Species-specific effects of earthworms on microbial communities and the
fate of litter-derived carbon

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13 **Abstract**

14 Soil respiration is frequently measured as a surrogate for biological activities and is
15 important in soil carbon cycling. The heterotrophic component of soil respiration is
16 primarily driven by microbial decomposition of leaf litter and soil organic matter, and is
17 partially controlled by resource availability. In North American temperate deciduous
18 forests, invasive European and Asian earthworms are known to variously affect soil
19 properties and resource availability through their feeding, burrowing, and casting
20 behaviors, and may affect different components of soil respiration through modulating
21 the microbial communities. By tracing litter-derived C from ^{13}C and ^{15}N double-enriched
22 leaf litter into soil and CO_2 efflux in a mesocosm experiment, we tested the hypothesis
23 that earthworms inhibit litter C-derived soil respiration by reducing resource availability
24 and microbial biomass, and further examined how species-specific effects of earthworms
25 on soil respiration are mediated by soil microbial community. We showed that while
26 earthworms generally had no effect on total soil respiration, the interaction between
27 *Octolasion lacteum* and *Lumbricus rubellus* had a significant negative non-additive
28 effect, presumably through affecting anaerobic microsites in the soil. Moreover, litter C-
29 derived soil respiration was reduced by the Asian *Amyntas hilgendorfi*, the European *L.*
30 *rubellus*, and the North American native species *Eisenoides lonnbergi*, but not by the
31 European species *O. lacteum*. Phospholipid fatty acid (PLFA) analysis and structural
32 equation modeling indicated that while soil bacteria and fungi abundances were affected
33 by earthworm species identities, the observed reduction of litter C-derived soil respiration
34 could not be fully explained by changes in microbial biomass. We attributed these effects
35 to earthworm-induced aggregate formation, reduction of microbial transformation of

36 labile carbon, and antimicrobial peptide activities, and concluded that the mechanisms
37 through which the four earthworm species affect the fate of litter-derived C and its
38 mineralization are species-specific.

39

40 **Keywords:** Earthworm; Phospholipid fatty acid analysis; Soil respiration; Stable isotope;
41 Structural equation modeling

1. Introduction

The heterotrophic component of soil respiration is primarily the result of decomposition and is driven by microbial activity. Soil macrofauna, such as earthworms, influence soil microbial communities and activities through complex direct and indirect processes. A recent meta-analysis concluded that the presence of earthworms increases soil respiration by an average of 33% (Lubbers et al., 2013), but the effect is transient and relatively short-term, usually observable only during short-term manipulation experiments or at the leading edge of the earthworm invasion front (Eisenhauer et al., 2011; Xia et al., 2011; Crumsey et al., 2013). The long-term field study by Fisk et al. (2004) supports this conclusion, showing no differences in soil respiration between plots with and without earthworms. However, these conclusions do not imply that the underlying processes contributing to soil respiration and their relative importance remain unchanged under earthworm invasion.

The overall effects of earthworms on soil microbial communities and activities could be positive, negative or neutral. In general, earthworms can indirectly affect soil microbes through changing resource availability by consuming leaf litter and soil organic matter (SOM), by casting, and by vertical translocation of C and N in the soil (Eisenhauer et al., 2007). Vertical mixing of leaf litter by litter-feeding species tends to increase microbial biomass in the soil as a result of the combination of the release of labile substrates and translocation of readily mineralizable C, while soil-feeding species tend to decrease microbial biomass in the soil due to their ingestion of recalcitrant organic matter (McLean et al., 2006). The drilosphere, which consists of earthworm burrows, casts and

middens, is rich in soluble C and earthworm mucus when fresh and is generally considered a hotspot of microbial activities (Aira et al., 2009; Stromberger et al., 2012). Bacteria are also known to be an important part of earthworm nutrition (Larsen et al., 2016). Earthworms may selectively feed on bacteria or bacteria-colonized patches (Jayasinghe and Parkinson, 2009; Zirbes et al., 2011), potentially leading to reduced bacteria abundance. Their burrowing activities can cause disruption of fungal hyphae (Butenschoen et al., 2007), leading to the reduction of fungi. Some of these behaviors and the associated impacts can be species-specific.

While the effects of earthworms on resource availability and soil microbes have been widely studied (Butenschoen et al., 2007; Jayasinghe and Parkinson, 2009; Eisenhauer et al., 2011; Dempsey et al., 2011, 2013; Sackett et al., 2013), less is known about how the different processes involved mediate the effects of earthworms on soil respiration and its different components. The earthworm-induced short-term increase in soil respiration, followed by gradual decrease back towards the baseline (e.g. Xia et al., 2011; Crumsey et al., 2013), is consistent with the theoretical responses of soil microbes following a C pulse event, and can be readily explained by the enzyme-driven theoretical models of microbial C and N limitation (Schimel and Weintraub, 2003; Waring et al., 2013).

Earthworms are also known to change the fate of litter-derived C through a combination of their feeding, burrowing, and casting behaviors (Fahey et al., 2013a; Chang et al., 2016). Therefore, the contribution of litter-derived C to soil respiration may still be altered even under situations where total soil respiration is not affected. Recently Snyder et al. (2009) suggested that soil macrofauna could reduce soil respiration derived from leaf litter. This new phenomenon has only been observed in two cases, one with the

millipede *Pseudopolydesmus erasus* native to the USA and the other with the Asian invasive earthworm *Amyntas corticis* (Snyder et al., 2009). It is unclear whether this phenomenon is common across different species with different feeding and burrowing behaviors, and the mechanisms leading to the decrease have never been investigated.

In temperate deciduous forests in eastern North America, European earthworm invasion into habitats with low native earthworm abundance or habitats previously devoid of earthworms has led to growing concerns from researchers and land managers. Through feeding, burrowing and casting behaviors, invasive European earthworms negatively affect the understory vegetation, reducing the leaf litter and organic layers and mixing the organic matter into the mineral soil (Hale et al., 2005, 2006; Nuzzo et al., 2009; Dempsey et al., 2011; Dobson and Blossey, 2015). Earthworm activities also lead to major changes in soil properties and biogeochemistry, including altered water retention capacity, pH, and soil C and N distribution and availability, as well as increased bulk density, aggregate formation, incorporation of organic matter, and CO₂ and N₂O efflux (Bohlen et al., 2004a; Hale et al., 2005, 2008; Eisenhauer et al., 2007; Szlavecz et al., 2011; Lubbers et al., 2013; Ma et al., 2013; Dobson and Blossey, 2015; Lyttle et al., 2015).

