholipids on silicic acid columns. Fatty Acid Methyl Esters (FAMEs) were quantified with nonadecanoic acid as internal standard and analyzed with a gas chromatograph (Agilent Technologies, Palo Alto, CA, USA), and MIDI Sherlock software (MIDI, Inc., Newark, DE, USA) was used to identify peaks. A total of 22 different PLFAs were detected and identified. The biomass of bacteria was determined using the combined weights of fatty acids i-14:0, i-15:0, a-15:0, 16:0, i-16:0, i-17:0, a-17:0, 16:1ω7c, cy-17:0ω7c, 18:1ω7c, while the two PLFA biomarkers 10me-16:0 and 10me-18:0 were used to quantify actinomycetes (Ruess and Chamberlain, 2010). Fungal PLFA was determined as the sum of 18:1ω9c and 18:2ω6 (Frostegård and Bååth, 1996).

*2.5. Enzyme activities*

Potential extracellular enzyme activities related to total C- and N-cycling were quantified by high throughput fluorometric assay in 96-well microtiter plates (Bell et al., 2013). Briefly, a homogenized soil slurry was prepared by shaking 2.75 g of field moist soil in 91 ml of 50 mM sodium acetate buffer (pH 6.8) in an Erlenmerver flask for 1 h. 800 µl soil slurry each were pipetted into a 96-deep-well (2 ml) micro-plate. Additional quench control replicates of soil slurry and 4-methylumbellfferone (MUB) or 7-amino-4-methylcoumarin (MUC) standard curves (0–100 µM concentrations) were included with each sample. α-1,4-glucosidase (AG), β-1,4-glucosidase (BG), and β-D- cellobiohydrolase (CB) represented C-degrading enzymesand β-1,4-N-acetylglucosaminidase (NAG) and leucine aminopeptidase (LAP) represented N-degrading enzymes (Sinsabaugh and Follstad Shah, 2012). Soil slurries with fluorometric substrates were sealed and incubated at 25°C for 3 h, centrifuged for 3 min at 2900 g, and 250 µl from each well transferred into corresponding wells of a black, flat-bottomed, 96-well plate and scanned on a TECAN Infinite M200

234 microplate reader at 365 nm and emission at 450 nm. Excitation values were
235 converted to nmol enzyme activity $g^{-1}$ dry soil $h^{-1}$ as units. The sum of AG + BG + CB
236 was calculated as a measure of overall C-degrading enzyme activity and the sum of
237 NAG + LAP was used to reflect overall N-degrading enzyme activity (Bell et al.,
238 2013).

239 *2.6. Data analysis*

240 All statistical analyses were carried out in R Version 3.3.0 (Team, 2013). To test our
241 first hypothesis, a two-way ANOVA was performed to test for the main and
242 interactive effects of earthworms and litter species on soil properties, followed by
243 Turkey's HSD test. The earthworm respired C were roughly estimated at 1.1% of
244 earthworm C per day according to Scheu, (1991). Structure equation modeling was
245 performed using package lavaan (Rosseel, 2012) in R to evaluate how earthworms
246 affect soil respiration by influcing resource availability and microbial communities.
247 The biomass of earthworm at the end of incubation and litter C:N ratio were used as
248 independent variable. Microbial community structure was indicated using fungal and
249 bacterial PLFA and microbial activity was indicated by AG, BG and CB. The
250 adequacy of models was determined using Chi-squared ($\chi^2$) test, the comparative fit
251 index (CFI) and the standardised root mean square residual (SRMR). To test the
252 second hypothesis that earthworms-induced soil C or N losses were related to enzyme
253 activities, ln(AG + BG + CB):ln(NAG + LAP) for each litter was calculated as an
254 index of microbial C:N acquisition effort (Sinsabaugh and Follstad Shah, 2012). A
255 ratio of C- to N-degrading enzyme activities greater than one indicated that microbe
256 had to increase their enzymatic activity to obtain C relatively to N. Non-metric
257 multidimensional scaling (NMDS) on Bray-Curtis distances of microbial communities
258 was performed under the vegan package (Oksanen et al., 2018) to distinguish soil

microbial community structure influenced by earthworms and litters. Data were natural log- or square root-transformed to achieve normality and homoscedasticity when necessary. Results were expressed by means and standard errors (SE).

## 3. Results

### *3.1. Earthworm growth and tissue element content*

All earthworms survived after 90 d and their biomass increased from 101.3% to 148.1% with litter amendment compared to their initial weight, but only remained 70.6% of their initial weight in the no litter treatment (Table 2). Earthworm biomass was the highest when the maize stover was mixed into soil (Table 2). Earthworm C content varied in a narrow range from 29.5 ± 0.5 to 30.5 ± 0.2 % dry mass and was not affected by litter treatments, while earthworm N content was significantly higher under clover than the other litters (Table 2). Earthworm tissue C:N ratio ranged from 3.52 ± 0.09 to 3.89 ± 0.08, and the slope of the earthworm C:N to soil C:N significantly deviated from the 1:1 line ($P < 0.05$; Fig. 1).

### *3.2. Effects of earthworms on soil C and N fractions*

The presence of earthworms increased cumulative $CO_2$ emissions between (14.3 % to 64.8%) and $N_2O$ emissions (between 3.2% to 48.7%) across all litter species (Fig. 2). $NO_3^-$-N was generally in the presence of earthworms regardless of litter species, while DON was decreased in the presence of earthworms except the two high lignin litters *Rumex* and bagasse fiber (Table S1). Earthworms further decreased DOC under maize stover compared to the corresponding no earthworm treatment ($P < 0.05$; Table S1). Compared to the earthworm free control, earthworm presence decreased SOC and POC, leading to a decreased SOC:TN ratio when clover, maize stover and wheat straw were amended to the soil (Table 3, Fig. 3). Meanwhile, the presence of

284 earthworms had negligible effects on PON and TN irrespective of litter species (Table
285 3, Fig. 3).

