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Amynthas agrestis invasion increases microbial biomass in Mid-Atlantic deciduous forests

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ABSTRACT

Earthworm species with different feeding, burrowing, and/or casting behaviors can lead to distinct microbial communities through complex direct and indirect processes. European earthworm invasion into temperate deciduous forests in North America has been shown to alter microbial biomass in the soil and reduce the fungi-to-bacteria ratios. It is unclear how changes in earthworm species composition due to interspecific competition may alter this dynamics, especially under the ongoing invasion by Asian *Amynthas* and *Metaphire* species. Furthermore, it is also poorly understood how interspecific interactions involving different species may have species-dependent, non-additive effects on different groups of soil microorganisms. By conducting a two-year field experiment in a deciduous forest in the Mid-Atlantic, we examined how the Asian species *Amynthas agrestis* and *Amynthas corticis*, the European species *Lumbricus rubellus* and *Octolasion lacteum*, and their interactions affect soil microbial communities, and tested the hypotheses that the Asian species and the interaction between the two European species negatively affect bacterial biomass. We showed that while *A. corticis* generally had no effect on soil microorganisms, *A. agrestis* had a positive effect on microbial biomass, primarily by increasing the biomass of Gram-positive and Gram-negative bacteria. Consistent with our hypothesis, the interaction between *L. rubellus* and *O. lacteum* had a negative effect on bacteria biomass. We concluded that the ongoing invasion of the Asian earthworm *A. agrestis* in forest soils and potential displacement of *L. rubellus* due to interspecific competition may lead to increased bacterial biomass through increasing resource availability and disrupting the interaction between *L. rubellus* and *O. lacteum*, potentially causing increased carbon mineralization and reducing soil carbon storage.

1. Introduction

Since European settlement, soils in temperate deciduous forests in North America have gone through major changes as a result of past land use, climate change and species gains and losses (Compton and Boone, 2000; Foster and Aber, 2003; Yesilonis et al., 2016). European earthworm invasion into habitats with low native earthworm abundance or previously devoid of earthworms in this region has resulted in major changes in soil carbon cycles. Through their feeding, burrowing, and casting behaviors, invasive earthworms increase litter decomposition rate, incorporate organic matter into aggregates, change the pool size and distribution of carbon and nitrogen in the soil profile, and affect carbon mineralization rates (Hale et al., 2005a; Eisenhauer et al., 2007; Szlavecz et al., 2011; Fahey et al., 2013; Ma et al., 2013, 2014; Yavitt et al., 2015). The altered forms and distribution of soil carbon and nitrogen can change resource availability to soil microbial communities, which in turn affect carbon transformation and mineralization (Schimel and Weintraub, 2003; Waring et al., 2013; Cotrufo et al., 2015). While the effects of invasive earthworms on soil carbon biogeochemistry in temperate deciduous forests have been the focus of many recent studies (e.g. Crumsey et al., 2015 and the studies cited above), the effects of earthworms on the structure of microbial communities in these forests have received less attention (but see Groffman et al., 2004).

It is generally assumed that the burrowing behaviors of earthworms disrupt fungal hyphae in the soil and therefore would shift

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soil food webs in temperate forests from a fungi-dominated system to a bacteria-dominated system (Bohlen et al., 2004b). This hypothesis has recently gained support from comparing forest stands invaded by European earthworms with those that are earthworm-free. Field observations in hardwood forests in Northeastern USA showed that earthworms decreased the fungi-to-bacteria ratio in the soil profile primarily by eliminating the organic horizon, where fungal biomass dominates (Dempsey et al., 2011, 2013). Consistent with the original hypothesis, the fungi-to-bacteria ratio also decreased in the surface organic soil, even though the impact was much weaker.

However, field observations focusing on total microbial biomass have reported contradictory results. Some deciduous forests in North America showed a net increase of microbial biomass in the overall soil profile (Groffman et al., 2004, 2015), while others showed significant net decrease (Eisenhauer et al., 2007, 2011). All of these studies documented consistent decrease in microbial biomass in the O horizon, but the inconsistency appeared to take place in the surface of the mineral soil, where microbial biomass decreased in some studies (Eisenhauer et al., 2007, 2011) while increased in others (Groffman et al., 2004, 2015). The relatively limited number of studies and the inconclusive results therein highlight our lack of understanding on the factors contributing to the contradictory impacts.

One potential explanation is the ecology of earthworm species involved. Earthworms affect soil microorganisms and activities through complex direct and indirect processes, and species with different feeding and/or burrowing behaviors can have potentially distinct effects (McLean et al., 2006). Earthworms change resource availability to soil microorganisms by consuming leaf litter and soil organic matter (SOM), by vertical translocation of C and N in the soil profile, and by promoting aggregate formation through casting (Eisenhauer et al., 2007; Dempsey et al., 2011). Fragmentation and vertical mixing of leaf litter by litter-feeding species into surface soil increase the concentration of readily mineralizable C and tend to increase microbial abundance in the bulk soil. In contrast, soil-feeding species may decrease microbial biomass in the bulk soil as they feed primarily on soil organic matter and reduce resource availability to microbes (McLean et al., 2006). The activity of soil living earthworms can disrupt fungal hyphae (Butenschoen et al., 2007), leading to the reduction of soil fungi. Some earthworm species selectively feed on bacteria (Jayasinghe and Parkinson, 2009; Larsen et al., 2016), reducing bacteria abundance. Newly produced earthworm casts, middens, and burrows are rich in soluble and readily accessible carbon and thus generally considered hotspots of microbial activity (Aira et al., 2009; Stromberger et al., 2012). However, the casting behavior also incorporates organic matter into aggregates, making it inaccessible to microorganisms. Altogether, these different processes highlight that earthworm communities with different species compositions can have contrasting overall effects on soil microbial communities, depending on the ecology of the involved species.

In North America, the majority of studies on invasive earthworm effects reported on less than 10 species, all European lumbricids. Invasion of several Asian species, including *Amynthas corticis*, *A. gracilis*, *A. agrestis*, *A. tokioensis*, and *Metaphire hilgendorfi* (also known as *A. hilgendorfi*), have received little attention until recent years. The last three species, *A. agrestis*, *A. tokioensis*, and *M. hilgendorfi*, although being present in most East Coast states for at least 80 years (Gates, 1982; Chang et al., 2016c), have recently been reported spreading within those states, as well as into Midwest and West Coast states, including Wisconsin in 2013 (Qiu and Turner, 2017) and Oregon in 2016 (Bruce Snyder, personal communication). Unlike most common European earthworms, which have a lifespan of more than a year, the three Asian species have an annual life cycle. They overwinter exclusively as cocoons, hatch in spring, grow rapidly to 5–15 cm long, reproduce at the end of summer, and die by the end of fall (Callaham et al., 2003; Görres et al., 2014). Their ongoing rapid spreading, which is likely facilitated by nursery plants and commercial mulch (Belliturk et al., 2015), especially in the form of cocoons, has caused increasing concern. The Asian species can reach extremely high abundance and biomass (Görres et al., 2014), are able to rapidly eliminate the leaf litter layer and change the structure of surface soil (Snyder et al., 2011; Greiner et al., 2012), and have significant impact on soil carbon pools and mineralization (Greiner et al., 2012; Chang et al., 2016a, b) and nutrient concentrations (Qiu and Turner, 2017). Moreover, they have been shown to negatively affect other organisms, such as millipedes and salamanders (Snyder et al., 2013; Ziemba et al., 2016). The Asian species are also superior competitors for leaf litter against one of the most common European earthworm, *Lumbricus rubellus*, (Zhang et al., 2010; Chang et al., 2016a) and, when present, are usually the dominant earthworm group (Davalos et al., 2015).

