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Resource Utilization by Native and Invasive Earthworms and Their Effects on Soil Carbon and Nitrogen Dynamics in Puerto Rican Soils

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Abstract: Resource utilization by earthworms affects soil C and N dynamics and further colonization of invasive earthworms. By applying ^{13}C-labeled *Tabebuia heterophylla* leaves and ^{15}N-labeled *Andropogon glomeratus* grass, we investigated resource utilization by three earthworm species (invasive endogeic *Pontoscolex corethrurus*, native anecic *Estherella* sp, and native endogeic *Onychochaeta borincana*) and their effects on soil C and N dynamics in Puerto Rican soils in a 22-day laboratory experiment. Changes of ^{13}C/C and ^{15}N/N in soils, earthworms, and microbial populations were analyzed to evaluate resource utilization by earthworms and their influences on C and N dynamics. *Estherella* spp. utilized the ^{13}C-labeled litter; however, its utilization on the ^{13}C-labeled litter reduced when cultivated with *P. corethrurus* and *O. borincana.* Both *P. corethrurus* and *O. borincana* utilized the ^{13}C-labeled litter and ^{15}C-labeled grass roots and root exudates. *Pontoscolex corethrurus* facilitated soil respiration by stimulating ^{13}C-labeled microbial activity; however, this effect was suppressed possibly due to the changes in the microbial activities or community when coexisting with *O. borincana.* Increased soil N mineralization by individual *Estherella* spp. and *O. borincana* was reduced in the mixed-species treatments. The rapid population growth of *P. corethrurus* may increase competition pressure on food resources on the local earthworm community. The relevance of resource availability to the population growth of *P. corethrurus* and its significance as an invasive species is a topic in need of future research.

Keywords: carbon and nitrogen mineralization; invasive earthworms; Luquillo mountains; microbial respiration; Puerto Rico; stable isotope; tropics

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1. Introduction

Invasive earthworms have caused significant effects on local biota and ecosystem processes (such as nutrient dynamics) in the invaded areas, e.g., European Lumbricids in North America [1–3]. Population declines of native earthworms, particularly in remote and non-fragmented forests, have contributed to a result of competitive exclusion by expanding invasive earthworm populations [2,4,5]. Lachnicht et al. [6] observed that invasive *Pontoscolex corethrurus* (Müller, 1856) earthworms, when incubated with native *Estherella* sp., utilized different N resources, possibly avoiding direct competition on food resource. Winsome et al. [7] found that invasive *Aporrectodea trapezoides* (Dugès, 1828) lost its competition advantage when co-existing with native *Argilophilus marmoratus* (Eisen, 1893) in the resource-poor habitat of a Californian grassland. Interactions between native and invasive earthworms varied with resource utilization of earthworm species and resource availability [6,7]. Earthworms are

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categorized into three ecological groups, epigeic, endogeic, and anecic, based on their preferences on space and food resources [8]. Epigeic earthworms mainly consume leaf litter (and microbial populations colonizing on it) and inhabit the litter layer, while endogeic earthworms occupy mineral soils and use soil organic matter as their main food resources. Anecic earthworms utilize mainly leaf litter but with the ability to build burrows deep in the soil [8]. Earthworms with same feeding strategies are expected to evolve stronger competitive interactions because they share the same food resources [2,9,10]. Hence, resource utilization of earthworms could serve as a determinant for the success of earthworm invasions and its effects on the native earthworm community [7].

Earthworm invasions have significantly altered nutrient dynamics (e.g., carbon (C) and nitrogen (N)) in invaded soils [1,11,12]. A mixed-species of European Lumbricid earthworm assemblage has been documented to lessen organic layers and relocate leaf litter and humus fragments (C) into the deeper mineral soils, as well as to cause an increase of N loss in the soil adjacent to plant roots in the temperate forests of North America [1]. The effects of earthworms on soil C and N dynamics may vary with the feeding strategies of earthworms and composition of earthworm assemblages [13]. For example, epigeic earthworms may have stronger effects on nutrient fluxes between leaf litter layers and microbial populations that colonized on it (detritusphere) from their comminution and digestion of the leaf litter substrate [1,11,12]. Endogeic/anecic earthworms, on the other hand, may play a significant role in regulating nutrient dynamics in mineral soil and plant root zones (rhizosphere) by their consumption of soil organic matter and root exudates (and depositions) and their active burrowing activity [14–16]. In an area inhabited by a mixture of earthworms (either different feeding strategies or native co-existing with invasive worms), whether earthworm effects on soil nutrient dynamics can be explained by a summation of individual earthworm effects or disproportionally dominated by one aggressive earthworm species is a topic of interest, yet still in need of more research.

Stable isotope ^{13}C and ^{15}N techniques, including ^{13}C- and ^{15}N-labeled plant materials and a natural abundance of ^{13}C and ^{15}N isotopes, have recently provided invaluable information for studying earthworm feeding strategies and their effects on soil C and N dynamics [6,17–20]. For example, Hendrix et al. [17] suggested an inter-specific competition for N resources based on their observation of overlapped natural abundance ^{15}N in both *Estherella* sp. and *P. corethrurus* in a lower altitude tabonuco forest, Puerto Rico. Neilson et al. [18] found that a natural abundance of ^{13}C and ^{15}N in earthworms can be used to assess the availability and diversity of food resources in the environment. With the application of ^{13}C- and ^{15}N-enriched plant materials, how earthworms utilize different type of food resources and the corresponding effects on soil C and N dynamics can be evaluated by tracking changes of δ^{13}C and δ^{15}N associated with ^{13}C and ^{15}N-labeled plant materials in soils, earthworms, and the microbial populations. In this study, we applied ^{13}C-labeled *Tabebuia heterophylla* (DC.) Britton leaves and ^{15}N-labeled *Andropogon glomeratus* (Walter) Britton, Sterns, & Poggenb. grass to investigate resource utilization of three earthworm species from Puerto Rico (invasive *Pontoscolex corethrurus*, native *Estherella* spp., and native *Onychochaeta borincana* (Borges, 1994)] and their effects on soil C and N dynamics in Puerto Rican soils.