286 *3.3. Effects of earthworms on microbial stoichiometry and the microbial community*

287 The MBC:MBN ratio varied two-fold between the no litter treatment and the bagasse
288 fiber treatment in absence of earthworms (Fig. 4). The presence of earthworms
289 generally enhanced all measured C- and N-related enzyme activities (Fig. 5).
290 Specifically, total C-degrading enzyme activity was increased 69% to 97% by
291 earthworms under clover, maize stover and wheat straw, while total N-degrading
292 enzyme activity was generally enhanced by earthworms from 3% to 33% across all
293 litters species (Fig. S3). Earthworms increased the ratio of C- to N-degrading enzyme
294 activities when clover, maize stover and wheat straw mixed into soil, while the ratio
295 was decreased by earthworms under *Rumex* and bagasse fiber (Fig. 6).

296 Earthworms changed microbial community structure by influencing the relative
297 abundance of gram positive bacteria, actinomycetes, and fungi (Table S3). Earthworm
298 presence decreased the fungi:bacteria ratio under maize stover, wheat straw and
299 *Rumex*, but increased it under bagasse fiber ($P < 0.05$; Table S3). NMDS analysis
300 confirmed a significant effect of earthworms on microbial community structure ($P <$
301 0.01, Fig. 7).

302 *3.4. Structural equation modeling results*

303 The final model adequately fit the data on soil respiration ($\chi^2_{11}$ =36.565, CFI= 0.906,
304 SRMR= 0.080). It explained 98% and 49% of resource availability and C-degrading
305 enzyme activity, respectively. Fungi, Bacteria and C loss were explained 51%, 23%
306 and 83%, respectively (Fig. 8). Earthworm had a direct positive effect on C-degrading
307 enzyme activity and a negative effect on resource availability, and the presence of
308 earthworms showed an opposite effect on fungi and bacteria abundance (Fig. 8). Soil

309 C loss was mainly attributed to the reduction of soil fungi abundance (Fig. 8).

310

311 **4. Discussion**

312 This study focused on how litter chemistry modify earthworm effects on soil C and N
313 turnover as well as associated microbial process. The presence of earthworms
314 translated into higher C-degrading enzyme activity, greater C mineralization and C
315 loss, except in the two low soluble compound and high lignin litter species (*Rumex*
316 and bagasse fiber). The SEM indicated that earthworm effects on C loss was mainly
317 attributed to their effects on soil microbial community structure, while much less
318 related to C-degrading enzyme activity. However, earthworm controlled microbial
319 C:N acquisition effort as C to N-degrading enzyme activity ratio were significantly
320 increased by earthworms in the low lignin litter species (clover, maize stover and
321 wheat straw), while it was decreased in the high lignin litter species (*Rumex* and
322 bagasse fiber). This highlights the role of litter chemistry in regulating earthworm
323 impact on C and N cycling as well as related microbial stoichiometry.

324 *4.1. Litter chemistry affected earthworm growth and tissue stoichiometry*

325 Litter chemistry is a primary controller of earthworm utilization, with litter
326 characterized by low N and high lignin content generally described as recalcitrant.
327 Our study confirmed the significant role of litter chemistry in driving earthworm
328 biomass, as indicated by earlier studies (Yatso and Lilleskov, 2016; Halvorson et al.,
329 2017; Sauvadet et al., 2017). Five different litter species from clover to bagasse fiber
330 generally showed an increasing trend for C:N ratio, cellulose and lignin concentration
331 and a declining trend for soluble C and N. However, contrary to our prediction that
332 earthworm growth would show an linear correlation from clover to bagasse fiber.
333 Earthworm biomass increased to a lesser extent with the most N rich and the lowest

334 lignin concentration clover litter in comparison to the other litter species. One possible
335 explanation is that beyond nutrient concentration earthworm growth might also
336 constrained by other elements (such as P, Ca and Mg) or plant secondary metabolites
337 (such as phenolics and condensed tannins) (Hättenschwiler and Jørgensen, 2010;
338 Cesarz et al., 2016).

339 Stoichiometric homeostasis of organisms refers to a relatively stable elemental
340 composition regardless of environmental imbalances in nutrient availability (Elser and
341 Urabe, 1999). The linear relationship between earthworm tissue C:N ratio relative to
342 soil C:N ratio indicated the plasticity of earthworm tissue stoichiometry. More
343 interestingly, earthworm tissue C content was rather stable compared with the
344 fluctuation in tissue N (Table 2), which implied that C is under stronger control and
345 associated with higher demand than N (Persson et al., 2010). Earthworms were not be
346 able to increase feeding rates to compensate for the physiological costs for acquiring
347 C and N under bagasse fiber. Meanwhile, litter species with greater content of
348 available resource favored earthworm effects on C and N cycling compared to high
349 lignin litter species. In brief, the fluctuation in earthworm tissue N suggesting
350 earthworm could have a greater influence on soil C compared to N, while the high
351 lignin litters constrained earthworm utilization and therefore might diminish
352 earthworm effects on C cycling process.