Several recent studies suggested that the common Asian and European invasive earthworms have distinct effects on soil microbial communities (Zhang et al., 2010; Chang et al., 2016a, b). The European species *Lumbricus rubellus* and *Octolasion lacteum* have been shown to have no effect on bacterial biomass, while the Asian species *A. agrestis* and *M. hilgendorfi* reduce the biomass of both Gram-positive and Gram-negative bacteria (Zhang et al., 2010; Chang et al., 2016a). Moreover, the interaction between *L. rubellus* and *O. lacteum* has a positive effect on soil carbon content, and a negative effect on soil respiration, suggesting reduction of carbon mineralization rates (Chang et al., 2016b), while the interaction between *M. hilgendorfi* and *L. rubellus* or *O. lacteum* has no effect. All of these contrasting findings between Asian and European earthworms were observed in short-term (<30 days), laboratory mesocosm experiments under well-controlled soil temperature and moisture with nearly no roots and mycorrhizal fungi. It is unclear if any of the changes in microbial communities observed in the laboratory will hold true in the field. Clearly, a sizable gap exists in our understanding between what has been learned from laboratory mesocosm experiments and those from field observational studies.

To fill this gap, and to understand species-specific effects and their interactions on soil microbial communities in temperate deciduous forest in Eastern US under the context of European and Asian earthworm invasion, we conducted a two-year, field experiment focusing on two Asian (*A. agrestis* and *A. corticis*) and two European (*L. rubellus* and *O. lacteum*) species in a temperate deciduous forest in the Mid-Atlantic region. We hypothesized that (1) earthworms reduce the fungi-to-bacteria ratio in the surface soil, (2) the effects of earthworms on soil microbial community are species-specific and non-additive, (3) the two Asian earthworms, *A. agrestis* and *A. corticis*, have negative effects on soil bacterial biomass, while the two European species, *L. rubellus* and *O. lacteum*, have no effect, and (4) the interaction between *L. rubellus* and *O. lacteum* has a negative effect on soil bacterial biomass.

2. Methods

2.1. Field site

A two-year field experiment was conducted in a forest stand called Treefall at the Smithsonian Environmental Research Center (SERC), Edgewater, Maryland, USA (38°53′17.0″N, 76°33′14.3″W; www.serc.si.edu). The upland secondary forests at SERC, ranging from 50 to 150 years old (Yesilonis et al., 2016), are dominated by tulip poplar (*Liriodendron tulipifera* L.), sweet gum (*Liquidambar styraciflua* L.), red maple (*Acer rubrum* L.), black cherry (*Prunus*

serotina Ehrh.), box elder maple (*Acer negundo* L.), American beech (*Fagus grandifolia* Ehrh.), oaks (e.g. *Quercus falcata* Michx., *Q. alba* L.) and hickories (e.g. *Carya tomentosa* (Lam. ex Poir.) Nutt., *C. glabra* (Mill.) Sweet) (Higman, 1968). Soils at our plot locations have been classified as Collington sandy loam (fine-loamy mixed, active, mesic Typic Hapludult) (Szlavecz et al., 2011), with an average pH of 5.1, 32% silt, 20% clay, and 5.6% organic matter content (Yesilonis et al., 2016). The forests have an established community of European earthworms, with *Lumbricus friendi* Cognetti, 1904, *L. rubellus* Hoffmeister, 1843, *Aporrectodea caliginosa* (Savigny, 1826), *Octolasion lacteum* (Orley, 1881), *O. cyaneum* (Savigny, 1826) and *Eisenoides lonnbergi* (Michaelsen, 1894) being the most common species. The forests are currently been invaded by four Asian earthworm species (Szlavecz and Csuzdi, 2007; Chang et al., 2016a; C.-H. Chang, personal observation), *viz. Amynthas agrestis* (Goto and Hatai, 1899), *Metaphire hilgendorfi* (Michaelsen, 1892), *A. tokioensis* (Beddard, 1892) and *A. corticis* (Kinberg, 1867).

2.2. Experimental design

Four species of earthworms were selected for the experiment: *A. agrestis*, *A. corticis*, *L. rubellus*, and *O. lacteum*. The species *A. agrestis* and *A. corticis* are both originally from Asia and epi-endogeic, but they have contrasting life history: *A. agrestis* is an annual species, reproduces at the end of summer, and dies by the end of fall, while *A. corticis* lives for more than a year (Chang et al., 2016c). The other two species, *L. rubellus* and *O. lacteum*, are among the most common European earthworms found in temperate deciduous forests in the US. *L. rubellus* is also epi-endogeic, while *O. lacteum* is usually considered polyhumic endogeic. All four species are found at SERC. Individuals of the two *Amynthas* species were collected from forests along the Stony Run Trail, Baltimore, MD; individuals of *L. rubellus* and *O. lacteum* were collected from SERC. All earthworm individuals collected for the experiment were mature (with a clitellum).

Six plots were established in the Treefall forest stand (Fig. 1). Each plot was 3 m × 4 m, at least 1 m away from the nearest trees, and at least 50 m away from the other plots. Each plot was equally divided into a 3 × 4 grid (Fig. 1). Field mesocosms were constructed by burying 23-L (30 cm in diameter, 35 cm in height) mesh metal wastebaskets 25 cm into the soil at each grid point. Since there was a total of eleven treatments (see below), one randomly chosen grid point in row 4 received no mesocosm. To improve drainage several holes were drilled in the bottom of the basket, which was then covered by a single layer of gravel. The interior of the baskets was then covered by a polyester mesh bag with a 0.8 mm pore size. The pore size allowed fine roots and fungal hyphae to grow into the mesocosms but kept earthworms from going through. Within one plot, the soil retrieved when installing the baskets was divided into the upper 10 cm and the lower 15 cm, which roughly equal the organic and mineral soils, respectively, sieved through a 5 mm sieve, and well mixed. Roots, earthworms, and other soil macrofauna were hand-sorted and removed during the process. Mesocosms were reconstructed by sequentially adding 7.4 kg mineral soil and 5.1 kg organic soils into the basket, and 30 g of tulip poplar leaf litter on the soil surface.

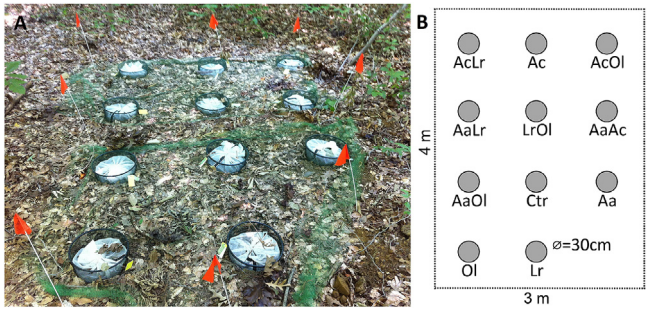

Fig. 1. One of the plots containing 11 mesocosms in the Treefall stand at the Smithsonian Environmental Research Center, Maryland, USA. (A) Photo of Plot 3. (B) Diagram of Plot 3 with dimension information and treatment assignment. Ctr: control; Aa: *A. agrestis*; Ac: *A. corticis*; Lr: *L. rubellus*; Ol: *O. lacteum*.

