

Dietary flexibility aids Asian earthworm invasion in North American forests

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Abstract. On a local scale, invasiveness of introduced species and invasibility of habitats together determine invasion success. A key issue in invasion ecology has been how to quantify the contribution of species invasiveness and habitat invasibility separately. Conventional approaches, such as comparing the differences in traits and/or impacts of species between native and/or invaded ranges, do not determine the extent to which the performance of invaders is due to either the effects of species traits or habitat characteristics. Here we explore the interaction between two of the most widespread earthworm invaders in the world (Asian *Amyntas agrestis* and European *Lumbricus rubellus*) and study the effects of species invasiveness and habitat invasibility separately through an alternative approach of “third habitat” in Tennessee, USA. We propose that feeding behaviors of earthworms will be critical to invasion success because trophic ecology of invasive animals plays a key role in the invasion process. We found that (1) the biomass and isotopic abundances ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of *A. agrestis* were not impacted by either direct effects of *L. rubellus* competition or indirect effects of *L. rubellus*-preconditioned habitat; (2) *A. agrestis* disrupted the relationship between *L. rubellus* and soil microorganisms and consequently hindered litter consumption by *L. rubellus*; and (3) compared to *L. rubellus*, *A. agrestis* shifted its diet more readily to consume more litter, more soil gram-positive (G+) bacteria (which may be important for litter digestion), and more non-microbial soil fauna when soil microorganisms were depleted. In conclusion, *A. agrestis* showed strong invasiveness through its dietary flexibility through diet shifting and superior feeding behavior and its indirectly negative effect of habitat invasibility on *L. rubellus* via changes in the soil microorganism community. In such context, our results expand on the resource fluctuation hypothesis and support the superior competitor hypothesis. This work presents additional approaches in invasion ecology, provides some new dimensions for further research, and contributes to a greater understanding of the importance of interactions between multiple invading species.

Key words: *Amyntas agrestis*; earthworm invasion; feeding effect; food web; Great Smoky Mountains National Park, Tennessee, USA; habitat invasibility; *Lumbricus rubellus*; phospholipid fatty acid; soil microbe; species invasiveness; stable isotopes; “third habitat” approach.

INTRODUCTION

Biological invasions are of utmost concern as drivers of ecosystems and essential components of global change (Vitousek 1990, Vitousek et al. 1997, Mack et al. 2000, Fukami et al. 2006). Unlike plant invasions, dietary flexibility of animals contributes greatly to their invasion success (Sol et al. 2002, Caut et al. 2008). However, whether and how trophic ecology of incon-

spicuous belowground invertebrates contributes to their invasion success is largely unknown, with the exception of a few ant species (LeBrun et al. 2007, Tillberg et al. 2007).

As one of the key belowground invertebrates, earthworms have played a most important part in the history of the world (Darwin 1881) and continue to alter the structure and function of ecosystems even more profoundly with the wide dispersal of exotic earthworm species (Bohlen et al. 2004a, Hendrix et al. 2008). Asian earthworms, such as those in the genus *Amyntas* Kinberg 1867, have successfully invaded many regions beyond the Oriental realm (within tropical or subtropical climate zones) from which they originated (Appen-

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dix A). However, there are no reports of exotic earthworms colonizing successfully in *Amyntas*-dominated natural forests. The mechanisms underlying the wide dispersal and territory-holding abilities of *Amyntas* are still unclear.

On a local scale, invasiveness of introduced species and invasibility of habitats together determine invasion success. Two common approaches of invasion ecology have been to compare the biological traits and/or impacts of an invasive species between native and invaded ranges and to compare these traits or impacts between invasive and native species within an invaded range (Bohlen et al. 2004b, Sánchez-de León and Zou 2004, Winsome et al. 2006). These trait differences alone may not necessarily be responsible for invasion success or failure, because the influence of habitat on a novel species could be positive, negative, or neutral. Even though certain traits have been shown to contribute to the invasion of a specific species in a specific habitat, conventional approaches have not shown whether these traits arose due to species' intrinsic invasiveness, habitat influences, or both.

For instance, if the resource use efficiency of Asian earthworms of *Amyntas* spp. is higher in North America than that in Asia and this trait difference causes negative impacts on native earthworm species in North America, we could conclude that higher efficiency of resource use contributes to the invasion of Asian *Amyntas* spp. in North America. However, we cannot tell that whether the invasion-associated difference in this trait of *Amyntas* spp. is facilitated by its intrinsic invasiveness or by the influence of North American habitats or by both. An alternative approach is to find a "third habitat" that is novel to all species of interest to isolate the effects of habitat and exclude the influences of native habitats on the biological and behavioral adaptations of native species (Appendix B), e.g., exploring the underlying mechanisms of biological invasion by investigating interaction between two exotic species.

In the present study, we selected the Great Smoky Mountains National Park (GSMNP), Tennessee, USA, as the "third habitat" in which the two common earthworm species, namely Asian *Amyntas agrestis* and European *Lumbricus rubellus*, are exotic. *Amyntas agrestis* (a subtropical/temperate species) was first recorded in Maryland, USA, in 1939 (Gates 1982), whereas *L. rubellus* (a temperate species) was introduced into North America during European settlement approximately 300 years ago (Frelich et al. 2006). These exotic species have coexisted for an undetermined period of time in the litter layer and surface soil of temperate deciduous forests in GSMNP. Field studies have shown that *A. agrestis* dominates in this ecosystem (Fig. 1), and European exotics, instead of native earthworm species, have been the majority of potential competitors of Asian exotics. This offers us an opportunity to explore the interaction between two of the most widespread

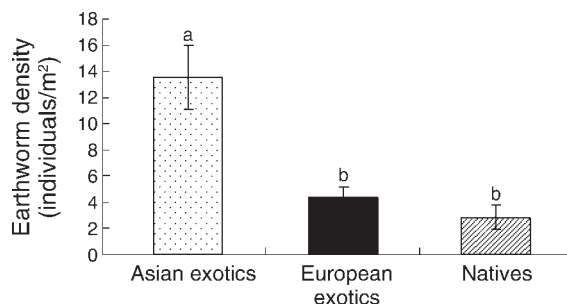


FIG. 1. Earthworm density (mean $\pm$ SE) in Great Smoky Mountains National Park, Tennessee, USA, surveyed in 2006. Earthworms were collected with the octet electroshocking method on five transects (45 plots) arrayed across an active *Amyntas agrestis* invasion front (details in Snyder [2008]). Asian earthworms included *A. agrestis* and *A. corticis*; European earthworms included *Lumbricus rubellus*, *Aporrectodea* spp., *Octolasion tyrtaeum*, *Dendrobaena octahedralis*, and *Eiseniella tetraedra*; native species included *Diplocardia* spp. and *Bimastos* spp. ($n = 5$ transects, $F_{2,12} = 21.08$, $P < 0.001$). Different letters above the bars indicate significant ($P < 0.05$) differences in mean earthworm density.

earthworm invaders in the world and to study the effects of species invasiveness and habitat invasibility separately.