In recent years, a group of Asian earthworms, *Amyntas*, has been widely reported invading forests already inhabited by European species, leading to a “second wave of invasion” where the soil ecosystem, already modified by European species, is going through another transition. Two of the invading species, *Amyntas agrestis* and *A. hilgendorfi*, are of special concern due to their high abundance and biomass (Callahan et

al., 2003; Görres and Melnichuk, 2012; Greiner et al., 2012), ability to spread with facilitation from human activities (Bellitürk et al., 2015), and potential to completely displace other earthworm species (Greiner et al. 2012; Chang et al., 2016). While recent studies have shown that the effect of *Amyntas* on soil C may be similar to that of the European species (Snyder et al., 2011, 2013; Greiner et al., 2012), our understanding on the mechanisms through which different *Amyntas* and European earthworm species affect soil C dynamics is still limited. One contrasting difference between the two *Amyntas* and most common European species in eastern North America is the annual life cycle of the two *Amyntas* species. This characteristic is accompanied by dietary flexibility, voracious foraging, fast growth, and dramatic seasonal biomass fluctuation (Callahan et al., 2003; Greiner et al. 2012; Chang et al., 2016), and may have distinct impacts on soil C biogeochemistry.

Redistribution of organic matter by earthworms through vertical mixing alters resource availability to soil microbes and therefore affects C sequestration and mineralization. Earthworms in different functional groups have distinct feeding and burrowing behaviors, and, in theory, can have their own characteristic soil mixing and C translocation patterns distinguishable from other functional groups. The most commonly used functional classification categorizes earthworms into three groups: ‘epigeic species’ are litter feeders and leaf litter/soil surface dwellers; ‘endogeic species’ are soil feeders and live predominantly in the soil; ‘anecic species’ are litter feeders that live in permanent vertical burrows extending deep into the soil (Bouché, 1977). A fourth group, ‘epi-endogeic species’, is sometimes separated from the true epigeic earthworms for species living in the litter-soil interface but also burrowing into the surface mineral

soil. Since all species in a functional group are assumed to exhibit functional equivalency (Blondel, 2003), the three or four-category classification has been widely used in recent research focusing on belowground processes (Bohlen et al., 2004b; Crumsey et al., 2013). However, several studies have demonstrated that using functional groups alone failed to reasonably explain and predict patterns of feeding, vertical mixing, and C translocation in a wide range of earthworm species (Neilson et al., 2000; Zicsi et al., 2011; Chang et al., 2016), stressing the importance of explicitly considering species identities and species-specific effects when studying earthworm impacts on soil C and N biogeochemistry.

Leaf litter with a unique ^{13}C signature, artificially altered through either enrichment or depletion, allows us to trace litter-derived C into soil and CO_2 , and to partition soil respiration into litter- and soil-derived components. In this study, we conducted a lab mesocosm experiment using ^{13}C and ^{15}N double-enriched leaf litter to address the complex processes mediating the earthworm effects on soil respiration and to evaluate how soil respiration is affected by four earthworm species with distinct behaviors. We hypothesized that (1) earthworms have an overall negative effect on litter C-derived soil respiration, (2) epi-endogeic earthworms negatively affect litter C-derived soil respiration by reducing leaf litter, (3) endogeic earthworms reduce litter C-derived soil respiration by reducing substrate availability and microbial biomass in the soil, and (4) the interspecific interactions have either additive, or positive non-additive effects on both litter C and soil C-derived soil respiration.

2. Methods

2.1. Experimental design

A laboratory experiment with ecologically different earthworm species and species combinations was conducted using ^{13}C and ^{15}N double-enriched leaf litter as a food resource (Chang et al., 2016). Four species of earthworms were selected based on their origin and functional groups: *Amyntas hilgendorfi*, an epi-endogeic species of Asian origin, *Lumbricus rubellus*, an epi-endogeic species from Europe, *Octolasion lacteum*, an endogeic species from Europe, and *Eisenoides lonnbergi*, an endogeic earthworm native to North America. All species and forest soil were collected at the Smithsonian Environmental Research Center (SERC), Edgewater, Maryland, USA (38°53'17.0"N, 76°33'14.3"W; www.serc.si.edu), where research on the impacts of European earthworm invasion has been conducted since 2004 (e.g. Szlavecz and Csuzdi, 2007; Filley et al., 2008; Crow et al., 2009; Szlavecz et al., 2011, 2013; Ma et al., 2013; Chang et al., 2016). Soils at the location have been classified as Collington sandy loam (fine-loamy mixed, active, mesic Typic Hapludult), with an average pH of 5.1, 32% silt, 20% clay, and 5.6% organic matter content (Yesilonis et al., 2016). Soil used for the experiment was collected from the depth of 0-15 cm and sieved through a 2 mm sieve. ^{13}C and ^{15}N double-enriched tulip poplar (*Liriodendron tulipifera*) leaf litter ($\delta^{13}\text{C} = 27.1 \pm 0.2 \text{ ‰}$, $\delta^{15}\text{N} = 890.3 \pm 11.0 \text{ ‰}$, C:N ratio = 33.8 ± 0.5 (mean $\pm$ SE)) produced in an enrichment chamber (Bernard et al. 2015) with petioles removed was broken by hand and sieved through a 4 mm sieve. Mesocosms consisted of 2 L white plastic containers with perforated lids (14.8 cm D x 14.9 cm H). 1450.0 g sieved and mixed soil (C content = $4.32 \pm 0.12\%$, N content = $0.35 \pm 0.01\%$, C:N ratio = 12.3 ± 0.1 , $\delta^{13}\text{C} = -27.3 \pm 0.1 \text{ ‰}$, $\delta^{15}\text{N} = 1.9 \pm 0.1 \text{ ‰}$ (mean $\pm$ SE)) was added into the mesocosms followed by 4.0 g of enriched litter on the surface.

Gravimetric water content was adjusted to 38%. Mesocosms were preconditioned in the dark at 40% RH and 17 °C for 16 days prior to the addition of earthworms.

A total of 10 treatments and one control ('Control'), all with six replicates, were set up: four were single-species treatments with each of the four earthworm species (*A. hilgendorfi*, *L. rubellus*, *O. lacteum*, and *E. lonnbergi* treatments, respectively), and six were two-species treatments with all combinations of the four species. The control contained both soil and litter but no earthworm. Four *A. hilgendorfi*, six *L. rubellus*, twelve *O. lacteum*, and six *E. lonnbergi* individuals were added into the single-species treatments with the respective species. For the two-species treatments, two *A. hilgendorfi*, three *L. rubellus*, six *O. lacteum*, and three *E. lonnbergi* were added into the respective treatments. The numbers of individuals were chosen for each species to take into account both density in the field and individual biomass differences to mimic potential co-occurrence conditions in the field (Chang et al., 2016). Three additional mesocosms with no leaf litter and no earthworms ("no-leaf treatment") were constructed for the purpose of isotopic measurements of CO₂ gas (see below). The mesocosms were incubated in the dark under 40% RH at 17 °C for 21 days. Gravimetric water content was adjusted to 35% on days 3, 6, 9, 12, 17 and 21.