Pontoscolex corethrurus has invaded multiple habitats in Puerto Rico, in contrast to the restricted distribution of the native earthworms in mature forests [21,22]. Competition pressure from invasive *P. corethrurus* to native earthworms has been suggested to be responsible for the absence of native earthworms in most disturbed areas, i.e., pasture and young forests [22–24]. Lachnicht et al. [6] observed that endogeic *P. corethrurus* and anecic *Estherella* sp. showed resource partitioning (in terms of space and food) to avoiding direct competition in a 19-day laboratory experiment. The interactions observed between *P. corethrurus* and *Estherella* sp. have also caused differential influences on soil C and N mineralization [6]. In this study, we investigated feeding strategies of endogeic *P. corethrurus*, anecic *Estherella* sp., and endogeic *Onychochaeta borincana* (single-species earthworm treatments) on ^{13}C-labeled *Tabebuia* leaves and ^{15}N-labeled *Andropogon* grass. Changes in resource utilization of individual earthworm species would be evaluated by comparing earthworm tissue ^{13}C and ^{15}N of single-species earthworm treatments to those of mixed-species earthworm treatments (co-existed with other anecic/endogeic earthworms). Influences of individual earthworm species and inter-specific earthworm interactions on soil C and N dynamics would be assessed by tracking the changes of ^{13}C and ^{15}N in soils, earthworms, and microbial populations in single- and mixed-species earthworm treatments. Anecic *Estherella* spp. was expected to utilize more ^{13}C-labeled *Tabebuia* leaves, as compared with endogeic *O. borincana* and endogeic *P. corethrurus*. Given that *P. corethrurus* is believed to exhibit flexible feeding behaviors and enhance soil mineralization [6,17], we expected that *P. corethrurus* would utilize more leaf litter (detritusphere) than plant roots (rhizosphere) resources, when incubated with endogeic *O. borincana*, to avoid competition with *O. borincana*. Higher population growth would be observed in a *P. corethrurus* population, which would enhance soil C and N mineralization. However, the presence of anecic *Estherella* sp. and endogeic *O. borincana* would weaken enhanced soil mineralization caused by *P. corethrurus*.

2. Materials and Methods

2.1. Experiment Design and Setup

The experiment was conducted at Sabana Field Research Station in Luquillo, Puerto Rico, from November to December 2006. A total of 60 soil mesocosms (Polyvinyl Chloride (PVC) material, 11 cm in diameter and 20 cm in depth) were set up with 15-cm-deep field soils with the bottoms sealed with a 1 mm mesh fiberglass window screen. Experimental treatments included (1) control mesocosms (n = 4, no earthworms; Control) with isotope-labeled *Tabebuia* litter and *Andropogon glomeratus* grass; and (2) seven earthworm treatments (each treatment: n = 4) with isotope-labeled *Tabebuia* litter and *A. glomeratus* grass: single and mixed earthworm treatments (two- and three-species earthworm combination; see below). Four soil mesocosms with no isotope-labeled plant materials and no earthworms (Soil; n = 4), four soil mesocosms with ^{15}N-labeled grass plants (Grass; n = 4), and four soil mesocosms with ^{13}C-labeled leaf litter (Litter; n = 4) were also analyzed as reference data to evaluate the efficiency of ^{13}C- and ^{15}N-labeled methods.

Experimental soil was collected from the forest at the Bisley Experimental Watersheds (BEW) in the Luquillo Mountains (18°18′ N; 65°50′ W). The forest at BEW is mostly dominated by a secondary growth of tabonuco trees, and its soils are clayey and well weathered Ultisols. Detailed description of BEW can be found in Scatena [25]. The collected soils were separated by three depths of 0–5, 5–10, and 10–15 cm to air-dry for 48 h and sieved through a 5 mm mesh size sieve to exclude plant roots, rocks, cocoons, and earthworms. Three depths of air-dry soils were used to set up the 0–5, 5–10, and 10–15 cm depth in the mesocosms. Total soil C and N in 0–5 cm were 3.96 ± 0.05% and 0.37%, respectively. Three *Andropogon glomeratus* seedlings (ca. 8 cm tall), the common grass species in Puerto Rico, were transplanted into each control and earthworm mesocosm a week before the beginning of the experiment. The *Andropogon* grass leaves were brushed with 2 atom % ^{15}N-urea solution every day to establish ^{15}N-labeled plant roots and root-derived substrates (the rhizosphere) during the experiment [26]. Seedlings of *Tabebuia heterophylla*, one of the common, native woody species (Family: Bignoniaceae) in Puerto Rico, were incubated in a growth chamber with pulse injection of 99 atom % $^{13}CO_2$ to acquire ^{13}C-labeled *Tabebuia* leaves through photosynthesis cycles during June–July 2006. After labeling procedures, *Tabebuia* senescent leaves were collected, air-dried for 48 hours, and then shredded into 1 cm^2 pieces ($\delta^{13}C$ varied from 385‰ to 804‰). A total of 3.7 g of dry ^{13}C-labeled *Tabebuia* litter (calculated based on field litterfall data) was applied to the soil surface of each control and earthworm mesocosm to establish ^{13}C-labeled litter and related microbial populations (detritusphere).

2.2. Earthworm Species and Collection

Three earthworm species from Puerto Rico were chosen for this experiment. Two native species, *Estherella* spp. and *Onychochaeta borincana*, were collected from the BEW forests (18.5°18′51.893″ N, 65.5°44′41.694″ W) and a riparian forest in Almirante Norte (18°41′ N, 65°38′ W; alluvial soil) in Puerto Rico [27], respectively; while *Pontoscolex corethrurus* was collected from the pasture at the Sabana

Field Research Station (18°18′ N, 65°50′ W) in the town of Luquillo, Puerto Rico. Anecic *Estherella* spp. has dark pigmentation on the dorsal side and stays in leaf litter and upper soil layers. Endogeic *O. borincana* has pale coloration and stays in the subsoil layer. The invasive earthworm species, *P. corethrurus*, as an endogeic species, is the dominant peregrine earthworm that has colonized most habitats of Puerto Rico [6,17]. Before introducing into the earthworm mesocosms, gut contents of all earthworms were voided for 24 h, and their fresh biomass was recorded as the initial biomass data at the beginning of the experiment. Earthworms were introduced to assigned single or mixed earthworm treatments as followed: single species treatments—*O. borincana* only (O; 4 worms), *Estherella* spp. only (E; 4 worms), and *P. corethrurus* only (P; 4–5 worms); two-species mixed treatments—*Estherella* and *P. corethrurus* (E + P; 3 worms from each species), *Estherella* and *O. borincana* (E + O; 4 *Estherella* worms and 3 *O. borincana* worms), and *O. borincana* and *P. corethrurus* (O + P; 3 worms from each species); and three-species mixed treatments—*Estherella*, *P. corethrurus*, and *O. borincana* (E + P + O; 2 *Estherella*, 2 *O. borincana*, and 3 *P. corethrurus*). Four soil mesocosms were assigned to the control and each earthworm treatment as experimental replicates. The earthworm species were introduced into the experimental mesocosms following the order of *O. borincana*, *Estherella* spp., and *P. corethrurus*. Average fresh biomass of earthworms for each earthworm treatment is listed in Table 1. Each mesocosm was watered with 35 mL of water every day to maintain soil moisture during the 22-day experiment. The mesocosms were rotated randomly every week during the experiment.