As the capability for nutrient absorption is vital to the survival and expansion of plant species (Funk and Vitousek 2007), food resource acquisition ability is vital to the invasiveness of earthworm species. We propose that feeding behaviors of earthworms will be critical to invasion success, and given that the natural dietary ranges of *A. agrestis* and *L. rubellus* do not usually overlap (see details in *Results*), Asian *A. agrestis* may outcompete European *L. rubellus* indirectly through altering the environment, e.g., changing the structure of the soil microbial community, which consequently negatively impacts the feeding activities of *L. rubellus*. Studies of the interactions between exotic species may offer distinct opportunities to understand invasion ecology from a unique perspective, but so far such work is scarce. Here we show how exotic earthworm species interact through their effects on the food web.

MATERIALS AND METHODS

Field survey and laboratory microcosm studies were conducted to investigate the mechanisms behind the invasion and dominance of *A. agrestis* in GSMNP by focusing on its feeding behavior. First, the natural abundances of ^{13}C and ^{15}N of field-collected samples of earthworms and their putative food sources (soil and leaf litter) in GSMNP were measured in order to understand the natural feeding habits of these earthworms. The microbial and nonmicrobial fauna community structure in earthworm intestines was also examined using phospholipid fatty acid (PLFA) analysis to ascertain whether unique groups of biota were harbored in the gut of *A. agrestis* or *L. rubellus*. Second, in microcosm studies, mean earthworm biomass change,

^{13}C and ^{15}N content in earthworm tissue, and microbial nonmicrobial fauna community structure (PLFA profile) in bulk soil were measured after a 28-d incubation. These characteristics were then compared between treatments with single and mixed earthworm species to investigate direct interspecific interactions. Importantly, we used soil preconditioned with *A. agrestis* (Asoil) and soil preconditioned with *L. rubellus* (Lsoil) to simulate the field conditions created by an exotic in its invaded habitat and then as media to examine habitat-induced indirect interspecific interaction through treatments of soil replacements (Asoil switched for Lsoil or vice versa). By this approach, the effect of each earthworm-modified habitat was separated and quantified. Thirdly, we tried to discern the key guilds of soil biota that were involved in the indirect interaction processes by sub-treatments of soil sterilization. Concurrently, we traced the feeding behavior of both earthworm species using ^{13}C -enriched oak litter ($\delta^{13}\text{C} = 453.97 \pm 15.66\text{‰}$). The ^{13}C enrichment factors (Δ) of earthworms were estimated and then the fractions of ^{13}C -enriched litter-derived carbon (as a percentage, f_{ltr}) in earthworm tissues were calculated to determine the variation in the amount of litter the earthworms consumed.

Field collections

To measure the natural abundance of ^{13}C and ^{15}N in soil, leaf litter, and earthworms, more than 30 samples were collected from five different subsites within an $\sim 2\text{-km}^2$ area on the western edge of GSMNP ($35^\circ 31' 30''$ – $35^\circ 33' 27''$ N; $83^\circ 59' 35''$ – $84^\circ 00' 36''$ W) during April and May 2007. Sixteen soil samples, 12 leaf litter samples, 10 *A. agrestis* individuals, and 11 *L. rubellus* individuals were selected randomly to analyze isotopic abundances (Appendices C–E). The rest of the collected earthworms were kept for use in the preconditioning experiment and other experimental microcosms not part of this study. Vegetation at the study site was dominated by *Acer* spp., *Quercus* spp., *Liquidambar styraciflua*, and *Liriodendron tulipifera* in valleys while the ridges were dominated by white pine (*Pinus strobus*). Ridge soils were a complex of moderately deep Junaluska and deep Brasstown series soils, fine-loamy, mixed, subactive, mesic Typic Hapludults. Valley soils were a complex of shallow Cataska series and moderately deep Sylco series soils, which are loamy-skeletal, mixed, active (Sylco) or semiactive (Cataska), mesic Typic Dystrudepts (Snyder 2008). Earthworms, soil (0–5 cm), and leaf litter were collected from this area for laboratory soil preconditioning and microcosm experiments.

Soil preconditioning

Field-collected soil was sieved (2 mm) to remove biota > 2 mm and subsequently air-dried and mixed thoroughly to create a homogenous soil substrate. Experimental units (54) were made from polyvinyl chloride pipes (15 cm in height, 10.4 cm in diameter) and cleaned with 75% ethanol. Two hundred grams of air-dried soil

was weighed into each microcosm and soil water content was adjusted to 60% of the maximum water-holding capacity. The entire unit was weighed every three days to maintain relatively constant water content; room temperature was maintained at 18°C . After one week of static culture, earthworms (either *A. agrestis* or *L. rubellus*) were inoculated into the soil (3 individuals/microcosm) and non-labeled oak litter (0.1 g/microcosm) was placed on the surface. Earthworm preconditioning took place for 23 d, after which three microcosms from each earthworm species were randomly chosen, destructively sampled, and the earthworms and soils were freeze-dried for PLFA and stable isotope analyses. For the remaining 48 microcosms, earthworms were removed by hand and soils were grouped by earthworm species (Asoil and Lsoil). These were each mixed thoroughly and half of each soil was sterilized (121°C , 30 min).