At the end of the experiment, earthworms, remaining leaf litter and soil were collected. Two mesocosms, one belonging to the *L. rubellus* treatment and one, *L. rubellus* + *E. lonnbergi* treatment, were excluded due to 50% earthworm mortality rate. The survival rates in the remaining mesocosms are 75% in two mesocosms, 83% or higher in the others, and 100% in more than two-thirds of the mesocosms. Soil samples

were divided into the 0-5 cm layer and the lower layer that roughly equaled 5-10 cm, sieved through a 4-mm sieve and homogenized. Subsamples of soil were stored at -20 °C for microbial analysis; another subset was dried at 60 °C, ground, and analyzed for C and N contents as described below.

2.2. Soil respiration measurement and CO₂ gas sampling

Soil CO₂ efflux was measured on days 3, 6, 9, 12, 17 and 21. The mesocosms were put into a customized closed cylinder chamber with a Vaisala GMP343 infrared CO₂ sensor (Vaisala, Finland) inserted at the top. CO₂ emitted from the soil was accumulated in the headspace of the chamber and the concentration was recorded every second for seven minutes.

CO₂ gas was sampled from five randomly selected mesocosms of each treatment on days 18-20 for stable isotope analysis. Up to 12 mesocosms were randomly selected and sampled at the same time using the same chambers used for CO₂ efflux measurements. Vaisala GMP343 infrared CO₂ sensors (Vaisala, Finland) were inserted at the top of three of the chambers. The sampling started once CO₂ concentration in all three chambers reached 1,000 ppm, and then occurred every 60 min for four hours. At each sampling point, CO₂ gas samples were taken using syringes and stored in 12 ml Exetainer vials (LabCo, UK) at room temperature.

2.3. Elemental and stable isotope analyses

The C and N elemental and stable isotope composition of soil from the mesocosm experiment were analyzed at the UC Davis Stable Isotope Facility, Davis, CA, USA using either a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) coupled with an Elementar Vario EL Cube or Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) or a PDZ Europa ANCA-GSL elemental analyzer (Sercon Ltd., Cheshire, UK). CO₂ gas from five randomly selected mesocosms of each treatment was analyzed at the same facility using a ThermoScientific PreCon-GasBench system coupled with a ThermoScientific Delta V Plus isotope ratio mass spectrometer (ThermoScientific, Bremen, DE). Stable isotope ratios of C and N (¹³C/¹²C and ¹⁵N/¹⁴N) were expressed using the delta (δ) notation: δ¹³C_{sample} or δ¹⁵N_{sample} = [R_{sample} / R_{standard} - 1] x 1000‰, where R_{sample} is the isotope ratio (¹³C/¹²C or ¹⁵N/¹⁴N) in the samples, and R_{standard} is the isotope ratios in the standard, which is Pee Dee Belemnite (PDB) for C and atmospheric nitrogen for N.

2.4. Phospholipid fatty acid analysis

Phospholipid fatty acid (PLFA) analysis was used to characterize soil microbial communities. Samples were prepared and analyzed as described by Buyer and Sasser (2012), using 19:0 phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) as an internal standard for quantitative analysis. Gas chromatography was conducted on an Agilent 6890 gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with autosampler, split-splitless injector, and flame ionization detector. The system was controlled with MIS Sherlock (Microbial ID, Inc., Newark, DE, USA) and Agilent ChemStation software. Fatty acids were identified using the PLFAD1 calibration

mix and PLFAD1 peak library (Microbial ID). Random samples were run on a Clarus 500 GC-MS (Perkin-Elmer, Waltham, MA, USA) to confirm fatty acid identifications.

2.5. CO₂ flux rate and isotopic calculations

CO₂ flux rate was calculated using the following equation:

$$F = \frac{\partial C}{\partial t} \left(\frac{PV}{RTS} \right)$$

where F is the gas flux in $\mu\text{mol m}^{-2} \text{s}^{-1}$, $\frac{\partial C}{\partial t}$ is the rate of CO₂ concentration change in the chamber in ppm s⁻¹ ($\mu\text{mol mol}^{-1} \text{s}^{-1}$), P is the pressure of headspace gas, V is the volume of the headspace, R is the ideal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is temperature in Kelvin, and S is the soil surface area.

$\delta^{13}\text{C}$ of CO₂ emitted from soil alone and soil plus leaf litter was estimated by applying Keeling plots to the $\delta^{13}\text{C}$ -CO₂ gas data following Pataki et al. (2003). The contribution of litter-derived C ($f_{\text{c-ltr}}$) to CO₂ efflux was estimated using a simple isotope mixing model:

$$\delta^{13}\text{C}_{\text{gas}} = \delta^{13}\text{C}_{\text{litter}} \times f_{\text{c-ltr}} + \delta^{13}\text{C}_{\text{CO}_2\text{-soil}} \times (1 - f_{\text{c-ltr}})$$

where $f_{\text{c-ltr}}$ is the proportions of litter-derived C in CO₂, $\delta^{13}\text{C}_{\text{gas}}$, $\delta^{13}\text{C}_{\text{litter}}$ and $\delta^{13}\text{C}_{\text{CO}_2\text{-soil}}$ are the ¹³C abundances in CO₂ flux, leaf litter (27.10‰, Chang et al. 2016) and CO₂ emitted from soil alone.

With the CO₂ gas sampling for ¹³C analysis happening on days 18-20, in order to reasonably estimate CO₂ flux rate for isotopic partitioning, we calculated the mean CO₂ flux rate of each treatment by taking the mean of the rates on days 17 and 21, the days before and after the CO₂ gas sampling, and partitioned the mean rates into the litter and soil components using estimated f_{c-ltr} .

$\delta^{13}\text{C}$ of leaf litter, earthworms, and soil and $\delta^{15}\text{N}$ of soil from the same mesocosms reported in Chang et al. (2016) were used to calculate the total amount of “excess” ¹³C and ¹⁵N in the respective pools at the end of the experiment (Fahey et al., 2013a, 2013b; Gilbert et al., 2014; Yavitt et al., 2015). Excess ¹³C and ¹⁵N (presented as $\mu\text{g } ^{13}\text{C}$ or ¹⁵N) were calculated by subtracting out the absolute amount of natural abundance isotopes in the respective pools.

2.6. Statistical analysis

All statistical tests were conducted in R v3.1.2 (R Core Team 2014) and, when relevant, using the biomass of each earthworm species as independent variables. Mixed effect models, beta regressions and structural equation modeling (SEM) were conducted using packages lme4 (Bates et al., 2015), betareg (Cribari-Neto and Zeileis, 2010) and lavaan (Rosseel, 2012), respectively. Data on earthworm survival and biomass, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of litter, soil and earthworms, and the absolute abundances of Gram-negative and Gram-positive bacteria PLFA biomarkers have been previously reported in Chang et al. (2016) and were included with the current dataset only when necessary.