Table 1. Average fresh biomass of *Estherella* spp. (E), *Onychochaeta borincana* (O), and *Pontoscolex corethrurus* (P) earthworms introduced into different earthworm mesocosm (g per mesocosm).

	Earthworm treatments				
Variables	Single species (E, O, P)	E + O	E + P	O + P	E + O + P
Estherella spp.					
Fresh weight (before)	5.3 (0.5)	4.2 (0.6)	3.2 (0.4)	n/a	2.2 (0.2)
Fresh weight (after)	4.6 (1.2)	3.9 (1.1)	2.7 (0.6)	n/a	2.3 (0.4)
Onychochaeta borincana					
Fresh weight (before)	4.9 (0.6)	3.6 (0.5)	n/a	2.7 (0.6)	2.2 (0.4)
Fresh weight (after)	2.9 (1.8)	2.4 (0.6)	n/a	2.3 (0.7)	1.6 (0.3)
Pontoscolex corethrurus					
Fresh weight (before)	2.0 (0.3)	n/a	1.5 (0.3)	1.4 (0.1)	1.2 (0.1)
Fresh weight (after)	1.8 (0.4)	n/a	1.5 (0.1)	1.7 (0.2)	1.4 (0.2)

Capital letters (E, O, and P) represent treatments with different earthworm assemblages. Single-species: E = *Estherella* spp.; O = *Onychochaeta borincana*; P = *Pontoscolex corethrurus*. Two-species: E + O = *Estherella* spp. and *O. borincana* assemblage; E + P = *Estherella* spp. and *P. corethrurus* assemblage; O + P = *O. borincana* and *P. corethrurus* assemblage. Three-species: E + O + P = *Estherella* spp., *O. borincana*, and *P. corethrurus* assemblage. Value is shown as mean (S.D.) (n = 4) at the beginning of the experiment (before) and after the 22-day experiment (after). "n/a" indicates the particular earthworm species was not introduced into the corresponding experimental mesocosm.

2.3. Experiment Responding Variables

2.3.1. Soil CO_2 and ^{13}C-CO_2

At Day 21 of the experiment, soil carbon dioxide (CO_2) evolution was collected using the alkali absorption technique [28]. At each sampling, a circular area (5 cm in diameter) in between the center and the edge of the mesocosm was randomly chosen for each mesocosm, and the *Tabebuia* litter within was gently removed to the side. A PVC chamber (10 cm tall and 5 cm in diameter) was inserted 1 cm into the soil surface of each mesocosm with a scintillation vial containing 10 mL of a 1 mol/L NaOH solution placed inside each PVC chamber. The chamber was sealed with plastic wrap and aluminum foil on the top for soil CO_2 absorption. Five NaOH solution vials (control) were kept closed during the 24 h absorption, except to open only at the beginning and the end of absorption to assess sampling contamination. Twenty-four hours later, each alkali solution was removed from the chamber, and 2 mL of 1 mol/L $BaCl_2$ was added to form $BaCO_3$ precipitate. Total CO_2 trapped by alkali solution was determined by titration with 1 mol/L HCl to reach a pH neutral point (phenolphthalein endpoint) [28]. $BaCO_3$ precipitate from each sample was air dried and packed in tin capsules for $^{13}C\text{-}CO_2$ analysis.

2.3.2. The Remaining Mass of the *Tabebuia* Litter

Soil mesocosms were deconstructed at Day 22 to collect final data of the experiment. *Tabebuia* litter was carefully picked up and oven-dried at 60 °C for 48 h. The litter samples were ground, and a subsample of 0.5 g litter was burned at 550 °C for 4 h to obtain ash-free dry matter (AFDM) data. The data were used to calculate the remaining litter mass at the end of the experiment.

2.3.3. Survivorship, Growth, and the ^{13}C and ^{15}N Composition of Earthworms

The number of earthworms that survived at the end of the experiment was used to determine earthworm survivorship. All earthworms were put into separate containers to void their gut contents for 24 h. Final fresh biomass was recorded after gut-voiding. Earthworms were killed by dipping in boiling water for 3 seconds. One-third of the earthworm body (tail part) was cut and rinsed with deionized water with the gut content removed. Earthworm tissue was then freeze-dried and ground. Two milligrams of earthworm tissue was packed into a tin capsule and analyzed by dry combustion on a Carlo Erba NA1500 CN analyzer (Thermo Scientific, Waltham, MA, USA) for earthworm total C, N, and ^{13}C and ^{15}N.

2.3.4. Soil and Soil Microbes

Soil was separated into three soil depths, 0–5, 5–10, and 10–15 cm. Ten grams of soil from each depth was oven-dried at 105 °C for 48 h to calculate soil moisture. Subsamples of soils were ground and packed into tin capsules (ca. 20 mg) for total soil carbon (C) and nitrogen (N) and isotopic analysis (^{13}C and ^{15}N) by dry combustion on a Carlo Erba NA1500 CN analyzer. Two sets of 20 g 0–5 cm soils were extracted with 60 mL of a 0.5 mol/L potassium sulfate (K_2SO_4) solution (3:1 solution to soil mass ratio) for soil microbial biomass analysis by using the fumigation–extraction method [29,30]. Total microbial biomass C and ^{13}C was analyzed from K_2SO_4-extracted samples using an OI analytical TIC/TOC analyzer (Shimaduz, Kyoto, Japan) coupled with a Thermo-Finnigan Delta Plus Isotope Ratio Mass Spectrometer (IRMS) (Thermo Scientific, Waltham, MA, USA). The persulfate digestion method was adapted to obtain microbial N data [31]. The K_2SO_4-extracted samples and persulfate digestion samples were analyzed with an Alpkem nitrogen autoanalyzer (OI analytical, College Station, TX, USA). Dissolved inorganic N (DIN; $NH_4{}^+$-N and $NO_3{}^-$-N) was calculated from a non-fumigated K_2SO_4 extract. Microbial biomass N (MBN) was calculated from the difference between total persulfate nitrogen from fumigated and non-fumigated samples. Total persulfate nitrogen from fumigated samples was used to determine total dissolved nitrogen (TDN).