Microcosm experimental design

These soils were used to set up a microcosm study. For each microcosm, 200 g equivalent dry soil was used and soil water content was adjusted to the initial level. The microcosms were static-cultured for 2 d. Then earthworms were inoculated into microcosms after 1 d of gut-voiding on wet filter paper, and immediately ^{13}C -enriched oak litter (shredded to ~ 4 mm size, $\delta^{13}\text{C} = 453.97 \pm 15.66\text{‰}$, $\delta^{15}\text{N} = 2.37 \pm 0.02\text{‰}$ [mean $\pm$ SE]) was placed on the soil surface. There were 48 microcosms; half of these were set up with non-sterilized soil and the other half with sterilized soil. The eight treatments were: three individuals of *A. agrestis* in Asoil, three individuals of *A. agrestis* plus one individual of *L. rubellus* in Asoil, three individuals of *A. agrestis* in Lsoil, three individuals of *L. rubellus* in Lsoil, three individuals of *L. rubellus* plus one individual of *A. agrestis* in Lsoil, three individuals of *L. rubellus* in Asoil, Asoil without earthworms, and Lsoil without earthworms. Each treatment has three replicates. At the beginning, biomasses of *A. agrestis* and *L. rubellus* were 0.97 ± 0.07 g/individual and 0.48 ± 0.02 g/individual, respectively. The experiment was ended after 28 d incubation when the litter on the soil surface disappeared markedly in microcosms with *L. rubellus*. All microcosms were destructively sampled. Earthworm numbers in each microcosm were recorded and earthworm biomass was measured after 1 d of gut-voiding. Earthworms were then euthanized by placement in a freezer for 2–5 min, and the posterior one-third of the body was removed, the gut cleaned with deionized water, and freeze-dried for isotopic and PLFA analyses. The anterior two-thirds of each earthworm was preserved in 70% ethanol for confirmation of taxonomic identity (by B. A. Snyder).

Isotopic analysis and PLFAs measurement

Stable isotope abundance was measured using a Thermo-Finnigan Delta Plus Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen, Germany) in the

Analytical Chemistry Laboratory in Odum School of Ecology, University of Georgia, Georgia, USA. Phospholipid fatty acids were extracted with methanol:chloroform:phosphate buffer solution (2:1:0.8 by volume) (Frostegård et al. 1991, 1993, Burke et al. 2003). Approximately 5 g and 100 mg freeze-dried soil and earthworm samples were used, respectively. Samples were analyzed with a Hewlett-Packard (Hewlett-Packard, Palo Alto, California) 6890 series gas chromatography with a flame ionization detector and a 30-m DB-5 (film thickness = 0.25 microns, internal diameter 0.32 mm; Agilent, Santa Clara, California, USA). Individual PLFA were quantified in relation to an external standard (20:0 ethyl ester) that was run in duplicate with every batch of samples. Compounds were identified by comparison of their retention times with those of a prepared mixture standard containing 38 fatty acid methyl esters (FAMES; Matreya, Pleasant Gap, Pennsylvania, USA; Sigma-Aldrich, St. Louis, Missouri, USA; Nu-Chek, Elysian, Minnesota, USA) that was run with each batch (Carrillo 2008). Fatty acid notation followed that in Frostegård and Bååth (1996). Those chosen to represent total bacterial PLFA were i15:0, a15:0, 15:0, i16:0, 16:1ω7c, 16:1ω7t, 17:0, i17:0, a17:0, cy17:0, and cy19:0; for gram-positive (G+) bacteria PLFA, i15:0, i16:0, 10Me16:0, a15:0, i17:0, and a17:0; for gram-negative (G-) bacteria PLFA, 16:1ω7c, cy17:0, and cy19:0; for fungi PLFA, 18:2ω6; for actinomycete PLFA, 10Me18:0; and for nonmicrobial microeucaryotes/microfauna (e.g., algae, protozoa) and other soil fauna, 20:4ω6, 20:5ω3, 20:3ω6, 20:2ω6, 20:3ω3, and 17:1ω7c.

Partitioning models of food sources for earthworms

Since both plant litter and soil are putative food sources for *A. agrestis* and *L. rubellus* (Doube et al. 1997, Scheu and Falca 2000; Fig. 2), a simple mixing model (Treseder et al. 1995, Schmidt et al. 2004, Staddon 2004) was used to estimate the ^{13}C enrichment factors (Δ) of these earthworm species with an isotopic data set from field sites in GSMNP. Based on the ^{13}C enrichment factors, we then calculated the fractions of earthworm tissue carbon derived from ^{13}C -enriched litter (f_{ltr}) in the microcosm experiment. The equations of the mixing model are presented as

$$\delta^{13}\text{C}_{\text{ew}} = \Delta + f_{\text{ltr}} \times \delta^{13}\text{C}_{\text{ltr}} + (1 - f_{\text{ltr}}) \times \delta^{13}\text{C}_{\text{soil}} \quad (1)$$

where $\delta^{13}\text{C}_{\text{ew}}$, $\delta^{13}\text{C}_{\text{ltr}}$, and $\delta^{13}\text{C}_{\text{soil}}$ are ^{13}C abundances in field-collected samples of earthworms, fallen litter, and soil, respectively. Δ represents the enrichment factors of earthworms from their putative diets; f_{ltr} is the fraction of carbon derived from forest litter into earthworm biomass. In order to calculate the Δ values of earthworms, we rearranged Eq. 1 as

$$\Delta = (\delta^{13}\text{C}_{\text{ew}} - \delta^{13}\text{C}_{\text{soil}}) - f_{\text{ltr}} \times (\delta^{13}\text{C}_{\text{ltr}} - \delta^{13}\text{C}_{\text{soil}}). \quad (2)$$

For *A. agrestis* and *L. rubellus*, the first part of Eq. 2 is as follows: $\delta^{13}\text{C}_{\text{ew}} - \delta^{13}\text{C}_{\text{soil}} = (-23.45\text{‰}) - (-26.53\text{‰}) =$