General linear models were used to investigate the effects of earthworm species and species interactions on soil C and N contents, cumulative CO₂ efflux, average CO₂ flux rates, $\delta^{13}\text{C}$ of CO₂ gas emitted from soil, and the partitioned CO₂ flux rate derived from only litter or soil C. Independent variables included are the mean biomass of individual species (the mean between initial and final biomass) and their interactions (Chang et al., 2016). The CO₂ flux rate at each sampling occasion was analyzed using mixed effect models with sampling occasions as random variables. Likelihood ratio tests were used to access significant differences between nested models. Separate regressions were conducted to illustrate the relationships between $\delta^{13}\text{C}$ of CO₂ gas and leaf litter remaining or excess ¹³C in the soil.

PLFA were combined into biomarker groups (Buyer and Sasser, 2012) and analyzed using three approaches. First, the effects of earthworms on total PLFA, anaerobe PLFA and fungi : bacteria ratios were assessed using general linear models. Second, PLFA data from Gram-negative bacteria, Gram-positive bacteria, Actinomycetes, total bacteria, fungi and protozoa were Hellinger-transformed (square root of proportion) and analyzed using beta regression, and likelihood ratio tests were used to access significant differences between nested models. Third, microbial community compositions were analyzed using redundancy analyses with the mean biomass of earthworm species as constrained variables. Permutation tests with 999 permutations were used to test the significances of the overall models, the constrained axes and the biomass of earthworm species.

In addition to general linear models, we used structural equation modeling (SEM) to investigate how the effects of earthworms on soil respiration were mediated by resource availability and microbial communities. SEM allows to test hypotheses inferred from causal diagrams under a graph theory framework (Grace et al., 2012; Eisenhauer et al., 2015), and has been increasingly used in studies dealing with complex systems, including soil ecology (Eisenhauer et al., 2013, 2015; Sackett et al., 2013). We used soil excess ^{15}N , soil excess ^{13}C and remaining litter as the proxies for N availability, fresh C availability, and litter availability, respectively. Soil microbial communities were represented using bacterial and fungal PLFA. PLFA data from the two depths (0-5 cm and 5-10 cm) were combined using the weighted means based on soil dry mass. The adequacy of models was determined using the likelihood ratio tests and the Akaike information criterion (AIC). Model modification indices were used to improve the models. Further modifications of the model were done by considering additional paths significant in general linear models.

3. Results

3.1. Soil C & N and CO_2

Data on leaf litter remaining, excess ^{13}C , soil C and N contents, and soil C:N ratios are listed in Table 1. Soil C and N contents were generally not affected by earthworm species. However, *L. rubellus* x *O. lacteum* interaction had a positive effect on C content in 0-5 cm ($F_{1,57} = 4.461$, $P = 0.039$) and a marginally significant negative effect on N content in 5-10 cm ($F_{1,58} = 3.993$, $P = 0.050$). The mean soil C:N ratio in mesocosms with leaf litter was 14.3 ± 0.1 . *L. rubellus* x *O. lacteum* interaction had a positive effect on soil C:N ratios ($F_{1,57} = 5.276$, $P = 0.025$).

Earthworm species generally did not significantly affect CO₂ flux rates and cumulative CO₂ effluxes (Fig. 1). However, interaction between *L. rubellus* and *O. lacteum* reduced both cumulative CO₂ efflux ($F_{1,58} = 5.188$, $P = 0.026$) and CO₂ flux rate ($\chi^2 = 7.510$, $P = 0.006$), and interaction between *O. lacteum* and *E. lonnbergi* negatively affected CO₂ flux rate ($\chi^2 = 4.872$, $P = 0.027$) (Cumulative CO₂ effluxes in treatments (mean $\pm$ SE): 106.7 $\pm$ 8.9 (*L. rubellus*), 115.4 $\pm$ 15.4 (*O. lacteum*), 69.8 $\pm$ 7.9 (*L. rubellus* + *O. lacteum*), and 74.3 $\pm$ 11.8 (Control) $\mu\text{mol CO}_2 \text{ g}^{-1} \text{ soil}$).

$\delta^{13}\text{C}$ of CO₂ gas was negatively correlated with excess ^{13}C in the soil ($F_{1,48} = 7.042$, $P = 0.011$, $r^2 = 0.11$) and positively correlated with leaf litter remaining at the end of the experiment ($F_{1,51} = 14.476$, $P < 0.001$, $r^2 = 0.22$). However, when non-measurable values of leaf litter remaining (<0.05g, recorded as 0 g) were excluded, the correlation between $\delta^{13}\text{C}$ of CO₂ gas and leaf litter remaining disappeared ($F_{1,32} = 1.445$, $P = 0.238$, $r^2 = 0.04$) (Fig. 2). $\delta^{13}\text{C}$ of CO₂ gas was significantly reduced by *A. hilgendorfi* ($F_{1,48} = 181.930$, $P < 0.001$), *L. rubellus* ($F_{1,48} = 33.049$, $P < 0.001$) and *E. lonnbergi* ($F_{1,48} = 40.119$, $P < 0.001$), and increased by *L. rubellus* x *E. lonnbergi* interaction ($F_{1,47} = 6.292$, $P = 0.016$) (Fig. 3). Between days 17 and 21, the mean CO₂ flux rate was significantly reduced by *A. hilgendorfi* ($F_{1,59} = 6.743$, $P = 0.012$), and the litter C-derived CO₂ flux rate was significantly reduced by *A. hilgendorfi* ($F_{1,48} = 30.329$, $P < 0.001$) and by *E. lonnbergi* ($F_{1,48} = 6.961$, $P = 0.011$). Earthworm species and their interactions had no significant effects on soil C-derived CO₂ flux rates.