353 *4.2. Earthworm-driven loss of different soil C fractions*

354 The changes of different soil C and N fractions revealed clear patterns of the
355 earthworms in acquiring necessary C and/or N under different litter species.
356 Earthworm presence increased $N_2O$ gas emissions and this is consistent with earlier
357 studies showing that earthworms enhance nitrification, subsequent $NO_3^-$-N levels in
358 soil, and further stimulate $N_2O$ emissions (Scheu, 1994; Whalen and Parmelee, 2000;

359 Drake and Horn, 2007; Wu et al., 2015). Although earthworms increased gaseous N
360 losses, they did not decrease PON and TN, further strengthening the argument that
361 earthworms did not incorporate these N pools as indicated by Lubbers et al. (2013).
362 Moreover, as dissolved organic matter could deliver bioavailable C and N to soil biota
363 (Cleveland et al., 2004; Dittman et al., 2007), the reduction in readily available C and
364 N sources by earthworms in the clover, maize stover and wheat straw treatment
365 bolstered the functional role of earthworm as bioavailable C- and N-consumer.
366 Meanwhile, in the presence of bagasse fiber, earthworm mineralized plant litter and
367 soil organic matter therefore enhanced DOC and DON levels, indicating earthworm
368 effects on labile organic C and N were dependent on litter chemistry.

369 So far, there is still uncertainty regarding to earthworm impacts on soil C pools.
370 Earthworms might concomitantly enhance C stabilization as well as mineralization
371 process (Bossuyt et al., 2005; Bernard et al., 2012). For example, earthworm could
372 incorporate labile organic matter into stable micro-aggregates in their casts thereby
373 promoting C sequestration (Zhang et al., 2013). Other studies in contrast
374 demonstrated a stimulation of microbial C mineralization and a loss of SOC by
375 earthworms when the earthworms mixed litter into soil (Crumsey et al., 2013;
376 Groffman et al. 2015). The paradox might result from the fact that the published
377 studies did not distinguish earthworm influence on the labile and recalcitrant C pool
378 which could explain the magnitude of earthworm-induced C mineralization or
379 stabilization (Bossuyt et al., 2005; Bernard et al., 2012; Crumsey et al., 2013; Zhang
380 et al., 2013). It was recently shown that earthworm assimilated more litter-derived C
381 than they defecated in soil aggregates (Lubbers et al., 2017). In a meta-analysis,
382 Lubbers et al. (2013) found that earthworms significantly increase $CO_2$ emission, but
383 there were no indications that earthworms affect soil C pool due to the large

384 background of soil C. In contrast to our study, the earthworm-induced C loss was
385 significant in the presence of low lignin litter species. Such conflict can be explained
386 by the abundant litters mixed into the soil therefore providing a huge amount of
387 relatively less stable C.

388 *4.3. Earthworm changed microbial acquisition for C*

389 Earthworms changed the soil C- and N-degrading enzyme activities and microbial
390 community structure, but microbial biomass remained relatively constant in the
391 presence and absence of earthworms. Several studies have shown significant
392 enhancement of microbial biomass by earthworms, while others have found the
393 opposite effect (Ferlian et al., 2017). This is largely due to these studies either focused
394 on differences between earthworm casts and bulk soil or the mixing of soil layers by
395 earthworms, as suggested by Groffman et al. (2015). The SEM revealed that
396 earthworm effects on C loss were mainly attributed to earthworm-induced reduction
397 of soil fungi abundance, as soil fungi characterized by higher microbial growth
398 efficiency and slower degradation rate of organic matter than bacteria (Six et al.,
399 2006).

400 It is generally acknowledged that earthworm stimulates the relatively inactive
401 microbial communities (as expressed in soil enzymatic activity) and accelerates soil C
402 and N cycling (Ferlian et al., 2017). Contrary to the common assumptions that
403 earthworms mainly facilitate microbial mineralization by releasing available C and N
404 locked away in organic matter. In our study, we found dissolved organic C and N
405 were decreased in the presence of earthworms when clover, maize stover and wheat
406 straw were mixed into the soil (Table S1). Furthermore, earthworm presence
407 significantly increased the enzyme activity per unit microbial biomass when clover,
408 maize stover and wheat straw were mixed into soil (Fig S3). In general, these results

indicated that earthworms decreased resource availability therefore stimulated microbial competition for C and N. There are three possibilities in explaining earthworm could enhance C competition with soil microbes. First, earthworms directly utilize available C in the soil by respiration and assimilation (Table S2). Second, earthworms facilitate C incorporation in soil aggregate fractions which decrease accessibility to soil microbes (Bossuyt et al., 2004; Chang et al., 2016). Third, earthworms increase the proportion of active microbes by the gut-associated process (Drake and Horn, 2007; Bernard et al., 2012). In contrast to earlier studies that earthworms could increase the decomposition rates of surface-applied litter therefore stimulating enzyme activity in the mineral soil, here we considered the fate of plant litter once it was incorporated into the soil. In addition, in the presence of bagasse fiber, earthworm effects on enzyme production bolstered earlier studies that earthworm stimulated enzyme production by enhancing resource availability. Earthworm presence increased the ratio of C- to N- degrading enzyme activities under clover, maize stover and wheat straw, indicating an increased enzymatic activity to obtain C relatively to N, while these effects were reversed under *Rumex* and bagasse fiber. This suggested the litter chemistry controlled earthworm effects on the direction of microbial investments in enzyme production. The increased index of microbial C:N acquisition effort can be explained if we assume that earthworms and microbes compete for the labile fraction of the C pool. According to our findings, earthworms would accelerate soil C turnover as found in litters like clover, maize stover and maize stover, but high recalcitrant compound litters (*Rumex* and bagasse fiber) constrained earthworm effects on soil C turnover and microbial C acquisition.