Earthworms were added into the field mesocosms in June 2012, two weeks after the mesocosms were constructed. A total of 10 treatments and one control were established in each of the six plots: four were single-species treatments with each of the four earthworm species and six were two-species treatments with all combinations of the four species. The control contained no earthworm. The treatments were randomly assigned to the grid points. Eight *A. agrestis*, six *A. corticis*, twelve *L. rubellus*, and twenty-four *O. lacteum* individuals were added into the single-species treatments with the respective species. For the two-species treatments, four *A. agrestis*, three *A. corticis*, six *L. rubellus*, and twelve *O. lacteum* were added into the respective treatments. The numbers of individuals were chosen to take into account both density in the field and individual biomass differences to mimic potential co-occurrence conditions in the field. The mean fresh biomass of individuals (including gut content) was 0.88 g for *A. agrestis*, 0.92 g for *A. corticis*, 0.58 g for *L. rubellus*, and 0.21 g for *O. lacteum*. After earthworm addition the mesh bags were closed, and the plot area was covered by bird nettings (Fig. 1). To supply food for earthworms, additional 60 g Tulip poplar litter was added in two increments, 30 g in October 2012 and 30 g in June 2013. Before the first litter addition, earthworm abundance in the mesocosms was monitored using electroshocking (Szlavecz et al., 2013). Before the second litter addition, electroshocking was used to remove all earthworms in each mesocosm, including all individuals without a clitellum, and the abundance of mature earthworms was reestablished to the initial conditions.

The mesocosms were destructively sampled in October 2013. The timing was chosen so that the sampling took place when the soil was moist, and before mature *A. agrestis* reached the end of its life cycle (Görres et al., 2014), and was also based on high microbial biomass previously reported in temperate deciduous forests in Eastern US (Dempsey et al., 2011, 2013). First, the remaining leaf litter was collected from the soil surface. Then, three soil cores were collected from each mesocosm using a plastic corer with a diameter of 3.5 cm and then divided into 0–2 cm, 2–5 cm, and 5–10 cm increments. Finally, all earthworms from the mesocosms were hand-sorted and preserved in 70% ethanol. The soil samples were sieved through a 2-mm sieve, homogenized, and stored at −20 °C for microbial analysis. Soils from 2 to 5 cm and 5–10 cm from one of the plot were lost during sample processing and were not analyzed. Soil pH was measured using a 1:2, soil:water slurry in 0.01 M $CaCl_2$.

2.3. Microbial community analysis

Phospholipid fatty acid (PLFA) analysis was used to characterize soil microbial communities, including Gram-positive bacteria, Gram-negative bacteria, actinomyctes, fungi, and protozoa. The PLFA commonly used as a biomarker for arbuscular mycorrhizal (AM) fungi, 16:1ω5, is also known to be found in Gram-negative bacteria, and is a poor indicator of AM fungi in soils with high bacterial biomass (Frostegard et al., 2011). Therefore, to better estimate changes in AM fungi, instead of using PLFA, we used the neutral lipid fatty acid (NLFA) 16:1ω5cis as the biomarker for AM fungi (Olsson, 1999), as suggested by Frostegard et al. (2011) and Sharma and Buyer (2015).

Samples were prepared and analyzed as described (Buyer and

Sasser, 2012; Sharma and Buyer, 2015) using 19:0 phosphatidylcholine (Avanti Polar Lipids, Alabaster, Alabama, USA) and trinonadecanoin glyceride (catalog # T-165, Nu-Chek Prep, Elysian, MN, USA) as internal standards for quantitative analysis. Gas chromatography was conducted on an Agilent 6890 gas chromatograph (Agilent Technologies, Wilmington, Delaware, USA) coupled 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.

2.4. Statistical analysis

Fatty acids were combined into groups of biomarkers for Gram-negative bacteria (monounsaturated fatty acids and cyclopropyl 17:0 and 19:0), Gram-positive bacteria (iso and anteiso saturated branched fatty acids), actinomycetes (10-methyl fatty acids), fungi (18:2 ω6 cis), AM fungi (NLFA 16:1ω5cis), and protozoa (20:3 and 20:4 fatty acids) following Buyer and Sasser (2012) and Sharma and Buyer (2015). These biomarkers are not completely universal for all members in each organism group, and are not entirely specific to each group, either. Therefore, they need to be interpreted with caution (Frostegard et al., 2011).

All statistical tests were conducted in R v3.1.2 (R Core Team, 2014). PLFA and NLFA were combined into biomarker groups (Buyer and Sasser, 2012) and analyzed using two approaches. First, the effects of earthworm species and species interactions on total PLFA, bacteria PLFA, AM fungi NLFA, fungi-to-bacteria ratios, and PLFA biomarkers for Gram-negative bacteria, Gram-positive bacteria, actinomycetes, fungi, and protozoa were analyzed using mixed effect models as implemented in the package lme4 (Bates et al., 2015). In the mixed effect models, 'plot' was the random effects and the biomass of each earthworm species was considered as fixed effects. Second, the data were Hellinger-transformed (square root of proportion), and microbial community compositions were analyzed using redundancy analyses as implemented in the package vegan (Oksanen et al., 2016) with soil moisture, soil pH, and the biomass of individual earthworm species as constrained variables. Permutation tests with 999 permutations were used to test the significances of the overall models, the constrained axes, soil pH, and the biomass of earthworm species. Linear regressions were used to investigate the correlations between the PLFA 16:1ω5cis (AM fungi biomarkers) and the NLFA biomarkers for AM fungi or the PLFA biomarkers for Gram-negative bacteria. The purpose of this regression analysis was to evaluate whether the PLFA 16:1ω5cis is an acceptable biomarker for AM fungi in our system, or if it has more contribution from Gram-negative bacteria.

3. Results

The overall survival rates were 12% for *A. agrestis*, 14% for *A. corticis*, 19% for *L. rubellus*, and 32% for *O. lacteum* (Table A1, Appendix). The mortality was most likely caused by extreme heat and drought during summer and was expected for an experiment conducted in field conditions without any control for temperature and soil moisture. Leaf litter remaining on the soil surface was highest in the control treatment (17.9 ± 5.6 g; mean ± SE), and lowest in the *A. corticis* + *O. lacteum* treatment (7.2 ± 2.1 g) (Table A2, Appendix).

Amynthas agrestis significantly increased total PLFA ($P = 0.003$) and Gram-negative ($P = 0.010$), Gram-positive ($P = 0.004$), and total bacteria ($P = 0.004$) PLFA biomarkers in the 0–2 cm soil layer (Fig. 2; Table 1), but not in the 2–5 cm and 5–10 cm soils. In contrast, *L. rubellus* x *O. lacteum* interaction had significant negative effects on total PLFA ($P = 0.012$) and Gram-negative ($P = 0.016$), Gram-positive ($P = 0.025$), and total bacteria ($P = 0.012$) PLFA biomarkers in the 0–2 cm soil layer (Table 1). In the 2–5 cm layer, *A. agrestis* x *O. lacteum* interaction had significant positive effects on total PLFA ($P = 0.047$) and Gram-negative ($P = 0.032$), Gram-positive ($P = 0.019$), and total bacteria ($P = 0.018$) PLFA biomarkers (Table A3-A5, Appendix).

In general, earthworms had non-significant effects on fungi PLFA and AM fungi NLFA (Fig. 3; Table 1; Table A3-A5, Appendix). The only significant effect was found in *A. corticis* x *O. lacteum* interaction, which had a positive effect on fungi PLFA ($P = 0.015$). Similarly, the fungi-to-bacteria ratios were not significantly affected either by earthworm species or their interactions (Fig. 3; Table 1) with only two exceptions. *A. agrestis* x *A. corticis* interaction and *A. corticis* x *O. lacteum* interaction had significant positive effects on the fungi-to-bacteria ratios in the 2–5 cm and 5–10 cm soils, respectively (Table 1). The PLFA 16:1ω5cis, frequently attributed to AM fungi, correlated relatively poorly to the NLFA biomarkers for AM fungi ($R^2 = 0.20$, 0.15 and 0.08 for 0–2 cm, 2–5 cm and 5–10 cm soils, respectively); in contrast, the PLFA 16:1ω5cis were highly correlated to the PLFA biomarkers for Gram-negative bacteria ($R^2 = 0.74$, 0.82 and 0.73 for 0–2 cm, 2–5 cm and 5–10 cm soils, respectively). These correlations also had lower AIC (Akaike information criterion) values when compared to the respective linear regressions with the NLFA biomarkers for AM fungi (ΔAIC = 68, 84 and 66 for 0–2 cm, 2–5 cm and 5–10 cm soils, respectively) (Fig. 4), confirming our choice of including NLFA for AM fungi analysis.