3.2. Microbial communities

Total PLFA concentration was used as a measure of total microbial biomass. PLFA biomarkers of specific groups were used as measures of biomass of the respective groups. However, these biomarkers are not entirely universal for all of the members of each taxonomic group, and are not completely specific to each taxonomic group. Therefore, they must be interpreted cautiously in the absence of independent corroborating evidence (Frostegård et al., 2011). Total PLFA concentration was negatively affected by *A. hilgendorfi* in 0-5 cm ($F_{1,56} = 11.341$, $P = 0.001$). The Hellinger-transformed Gram-negative bacteria PLFA concentration was positively affected by *A. hilgendorfi* in 5-10 cm; the Hellinger-transformed Gram-positive bacteria PLFA concentration was negatively affected by *A. hilgendorfi* but positively affected by *O. lacteum* in 5-10 cm; the Hellinger-transformed Actinomycete PLFA concentration was negatively affected by *A. hilgendorfi* and by *O. lacteum* in 5-10 cm (Table 2). The Hellinger-transformed fungi PLFA concentration was positively affected by *A. hilgendorfi* in 5-10 cm and by *O. lacteum* in 0-5 cm (Table 2); similarly, the fungi : bacteria ratios were increased by *A. hilgendorfi* at 5-10 cm ($F_{1,59} = 9.268$, $P = 0.003$) and by *O. lacteum* at 0-5 cm ($F_{1,57} = 4.998$, $P = 0.029$). The Hellinger-transformed protozoan PLFA concentration was positively affected by *A. hilgendorfi* in 5-10 cm (Table 2). Anaerobe PLFA concentration in 5-10 cm was positively affected by *L. rubellus* x *O. lacteum* interaction ($F_{1,58} = 4.294$, $P = 0.043$). The redundancy analysis (RDA) of microbial community composition indicated that in 0-5 cm the constrained axes (biomass of individual earthworm species) explained 10.9% of the variance, although only axis 1 was significant ($P=0.003$). Among the constrained variables, *A. hilgendorfi* had significant effects ($P=0.01$), while the effects of other earthworm species were not significant (Fig. 4a). The biomass of *A.*

hilgendorfi was positively associated with Gram-positive bacteria, and negatively correlated with Gram-negative bacteria. RDA of microbial community composition in 5-10 cm showed that the constrained axes explained 22.3% of the variance; both axis 1 ($P=0.001$) and axis 2 ($P=0.005$) were significant. Among the constrained variables, *A. hilgendorfi* ($P=0.001$) and *O. lacteum* ($P=0.02$) had significant effects (Fig. 4b). The biomass of *A. hilgendorfi* was positively associated with Gram-negative bacteria, fungi and protozoa, and negatively associated with Gram-positive bacteria. The biomass of *O. lacteum* was positively associated with Gram-positive bacteria.

3.3. SEM results

The final model adequately fit the data on soil respiration ($\chi^2_{30} = 31.04$, $P = 0.413$; Table 3). It explained 57% and 22% of soil respiration derived from litter C and soil C, respectively, 14% of the variance in bacteria PLFA, 13% of fungi PLFA, 55% of soil excess ^{13}C , 83% of soil excess ^{15}N , and 82% of leaf litter remaining (Fig. 5). *A. hilgendorfi*, *L. rubellus* and *E. lonnbergi* had direct negative effects on litter C-derived soil respiration, while only *A. hilgendorfi* negatively influenced soil C-derived soil respiration through a direct path. The four earthworm species had direct negative effects on leaf litter biomass on the soil surface, leading to an increase in ^{13}C and ^{15}N in the soil. This increased ^{13}C led to increased fungi and then bacteria biomass; the former negatively influenced soil C-derived soil respiration, while the latter had positive effects on litter C-derived soil respiration.

4. Discussion

By combining soil respiration measurement, isotopically enriched leaf litter, and ^{13}C analysis of CO_2 gas, we showed that after the transient initial pulse of CO_2 efflux, three earthworm species, including *A. hilgendorfi*, *L. rubellus*, and *E. lonnbergi* reduced the proportions of litter C-derived soil respiration even when there were no apparent overall changes in CO_2 efflux. In contrast, *O. lacteum* had no effects on either the litter C or the soil C-derived soil respiration. Altogether, these results partially support our first hypothesis that earthworms reduced litter C-derived soil respiration, but the effects are not universal and are species-specific.

While litter C-derived soil respiration was reduced by earthworms, the final structural equation (SEM) model indicated a more complex relationship that includes both direct negative and indirect positive connections between earthworms and soil respiration, with the direct negative connections being the stronger of the two. The indirect connections suggested that earthworms positively affected litter C-derived soil respiration through increasing substrate availability and consequently microbial biomass in the soil, while the reduction of leaf litter by earthworms had no direct effect. This result provided no support for either the reduction of leaf litter by epi-endogeic species or the decrease of microbial biomass by endogeic species as causes of the observed reduction in litter C-derived soil respiration, and therefore do not support our hypotheses 2 and 3.

In contrast to the indirect positive connections, direct paths with strong negative effects between earthworms and litter C-derived soil respiration indicated that processes not explicitly identified in the final SEM model might be important. Recent advances in our understanding of the connections among soil microbes, exoenzymes, and resource

stoichiometry (Schimel and Weintraub, 2003; Waring et al., 2013; Cotrufo et al., 2015) suggest that earthworms may reduce soil respiration through four different processes: (1) increasing physical protection of SOM by aggregate formation, (2) increasing physical protection of SOM by mediating microbial SOM transformation, (3) reducing microbial biomass and the associated maintenance and enzyme production, and (4) decreasing overflow metabolism respiration by increasing N availability. The last mechanism happens in N-limited systems with high soil C:N ratios and requires N addition as treatments, and therefore is unlikely to be the explanation for our observed change.

The negative correlation between $\delta^{13}\text{C}$ of CO_2 gas and excess ^{13}C in the soil suggest that C sequestration may play an important role in the reduction of litter C-derived soil respiration. Casts produced by earthworms may increase the formation of soil macro- and micro-aggregates and potentially the incorporation of litter-derived C within them (Bossuyt et al., 2005, 2006; Fahey et al., 2013b), which provides physical protection for the enclosed SOM, making it inaccessible for microbes (Mummey et al., 2006). Both *A. hilgendorfi* and *L. rubellus* have been shown to increase the formation of water-stable aggregates (Greiner et al., 2012; Sanchez-de Leon et al., 2014). Moreover, Zhang et al. (2013) showed that *L. rubellus* reduced litter C-derived soil respiration even though the litter was actually added after removing earthworms from the system, leaving no chance for earthworms to incorporate litter-derived C into aggregates. This suggests the presence of a separate pathway to our observed changes. Accordingly, in addition to increasing aggregate formation, we hypothesized that *L. rubellus* also leads to a microbial community that increases transformation of labile C and the subsequent formation and sequestration of SOM. In contrast, *A. hilgendorfi* has strong negative effects on bacteria

biomass (Chang et al., 2016), and it is more likely that in addition to increasing aggregate formation, the decrease in litter C-derived soil respiration may be attributed to the reduction of microbial biomass in the case of *A. hilgendorfi*. The two microbial hypotheses outlined above are consistent with the observations that microbial respiration tends to remain unchanged or decrease during the processes of earthworm invasion (McLean and Parkinson, 1997a, 1997b; Welke and Parkinson, 2003; Eisenhauer et al., 2011), and further provide potential mechanistic explanations for previously documented patterns.

By tracing ^{13}C -labeled leaf litter C in a sugar maple forest into soil for two years, Fahey et al. (2013a, 2013b) demonstrated that when compared to plots with no earthworms, soil in plots dominated by *L. rubellus* had lower soil carbon storage, and a much lower proportion of the added ^{13}C was recovered in the *L. rubellus*-dominated plots. These observations suggest that in the long-term, C loss due to acceleration of litter C-mineralization induced by earthworms during the early stage of litter decomposition cannot be offset by the reduction in litter C-derived soil respiration we observed, and may be further exacerbated by leaching in field conditions (Bohlen et al., 2004a; Crumsey et al., 2013).