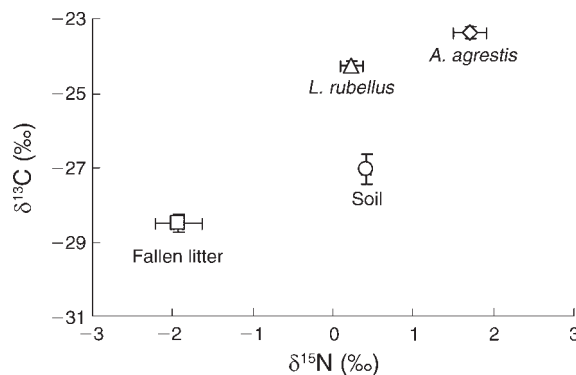


FIG. 2. Plot showing $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (mean $\pm$ SE) of Asian *Amyntas agrestis* and European *Lumbricus rubellus*, the soil, and fallen litter from temperate forests in Great Smoky Mountains National Park. More than 30 samples were collected from five different subsites during April and May 2007, and some of these were selected randomly for analysis. Independent samples *t* tests ($n = 10$ for *A. agrestis* and $n = 11$ for *L. rubellus*) demonstrate significant differences of the $\delta^{13}\text{C}$ (two-tailed, $df = 19$, $t = 4.69$, $P < 0.001$) and $\delta^{15}\text{N}$ (two-tailed, $df = 19$, $t = 5.93$, $P < 0.001$) values between Asian *A. agrestis* and European *L. rubellus*. ANOVA ($n = 16$ for soil, and $n = 12$ for litter) indicates significant differences of the $\delta^{13}\text{C}$ ($F_{3,45} = 57.56$, $P < 0.001$) and $\delta^{15}\text{N}$ ($F_{3,45} = 64.09$, $P < 0.001$) values among earthworms, soil, and litter.

3.08‰ and $\delta^{13}\text{C}_{\text{ew}} - \delta^{13}\text{C}_{\text{soil}} = (-24.30\text{‰}) - (-26.53\text{‰}) = 2.23\text{‰}$, respectively. As for the second part of Eq. 2, i.e., $f_{\text{ltr}} \times (\delta^{13}\text{C}_{\text{ltr}} - \delta^{13}\text{C}_{\text{soil}})$, it should be less than zero since f_{ltr} is in the range of 0–1 and $\delta^{13}\text{C}_{\text{ltr}}$ is more negative than $\delta^{13}\text{C}_{\text{soil}}$. Therefore, the Δ values of *A. agrestis* and *L. rubellus* must be greater than 3.08‰ and 2.23‰ , respectively. Considering that the Δ values of earthworms reported in the literature were in the range of 2–4.3‰ (Martin et al. 1992a, b, Schmidt et al. 1997), we think the Δ values of *A. agrestis* and *L. rubellus* are most likely in the ranges of 3.08‰ – 4.3‰ and 2.23‰ – 4.3‰ , respectively. It is also noteworthy that Eq. 1 may not be used directly to calculate the actual fraction of earthworm tissue carbon derived from litter (f_{ltr}) in our short-term microcosm experiment because the 28-d incubation was insufficient to complete the whole turnover cycle of the earthworm tissue carbon. According to previous reports (Scheu 1991, Martin et al. 1992b, Whalen and Janzen 2002), the turnover rate of carbon in earthworm tissues was assumed to be 0.25 in the 28-d incubation. Thus, the actual amount of ^{13}C fixed in earthworm tissues from the ^{13}C -enriched litter and non-enriched soil during the period of our microcosm experiment could be calculated by the following modified equation:

$$\delta^{13}\text{C}_{\text{ew}'} = [\delta^{13}\text{C}_{\text{ewt}} - \delta^{13}\text{C}_{\text{ewt}}(1 - f_{\text{turn}})]/f_{\text{turn}} \quad (3)$$

where $\delta^{13}\text{C}_{\text{ew}'}$ is the estimated ^{13}C abundance of earthworm tissue derived from the ^{13}C -enriched litter and soil, $\delta^{13}\text{C}_{\text{ewt}}$ is the actual measurement of the ^{13}C abundance in earthworm tissue after the 28-d incuba-

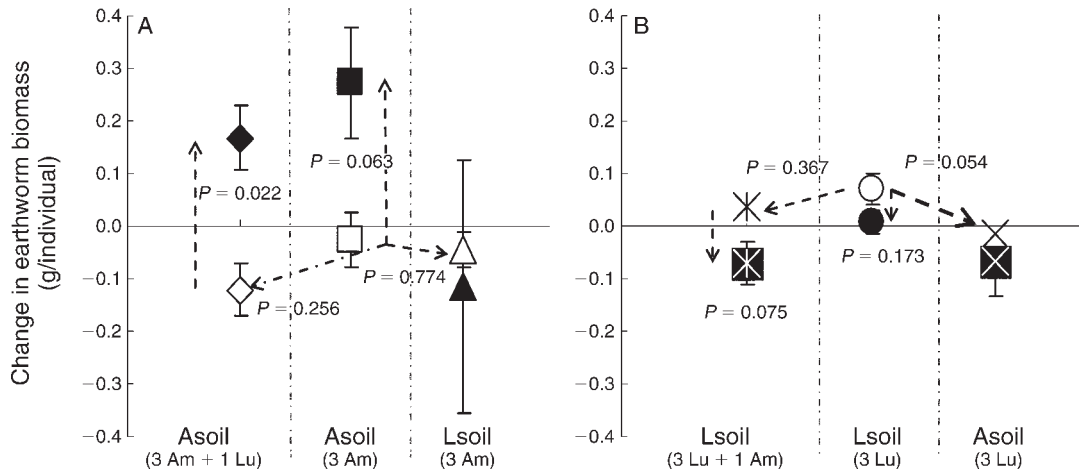


FIG. 3. Change in earthworm biomass (mean $\pm$ SE, $n = 3$) during the 28-d experiment for (A) *Amynthus agrestis* (Am) and (B) *Lumbricus rubellus* (Lu). Individual numbers of earthworms in each microcosm appear in parentheses. Open and solid symbols represent treatments in non-sterilized soil and sterilized soil, respectively. Arrows show the change of parameters between treatments, accompanied by statistical P values; dash-dotted lines were used to separate the three treatments. Soil treatment abbreviations are: Asoil, *Amynthus*-preconditioned soil; Lsoil, *Lumbricus*-preconditioned soil.