Delta ^{15}N data for each portion (DIN, MBN, and TDN) were obtained by running the samples through the isotope diffusion method [32]. The $\delta^{13}C$/ $\delta^{15}N$ value is calculated based on the measure isotope ratios between the samples and the standard:

$$\delta\ ^{13}C\ (‰) = \left((R_{\text{sample}} - R_{\text{standard}})\ /\ R_{\text{standard}}\right) \times 10^3 \tag{1}$$

$$\delta\ ^{15}N\ (‰) = \left((R_{\text{sample}} - R_{\text{standard}})\ /\ R_{\text{standard}}\right) \times 10^3 \tag{2}$$

where $\delta^{13}C$ ($\delta^{15}N$) unit is the parts per thousand and R is the mass ratio of $^{13}C/^{12}C$ ($^{15}N/^{14}N$) in the sample and standard [33].

For DIN (K_2SO_4) extracts, KCl was added along with MgO and Devarda's alloy to increase the ionic strength of the solution. For microbial N and TDN (persulfate digests) samples, 10 M NaOH was added to raise pH (>13) of the solution instead. Pairs of glass filter disks (Whatman GF/D) were prepared by baking in a muffle furnace at 500 °C for 4 h. They were acidified with 35 µl of 2M H_2SO_4 and then wrapped with Teflon tape. The Teflon-filter packages were incubated in the solutions for 6 days. After the incubation, the packages were dried over concentrated H_2SO_4 for at least 48 h, then packed in silver capsules for dry combustion on a Carlo Erba NA1500 CN analyzer and IRMS for total N and ^{15}N data.

2.4. Statistic Analysis

The differences of litter remaining mass (data transformed), soil respiration (C-CO_2, ^{13}C-CO_2, and δ^{13}C), total C/N concentration, atom percentage of ^{13}C/^{15}N, and δ^{13}C/ δ^{15}N in soil, microbial biomass, and earthworm tissue, dissolved inorganic nitrogen (DIN), DIN-^{15}N, and total dissolved nitrogen (TDN) between control and earthworm treatments were analyzed by a one-way ANOVA procedure (a generalized linear model (GLM) was used if data were not balanced) in SAS statistical software [34]. A GLM was also used to compare the differences of earthworm biomass and survivorship (data transformed) between earthworm treatments. If significantly different, Tukey's HSD method was applied for the comparisons between treatments. Student's *t*-test and GLM were applied to compare worm ^{13}C and ^{15}N differences between earthworm species in two-species and three-species mixed earthworm treatments, respectively. The significance level was set as $\alpha = 0.05$.

3. Results

3.1. Litter Mass Loss and Soil C and N

The remaining mass of the *Tabebuia* litter (ash-free dry weight), ranging from 21.6% in the control treatment to 45.3% in the E + O earthworm mesocosms, was not significantly different between control and earthworm treatments (data transformed; GLM, $F_{7,31} = 2.1$, $p = 0.08$). At the end of the experiment, soil total C and total N concentrations were not significantly different between the initial soil, the soil samples (with no worms), and earthworm treatments (soil C: $F_{8,27} = 1.0$, $p = 0.43$; soil N: $F_{8,27} = 0.2$, $p = 0.9$; Table 2). Soil carbon in the O + P earthworm mesocosms showed a significantly higher soil ^{13}C percentage (1.0786 ± 0.002%) and soil ^{15}N percentage (0.36915 ± 0.00072%), as compared with those in the initial soil (soil ^{13}C = 1.0753 ± 0.0001% and soil ^{15}N = 0.36815 ± 0.00005%) and the control soil (soil ^{13}C = 1.0752 ± 0.0002% and soil ^{15}N = 0.36810 ± 0.00005%) (both $p < 0.01$; Table 2). Soil C and N from the earthworm treatments showed stronger δ^{13}C (average = −25.9 ± 0.9‰) and δ^{15}N (average = 6.5 ± 1.0‰) signatures as compared with the control soil (δ^{13}C= −27.9 ± 0.2‰ and δ^{15}N = 4.5 ± 0.1‰).

Table 2. Total soil carbon (mg C/ g soil) and nitrogen (μg N/ g soil), atom percentages of ^{13}C and ^{15}N (%) and delta ^{13}C (δ^{13}C; ‰) and delta ^{15}N (δ^{15}N; ‰) from the initial soil samples (no isotope-labeled materials and no worms at Day 0; Initial), control treatment (no isotope-labeled materials and no worms at Day 22; Soil) and earthworm treatments at the end of the 22-day mesocosm experiment with Puerto Rican soils.

Earthworm Treatment										
Variables	**Initial**	**Soil**	**E**	**O**	**P**	**E + O**	**E + P**	**O + P**	**E + O + P**	**Statistics**
Soil Carbon										
Total C	39.6 (0.5)	43.5 (2.5)	42.5 (3.7)	43.8 (2.0)	41.0 (1.6)	42.9 (3.8)	42.1 (2.7)	42.0 (1.5)	42.1 (2.2)	$F_{8,27} = 1.0$; $p = 0.43$
Atom ^{13}C (%)	1.0753 a (0.0001)	1.0752 a (0.0002)	1.0767 bc (0.0003)	1.0771 abc (0.0009)	1.0767 ac (0.0004)	1.0768 abc (0.0002)	1.0776 bc (0.0005)	1.0786 b (0.0020)	1.0769 abc (0.0007)	$F_{8,27} = 7.2$; $p < 0.0001$
δ^{13}C	−27.8 a (0.1)	−27.9 a (0.2)	−25.8 bc (0.3)	−26.1 abc (0.9)	−26.5 ab (0.4)	−26.4 abc (0.2)	−25.6 bc (0.4)	−24.8 c (1.8)	−26.3 abc (0.6)	$F_{8,27} = 7.2$; $p < 0.0001$
Soil Nitrogen										
Total N	371.5 (2.3)	367.9 (19.0)	375.0 (19.5)	370.8 (21.4)	364.3 (11.0)	369.1 (19.6)	362.3 (19.7)	367.4 (11.4)	365.6 (8.3)	$F_{8,27} = 0.2$; $p = 0.9$
Atom ^{15}N (%)	0.36815 a (0.00005)	0.36810 a (0.00005)	0.36885 ab (0.00042)	0.36858 ab (0.00012)	0.36866 ab (0.00036)	0.36889 ab (0.00025)	0.36885 ab (0.00004)	0.36915 b (0.00072)	0.36882 ab (0.00033)	$F_{8,27} = 4.3$; $p = 0.002$
δ^{15}N	4.6 a (0.1)	4.5 a (0.2)	6.5 ab (1.1)	5.8 ab (0.3)	6.0 ab (1.0)	6.6 ab (0.7)	6.5 ab (0.1)	7.3 b (2.0)	6.4 ab (0.9)	$F_{8,27} = 4.3$; $p = 0.002$

Capital letters (E, O, and P) represent treatments with different earthworm assemblages. Single-species: E = Estherella spp.; O = Onychochaeta borincana; P = Pontoscolex corethrurus. Two-species: E + O = Estherella spp. and O. borincana assemblage; E + P = Estherella spp. and P. corethrurus assemblage; O + P = O. borincana and P. corethrurus assemblage. Three-species: E + O + P = Estherella spp., O. borincana and P. corethrurus assemblage. Value is shown as mean (S.D.) ($n = 4$). Statistics shows the statistical results (F ratios and p values) from one-way ANOVA (GLM for unbalanced data). Different letters indicate significant difference among earthworm treatments (Tukey's HSD, $p < 0.05$).