The contrasting results from *L. rubellus* and *O. lacteum* are intriguing. Although often categorized in two different functional groups, both species live in the surface soil, feed directly and rely heavily on leaf litter, and lead to comparable C and N translocation into soil (Chang et al., 2016), but yet *O. lacteum* is the only species that did not negatively affect litter C-derived soil respiration. In contrast, the other endogeic species, *E.*

449 *lonnbergi*, the only native species in our experiment and the only species that does not
450 feed on leaf litter (Chang et al., 2016), has a negative effect on litter C-derived respiration,
451 one as strong as in the litter feeding *L. rubellus*. Unlike *A. hilgendorfi* and *L. rubellus*, *E.*
452 *lonnbergi* does not lead to increased incorporation of leaf litter C into soil (Chang et al.,
453 2016). Therefore, the two mechanisms that require an increased mineral-organic matter
454 interaction cannot satisfactorily explain our observation. At the end of the experiment,
455 70% of the initial leaf litter remained in the *E. lonnbergi*-only treatment in contrast to the
456 16% observed in the *O. lacteum*-only treatment. The 70% remaining leaf litter was
457 second to the 85% in the control. We expected that in these two treatments the large
458 amount of wet surface litter would be the major contributor of total soil respiration.
459 However, this was the case only in the control, but not in the *E. lonnbergi*-only treatment.
460 Although categorized as an endogeic earthworm, *E. lonnbergi* has been observed living
461 near the litter-soil interface in the field, a behavior similar to those observed in another
462 endogeic species, *Aporrectodea caliginosa* (C.-H. Chang, personal observation).
463 However, unlike *E. lonnbergi*, *Ap. caliginosa* has been shown to induce priming effects
464 on plant residues through the mucus it secretes (Bityutskii and Orlova, 2012), and have a
465 positive effect on litter C-derived soil respiration (Zareitalabad et al., 2010). Presumably,
466 *E. lonnbergi* may have reduced litter C-derived soil respiration through negatively
467 affecting the microbial community living in the litter-soil interface by secreting anti-
468 microbial peptides (Cameron, 1932; Valembois et al., 1982) through its dorsal pores.
469 With our extremely limited knowledge on *E. lonnbergi*, a common native species in
470 eastern USA, this explanation, albeit the only one consistent with our observations, is
471 highly speculative and needs to be further tested.

Comparison of the standardized path coefficients of direct paths between earthworms and litter C-derived soil respiration suggested that the effect of *A. hilgendorfi* is larger than those of *L. rubellus* and *E. lonnbergi*. Although *A. hilgendorfi* is larger than the other two species, our statistical analyses have taken earthworm biomass into account to some degree, and therefore the differences cannot be explained by size alone. *A. hilgendorfi* produces large quantities of granular casts rich in NH_4^+ and labile C at the time of excretion. While fresh casts produced by *A. hilgendorfi* have high microbial activity, it starts to decrease after seven days (Kawaguchi et al., 2011). The casts become water-stable within 24 hours after formation (Kawaguchi et al., 2011) and increase the prevalence of large soil aggregates in the field, even when compared with *L. rubellus* (Greiner et al., 2012). While it is unclear how aggregate turnover (e.g. Yavitt et al., 2015) will affect the protected carbon in the case of *A. hilgendorfi* for the long term, the consistent observations mentioned above support our interpretation that the strong effect of *A. hilgendorfi* on stable aggregate formation is responsible for the large negative path coefficient leading from *A. hilgendorfi* to litter C mineralization. Altogether, our growing understanding on the Asian *A. hilgendorfi* unveils an invasive species that is large, reaches high density, eats a wide range of food resources voraciously, moves fast, grows rapidly, and completes its life cycle in a year. Compared to its most common European “ecological equivalent”, *L. rubellus*, it causes stronger bacteria biomass reduction (Chang et al., 2016), macro-aggregate formation (Greiner et al., 2012), and reduction of litter C-derived soil respiration. All of these effects are linked potentially to the voracious feeding behavior, a requirement in the case of *A. hilgendorfi* to complete its short life cycle. While it is unclear whether the superior competitor *A. hilgendorfi* will eventually

outcompete *L. rubellus* (Chang et al., 2016), further research is need to understand the long-term effects of *A. hilgendorfi* on soil C biogeochemistry.

The complex microbial responses revealed in the SEM model are consistent with the expectations under the theoretical models of C and N stoichiometry (Schimel and Weintraub 2003; Waring et al. 2013). Bacteria are more likely than fungi to be N-limited. Increasing N favors bacteria over fungi and can have a negative effect on the latter (Waring et al., 2013), which explains the observed negative path from soil excess ¹⁵N to fungi. Moreover, the incorporation of leaf litter into SOM increases C:N ratio, a situation where bacteria may benefit from N mineralization by fungi (Romani et al., 2006; Waring et al., 2013), consistent with the positive path from fungi to bacteria in our model. Fungi are generally more efficient in assimilating C compared to bacteria (Sakamoto and Oba, 1994). The increase in fungal abundance and fungi : bacteria ratios can lead to lower C mineralization per unit biomass (Sakamoto and Oba ,1994; McLean et al., 2006), resulting in reduced soil respiration, as seen in the negative path from fungi to soil C-derived soil respiration.

In agreement with our hypothesis 4, most interspecific interactions have additive effects on litter C and soil C-derived soil respiration. However, the negative non-additive effect caused by *L. rubellus* x *O. lacteum* interaction was unexpected. *O. lacteum* has been reported to reduce microbial respiration (Eisenhauer et al. 2007). In our case, it caused reduced soil respiration only when co-occurring with *L. rubellus*. The results were consistent with the positive effects of the interaction between *O. lacteum* and *L. rubellus* on soil C content, but contradicted the conclusion by Xia et al. (2011), where interactions

between *L. rubellus* and *O. lacteum* increased soil respiration. Two apparent differences between our and Xia et al.'s (2011) experimental designs are the relatively long pre-conditioning period and larger soil mass in our experiment. These conditions, combined with activity of specific earthworm species, could potentially lead to increased anaerobic microsites in the soil, causing reduced soil respiration and increased C content. This explanation is supported by the increased anaerobe PLFAs by the *L. rubellus* x *O. lacteum* interaction, and is consistent with the observations by Giannopoulos et al. (2010), who demonstrated that interspecific interactions between *L. rubellus* and a different endogeic species, *Aporrectodea caliginosa*, increase N₂O emission from soil, a process requiring anaerobic conditions. These findings indicate that species-specific interactions of earthworms could lead to changes in soil aerobic conditions, potentially impacting aerobic (e.g. CO₂) and anaerobic (e.g. N₂O) greenhouse gas emissions (Lubbers et al., 2013). The opposite effects on soil respiration and soil C content also suggest that the interaction between *L. rubellus* and *O. lacteum* may lead to increased C stabilization through mineral-organic matter interactions and/or aggregate formation. These alternative but not mutually exclusive hypotheses indicate potential long-term consequences on C sequestration and are worth further investigation.