In addition to the biomarkers for bacteria, fungi, and protozoa, our samples also contained compounds that matched entries in the PLFAD1 library as dimethyl acetals, which are biomarkers for anaerobes. These compounds are produced from plasmalogens, the class of phospholipids found in anaerobes, by acid-catalyzed transesterification. However, we used base-catalyzed transesterification in the PLFA procedure, and dimethyl acetals would not be produced under basic conditions (Christie, 2003). These identifications are therefore incorrect. The concentrations of these unknown compounds were positively affected in the 0–2 cm soils by *A. agrestis* x *A. corticis* interaction ($P = 0.003$) and in the 5–10 cm soils by *L. rubellus* x *O. lacteum* interaction ($P < 0.001$) (Fig. 5; Table 1).

The redundancy analysis (RDA) of microbial community composition showed that in 0–2 cm soil the constrained axes (soil pH and the biomass of individual earthworm species) explained 20.2% of the variance. The overall model was significant ($P = 0.016$), and so was axis 1 ($P = 0.001$). Among the constrained variables, soil pH ($P = 0.004$) and *A. agrestis* had significant effects ($P = 0.034$), while the effects of the other three earthworm species were not significant (Fig. 6). Soil pH was positively associated with the proportion of AM fungi, and negatively associated with the proportion of Gram-positive bacteria. The biomass of *A. agrestis* was positively associated with AM fungi, and negatively associated with the proportion of Gram-negative bacteria. RDA of microbial community composition in 2–5 and 5–10 cm soils showed that the constrained axes explained 10.0% and 12.0% of the variance, respectively, but the overall models were not significant ($P = 0.366$ for 2–5 cm; $P = 0.247$ for 5–10 cm).

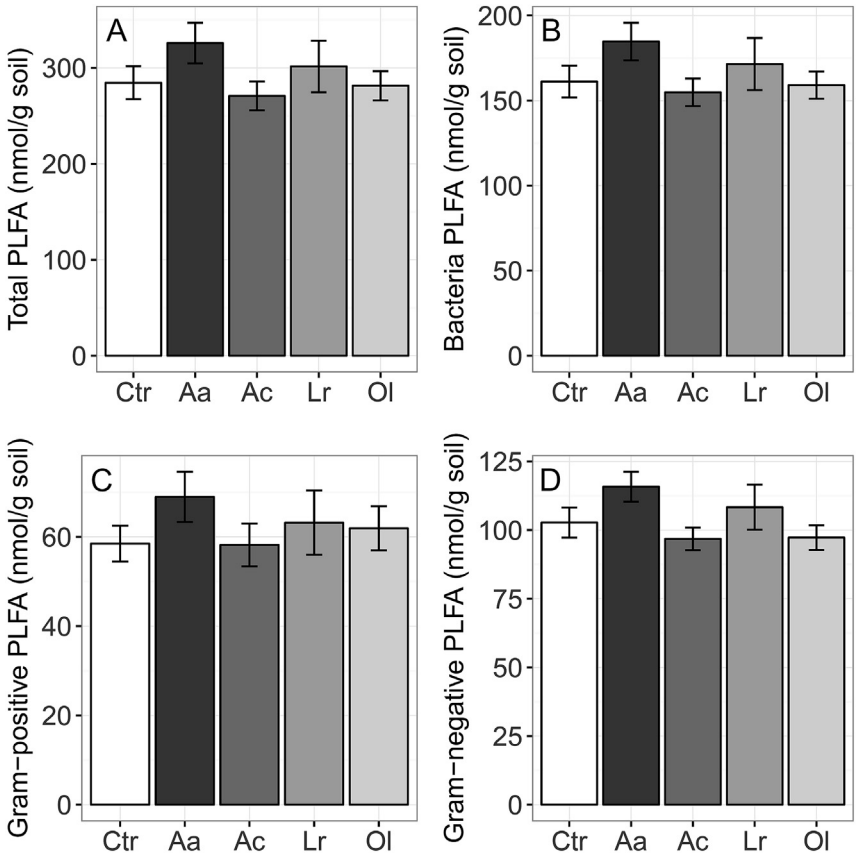

Fig. 2. Bulk soil (0–2 cm) phospholipid fatty acid (PLFA) biomarker concentrations in the control (Ctr) and in the single-species treatments containing *A. agrestis* (Aa), *A. corticis* (Ac), *L. rubellus* (Lr), or *O. lacteum* (Ol), showing increased PLFA biomarker concentration in the *A. agrestis* treatment for (A) total PLFA, (B) total bacteria PLFA, (C) Gram-positive bacteria, and (D) Gram-negative bacteria. The effects of *A. agrestis* were significant (positive; $P < 0.05$) in all four groups of PLFA biomarkers, while all of the other three species were not significant. Error bars show SE.

Table 1
Results of mixed models testing the effects of species and their interactions on PLFA or NLFA biomarkers for bacteria, fungi, and protozoa in the soil. *P*-values from likelihood ratio tests with a χ^2 distribution and a $df = 1$.