3.2. Earthworm Populations

3.2.1. Earthworm Biomass and Survivorship

Average fresh biomass of the surviving earthworms for each earthworm treatment at the end of the 22-day mesocosm experiment is listed in Table 1. The endogeic earthworm, *Onychochaeta borincana*, showed significantly lower survivorship (71.8 ± 25.0%) than the other two earthworm species (epi-endogeic *Pontoscolex corethrurus*: 96.9 ± 8.3%; anecic *Estherella* spp.: 93.8 ± 13.0%) (data-transformed, GLM, $F_{2,\,47} = 9.56$, $p = 0.0003$). However, the survivorship of individual earthworm species did not significantly differ between the single or the mixed-earthworm treatments (GLM, *Estherella*: $F_{3,\,15} = 1.6$, $p = 0.2$; *O. borincana*: $F_{3,\,15} = 0.4$, $p = 0.8$; *P. corethrurus*: $F_{3,\,15} = 0.7$, $p = 0.6$), nor did the biomass changes (%) of individual earthworm species (*Estherella*: $F_{3,\,15} = 0.6$, $p = 0.7$; *O. borincana*: $F_{3,\,15} = 1.1$, $p = 0.4$; *P. corethrurus*: $F_{3,\,15} = 2.1$, $p = 0.2$). A total of eight *P. corethrurus* were reproduced during the 22-day mesocosm experiment.

3.2.2. Tissue C/^{13}C and N/^{15}N in Native *Estherella* spp.

Percentage of tissue biomass C of anecic *Estherella* spp. showed no significant difference between its single species treatment (cultivated alone; tissue C = 46.3 ± 0.3%) and the mixed-species treatments (cultivated with *O. borincana* and/or *P. corethrurus*; tissue C (%) = 45.7%–47.2%) ($F_{3,\,39} = 2.2$, $p = 0.1$; Table 3). However, *Estherella* spp. when cultivated alone was found to have significantly higher ^{13}C enrichment (as in δ^{13}C and atom percentage of ^{13}C) as compared with the mixed-species treatments (for δ^{13}C: E + P and E + O + P mesocosms; $F_{3,\,39} = 2.0$, $p = 0.04$) (for tissue ^{13}C (%): E + P mesocosms; $F_{3,\,39} = 2.9$, $p = 0.047$) (Table 3). *Estherella* spp. did not show a significant difference in worm tissue N (%), δ^{15}N, and ^{15}N (%) between its single species and the mixed-species mesocosms (all $p > 0.4$; Table 3).

Table 3. Earthworm tissue total carbon (C) and nitrogen (N) percentages (%), atom percentages of ^{13}C and ^{15}N (%), and delta ^{13}C (δ^{13}C; ‰) and delta ^{15}N (δ^{15}N; ‰) in native earthworms *Estherella* spp. (E) and *Onychochaeta borincana* (O) at each earthworm mesocosm from different earthworm treatments at the end of the 22-day experiment with Puerto Rican soils.

	Earthworm Treatments					
Variables	**Single species (E or O)**	**E + O**	**E + P**	**O + P**	**E + O + P**	**Statistics**
Estherella spp.						
Total C (%)	46.3 (0.8)	45.7 (1.3)	46.2 (1.1)	n/a	47.2 (1.8)	$F_{3,\,39} = 2.2$; $p = 0.10$
Atom^{13}C (%)	1.0805 a (0.0039)	1.0785 ab (0.0004)	1.0781 b (0.0006)	n/a	1.0788 ab (0.0007)	$F_{3,\,39} = 2.9$; $p = 0.047$
δ^{13}C	−23.0 a (3.5)	−24.8 ab (0.4)	−25.2 b (0.5)	n/a	−24.6 b (0.7)	$F_{3,\,39} = 2.9$; $p = 0.040$
Total N (%)	12.4 (0.5)	12.3 (0.8)	12.2 (1.0)	n/a	12.7 (0.4)	$F_{3,\,39} = 0.8$; $p = 0.5$
Atom^{15}N (%)	0.3690 (0.0002)	0.3688 (0.0003)	0.3689 (0.0004)	n/a	0.3688 (0.0002)	$F_{3,\,39} = 1.0$; $p = 0.4$
δ^{15}N	6.8 (0.6)	6.2 (0.9)	6.6 (1.0)	n/a	6.5 (0.6)	$F_{3,\,39} = 1.0$; $p = 0.4$
Onychochaeta borincana						
Total C (%)	46.0 (1.2)	46.6 (1.5)	n/a	46.6 (1.3)	46.5 (1.2)	$F_{3,\,25} = 0.4$; $p = 0.8$
Atom^{13}C (%)	1.0823 (0.0046)	1.0812 (0.0016)	n/a	1.0845 (0.0102)	1.0812 (0.0006)	$F_{3,\,25} = 0.5$; p = 0.7
δ^{13}C	−21.4 (4.2)	−22.4 (1.5)	n/a	−19.3 (9.4)	−22.3 (0.5)	$F_{3,\,25} = 0.5$; p = 0.7
Total N (%)	11.8 (0.8)	12.5 (0.7)	n/a	12.3 (0.7)	12.4 (0.5)	$F_{3,\,25} = 1.8$; p = 0.2

Table 3. *Cont.*

	Earthworm Treatments					
Variables	**Single species (E or O)**	**E + O**	**E + P**	**O + P**	**E + O + P**	**Statistics**
Atom^{15}N (%)	0.3693 (0.0013)	0.3694 (0.0006)	n/a	0.3705 (0.0035)	0.3697 (0.0004)	$F_{3,\,25}$ = 0.4; p = 0.7
δ^{15}N	8.69 (3.6)	8.2 (1.6)	n/a	11.0 (9.5)	8.9 (1.0)	$F_{3,\,25}$ = 0.4; p = 0.7

Capital letters (E, O, and P) represent treatments with different earthworm assemblages. Single-species: E = *Estherella* spp.; O = *Onychochaeta borincana*; P = *Pontoscolex corethrurus*. Two-species: E + O = *Estherella* spp. and *O. borincana* assemblage; E + P = *Estherella* spp. and *P. corethrurus* assemblage; O + P = *O. borincana* and *P. corethrurus* assemblage. Three-species: E + O + P = *Estherella* spp., *O. borincana* and *P. corethrurus* assemblage. Value is shown as mean (S.D.). Statistics shows the statistical results (*F* ratios and *p* values) from one-way ANOVA (GLM for unbalanced data). Different letters indicate significant difference among earthworm treatments (Tukey's HSD, $p < 0.05$).