## 5. Conclusions

In our study, earthworm showed negligible effects on soil N, while litter chemistry modified earthworm effects on soil C as earthworms reduced POC and SOC under high soluble compounds litter species but no significant effects under high lignin litter species. The result of earthworm tissue stoichiometry also supported the idea that C was under stronger control and associated with higher demand than N. The SEM indicated that earthworm effects on C loss was mainly attributed to earthworm-induced soil fungi abundance decrease, while much less related to C-degrading enzyme activity. However, earthworm controlled microbial C:N acquisition effort as C to N-degrading enzyme activity ratio was significantly increased by earthworms in the low lignin litter species (clover, maize stover and wheat straw), while such effect was reversed in the high lignin litter species (*Rumex* and bagasse fiber). In conclusion, it is important to distinguish a recalcitrant and labile C pool in determining the functional role of soil fauna in soil biogeochemical cycling (Buchkowski et al., 2017; Dias et al., 2017).

**Acknowledgments**

This work was supported by the Natural Science Foundation of China (41371263 and 41671255), National Key R&D program (2016YFD0200305), Fundamental Research Funds for the Central Universities (KYTZ201404) and Innovative Foreign Experts Introduction Plan for National Key Discipline of Agricultural Resources and Environment (B12009). Bryan Griffiths acknowledges funding from the Scottish Government's Rural and Environment Science and Analytical Services Division. We are grateful to the two anonymous reviewers for insightful reviews of the manuscript. We also thank Dr. Di Wu and Mr. Ruihao Wu for their help in the laboratory and helpful comments from Dr. Ting Liu and Professor Joann K. Whalen.

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**Table 1**

Initial properties of different litters used as soil amendments in the experiment (Mean ± standard error, n = 5). Litters are presented in the order of increasing C:N ratio.

| | Clover | Maize stover | Wheat straw | Rumex | Bagasse fiber |
|---|---|---|---|---|---|
| Total C (%DM) | 35.3 (± 0.3) | 37.5 (± 0.5) | 36.3 (± 0.6) | 35.4 (± 0.7) | 38.0 (± 0.4) |
| Total N (%DM ) | 1.75 (± 0.02) | 0.84 (± 0.02) | 0.67 (± 0.01) | 0.56 (± 0.02) | 0.25 (± 0.01) |
| Total C:N | 20.4 (± 0.3) | 44.2 (± 1.3) | 54.6 (± 2.1) | 63.4 (± 1.4) | 150.8 (± 7.7) |
| Soluble C (g $kg^{-1}$) | 23.88 (± 0.95) | 8.46 (± 0.29) | 7.29 (± 0.33) | 9.22 (± 0.23) | 2.31 (± 0.10) |
| Soluble N (mg $kg^{-1}$) | 276.4 (± 11.0) | 90.9 (± 3.0) | 42.4 (± 0.6) | 43.7 (± 0.8) | 25.3 (± 2.2) |
| Soluble C:N | 87.0 (± 6.9) | 93.4 (± 5.9) | 180.5 (± 11.8) | 216.6 (± 8.3) | 95.2 (± 6.8) |
| Cellulose (g $kg^{-1}$) | 89.2 (± 6.8) | 103.8 (± 5.1) | 226.7 (± 11.3) | 195.0 (± 5.9) | 385.4 (± 11.2) |
| Lignin (g $kg^{-1}$) | 44.8 (± 5.9) | 187.9 (± 8.2) | 152.6 (± 10.2) | 233.2 (± 4.1) | 250.6 (± 14.5) |

**Table 2**

Percent of initial biomass and earthworm tissue stoichiometry under different litters after 90 d incubation (Mean ± standard error, n = 5).

| | No litter | Clover | Maize stover | Wheat straw | Rumex | Bagasse fiber |
|---|---|---|---|---|---|---|
| **Earthworm growth** | | | | | | |
| Biomass | 70.6 (± 2.3) d | 104.0 (± 1.4) c | 148.1 (± 2.7) a | 128.9 (± 7.2) b | 124.3 (± 5.8) b | 101.3 (± 3.9) c |
| **Earthworm tissue stoichiometry** | | | | | | |
| C (%DM) | 29.8 (± 0.7) a | 30.0 (± 0.1) a | 29.5 (± 0.5) a | 29.8 (± 0.4) a | 30.0 (± 0.7) a | 30.5 (± 0.2) a |
| N (%DM) | 7.70 (± 0.13) b | 8.52 (± 0.23) a | 7.72 (± 0.12) b | 7.72 (± 0.04) b | 7.75 (± 0.11) b | 7.83 (± 0.07) b |
| C:N | 3.85 (± 0.12) a | 3.52 (± 0.09) a | 3.83 (± 0.05) a | 3.86 (± 0.07) a | 3.87 (± 0.13) a | 3.89 (± 0.08) a |

Different letters within the same row indicate significant differences at $P < 0.05$ by Turkey's HSD test for each variable including earthworm biomass or element content across different species litter.