	Gram-negative bacteria			Gram-positive bacteria			Actinomycetes			Total bacteria			Total PLFA		
Factor*	0–2 cm	2–5 cm	5–10 cm	0–2 cm	2–5 cm	5–10 cm	0–2 cm	2–5 cm	5–10 cm	0–2 cm	2–5 cm	5–10 cm	0–2 cm	2–5 cm	5–10 cm
Aa	↑**0.010**	0.349	0.117	↑**0.004**	0.937	0.137	↑**0.005**	0.817	0.145	↑**0.004**	0.633	0.115	↑**0.003**	0.528	0.166
Ac	0.184	0.952	0.842	0.946	0.398	0.512	0.871	0.427	0.327	0.427	0.647	0.877	0.374	0.860	0.993
Lr	0.445	0.301	0.407	0.216	0.746	0.995	0.397	0.772	0.897	0.303	0.464	0.611	0.320	0.481	0.610
Ol	0.200	0.432	0.699	0.854	0.140	0.892	0.535	0.153	0.906	0.474	0.241	0.770	0.495	0.285	0.808
Aa x Ac	0.302	0.530	0.418	0.684	0.235	0.455	0.765	0.598	0.638	0.410	0.347	0.419	0.521	0.609	0.425
Aa x Lr	0.869	0.272	0.966	0.279	0.840	0.944	0.415	0.979	0.681	0.555	0.617	0.995	0.451	0.775	0.927
Aa x Ol	0.264	↑**0.032**	0.999	0.582	↑**0.019**	0.952	0.413	↑0.089	0.984	0.348	↑**0.018**	0.980	0.380	↑**0.047**	0.814
Ac x Lr	0.295	0.973	0.229	0.326	0.255	↑0.053	0.188	0.593	↑0.056	0.275	0.583	0.123	0.276	0.613	0.234
Ac x Ol	0.722	0.434	0.550	0.601	0.406	0.962	0.652	0.309	0.375	0.647	0.397	0.700	0.679	0.489	0.462
Lr x Ol	↓**0.016**	0.323	0.441	↓**0.025**	↓**0.016**	0.947	↓**0.022**	↓**0.040**	0.540	↓**0.012**	↓0.082	0.659	↓**0.012**	0.106	0.684
	Fungi			AM fungi			Protozoa			Fungi-to-bacteria ratio			Unknown compounds#		
Factor*	0–2 cm	2–5 cm	5–10 cm	0–2 cm	2–5 cm	5–10 cm	0–2 cm	2–5 cm	5–10 cm	0–2 cm	2–5 cm	5–10 cm	0–2 cm	2–5 cm	5–10 cm
Aa	0.894	0.291	0.764	↑0.059	0.198	0.212	0.189	0.603	0.379	0.125	0.344	0.992	0.213	↑0.097	0.473
Ac	0.191	0.329	0.555	0.319	0.890	0.479	↓**0.048**	0.813	0.395	0.252	0.249	0.591	0.493	0.613	0.498
Lr	0.700	0.533	0.733	0.280	0.961	0.434	0.902	0.824	0.298	0.996	0.695	0.749	0.661	0.754	0.474
Ol	0.288	0.693	0.662	0.207	0.559	0.228	↓**0.014**	0.332	0.549	0.443	0.844	0.754	0.669	0.161	0.704
Aa x Ac	0.856	↑0.069	0.752	0.472	0.215	↓0.079	0.404	↑0.091	0.389	0.597	↑**0.026**	0.945	↑**0.003**	0.439	0.631
Aa x Lr	↑0.092	0.778	0.820	0.800	0.135	0.226	↑**0.035**	0.797	0.469	0.311	0.810	0.862	0.381	0.579	0.503
Aa x Ol	0.497	0.577	0.383	0.907	0.405	0.819	0.406	0.637	0.781	0.787	0.259	0.321	0.386	0.128	↓0.066
Ac x Lr	0.914	0.495	0.449	0.442	0.268	0.808	0.578	0.691	0.232	0.541	0.323	0.211	0.613	0.330	0.926
Ac x Ol	0.672	0.473	↑**0.015**	0.439	0.369	0.770	0.317	0.282	0.808	0.538	0.306	↑**0.012**	0.152	0.983	0.536
Lr x Ol	0.187	0.871	0.965	0.597	0.363	0.283	0.321	0.411	0.722	0.940	0.575	0.874	0.909	0.850	↑<**0.001**

Notes: ↑, positive effect; ↓, negative effect; significant effects ($P < 0.05$) are given in bold.
*Biomass of *Amynthas agrestis* (Aa), *Amynthas corticis* (Ac), *Lumbricus rubellus* (Lr), *Octolasion lacteum* (Ol) and their interactions.
Unknown compounds matching entries for anaerobic biomarkers in the PLFAD1 library.

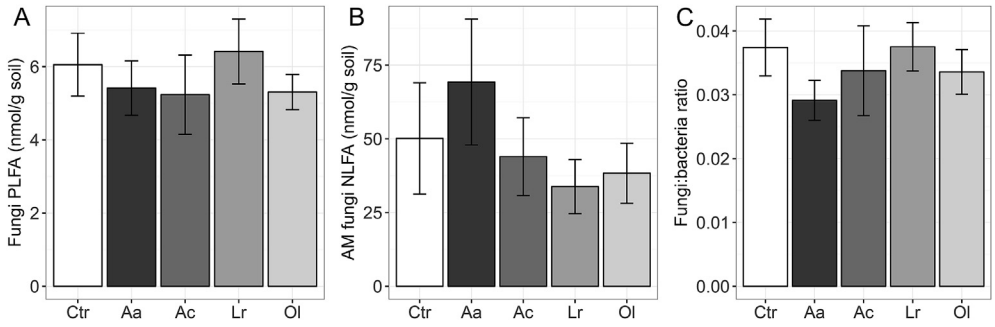

Fig. 3. Bulk soil (0–2 cm) fungi biomarkers and fungi-to-bacteria ratio in the control (Ctr) and in the single-species treatments containing *A. agrestis* (Aa), *A. corticis* (Ac), *L. rubellus* (Lr), or *O. lacteum* (Ol). The effects of all four earthworm species were not significant ($P > 0.05$) for fungi PLFA (A), AM fungi NLFA (B), and fungi-to-bacteria ratio (C). Error bars show SE.

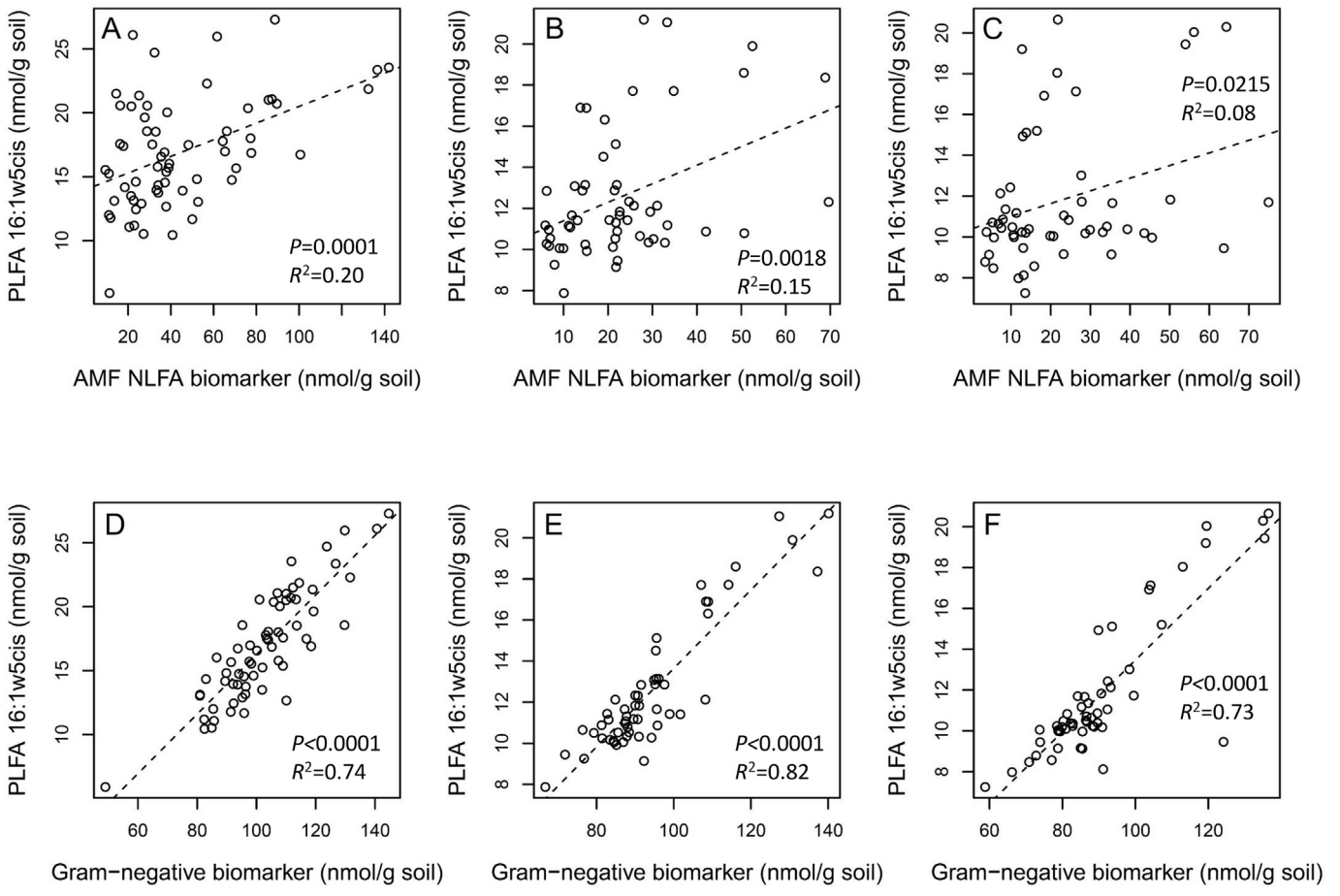

Fig. 4. Correlations between the PLFA 16:1ω5cis and the NLFA AM fungi biomarker (A–C) or the PLFA biomarkers for Gram-negative bacteria (D–F) in 0–2 cm (A and D), 2–5 cm (B and E), and 5–10 cm (C and F) soils. All correlations were significant, but the correlations were stronger (higher R^2 values) with the Gram-negative bacteria than with the AM fungi biomarkers.