3.2.3. Tissue C/^{13}C and N/^{15}N in Native *O. Borincana*.

For endogeic *O. borincana*, there was no significant difference in worm tissue C and N (%), δ^{13}C and δ^{15}N signatures, and tissue ^{13}C and ^{15}N (%) between its own single species and the mixed-species mesocosms (all $p > 0.2$; see Table 3).

3.2.4. Tissue C/^{13}C and N/^{15}N in Invasive *P. corethrurus*

Invasive *P. corethrurus* earthworms did not show significant differences in worm tissue C and N (%), δ^{13}C and δ^{15}N enrichments, and tissue ^{13}C and ^{15}N (%) between its single species and the mixed-species mesocosms (all $p > 0.2$; Table 4). However, the juvenile *P. corethrurus* reproduced during this 22-day mesocosm experiment did show significant lower tissue C (41.0 ± 4.7%) and N percentages (9.2 ± 2.0%), as compared with the adult *P. corethrurus* worms (tissue C (%): $F_{4,\,52} = 3.9$, $p = 0.007$; tissue N (%): $F_{4,\,52} = 6.0$, $p < 0.001$) (Table 4). Juvenile *P. corethrurus* worms also showed lower enrichment of δ^{13}C (−24.3 ± 1.4‰) and tissue ^{13}C (1.0791 ± 0.0011%), as compared with the adult *P. corethrurus* worms (δ^{13}C: $F_{4,\,52} = 4.7$, $p = 0.002$; tissue ^{13}C (%): $F_{4,\,52} = 4.8$, $p = 0.002$) (Table 4). There was a significantly higher enrichment of ^{15}N (as in δ^{15}N = 7.6 ± 0.9‰ and an atom percentage of ^{15}N = 0.3692 ± 0.0003%), as compared with the adult *P. corethrurus* worms (δ^{15}N: $F_{4,\,52} = 7.2$, $p < 0.001$; atom percentage ^{15}N: $F_{4,\,52} = 7.2$, $p < 0.001$) (Table 4).

Table 4. Earthworm tissue total carbon (C) and nitrogen (N) percentages (%), atom percentages of ^{13}C and ^{15}N (%) and delta ^{13}C (δ^{13}C; ‰) and delta ^{15}N (δ^{15}N; ‰) in native earthworms *Estherella* spp. (E) and *Onychochaeta borincana* (O) at each earthworm mesocosm from different earthworm treatments at the end of the 22-day experiment with Puerto Rican soils.

	Earthworm Treatments					
Variables	**Single Species (P)**	**PJ**	**E + P**	**O + P**	**E + O + P**	**Statistics**
Pontoscolex corethrurus						
Total C (%)	47.0 (1.0) a	41.0 (4.7) b	44.7 (6.1) ab	46.3 (1.6) a	46.9 (0.3) a	$F_{4,52} = 3.9$; $p = 0.007$
Atom^{13}C (%)	1.0809 a (0.0008)	1.0791 b (0.0011)	1.0812 a (0.0009)	1.0813 a (0.0012)	1.0812 a (0.0005)	$F_{4,52} = 4.8$; $p = 0.002$
δ^{13}C	−22.6 a (0.8)	−24.3 b (1.4)	−22.4 a (0.8)	−22.2 a (1.1)	−22.3 a (0.5)	$F_{4,52} = 4.7$; $p = 0.002$
Total N (%)	11.9 (0.5) a	9.2 (2.0) b	11.3 (1.5) a	11.4 (1.1) a	11.9 (0.4) a	$F_{4,52} = 6.0$; $p < 0.001$
Atom^{15}N (%)	0.3686 a (0.0001)	0.3692 b (0.0003)	0.3686 a (0.0002)	0.3687 a (0.0003)	0.3686 a (0.0001)	$F_{4,52} = 7.2$; $p < 0.001$
δ^{15}N	5.9 (0.4) a	7.6 (0.9) b	5.9 (0.5) a	6.1 (0.9) a	5.9 (0.3) a	$F_{4,52} = 7.2$; $p < 0.001$

Capital letters (E, O, and P) represent treatments with different earthworm assemblages. Single-species: E = *Estherella* spp.; O = *Onychochaeta borincana*; P = *Pontoscolex corethrurus*. Two-species: E + O = *Estherella* spp. and *O. borincana* assemblage; E + P = *Estherella* spp. and *P. corethrurus* assemblage; O + P = *O. borincana* and *P. corethrurus* assemblage. Three-species: E + O + P = *Estherella* spp., *O. borincana* and *P. corethrurus* assemblage. Value is shown as mean (S.D.). Statistics shows the statistical results (*F* ratios and *p* values) from one-way ANOVA (GLM for unbalanced data). Different letters indicate significant difference among earthworm treatments (Tukey's HSD, $p < 0.05$).

3.3. Microbial Biomass Carbon and Soil Respiration

There was no significant difference in microbial biomass carbon (MBC) and MBC-^{13}C between the soil (MBC = 741.3 ± 103.0 ug C g^{-1} soil; MBC-^{13}C = 8.0 ± 1.1 ug C g^{-1} soil), control (MBC = 332.1 ± 183.2 ug C g^{-1} soil; MBC-^{13}C = 3.6 ± 1.3 ug C g^{-1} soil), and earthworm treatments (MBC ranged from 340.1 to 532.1 ug C g^{-1} soil; MBC-^{13}C from 3.7 to 7.2 ug C g^{-1} soil) (MBC: $F_{10,29} = 1.3$, $p = 0.26$; MBC-^{13}C: $F_{10,29} = 1.3$, $p = 0.27$; Table 5). Microbial biomass ^{13}C (%) was significantly higher in the control treatments, as compared with those in the Soil Only or Grass mesocosms ($F_{10,29} = 2.8$, $p = 0.015$; Table 5), which suggested the microbes utilized and incorporated the ^{13}C-labeled litter into their biomass. The microbial biomass δ^{13}C enrichment from P (−28.8 ± 3.4‰), O + P (−28.8 ± 3.4‰), and E + O + P (−29.1 ± 2.5‰) treatments were significantly higher than the Soil Only treatment (−36.1 ± 1.4‰) ($F_{10,29} = 3.3$, $p = 0.006$; Table 5).