tion, $\delta^{13}\text{C}_{\text{ew}0}$ is the ^{13}C natural abundance in earthworm tissue, and f_{turn} is 0.25, the assumed turnover rate of earthworm tissue carbon. Based on the Δ and $\delta^{13}\text{C}_{\text{ew}}$ value calculated from the above equations, the actual fraction of earthworm carbon derived from ^{13}C -enriched litter (f_{ltr}) in the microcosm system was calculated using the following equation:

$$f_{\text{ltr}} = (\delta^{13}\text{C}_{\text{ew}} - \Delta - \delta^{13}\text{C}_{\text{soil}}) / (\delta^{13}\text{C}_{\text{ltr}} - \delta^{13}\text{C}_{\text{soil}}) \quad (4)$$

where f_{ltr} is the actual fraction of earthworm tissue carbon derived from ^{13}C -enriched litter, $\delta^{13}\text{C}_{\text{ltr}}$ (453.97‰) is the isotope abundance of the ^{13}C -enriched litter, and Δ is the enrichment factor of earthworms, which was 3.08–4.3‰ and 2.23–4.3‰ for *A. agrestis* and *L. rubellus*, respectively. $\delta^{13}\text{C}_{\text{soil}}$ is the isotope abundance in the earthworm-preconditioned soil.

Statistical analysis

One-way ANOVA was used to compare the population density of different categories of earthworms in the field sites, stable isotope abundances among earthworms, soil, and leaf litter, and the PLFA profiles among treatments of non-sterilized or sterilized Asoil and Lsoil with or without earthworms. An independent-samples two-tailed t test was used to examine the differences of the natural abundance of earthworms' ^{13}C and ^{15}N and the variations of the mean earthworm biomass and isotopic values between different microcosm treatments. A paired-samples two-tailed t test was conducted to determine the variation of the values of f_{ltr} in response to soil replacement or sterilization. Absolute mole concentration of PLFA was log-transformed before analysis. All statistical analyses were performed with SPSS version 13.0; the significance level was set at $P < 0.05$.

RESULTS

The natural ranges of both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ scarcely ever overlapped and were significantly higher (two-tailed t test, $t = 4.69$, $P < 0.001$ for $\delta^{13}\text{C}$; $t = 5.93$, $P < 0.001$ for $\delta^{15}\text{N}$) in *A. agrestis* than in *L. rubellus* (Fig. 2). Hence, the dietary range of field-collected *A. agrestis* and *L. rubellus* did not usually overlap, although they lived in the same horizon of surface soil. There was no overlap in the combined levels of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between species.

However, in the microcosm study, we observed strong interspecific interaction between *A. agrestis* and *L. rubellus*. We found that the mean biomass change (compared to initial biomass) of *A. agrestis* was not affected by either Lsoil or the addition of *L. rubellus* in Asoil (Fig. 3A), whereas the mean biomass change (compared to initial biomass) of *L. rubellus* declined considerably (two-tailed t test, $t = -2.69$, $\text{df} = 4$, $P = 0.054$) in Asoil but did not change with the addition of *A. agrestis* in Lsoil (Fig. 3B). Furthermore, when soil was sterilized the mean biomass change of *A. agrestis* increased in Asoil (two-tailed t test, $\text{df} = 4$, $t = -3.66$, $P = 0.022$ and $t = -2.55$, $P = 0.063$) but did not change in Lsoil (Fig. 3A), whereas the mean biomass change of *L. rubellus* decreased in all cases (Fig. 3B). We repeated these analyses using relative changes in biomass instead of absolute changes in biomass and obtained the same results.

Isotopic signatures in earthworm tissues clearly reflected the mean biomass change and the corresponding dietary variation pattern of these earthworms. The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of *A. agrestis* were not affected by Lsoil or the addition of *L. rubellus* (Fig. 4A) in non-sterilized soil, which was consistent with the variation pattern of the mean biomass change of earthworms (Fig.

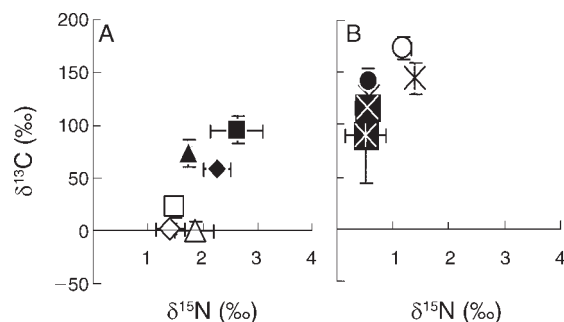


FIG. 4. Abundance of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in earthworms (mean $\pm$ SE, $n = 3$) after the 28-d experiment for (A) *Amyntas agrestis* and (B) *Lumbricus rubellus*. Abbreviations and symbols for treatments are as in Fig. 3.

3A). However, $\delta^{13}\text{C}$ value of *A. agrestis* increased significantly (two-tailed t test, $df = 4$, $t = -9.86$, $P = 0.001$, $t = -4.61$, $P = 0.010$, and $t = -4.58$, $P = 0.010$) and its $\delta^{15}\text{N}$ changed slightly in sterilized soil (Fig. 4A). Correspondingly, the fraction of ^{13}C -enriched litter-derived carbon (f_{ltr}) in *A. agrestis* increased significantly (paired-samples two-tailed t test, $df = 17$, $t = -23.72$, $t = -24.08$, and $t = -10.17$, all $P < 0.001$) from 4.9–9.8% to 17.0–24.9% in response to soil sterilization, but was not affected by the addition of *L. rubellus* and soil replacement (replacement of Asoil with Lsoil; Fig. 5A). As for *L. rubellus*, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ decreased considerably in response to soil replacement (replacement of Lsoil with Asoil) (two-tailed t test, $df = 4$, $t = -2.95$, $P = 0.042$ and $t = -3.38$, $P = 0.028$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively) and soil sterilization especially for the monoculture in Lsoil (two-tailed t test, $df = 4$, $t = 2.69$, $P = 0.055$ and $t = 4.25$, $P = 0.013$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively; Fig. 4B). Correspondingly, f_{ltr} in *L. rubellus* decreased from 40.8–41.2% to 31.1–31.5% in response to soil replacement

(paired-samples two-tailed t test, $df = 29$, $t = 10.14$, $P < 0.001$) and from 31.1–41.2% to 23.7–34.5% in response to soil sterilization (paired-samples two-tailed t test, $df = 29$, $t = 4.04$, $P < 0.001$, $t = 14.01$, $P < 0.001$, and $t = 0.80$, $P = 0.43$; Fig. 5B). Interestingly, the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and f_{ltr} values of *L. rubellus* in Asoil were low compared to that in Lsoil and were not affected by soil sterilization (Figs. 4B and 5B).