**Table 3**

The *F*-value of two-way ANOVA results showing the effects of earthworms, litter and their interaction on soil C and N fractions.

| | d.f.[a] | SOC | POC | DOC | MBC | TN | PON | DON | $NO_3^-$-N | MBN |
|---|---|---|---|---|---|---|---|---|---|---|
| Earthworm (E) | 1 | **39.0**$^{***}$ | **19.5**$^{***}$ | **5.2**$^{*}$ | 0.2 ns | 2.2 ns | **8.3**$^{**}$ | 3.0 | **15.7**$^{***}$ | 2.7 ns |
| Litter (L) | 5 | **146.8**$^{**}$ | **145.5**$^{**}$ | **85.3**$^{***}$ | **48.5*****| **211.2**$^{***}$ | **23.5**$^{***}$ | **5.1**$^{***}$ | **105.0**$^{***}$ | **37.4***** |
| E × L | 5 | **4.4**$^{**}$ | 2.1 ns | **4.8**$^{**}$ | 2.2 ns | 0.5 ns | 0.8 ns | 0.8 | **5.2**$^{***}$ | 1.6 ns |
| Residuals | 48 | | | | | | | | | |

*, ** and *** indicate significant effects at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. ns, non significant effect. Bolded *F*-value are significant ($p < 0.05$).

SOC = soil organic carbon, POC = particulate organic carbon, DOC = dissolved organic carbon, MBC = microbial biomass carbon, TN = total soil nitrogen, PON = particulate organic nitrogen, DON = dissolved organic nitrogen, $NO_3^-$-N = nitrate nitrogen, MBC = microbial biomass nitrogen

[a] d.f.: degree of freedom.

**Figure captions**

**Fig. 1.** Scatter plots of earthworm tissue C:N ratio relative to soil C:N ratio (SOC:TN). The inserted subgraph presents the relative positions between earthworm tissue and soil C:N relationship in comparison to the 1:1 line. Litters are labeled according to clover (CL), maize stover (MA), wheat straw (WH), *Rumex* (RU) and bagasse fiber (BA). The grey polygon indicates the 95% confidence interval.

**Fig. 2.** Influences of earthworms and plant litter on cumulative $CO_2$ emissions (A) and $N_2O$ emissions (B) during the 90 d incubation period. The error bars represent standard errors (n = 5).

**Fig. 3.** Influences of earthworms and plant litter on soil organic carbon (A), total nitrogen (B), particulate organic carbon (C), particulate organic nitrogen (D), SOC:TN (E) and POC:PON (F). The error bars represent standard errors (n = 5).

**Fig. 4.** Influence of earthworms and plant litter on microbial biomass carbon (MBC), microbial biomass nitrogen (MBN) and microbial biomass carbon to nitrogen ratio (MBC:MBN). The error bars represent standard errors (n = 5).

**Fig. 5.** Influences of earthworms and plant litter on α-1,4-D-glucosidase (AG), β-1,4-glucosidase (BG), β-D-cellobiohydrolase (CB), β-1,4-N-acetylglucosaminidase (NAG), L-leucine aminopeptidase (LAP). The error bars represent standard errors (n = 5).

**Fig. 6.** Relationships between enzyme stoichiometry and soil C:N ratio. The ln(AG + BG + CB):ln(NAG + LAP) is an indicator of microbial C:N acquisition effort. The horizontal dashed line indicates 1:1 enzyme stoichiometry, and the error bar represent standard errors (n = 5). The different color represented different litter, i.e. the yellow, green, red, purple, blue and dark green corresponded to NL, CL, MA, ST, RU and BA, respectively. The symbol ○ represent without earthworm, ■ represent with earthworm.

**Fig. 7.** Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance analysis of relative abundances of phospholipid fatty acid (PLFA) markers. Circles represent 95% confidence intervals of microbial communities associated with distinct litter species.

**Fig. 8.** Structural equation model showing potential causal effects of earthworm, resource availability and soil microbial communities on $CO_2$. Arrow thickness is scaled proportionally to the standardized path coefficients (numbers on arrows). Solid and dashed lines indicate significant ($P < 0.05$) and marginally significant effects ($P < 0.1$), respectively; Dotted lines represent non-significant paths. The proportion of variation explained by the model ($R^2$) are shown next to each endogenous variable.

**Figure 1**

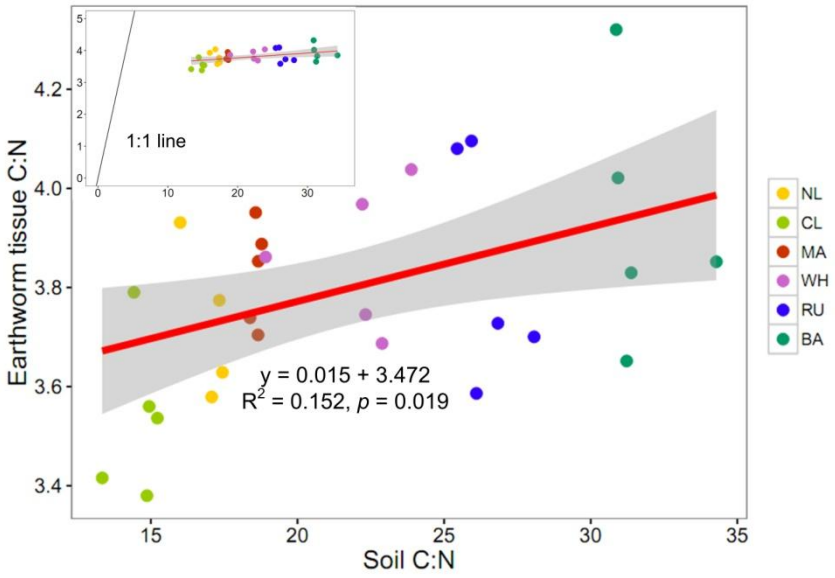


**Fig. 1.** Scatter plots of the earthworm tissue C:N ratio relative to soil C:N ratio (SOC:TN). The inserted subgraph shows the linear relationship between the earthworm tissue C:N ratio relative to soil C:N ratio in comparison to the 1:1 line. Litters are labeled according to clover (CL), maize stover (MA), wheat straw (WH), rumex (RU) and bagasse fiber (BA). The grey polygon indicates the 95% confidence interval.