At the end of the experiment (Day 21), soil respiration C-CO$_2$ and ^{13}C-CO$_2$ (%) from the control (soil with both ^{13}C- and ^{15}N-labeled materials but no worms) and the earthworm treatments were significantly higher than the Soil Only and Grass treatments (both $p < 0.0001$; Table 5). This suggested that the input of ^{13}C-labeled leaf-litter and earthworms facilitated microbial respiration. However, different earthworm treatments showed differential effects on ^{13}C-CO$_2$ (%) evolved in the microbial respiration. The *P. corethrurus* earthworm treatment showed higher ^{13}C evolved from the microbial respiration (as in ^{13}C-CO$_2$ and δ^{13}C; Table 5) as compared with that from the O + P earthworm treatment at the end of the experiment (both $p < 0.0001$; Table 5).

Table 5. Microbial biomass total carbon (MBC, μg C /g soil), carbon-^{13}C (MBC-^{13}C; μg ^{13}C /g soil), atom percentage of ^{13}C (%), and soil delta ^{13}C (δ^{13}C; ‰), soil respiration C-CO_2 (μg C per day), atom percentage of ^{13}C-CO_2 (%), and delta ^{13}C-CO_2 (δ^{13}C; ‰) from the control treatments [Soil Only, soil with ^{15}N-labeled grass (Grass), soil with ^{13}C-labeled leaf litter (Litter), and Control (soil with both grass and leaf litter but no worms)] and earthworm treatments at the end of the 22-day mesocosm experiment with Puerto Rican soils.

					Earthworm treatments							
Variables	**Soil Only**	**Grass**	**Litter**	**Control**	**E**	**O**	**P**	**E + O**	**E + P**	**O + P**	**E + O + P**	**Statistics**
Microbial biomass												
MBC	741.3 (103.0)	570.2 (167.9)	568.3 (166.3)	332.1 (183.2)	659.8 (119.2)	340.1 (115.3)	528.8 (91.4)	479.4 (119.2)	448.1 (110.3)	483.4 (199.5)	431.7 (224.4)	$F_{10,29} = 1.3$; $p = 0.26$
MBC-^{13}C	8.0 (1.1)	6.2 (1.3)	6.2 (1.1)	3.6 (1.3)	7.2 (1.3)	3.7 (1.2)	5.7 (1.0)	5.2 (1.3)	4.9 (1.1)	5.3 (2.2)	4.7 (1.1)	$F_{10, 29} = 1.3$; $p = 0.27$
Atom^{13}C (%)	1.078 a (0.002)	1.079 a (0.0004)	1.083 ab (0.004)	1.09 b (0.009)	1.083 ab (0.003)	1.082 ab (0.003)	1.086 ab (0.004)	1.085 ab (0.002)	1.083 ab (0.002)	1.086 ab (0.004)	1.086 ab (0.003)	$F_{10, 29} = 2.8$; $p = 0.015$
δ^{13}C	−36.1 a (1.4)	−34.9 ab (0.4)	−31.1 ab (3.5)	−30.6 ab (1.4)	−31.1 ab (2.3)	−32.0 ab (2.9)	−28.8 b (3.4)	−29.4 ab (1.8)	−31.5 ab (1.6)	−28.8 b (3.4)	−29.1 b (2.5)	$F_{10, 29} = 3.3$; $p = 0.006$
Variables	Soil Only	Grass	Litter	Control	E	O	P	E + O	E + P	O + P	E + O + P	Statistics
Soil respiration at day 21												
C-CO_2	1.73 a (0.79)	3.87 ab (0.91)	5.24 abc (0.92)	9.51 c (4.95)	8.01 bc (2.05)	6.35 abc (1.58)	9.32 bc (1.50)	9.50 c (0.99)	8.90 bc (1.09)	9.99 c (3.17)	7.88 bc (2.23)	$F_{10, 32} = 5.2$; $p < 0.001$
^{13}C- CO_2 (%)	1.085 a (0.002)	1.088 a (0.004)	1.228 b (0.014)	1.223 b (0.012)	1.206 bc (0.020)	1.209 bc (0.004)	1.220 b (0.023)	1.205 bc (0.007)	1.215 bc (0.018)	1.183 c (0.008)	1.195 bc (0.010)	$F_{10, 32} = 53.6$; $p < 0.0001$
δ^{13}C	−18.8 a (2.1)	−16.5 a (3.6)	111.9 b (13.1)	107.5 b (11.3)	91.7 bc (18.7)	94.3 bc (3.3)	104.9 b (21.2)	91.3 bc (6.9)	100.2 bc (16.9)	71.1 c (7.1)	81.8 bc (8.9)	$F_{10, 32} = 53.5$; $p < 0.0001$

Capital letters (E, O, and P) represent treatments with different earthworm assemblages. Single-species: E = *Estherella* spp.; O = *Onychochaeta borincana*; P = *Pontoscolex corethrurus*. Two-species: E + O = *Estherella* spp. and *O. borincana* assemblage; E + P = *Estherella* spp. and *P. corethrurus* assemblage; O + P = *O. borincana* and *P. corethrurus* assemblage. Three-species: E + O + P = *Estherella* spp., *O. borincana* and *P. corethrurus* assemblage. Value is shown as mean (S.D.) ($n = 4$, except data with: $n = 3$). Statistics shows the statistical results (F ratios and p values) from one-way ANOVA (GLM for unbalanced data). Different letters indicate significant difference among earthworm treatments (Tukey's HSD, $p < 0.05$).

Table 6. Soil microbial total nitrogen (MBN; µg N/g soil), atom percentage of ^{15}N (MBN-^{15}N; %), and delta^{15}N (δ^{15}N; ‰) signature and dissolved inorganic nitrogen (DIN; µg N/g soil), and atom percentage of ^{15}N (DIN-^{15}N; %) in DIN from the control treatments [Soil Only, soil with ^{15}N-labeled grass (Grass), soil with ^{13}C-labeled leaf litter (Litter), and Control (soil with grass and leaf litter but no worms)] and earthworm treatments at the end of the 22-day mesocosm experiment with Puerto Rican soils. See Table 5 for definitions of abbreviations.