It was also notable that the mean biomass change of *A. agrestis* (0.233 ± 0.098 g) in treatment with only one individual of *A. agrestis* (i.e., one individual *A. agrestis* plus three *L. rubellus* in non-sterilized *Lumbricus*-preconditioned soil) was the highest and did not decline (0.237 ± 0.035 g) in response to soil sterilization compared to treatments with three individuals of *A. agrestis* (Fig. 3A). Correspondingly, the $\delta^{13}\text{C}_{\text{ew}}$ of *A. agrestis* ($-7.25 \pm 2.16\text{‰}$) in treatment with one individual of *A. agrestis* was the lowest and least variable in response to soil sterilization ($5.02 \pm 7.59\text{‰}$). In contrast, the mean biomass change of *L. rubellus* (0.097 ± 0.018 g) in treatment with only one individual of *L. rubellus* (i.e., one individual *L. rubellus* plus three *A. agrestis* in non-sterilized *Amyntas*-preconditioned soil) declined (-0.142 ± 0.226 g) in response to soil sterilization. Similarly, the $\delta^{13}\text{C}_{\text{ew}}$ of *L. rubellus* ($210.43 \pm 11.97\text{‰}$) in treatment with one individual of *L. rubellus* decreased in response to soil sterilization ($148.47 \pm 8.36\text{‰}$).

The PLFAs profiles in earthworms (with or without gut) and bulk soil showed that no unique guilds of biota were present in both earthworms (data not shown) and soil biota was affected differently by *A. agrestis* and *L. rubellus*. In non-sterilized soil, *A. agrestis* significantly decreased soil total bacteria PLFAs (measured in nanomoles per gram of soil; $n = 3$, $P = 0.024$) but *L. rubellus* did not affect total bacteria PLFAs (Fig. 6).

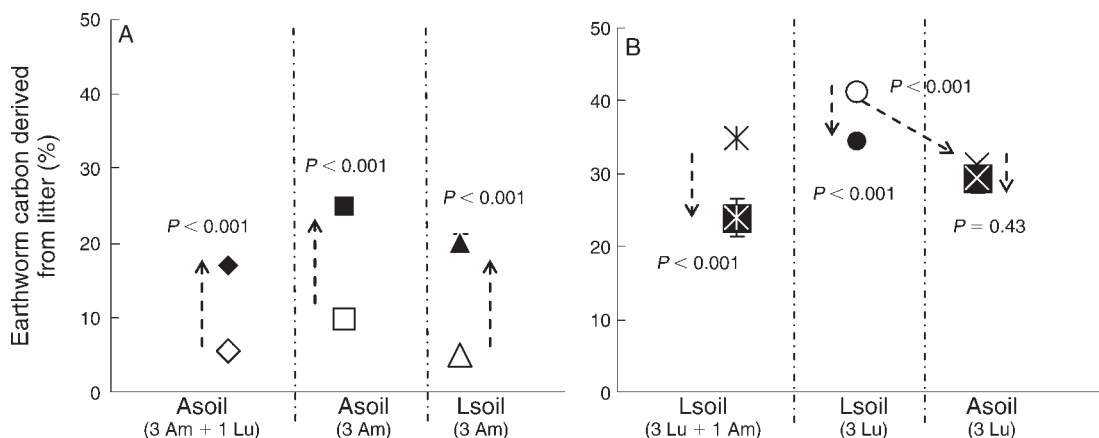


FIG. 5. The carbon fractions of earthworms (mean $\pm$ SE) derived from ^{13}C -enriched oak litter (f_{ltr}) after the 28-d experiment for (A) *Amyntas agrestis* (Am; $n = 18$) and (B) *Lumbricus rubellus* (Lu; $n = 30$). The number of earthworm individuals in each microcosm is indicated in parentheses. Abbreviations and symbols for treatments are as in Fig. 3. The fractions of earthworm tissue carbon derived from litter are calculated by Eq. 4, using ranges of ^{13}C enrichment factors (Δ) of earthworms obtained from Eq. 2. Six values of Δ (3.1–4.2‰) were used for *A. agrestis*, and 10 values of Δ (2.3–4.2‰) for *L. rubellus*. A paired-samples two-tailed t test was performed.

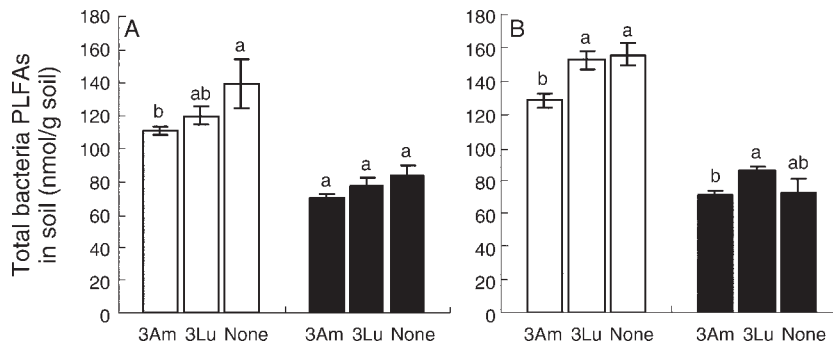


FIG. 6. Earthworm effects on the total bacteria phospholipid fatty acid (PLFA; mean $\pm$ SE, $n = 3$) in earthworm-preconditioned soils: (A) *Amynthus*-preconditioned soil (Asoil) and (B) *Lumbricus*-preconditioned soil (Lsoil). Open and solid bars represent non-sterilized soil and sterilized soil, respectively. Key for terms used: “3Am” and “3Lu” refer to three individuals of *A. agrestis* and three individuals of *L. rubellus*, respectively; “None” indicates no earthworm was inoculated in Asoil or Lsoil. Different letters above the bars indicate significant ($P < 0.05$) differences between treatments in non-sterilized soil or sterilized soil according to one-way ANOVA.