**Figure 2**

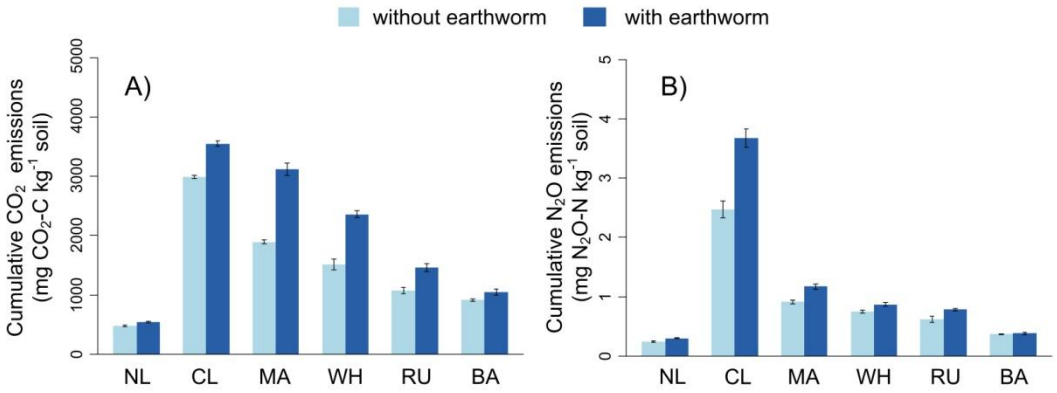


**Fig. 2.** Influences of earthworms and plant litter on cumulative $CO_2$ emissions (A) and $N_2O$ emissions (B) during the 90 d incubation period. The error bars represent standard errors (n = 5).

**Figure 3**

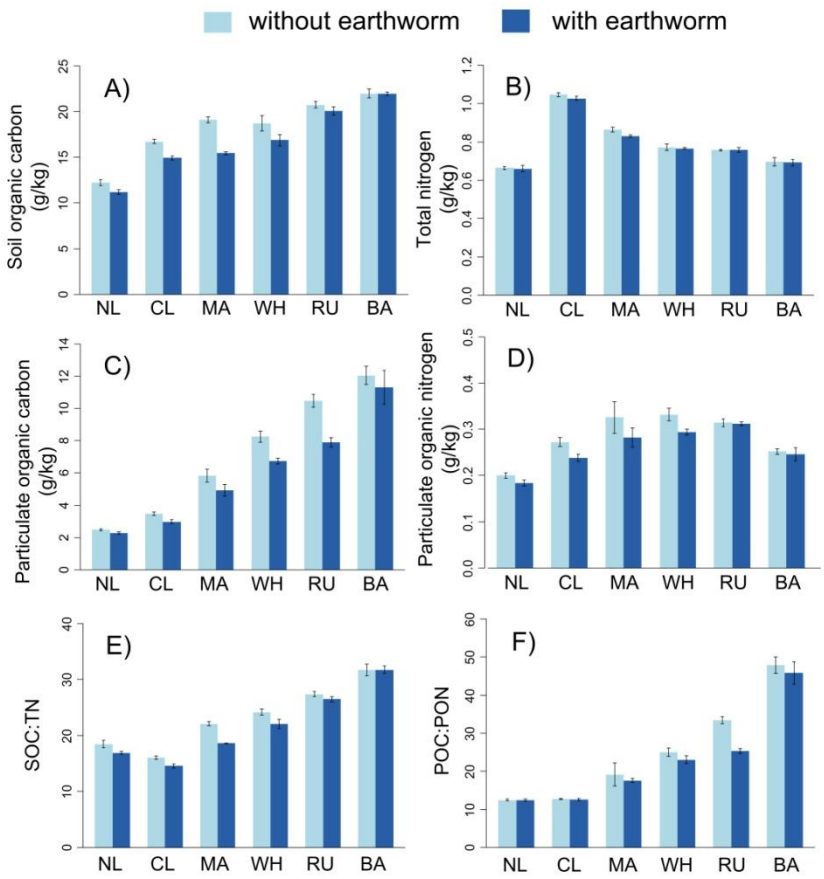


**Fig. 3.** Influences of earthworms and plant litter on soil organic carbon (A), total nitrogen (B), particulate organic carbon (C), particulate organic nitrogen (D), SOC:TN (E) and POC:PON (F). The error bars represent standard errors (n = 5).