In summary, our results documented suppression of litter C-derived soil respiration by several species of earthworms, and highlighted that four species of earthworms, *A. hilgendorfi*, *L. rubellus*, *O. lacteum*, and *E. lonnbergi*, affect soil respiration through different mechanisms and pathways. Moreover, interspecific interactions of species pairs generally have additive effects on litter C mineralization and total soil respiration. However, two common species, *L. rubellus* and *O. lacteum*, have a strong, non-additive

interaction on soil respiration and C dynamics, leading to reduced CO₂ fluxes and increased soil C content. Altogether, we concluded that the mechanisms through which the four earthworm species affect the fate of litter-derived C and its mineralization are species-specific. These species-specific responses, which differ in mechanisms and/or magnitude, are further complicated by unexpected interspecific interactions, and by the complex responses of soil microbial communities to resource availabilities.

Acknowledgements

We are grateful to the people who helped in the field and during the lab experiments, especially Kelly Baker, Zachary Ferguson, Adam Dec and Jia-Hsing Wu. We also thank Stanley Tesch for helping with PLFA, Chuan-Chin Huang for insights on data analysis, and Jim Grace for suggestions on SEM. We appreciate two anonymous reviewers for providing positive, insightful, and helpful comments on an earlier version of this manuscript. Funding for this study was partially provided by the EPS Field Funds, the National Science Foundation (EEC-0540832, ACI 1244820, and EAR-0748574), and Microsoft Research.

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786

Figure legends

Fig. 1. CO₂ flux rates (a and b) and cumulative CO₂ efflux (c and d) in single-species treatments (a and c), and two-species treatments (b and d). Control (without earthworms) is shown on each graph. Mean values measured on days 3, 6, 9, 12, 17, and 21 are shown; standard errors are omitted for clarity. Ctr: Control; Ah: *Amyntas hilgendorfi*; Lr: *Lumbricus rubellus*; Ol: *Octolasion lacteum*; El: *Eisenoides lonnbergi*. The interaction between *O. lacteum* and *L. rubellus* has significant negative effects ($P < 0.05$) on CO₂ efflux (d, arrows) compared to the respective single-species treatments (c, arrows) and Control.

Fig. 2. Correlations between $\delta^{13}\text{C}$ of CO₂ gas emitted from the mesocosms and litter remaining on the soil surface (a) or excess ^{13}C in the soil (b). Each point represents a mesocosm. Solid points in (a) represents mesocosms where the remaining leaf litter was not measurable (< 0.05 g) at the end of the experiment. Correlation between $\delta^{13}\text{C}$ of CO₂ gas and leaf litter remaining was significant only when the non-measurable samples were treated as 0 (solid line; $F_{1, 51} = 14.476$, $P < 0.001$, $r = 0.470$), but not when the non-measurable samples were not included (dashed line; $F_{1, 32} = 1.445$, $P = 0.238$, $r = 0.208$). $\delta^{13}\text{C}$ of CO₂ gas was negatively correlated with excess ^{13}C in the soil (solid line in b; $F_{1, 48} = 7.042$, $P = 0.011$, $r = 0.332$).

Fig. 3. Percentage of litter-derived C in respired CO₂ in Control, single species treatments, and two species treatments. Each box shows the mean and SE. Ctr: Control;

Ah: *Amyntas hilgendorfi*; Lr: *Lumbricus rubellus*; Ol: *Octolasion lacteum*; El: *Eisenoides lonnbergi*.

Fig. 4. Redundancy analysis of soil microbial community structures in 0-5 cm (a) and 5-10 cm (b) soils. Each cross represents a mesocosm. GN: Gram-negative bacteria; GP: Gram-positive bacteria; AT: Actinomycetes; FG: fungi; PZ: protozoa. Significant vectors for earthworm species were highlighted using thick arrows, and vectors for microbes were eliminated for clarity. In 0-5 cm, *Amyntas hilgendorfi* is positively associated with Gram-positive bacteria, and negatively associated with Gram-negative bacteria. In 5-10 cm, *Octolasion lacteum* is positively associated with Gram-positive bacteria; *A. hilgendorfi* is positively associated with Gram-negative bacteria, fungi and protozoa, and negatively associated with Gram-positive bacteria.

Fig. 5. Structural equation model showing potential causal effects of earthworm species (exogenous variables; gray rectangles), resource availability and soil microbial abundance on CO₂ efflux derived from litter C (CO₂ - Litter C) or soil C (CO₂ - Soil C) (endogenous variables; white rectangles). Ah: *Amyntas hilgendorfi*; Lr: *Lumbricus rubellus*; Ol: *Octolasion lacteum*; El: *Eisenoides lonnbergi*; Bacteria: bacterial PLFA; Fungi: fungal PLFA; ¹⁵N: soil excess ¹⁵N; ¹³C: soil excess ¹³C; Litter: remaining litter. Arrow thickness is scaled proportionally to the standardized path coefficients (numbers on arrows). Arrows with solid and dashed lines indicate significant ($P < 0.05$) and marginally significant effects ($P < 0.1$), respectively; arrows with dotted lines indicate

832 non-significant paths. Variances explained by the model (R^2) are shown next to each
833 endogenous variable.

834

Table 1

Mean (SE) of litter remaining, excess ^{13}C (^{13}C surplus above natural abundance), and soil C and N in Control, single-species and two-species treatments at the end of the experiment.