4. Discussion

By conducting a two-year field manipulation, we showed that the Asian species *Amynthas agrestis* had a consistent positive effect on total microbial and bacterial biomass in the surface soil, including the biomass of Gram-positive bacteria, Gram-negative bacteria, and Actinomycetes, as indicated by PLFA biomarkers, while the other Asian species and the two European species had no significant effect. In contrast, the interspecific interaction between *Lumbricus rubellus* and *Octolasion lacteum* had negative effects on total microbial biomass and the biomass of both Gram-positive and Gram-negative bacteria. These results partially support our second hypothesis that the effects of earthworms are species-specific and non-additive, but contradict our third hypothesis regarding the negative relationship between microbial biomass and the presence of *A. agrestis*.

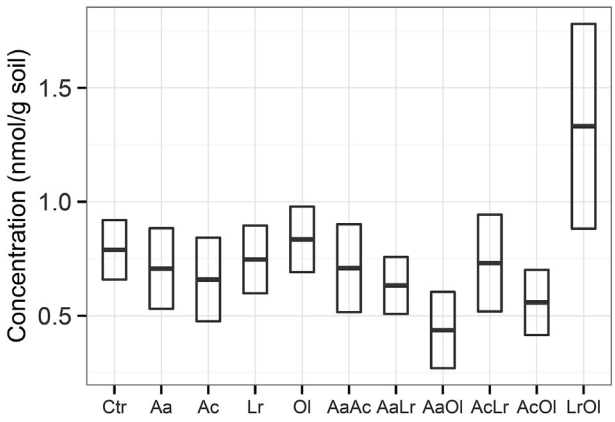

Fig. 5. Concentrations of unknown compounds matching the entries of dimethyl acetals in the PLFAD1 library in the 5–10 cm soil, showing concentrations in the control (Ctr), in the single-species treatments containing *A. agrestis* (Aa), *A. corticis* (Ac), *L. rubellus* (Lr), or *O. lacteum* (Ol), and in the two-species treatments. The effect of *L. rubellus* x *O. lacteum* interaction was significant (positive; $P < 0.05$), while all of the other factors (species and their interactions) were not significant. Error bars show SE.

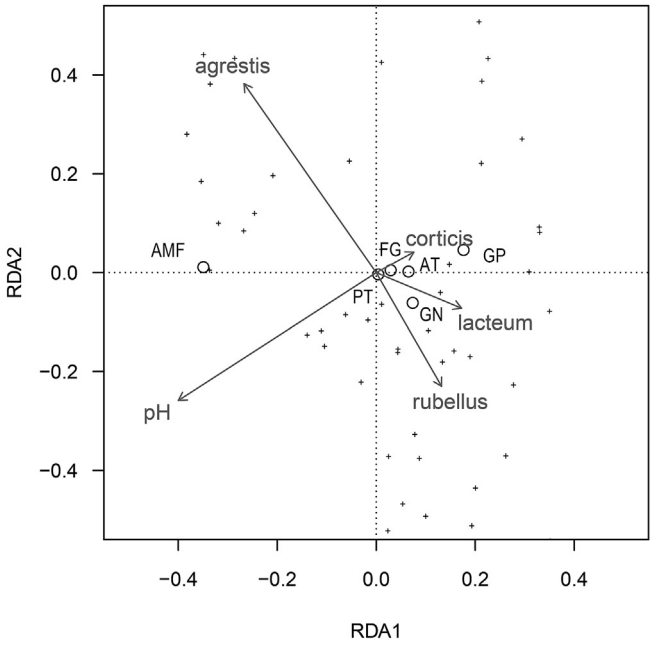

Fig. 6. Redundancy analysis of soil microbial community structures in 0–2 cm soil based on phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA) biomarkers. GN: Gram-negative bacteria; GP: Gram-positive bacteria; AT: Actinomycetes; FG: fungi; AM: arbuscular mycorrhizal fungi; PT: protozoa; pH: soil pH; agrestis: *Amynthas agrestis*; corticis: *A. corticis*; rubellus: *Lumbricus rubellus*; lacteum: *Octolasion lacteum*. Among the five constrained variables, only soil pH and *A. agrestis* were significant. Vectors for microbes were not shown for clarity.

4.1. The impact of Amynthas agrestis invasion

Our finding that *A. agrestis* increased microbial and bacterial biomass in the surface soil is consistent with that reported on non-native European earthworms in northern hardwood forests in Northeastern US (Dempsey et al., 2011, 2013; Groffman et al., 2004, 2015), but is contradictory to a previous laboratory mesocosm study documenting that *A. agrestis* reduces total microbial biomass and the biomass of Gram-negative and Gram-positive bacteria (Zhang et al., 2010). These contradictory results highlighted that data from short-term, laboratory mesocosm experiments need to be interpreted with caution before they are confirmed in the field. *A. agrestis* is an epi-endogeic species that is primarily active and casting in the surface soil. Its activity has been known to increase extracellular enzyme activity (Belliturk et al., 2015) and the concentrations of nitrate, inorganic nitrogen, and dissolved organic carbon in the soil (Qiu and Turner, 2017). These observations suggest that the activity of *A. agrestis* increases carbon and nitrogen readily available to microorganisms, leading to our observed increase in bacterial biomass. Altogether, the increase in microbial biomass and resource availability to microorganisms can potentially increase carbon mineralization and lead to higher carbon losses (Schimel and Weintraub, 2003). Our result further suggests that previous field observations showing no apparent correlation between microbial biomass and *A. agrestis* presence (Snyder et al., 2011) in the Great Smoky Mountains might be caused by confounding factors that are common in heterogeneous field environments, such as soil texture, soil moisture, and topography.

The fact that *A. agrestis* increases, as opposed to decreases, bacterial biomass in the field lead us to reevaluate how it may interact with another epi-endogeic species, the European *Lumbricus rubellus*, which is common in temperate deciduous forests in North America. Zhang et al. (2010) suggested that *A. agrestis* competes with *L. rubellus* by negatively affecting soil bacteria, which were thought to be important for *L. rubellus* nutrition or for conditioning leaf litter (Zhang et al., 2010). The fact that both Gram-negative and Gram-positive bacterial biomass increased in the field does not support that hypothesis. An alternative hypothesis regarding interspecific competition in earthworms has been proposed for another Asian invasive species, *Metaphire hilgendorfi*, often referred to as *Amynthas hilgendorfi* in North American literature (Chang et al., 2016c). *Metaphire hilgendorfi* is a species closely related and ecologically similar to *A. agrestis* (Chang et al., 2016c). It has been shown to directly compete with *L. rubellus* for leaf litter, and as the superior competitor, it has the potential to outcompete the latter (Chang et al., 2016a). Considering the similarities between *A. agrestis* and *M. hilgendorfi*, including their body size, annual life cycle, reproduction, and their moving, feeding, burrowing and casting behaviors (Callaham et al., 2003; Richardson et al., 2009; Snyder et al., 2011, 2013; Greiner et al., 2012; Görres and Melnichuk, 2012, 2014; Chang et al., 2016a, b, c; Qiu and Turner, 2017), we believe that the mechanisms of competition between *A. agrestis* and *L. rubellus* are similar to those reported for *M. hilgendorfi* (Chang et al., 2016a), with *A. agrestis* being the superior competitor.