**Figure 4**

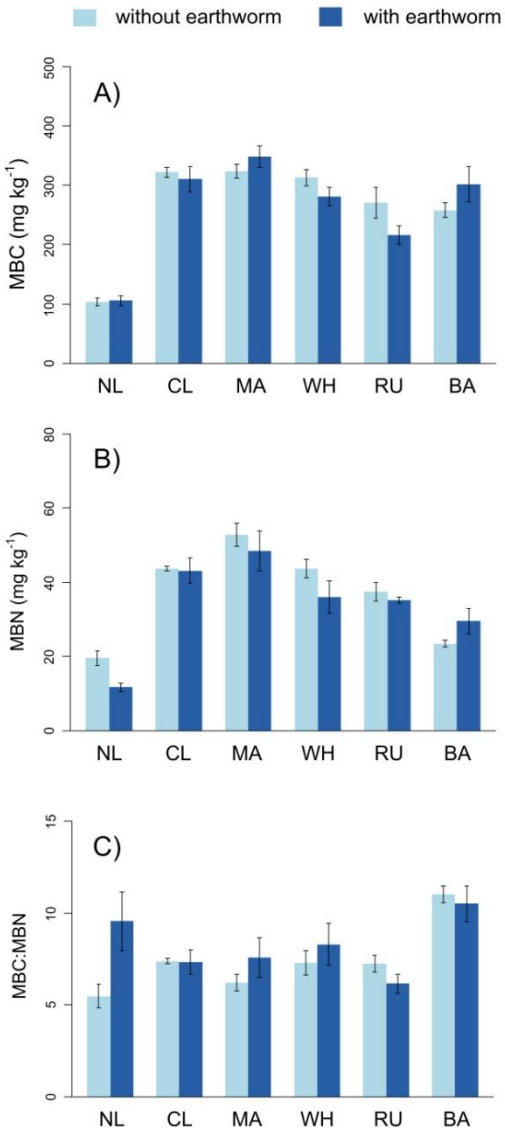


**Fig. 4.** Influence of earthworms and plant litter on microbial biomass carbon (MBC), microbial biomass nitrogen (MBN) and microbial biomass carbon to nitrogen ratio (MBC:MBN). The error bars represent standard errors (n = 5).

**Figure 5**

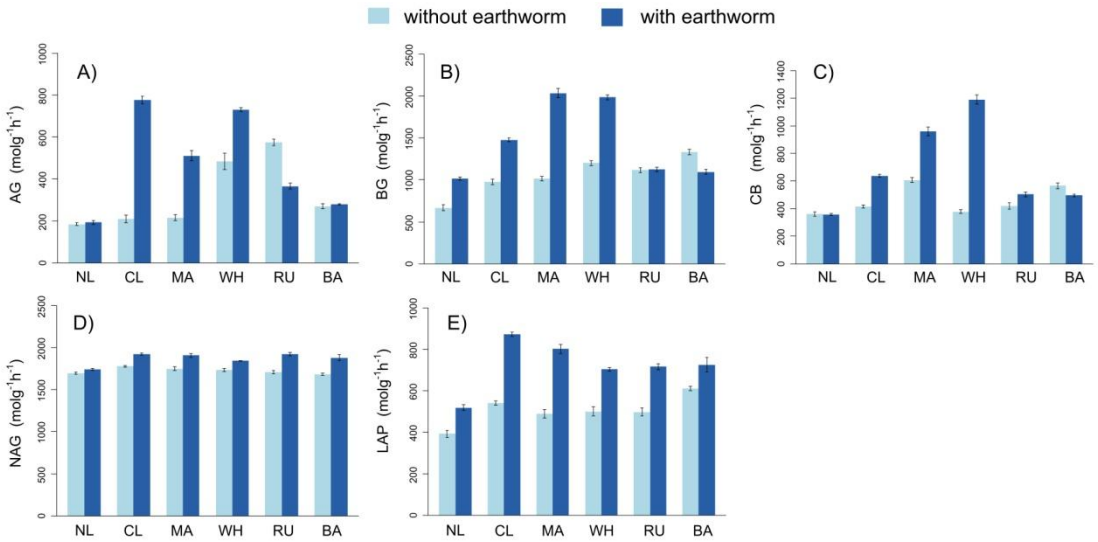


**Fig. 5.** Influences of earthworms and plant litter on α-1,4-D-glucosidase (AG), β-1,4-glucosidase (BG), β-D-cellobiohydrolase (CB), β-1,4-N-acetylglucosaminidase (NAG), L-leucine aminopeptidase (LAP). The error bars represent standard errors (n = 5).