Interestingly, both earthworm species decreased G+ bacteria (percentage of moles) significantly in Lsoil ($n = 3$, $P < 0.001$) but did not change G+ bacteria in Asoil (Fig. 7A, B). In sterilized soil, however, *A. agrestis* decreased G+ bacteria significantly in both Asoil ($n = 3$, $P = 0.021$) and Lsoil ($n = 3$, $P < 0.001$), while *L. rubellus* did not reduce G+ bacteria in either Asoil or Lsoil. In addition, *A. agrestis* also decreased nonmicrobial fauna PLFAs in sterilized Asoil ($n = 3$, $P = 0.017$) and sterilized Lsoil ($n = 3$, $P = 0.072$), while *L. rubellus* had no negative effects on these PLFAs in all cases (Fig. 7C, D).

DISCUSSION

Conventional approaches in invasion ecology, namely comparing the biological traits and/or impacts of invasive species between their native and invaded ranges

and comparing these traits or impacts between invasive and native species within an invaded range, are useful ways to explore the traits that contribute to invasion success. However, these approaches are limited in their ability to determine why such traits arise and contribute to invasion success. One of the main obstacles to understanding this process is our ability to separate the contributions of species invasiveness from those of habitat influences in invasion processes. The alternative approach of “third habitat” employed here was helpful in separating these factors (Appendix B). Although the third-habitat approach was used here to focus on the interaction between two exotic earthworm species, this approach can be applied to an interaction between any two species, regardless of whether they are exotic or native where they co-occur.

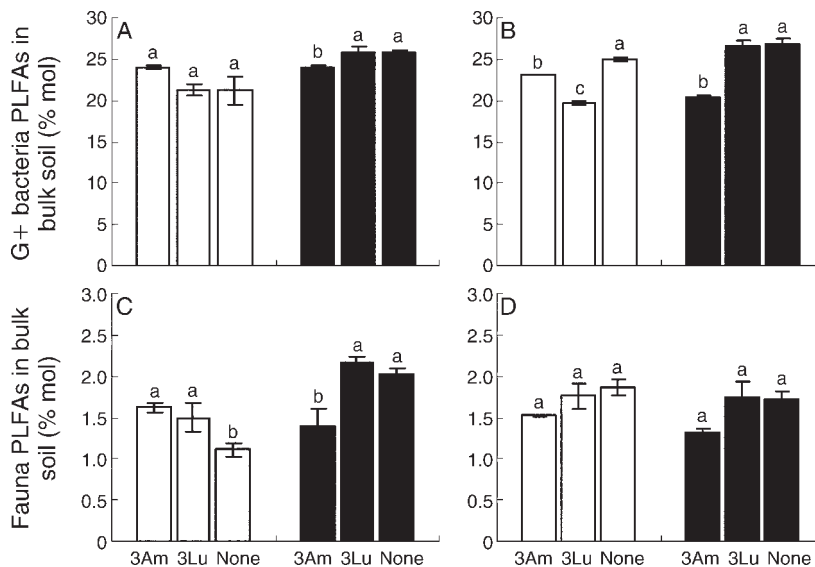


FIG. 7. Earthworm effects on mole percentage of gram-positive (G+) bacteria and nonmicrobial fauna phospholipid fatty acids (PLFAs; mean $\pm$ SE; $n = 3$) in earthworm-preconditioned soil: (A, C) *Amynthus*-preconditioned soil (Asoil) and (B, D) *Lumbricus*-preconditioned soil (Lsoil). Abbreviations are as in Fig. 6.

In the field, the effects of species invasiveness and habitat invasibility were interwoven because both *A. agrestis* and *L. rubellus* lived in the litter layer and surface soil. The natural isotopic abundances of earthworms in GSMNP suggested that *A. agrestis* fed more on soil biota, i.e., the diet of *A. agrestis* had a greater proportion of soil organic matter with microbial-incorporated ^{13}C , and consumed less leaf litter than *L. rubellus* (Ehleringer et al. 2000, Curry and Schmidt 2006, Hyodo et al. 2008). Hence, interspecific competition for food resources between the two earthworm species is unlikely to be direct most of the time. However, the mean biomass change patterns of the two earthworm species (Fig. 3) implied significant indirect interspecific earthworm interaction during the 28-d microcosm experiment. The mean biomass change of earthworms suggested that *A. agrestis* not only was released from the impacts of *L. rubellus* either directly (by addition of *L. rubellus*) or indirectly (by Lsoil), but also negatively affected *L. rubellus* in an indirect way. In other words, *A. agrestis* not only encountered nonnegative influence of habitat invasibility from Lsoil, but also showed stronger invasiveness over *L. rubellus*. The consistently lower biomass of *L. rubellus* in Asoil (compared to Lsoil) and all sterilized soil (Fig. 3B) suggested that the higher invasiveness of *A. agrestis* was related to its negative effect of habitat invasibility on *L. rubellus* and soil biota may be involved in these processes.

Isotopic and PLFAs analyses showed in greater detail how and which soil microbes were involved in earthworm interaction processes. First, the comparatively stable values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in *A. agrestis* tissue implied that the feeding processes of *A. agrestis* were not significantly affected by either the addition of *L. rubellus* (invasiveness of *L. rubellus*) or *Lumbricus*-preconditioned soil (habitat invasibility; Fig. 4A). Second, the variation pattern of ^{13}C (Figs. 4 and 5) abundance in earthworm tissues that respond to soil sterilization indicated that *A. agrestis* shifted its diet to consume more litter as a result of reduction in the abundance of soil microbes; in contrast, *L. rubellus* failed to adapt to such environmental stress, suggesting litter consumption by *L. rubellus* was closely related to soil biota. The distinct responses of *A. agrestis* and *L. rubellus* to extreme environmental change such as soil sterilization highlighted that *A. agrestis* was more adaptable than *L. rubellus*, although such critical stress rarely occurs in natural forests. We thought that the high adaptability of *A. agrestis* would certainly enhance its invasiveness. This result was consistent with that of Tillberg et al. (2007) in which the diet shifting of Argentine ants contributed to their successful invasion. These findings suggested that invasive species with high dietary flexibility could create new resource openings or opportunities, that access to resources is not only influenced by disturbance and varies with space and time, but is also dependent on feeding behavior; in this context our finding expands on the resource fluctuation hypothesis (Davis et al. 2000)