In a short-term, laboratory mesocosm experiment, Chang et al. (2016b) reported that the interaction between *L. rubellus* and *O. lacteum* has a positive effect on soil carbon content and a negative effect on soil respiration. Consistent with these reports, our data from the field experiment supported our last hypothesis that the interaction between the two European species has a negative effect on the biomass of both Gram-negative and Gram-positive bacteria, and further suggested that decreases in microbial biomass may be responsible for the previously observed changes in soil respiration and soil carbon content. The fact that the same type of interaction did not happen between *O. lacteum* and the other two epi-endogeic species in our experiment, *A. agrestis* and *A. corticis*, further strengthens the idea that when soil carbon biogeochemistry is concerned, earthworm species

composition, and more importantly, species identities, need to be taken into account (Neilson et al., 2000; Zicsi et al., 2011; Chang et al., 2016a).

When taking into account both the effects of *A. agrestis* and the interspecific interaction between epi-endogeic species and *O. lacteum* (endogeic), the ongoing invasion of *A. agrestis* has an unexpected consequence. Most of the forests at SERC, as well as many temperate deciduous forests in Eastern and Northeastern US, are currently dominated by the epi-endogeic species *L. rubellus*, the anecic species *Lumbricus friendi* or *L. terrestris*, and one or two of the common endogeic species, including *Eisenoides lonnbergi*, *Aporrectodea. caliginosa*, *Octolasion. cyaneum*, and *O. lacteum* (Bohlen et al., 2004a; Hale et al., 2005b; Szlavecz and Csuzdi, 2007; Filley et al., 2008; Nuzzo et al., 2009). Recent studies have collectively indicated displacement of *L. rubellus* by *A. agrestis* as a result of interspecific competition (Zhang et al., 2010; Greiner et al., 2012; Davalos et al., 2015). Adding *A. agrestis* into the system may result in replacing *L. rubellus* by *A. agrestis* as a result of competition, and thus disrupting the existing interaction between *L. rubellus* and *O. lacteum*. This shift in community composition may then increase carbon mineralization rates and lead to future carbon loss through increasing soil microbial biomass. This conclusion, albeit unexpected, would not have been possible without knowing the interaction between *L. rubellus* and *O. lacteum*, or conducting field experiments.

4.2. The effect of earthworms on fungi-to-bacteria ratio

In general, our data showed little effect of earthworms on the fungi-to-bacteria ratio in the soil, and did not support our first hypothesis. These results are inconsistent with field observations reported by Dempsey et al. (2011, 2013), which is most likely due to the different initial conditions at the onset of the experiment. Dempsey et al. (2011, 2013) documented how earthworms changed soil microbial communities in a previously earthworm-free ecosystem with a thick O horizon rich in fungal biomass. Our experiment captured the dynamics of microorganisms under the presence or absence of different earthworm species in forest soils with a long history of land use and earthworm legacy (Szlavecz and Csuzdi, 2007; Szlavecz et al., 2011). The soil surface in our forest has not redeveloped a thick O horizon even after 150 years of agricultural abandonment (Yesilonis et al., 2016). As soil sieving at the beginning of our experiment destroyed fungal hyphae and bacteria were probably feeding on the dead fungal biomass, the system was in a state of recovery for the duration of the experiment. The lack of the O horizon may have limited fungi growth into the mesocosms, including the control.

Positive interspecific interactions between *A. agrestis* and *A. corticis* (in 2–5 cm soil) and between *A. corticis* and *O. lacteum* (in 5–10 cm soil) on fungi-to-bacteria ratios are intriguing, and it appears that the effects under these species interactions were mainly caused by positive effects on fungal biomass. The treatment with both *A. corticis* and *O. lacteum* had the highest fungal biomass in the 5–10 cm soil among all treatments, but its AM fungal biomass is the lowest, suggesting that the effects we observed were primarily contributed by increasing biomass of saprotrophic and/or ectomycorrhizal fungi, as opposed to AM fungi. This result is consistent with our observations that fine roots from the surrounding trees and saplings, primarily *Fagus grandifolia* (American beech, associated with ectomycorrhizal fungi), were able to successfully grow back into most of our mesocosms after the initial disruption of soil structure, presumably providing the source of ectomycorrhizal fungi.

4.3. Technical consideration for PLFA

The present study also documented the importance of choosing the appropriate biomarkers for AM fungi. The PLFA 16:1ω5cis, commonly used as a biomarker for AM fungi, has also been found repeatedly in Gram-negative bacteria (Frostegard et al., 2011), and may be appropriate as a biomarker in some systems (e.g. Dempsey et al., 2013) while not in others (Frostegard et al., 2011; Sharma and Buyer, 2015). Our data clearly showed that the assumed AM fungi biomarker PLFA 16:1ω5cis had a stronger correlation with Gram-negative bacteria PLFA biomarkers than with the reliable AM fungi biomarker NLFA 16:1ω5cis (Olsson, 1999; Frostegard et al., 2011; Sharma and Buyer, 2015), suggesting that the assumed biomarker reflects more of the biomass of Gram-negative bacteria rather than that of AM fungi. Accordingly, in our forest plots at SERC, where the dominant tree species are *Liriodendron tulipifera* (associated with AM fungi) and *Fagus grandifolia* (associated with ectomycorrhizal fungi), choosing a reliable AM fungi fatty acid biomarker is crucial in estimating the relative biomass change among different treatments, and the PLFA 16:1ω5cis should never be used.

Chang et al. (2016b) claimed that the interaction between *L. rubellus* and *O. lacteum* has a positive effect on the PLFA biomarkers of anaerobes. However, we recently discovered that the assertion is unfounded. As noted earlier, the biomarkers for anaerobes, dimethyl acetals, are not produced under the base-catalyzed transesterification procedure we adopted (Christie, 2003). Therefore, the compounds in our samples are not dimethyl acetals. We have not been able to identify the compounds due to their low concentrations in the soil. However, the concentration of these unknown compounds is consistently increased in both laboratory (Chang et al., 2016b) and field (this study) conditions when both *L. rubellus* and *O. lacteum* are present. Further study is needed to understand the ecological implication of this increase.

5. Conclusions

Our study documented microbial communities in a field experiment with different earthworm species and species combinations in a temperature deciduous forest in Eastern US. Our results highlighted that three different epi-endogeic species and their interactions with the endogeic earthworm *O. lacteum* have distinct effects on bacterial biomass. The ongoing invasion of the Asian earthworm *A. agrestis* in forest soils will lead to increased microbial and bacterial biomass. Moreover, through outcompeting the common European species L. *rubellus* and changing interspecific interactions with *O. lacteum*, *A. agrestis* can further exacerbate the condition, potentially causing increased carbon mineralization and reducing soil carbon storage.

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Appendix

Table A1
Number and biomass (g) of earthworm species after earthworm addition (June 2012 and 2013) and at the end of the year (October 2012 and 2013) under different treatments.