**Figure 6**

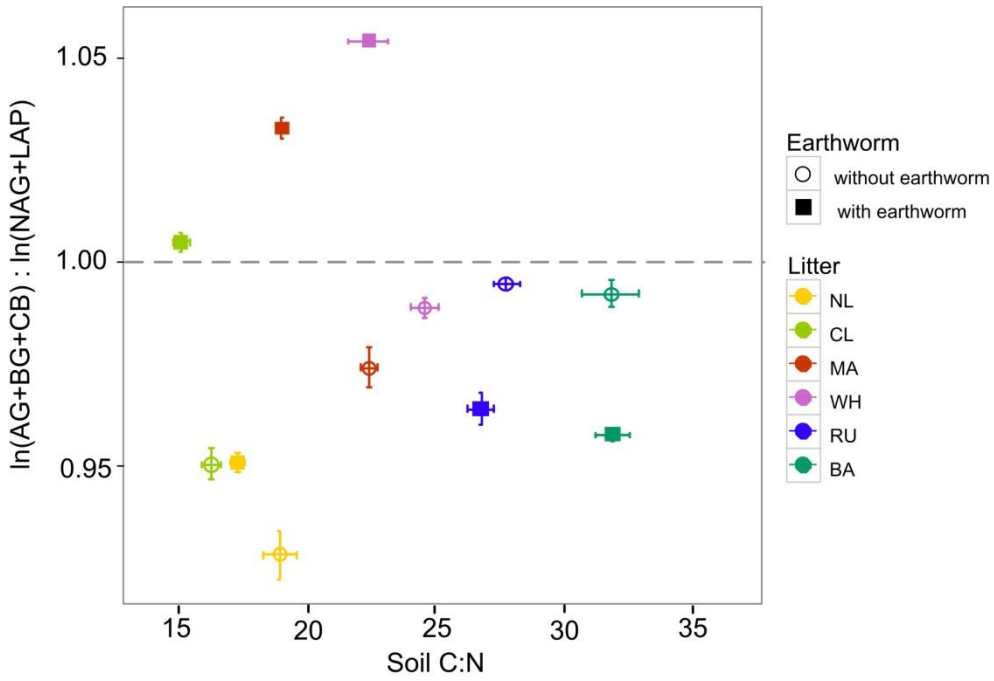


**Fig. 6.** Relationships between enzyme stoichiometry and Soil C:N ratio. The ratio ln(AG + BG + CB):ln(NAG + LAP) is an indicator of microbial C:N acquisition effort. The horizontal dashed line indicates 1:1 enzyme stoichiometry, and the error bars represent standard errors (n = 5). The different color represented different litter, i.e. the yellow, green, red, purple, blue and dark green corresponded to NL, CL, MA, ST, RU and BA, respectively. The symbol ○ represent without earthworm, ■ represent with earthworm.

Figure 7

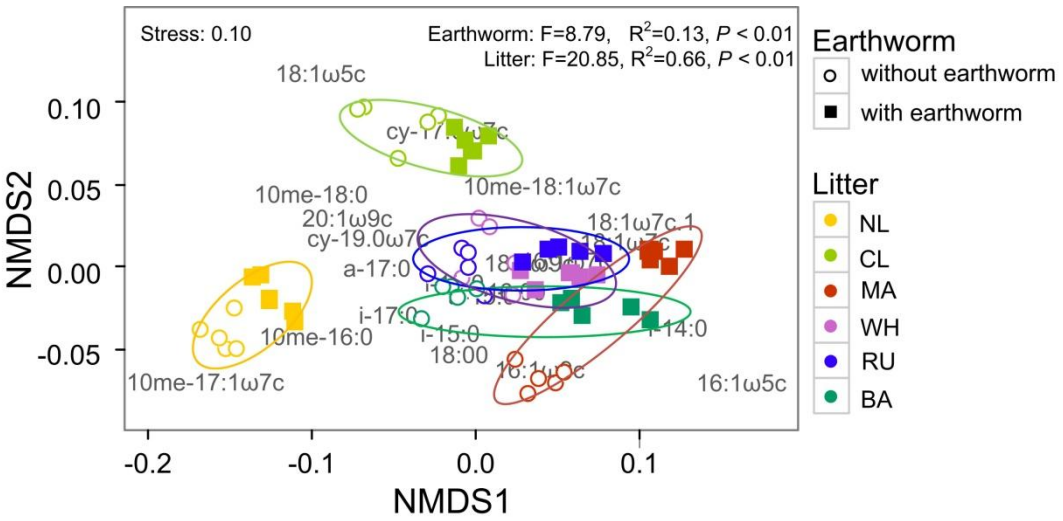


**Fig. 7.** Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distance analysis of relative abundances of phospholipid fatty acid (PLFA) markers. Circles represent 95% confidence intervals of microbial communities associated with distinct litter species.

**Figure 8**

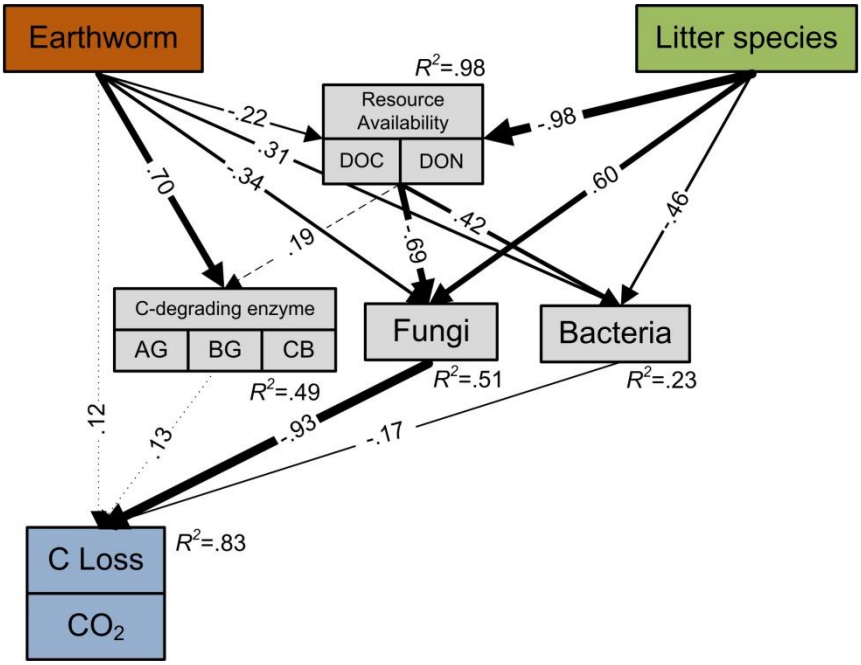


**Fig. 8.** Structural equation model showing potential causal effects of earthworm, resource availability and soil microbial communities on $CO_2$. Arrow thickness is scaled proportionally to the standardized path coefficients (numbers on arrows). Solid and dashed lines indicate significant ($P < 0.05$) and marginally significant effects ($P < 0.1$), respectively; Dotted lines represent non-significant paths. The proportion of variation explained by the model ($R^2$) are shown next to each endogenous variable.