and supports the niche opportunities hypothesis (Shea and Chesson 2002). Third, the highest and stable values of the mean biomass change and ^{13}C of *A. agrestis* in the treatment with only one individual of *A. agrestis* implied that (1) if population density was low, it was not necessary for *A. agrestis* to shift its diet to consume more litter and *A. agrestis* could grow well under severe environmental stress such as disruption of the soil microbial community; and (2) the growth of *A. agrestis* may be facilitated by the activity of *L. rubellus*, which stimulates the growth of soil bacteria during the processes of litter consumption and incorporation into soil (Fig. 6), i.e., “invasional meltdown” as Tiunov et al. (2006) had suggested may also occur and contribute to dispersal of *A. agrestis*. The habitat invasibility effect on *A. agrestis* from Lsoil may be positive, thus we postulated that *A. agrestis* may thrive in previously earthworm-free forests in North America where *L. rubellus* consumed 10 cm or more thickness of intact forest floor within one growing season (Frelich et al. 2006). In contrast, the decline of the values of the mean biomass change and ^{13}C of *L. rubellus* in the treatment with only one individual of *L. rubellus* indicated that *L. rubellus* was affected negatively by *A. agrestis* even though the population density of *L. rubellus* was low. Finally, the low values for biomass (Fig. 3B), isotopic abundance (Fig. 4B), and f_{tr} (Fig. 5B) of *L. rubellus* in Asoil (compared to Lsoil), which were all as low as that in treatments of sterilized soil, suggested that the mechanism underlying the negative effects of Asoil on *L. rubellus* is similar to that of soil sterilization. Thus, long-term occupation of *A. agrestis* is likely to have a strong negative impact on *L. rubellus* growth in a given habitat, i.e., the habitat invasibility effect from Asoil on *L. rubellus* is greatly negative. Therefore, without regard to other environmental factors, *A. agrestis* has the potential to not only invade and establish in previously earthworm-free areas, but also in *L. rubellus*-dominated habitats.

Since there were no unique PLFAs in both earthworms' guts, we concluded it was extremely unlikely that it was earthworm-associated biota but rather biota in the soil that mediated the dietary process of earthworms. In fact, our results indicated that soil bacteria may be important food sources to *A. agrestis* but not to *L. rubellus* (Fig. 6). Further analysis of PLFA profiles in bulk soil (Figs. 5B and 7A, B), however, indicated that G+ bacteria were significantly reduced by *L. rubellus* in non-sterilized Lsoil, where the greatest amount of litter was consumed. On the contrary, *L. rubellus* did not affect G+ bacteria in treatments of Asoil and sterilized soil where much less litter was consumed. In addition, *A. agrestis* also decreased G+ bacteria significantly in all sterilized soil in which it consumed more litter. These data suggested that G+ bacteria may be essential to earthworms for litter digestion and *A. agrestis* showed superior feeding on G+ bacteria over *L. rubellus* when necessary. In other words, *A. agrestis* could negatively

affect *L. rubellus* through its negative effects on soil microbes, especially G+ bacteria. Nevertheless, it was also possible that G+ bacteria were consumed by *A. agrestis* as a food source (not for litter digestion), because *A. agrestis* reduced G+ bacteria in non-sterilized Lsoil where it consumed as much litter as in non-sterilized Asoil. Also, the decrease of nonmicrobial fauna PLFAs in sterilized Asoil and Lsoil suggested that *A. agrestis* could enhance its feeding on other soil fauna as well to offset food shortage. This behavior was also reflected in the high $\delta^{15}\text{N}$ values in *A. agrestis* in sterilized soil (Fig. 4A). We believe these patterns together are strong evidence that *A. agrestis* has stronger dietary flexibility than *L. rubellus*.

Overall, Asian *A. agrestis* disrupted the important relationship between European *L. rubellus* and soil microbes and consequently affected the feeding processes of *L. rubellus* negatively. As a result, *A. agrestis* became a superior competitor for resources (soil biota and leaf litter) over *L. rubellus*, which supports the superior competitor hypothesis (SCH), although it was initially proposed to explain competition between planktonic algae (Tilman 1977). Interestingly, this finding also suggested that the availability or use efficiency of resources of earthworms was sometimes controlled by interactions within the food web; therefore, the structure and variation of food webs at invaded habitats should be considered carefully when investigating the contributions of species invasiveness and habitat invasibility during invasion processes.

In conclusion, *A. agrestis* showed strong invasiveness through its dietary flexibility through diet shifting and superior feeding behavior and through its indirectly negative effect of habitat (Asoil) invasibility on *L. rubellus* via changes in the soil microorganism community. We consider that dietary flexibility might be a common "weapon" of invasive animals, not only for vertebrates (e.g., birds and rats) but also for belowground invertebrates. The variations in trophic ecology of invading animals induced by dietary flexibility poses a challenge to scientists but also provides a new dimension of further research that may be important for development of successful control methods. Importantly, compared to aboveground biological invasion, the serious problem of belowground invasion might accumulate over time and become more and more detrimental due to the potential for facilitation of invasions by established exotic species. This situation may be exacerbated by global change (Hendrix et al. 2008), which increases the importance of belowground invasion to scientists, the public, and policy makers.

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APPENDIX A

The distribution of some prominent exotic Asian earthworm species belonging to genus *Amyntas* (*Ecological Archives* E091-143-A1).

APPENDIX B

Diagram of the “third habitat” approach in invasion ecology (*Ecological Archives* E091-143-A2).

APPENDIX C

The natural abundance of ^{13}C in earthworm tissues (*Ecological Archives* E091-143-A3).

APPENDIX D

The natural abundance of ^{15}N in earthworm tissues (*Ecological Archives* E091-143-A4).

APPENDIX E

The natural abundances of both ^{13}C and ^{15}N in earthworm tissues (*Ecological Archives* E091-143-A5).