Treatment*	Litter remaining (g)		Excess ^{13}C - litter (μg)		Excess ^{13}C - earthworms (μg)		Excess ^{13}C - soil (μg)		Excess ^{13}C - total (μg)		Soil C content (%)		Soil N content (%)		Soil C:N ratio	
Control	3.38	(0.14)	1007	(40)	na		257	(11)	1264	(40)	4.68	(0.11)	0.33	(0.01)	14.0	(0.1)
Ah	<0.01		na		16	(1)	819	(54)	835	(54)	4.88	(0.09)	0.34	(0.00)	14.3	(0.1)
Lr	0.08	(0.04)	5	(2)	11	(0)	742	(48)	758	(48)	4.87	(0.16)	0.34	(0.01)	14.3	(0.2)
Ol	0.65	(0.08)	71	(9)	8	(1)	735	(38)	813	(36)	4.87	(0.11)	0.34	(0.01)	14.3	(0.1)
El	2.80	(0.14)	545	(27)	3	(0)	265	(20)	813	(24)	4.73	(0.04)	0.33	(0.00)	14.2	(0.1)
Ah + Lr	<0.01		na		13	(1)	881	(33)	895	(33)	4.65	(0.14)	0.33	(0.01)	14.2	(0.1)
Ah + Ol	0.07	(0.03)	3	(1)	13	(1)	557	(255)	572	(255)	4.69	(0.20)	0.34	(0.01)	13.9	(0.4)
Ah + El	0.02	(0.02)	1	(1)	12	(0)	1002	(202)	1015	(202)	4.69	(0.10)	0.34	(0.00)	14.0	(0.3)
Lr + Ol	0.32	(0.18)	22	(12)	10	(1)	1112	(170)	1144	(172)	5.05	(0.34)	0.33	(0.00)	15.3	(0.8)
Lr + El	0.26	(0.07)	17	(4)	7	(2)	497	(436)	520	(437)	4.45	(0.15)	0.32	(0.00)	13.8	(0.6)
Ol + El	1.82	(0.28)	292	(44)	7	(1)	718	(83)	1017	(89)	4.60	(0.06)	0.32	(0.00)	14.5	(0.2)

* Ah: *Amyntas hilgendorfi*, Lr: *Lumbricus rubellus*, Ol: *Octolasion lacteum*, El: *Eisenoides lonnbergi*.

841 **Table 2**

842 Results of beta regressions testing the effects of species and their interactions on Hellinger-transformed PLFA biomarkers for
 843 bacteria, fungi and protozoa.

Factor*	Gram-negative bacteria				Gram-positive bacteria				Actinomycete			
	0-5 cm		5-10 cm		0-5 cm		5-10 cm		0-5 cm		5-10 cm	
	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Ah	2.887	↓0.089	16.181	<0.001	2.687	0.101	7.402	↓ 0.007	0.282	0.596	8.142	↓ 0.004
Lr	0.009	0.926	1.014	0.314	0.226	0.634	1.080	0.299	2.259	0.133	0.284	0.594
Ol	2.121	0.145	0.585	0.445	1.612	0.204	4.383	0.036	0.550	0.458	9.040	↓ 0.003
El	0.003	0.959	0.247	0.619	<0.001	0.986	0.018	0.893	0.295	0.587	3.588	0.058
Ah x Lr	4.352	↓ 0.037	0.422	0.516	1.206	0.272	0.246	0.620	2.053	0.152	0.821	0.365
Ah x Ol	1.251	0.263	0.051	0.822	0.515	0.473	0.637	0.425	0.800	0.370	1.372	0.241
Ah x El	0.916	0.339	3.000	0.083	0.086	0.770	0.941	0.332	1.211	0.271	0.696	0.404
Lr x Ol	1.493	0.222	0.383	0.536	4.517	↓ 0.034	0.599	0.439	1.810	0.179	0.095	0.758
Lr x El	1.221	0.269	1.588	0.208	0.112	0.738	3.195	0.074	2.440	0.118	1.373	0.241
Ol x El	2.171	0.141	0.112	0.738	1.157	0.282	0.129	0.719	0.024	0.877	2.015	0.156
Factor*	Total bacteria				Fungi				Protozoa			
	0-5 cm		5-10 cm		0-5 cm		5-10 cm		0-5 cm		5-10 cm	
	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Ah	0.187	0.665	1.450	0.229	0.380	0.538	8.835	0.003	0.002	0.965	7.670	0.006
Lr	1.114	0.291	0.095	0.758	1.021	0.312	2.430	0.119	1.271	0.260	2.183	0.140
Ol	0.003	0.954	5.837	0.016	4.840	0.028	0.324	0.569	2.244	0.134	2.753	0.097
El	<0.001	0.998	0.768	0.381	2.440	0.118	3.542	0.060	0.768	0.381	3.058	0.080
Ah x Lr	1.152	0.283	0.016	0.899	0.620	0.431	0.214	0.643	5.577	↓ 0.018	3.560	0.059
Ah x Ol	0.135	0.713	0.974	0.324	0.247	0.619	1.461	0.227	4.129	↓ 0.042	0.363	0.547
Ah x El	0.726	0.394	0.512	0.474	0.404	0.525	0.181	0.671	2.876	0.090	0.051	0.822
Lr x Ol	3.817	↓0.051	0.154	0.695	1.440	0.230	0.881	0.348	1.110	0.292	0.533	0.465
Lr x El	1.366	0.243	1.437	0.231	0.014	0.906	0.054	0.817	1.926	0.165	0.015	0.903
Ol x El	0.074	0.786	1.079	0.299	0.143	0.705	1.331	0.249	0.217	0.641	<0.001	0.977

844 Notes: Significant effects ($P < 0.05$) are given in bold; ↓, significant or marginally significant ($0.05 < P < 0.1$) negative effect.

845 * Biomass of *Amyntas hilgendorfi* (Ah), *Lumbricus rubellus* (Lr), *Octolasion lacteum* (Ol), *Eisenoides lonnbergi* (El) and
 846 their interactions.

847 **Table 3**

848 Summary of the final structural equation model showing the unstandardized path coefficients, their standard errors, and *P*-
 849 values.

	Path	Unstandardized path coefficient	Standard error	<i>P</i> -value
Litter – CO ₂	← Bacteria PLFA	0.079	0.043	0.065 ¹
	← Fungi PLFA	-0.182	0.110	0.099 ¹
	← <i>Amyntas hilgendorfi</i>	-0.554	0.074	<0.001
	← <i>Lumbricus rubellus</i>	-0.678	0.089	<0.001
	← <i>Eisenoides lonnbergi</i>	-0.438	0.055	<0.001
Soil – CO ₂	← Bacteria PLFA	0.122	0.079	0.121 ²
	← Fungi PLFA	-0.497	0.203	0.014
	← <i>Amyntas hilgendorfi</i>	-0.292	0.134	0.029
Bacteria PLFA	← Fungi PLFA	0.799	0.354	0.024
	← <i>Amyntas hilgendorfi</i>	-0.382	0.240	0.111
Fungi PLFA	← Excess ¹³ C	0.335	0.129	0.009
	← Excess ¹⁵ N	-0.163	0.080	0.041
Excess ¹³ C	← Litter remaining	-2.001	0.264	<0.001
Excess ¹⁵ N	← Litter remaining	-3.973	0.258	<0.001
Litter remaining	← <i>Amyntas hilgendorfi</i>	-0.358	0.031	<0.001
	← <i>Lumbricus rubellus</i>	-0.878	0.087	<0.001
	← <i>Octolasion lacteum</i>	-0.829	0.102	<0.001
	← <i>Eisenoides lonnbergi</i>	-0.143	0.054	0.008

850 1. Marginally significant (0.05 < *P* < 0.1).

851 2. Paths not significant but kept in the model.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5