Treatment	*Amynthas agrestis*		*Amynthas corticis*		*Lumbricus rubellus*		*Octolasion lacteum*	
	Number	Biomass	Number	Biomass	Number	Biomass	Number	Biomass
June 2012								
Aa	8	5.3	–	–	–	–	–	–
Ac	–	–	6	6.2	–	–	–	–
Lr	–	–	–	–	12	5.4	–	–
Ol	–	–	–	–	–	–	24	5.3
Aa + Ac	4	2.9	3	3.2	–	–	–	–
Aa + Lr	4	3.1	–	–	6	3	–	–
Aa + Ol	4	2.8	–	–	–	–	12	2.5
Ac + Lr	–	–	3	2.8	6	2.7	–	–
Ac + Ol	–	–	3	2.9	–	–	12	2.6
Lr + Ol	–	–	–	–	6	2.4	12	2.5
October 2012								
Aa	1.8	1.2	–	–	–	–	–	–
Ac	–	–	1.2	1.4	–	–	–	–
Lr	–	–	–	–	1.8	1.1	–	–
Ol	–	–	–	–	–	–	3.7	1.2
Aa + Ac	0.3	0.3	0.3	0.5	–	–	–	–
Aa + Lr	0.8	0.6	–	–	1.2	0.4	–	–
Aa + Ol	0.7	0.6	–	–	–	–	2	0.7
Ac + Lr	–	–	0	0	1.5	0.8	–	–
Ac + Ol	–	–	0.3	0.5	–	–	3.5	1
Lr + Ol	–	–	–	–	1.5	0.8	3.2	0.9
June 2013								
Aa	8	8.8	–	–	–	–	–	–
Ac	–	–	6	3.9	–	–	–	–
Lr	–	–	–	–	12	8.8	–	–
Ol	–	–	–	–	–	–	24	5
Aa + Ac	4	4.2	3	2.9	–	–	–	–
Aa + Lr	4	4	–	–	6	4	–	–
Aa + Ol	4	4.3	–	–	–	–	12	2.6
Ac + Lr	–	–	3	2.9	6	4.5	–	–
Ac + Ol	–	–	3	2.9	–	–	12	2.5
Lr + Ol	–	–	–	–	6	4.3	12	2.4
October 2013								
Aa	0.3	0.3	–	–	–	–	–	–
Ac	–	–	0.8	0.45	–	–	–	–
Lr	–	–	–	–	2.3	1	–	–
Ol	–	–	–	–	–	–	9.8	1.8
Aa + Ac	0.2	0.3	0.8	0.5	–	–	–	–
Aa + Lr	0.2	0.3	–	–	1	0.5	–	–
Aa + Ol	0.3	0.5	–	–	–	–	3.3	0.6
Ac + Lr	–	–	0.2	0.1	1.7	0.9	–	–
Ac + Ol	–	–	0.5	0.3	–	–	6.2	1.3
Lr + Ol	–	–	–	–	0.7	0.3	6.2	1.3

*Aa: *Amynthas agrestis*, Ac: *Amynthas corticis*, Lr: *Lumbricus rubellus*, Ol: *Octolasion lacteum*; n = 6 for all treatments.

Table A2
Mean leaf litter remaining and soil pH in all treatments and the control.

Treatment*	Litter remaining (g)	pH (0–2 cm)	pH (2–5 cm)	pH (5–10 cm)
Ctr	17.9	5.2	4.1	4.1
Aa	8.3	5.3	4.6	4.1
Ac	7.2	5.0	4.2	4.1
Lr	8.2	5.4	4.2	4.1
Ol	14.4	5.0	4.5	3.9
Aa + Ac	15.1	5.0	4.4	4.1
Aa + Lr	8.0	5.3	4.3	4.1
Aa + Ol	13.0	5.6	4.5	4.1
Ac + Lr	10.5	5.1	4.5	4.3
Ac + Ol	7.2	5.1	4.5	4.2
Lr + Ol	11.8	5.4	4.6	4.1

*Ctr: control, Aa: *Amynthas agrestis*, Ac: *Amynthas corticis*, Lr: *Lumbricus rubellus*, Ol: *Octolasion lacteum*; n = 6 for litter remaining and 0–2 cm pH, n = 5 for 2–5 cm and 5–10 cm pH.

Table A3
Mean PLFA (all microbial groups except AM fungi) and NLFA (AM fungi only) concentrations (nmol/g) of 0–2 cm bulk soil.

Treatment*	Gram-negative	Gram-positive	Actinomycetes	Fungi	AM fungi	Protozoa
Ctr	102.751	58.474	28.719	6.053	50.113	2.323
Aa	115.799	68.923	32.001	5.414	69.257	1.786
Ac	96.760	58.169	27.853	5.233	43.948	1.852
Lr	108.334	63.167	29.640	6.412	33.775	2.286
Ol	97.255	61.906	28.441	5.304	38.326	1.727
Aa + Ac	100.431	63.082	30.102	5.536	47.967	2.045
Aa + Lr	111.916	69.784	31.819	7.367	57.389	2.570
Aa + Ol	109.657	66.145	30.671	6.037	53.882	2.028
Ac + Lr	103.560	64.232	30.308	5.633	40.577	1.999
Ac + Ol	93.238	57.738	27.421	5.291	28.213	1.911
Lr + Ol	89.026	55.006	26.176	5.241	23.401	1.849

*Ctr: control, Aa: *Amynthas agrestis*, Ac: *Amynthas corticis*, Lr: *Lumbricus rubellus*, Ol: *Octolasion lacteum*; n = 6 for all treatments.

Table A4
Mean PLFA (all microbial groups except AM fungi) and NLFA (AM fungi only) concentrations (nmol/g) of 2–5 cm bulk soil.

Treatment*	Gram-negative	Gram-positive	Actinomycetes	Fungi	AM fungi	Protozoa
Ctr	92.280	69.512	33.512	5.381	19.567	2.084
Aa	96.747	67.741	32.686	5.835	27.168	1.778
Ac	96.916	69.340	33.868	5.041	27.454	2.039
Lr	101.244	73.033	35.457	6.253	18.449	2.026
Ol	88.814	64.348	31.748	4.683	19.564	1.790
Aa + Ac	93.234	64.341	32.246	7.613	21.147	2.274
Aa + Lr	94.076	70.181	33.654	5.663	34.688	1.936
Aa + Ol	99.570	72.599	34.218	5.054	27.852	1.967
Ac + Lr	95.182	70.911	33.689	5.601	27.411	1.938
Ac + Ol	89.299	62.793	30.486	6.052	15.759	1.695
Lr + Ol	90.728	60.633	30.130	5.034	14.469	2.056

*Ctr: control, Aa: *Amynthas agrestis*, Ac: *Amynthas corticis*, Lr: *Lumbricus rubellus*, Ol: *Octolasion lacteum*; n = 5 for all treatments.

Table A5
Mean PLFA (all microbial groups except AM fungi) and NLFA (AM fungi only) concentrations (nmol/g) of 5–10 cm bulk soil.

Treatment*	Gram-negative	Gram-positive	Actinomycetes	Fungi	AM fungi	Protozoa
Ctr	90.000	70.162	34.035	5.650	18.882	1.677
Aa	96.481	74.129	35.900	6.252	34.021	1.856
Ac	84.222	63.191	30.936	4.555	27.033	1.683
Lr	92.835	67.010	33.252	6.484	29.761	1.825
Ol	89.881	68.947	34.277	5.221	16.983	1.717
Aa + Ac	88.739	67.691	32.862	5.805	15.469	1.681
Aa + Lr	92.746	70.237	33.511	6.270	41.371	1.694
Aa + Ol	91.835	70.783	34.389	4.684	20.938	1.797
Ac + Lr	94.675	71.474	34.348	5.183	21.145	2.002
Ac + Ol	89.592	65.681	30.424	9.472	12.215	1.697
Lr + Ol	85.835	67.764	32.025	5.716	13.700	1.623

*Ctr: control, Aa: *Amynthas agrestis*, Ac: *Amynthas corticis*, Lr: *Lumbricus rubellus*, Ol: *Octolasion lacteum*; n = 5 for all treatments.

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