					Earthworm treatments							
Variables	**Soil Only**	**Grass**	**Litter**	**Control**	**E**	**O**	**P**	**E + O**	**E + P**	**O + P**	**E + O + P**	**Statistics**
Microbial biomass												
MBN	124.6 (30.1)	96.8 (26.8)	110.2 (25.5)	129.1 (54.5)	190.4 (110.2)	114.5 (31.0)	162.1 (65.0)	92.3 (27.9)	111.0 (39.0)	90.4 (21.9)	136.9 (74.3)	$F_{10,31} = 1.2$; $p = 0.3$
MBN-^{15}N (%)	0.3691 a (0.0005)	0.3747 b (0.0017)	0.3693 a (0.0007)	0.3708 a (0.0019)	0.3709 a (0.0019)	0.3694 a (0.0012)	0.3711 a (0.0017)	0.3721 ab (0.0015)	0.3709 a (0.0004)	0.3711 a (0.0008)	0.3698 a (0.0001)	$F_{10,31} = 6.0$; $p < 0.0001$
δ^{15}N	7.5 a (1.3)	23.0 b (4.8)	8.1 a (1.9)	12.3 a (5.3)	12.7 a (5.3)	8.5 a (3.4)	12.0 a (4.7)	15.7 ab (4.1)	12.5 a (1.2)	13.2 a (2.3)	9.4 a (0.1)	$F_{10,31} = 6.0$; $p < 0.0001$
Dissolved inorganic N												
DIN	62.9 a (12.8)	37.0 b (8.9)	22.6 b (8.4)	18.4 b (4.1)	40.4 ab (8.6)	38.8 ab (20.2)	23.8 b (7.7)	31.1 b (6.7)	25.7 b (6.4)	28.8 b (7.1)	33.0 b (6.2)	$F_{10,31} = 5.7$; $p < 0.0001$
DIN-^{15}N (%)	0.3692 a (0.0008)	0.3958 b (0.0149)	0.3687 a (0.0003)	0.3740 a (0.0003)	0.3770 a (0.0041)	0.3749 a (0.0025)	0.3751 a (0.0087)	0.3784 a (0.0046)	0.3749 a (0.0025)	0.3813 ab (0.0076)	0.3776 a (0.0043)	$F_{10,31} = 6.4$; $p < 0.0001$

Value is shown as mean (S.D.) ($n = 4$, except data with: $n = 3$). Statistics shows the statistical results (F ratios and p values) from one-way ANOVA (GLM for unbalanced data; significant level $\alpha = 0.05$).

3.4. Soil and Microbial Nitrogen Dynamics

There was no significant difference in microbial biomass nitrogen (MBN) between the control and earthworm treatments. However, the higher MBN-^{15}N and microbial δ^{15}N signature from the Grass treatment, compared with those in the control and earthworm treatments (except E + O treatment), indicated that the microbes did utilize and incorporate the ^{15}N-labeled grass resources (plant roots or root exudates) into the microbial biomass (both $p < 0.0001$; Table 6).

At the end of experiment (Day 21), lower soil dissolved inorganic nitrogen (DIN) was found in the control and the earthworm treatments, except native *Estherella* spp. (E) and *O. borincana* (O) treatments, as compared with the Soil Only mesocosms ($F_{10, 31} = 5.7$, $p < 0.0001$; Table 6). Earthworms not only reduced the DIN in the experimental soil but also reduced the ^{15}N percentage in DIN (except O + P treatment) ($F_{10, 31} = 6.4$, $p < 0.0001$; Table 6). There was no significant difference total dissolved nitrogen (TDN) between the control (10.8 ± 1.5 μg N/g soil) and the earthworm treatments (ranged from 12.0–17.1 μg N/g soil) ($F_{10, 31} = 1.3$, $p = 0.3$).

4. Discussion

In this study, newly added ^{13}C-labeled leaf litter and ^{15}N-labeled grass were sufficiently incorporated into 10 cm of top soil, soil microbial biomass, and earthworm tissue. Natural abundance of δ^{13}C in earthworms was suggested to be 1−3‰ heavier than its dietary sources (such as leaf litter, root exudates, and microbial populations in the soil) [18,35]. In this study, earthworm δ^{13}C showed on average 1.4‰ heavier in *Estherella* spp., 3.5‰ heavier in *P. corethrurus*, and 5‰ heavier in *O. borincana*, with respect to the soil δ^{13}C, while earthworm tissue showed on average 5.9‰ heavier δ^{13}C in *Estherella* spp., 7.2‰ heavier in *P. corethrurus*, and 8.5‰ heavier in *O. borincana* than the microbial biomass δ^{13}C in which they inhabited (Tables 2–5).

We did not observe any competition exclusion among three earthworm species based on the survivorship and biomass gain among the single-species and the mixed-species treatments for each individual species. However, anecic *Estherella* spp., when cultivated alone, did show higher tissue—^{13}C (%) and δ^{13}C—compared with when it was cultivated with other earthworm species. This suggested that *Estherella* spp. might change its feeding strategy by reducing its utilization of ^{13}C-labeled litter materials and/or the microbial community that was related to the ^{13}C-labeled litter when cultivated with *P. corethrurus* or both *P. corethrurus* and *O. borincana.* Lachnicht et al. [6] observed that *P. corethrurus* and *Estherella* spp., while cultivated together, excluded each other in the bottom and upper layers of soil, respectively, in a 19-day laboratory experiment in Puerto Rican soils. The authors also found that *P. corethrurus* acquired more ^{15}N-labeled leaf litter when co-occurring with *Estherella* spp. [6]. We did not find that *P. corethrurus* changed its feeding preference in this 22-day experiment based on worm tissue ^{13}C and δ^{13}C as well as tissue ^{15}N and δ^{15}N between the single-species and mixed-species earthworm treatments, nor did *O. borincana*. In this study, cultivating live *A. glomeratus* grass plants could provide a steady, continuous supply of root exudates and rhizodeposits for soil microbes and earthworms, as compared to the one-time application of ^{13}C-labeled glucose and ^{15}N-labeled leaf litter adopted by Lachnicht et al. [6]. Such a continuous supply of food resources might relieve potential inter-specific competitive pressure derived from limited food resources in short-term experiments, especially for endogeic earthworms like *O. borincana* and *P. corethrurus* that strongly rely on rhizosphere resources.

Both endogeic *O. borincana* and *P. corethrurus* showed 5‰ or higher δ^{13}C signature than their food resources (soil organic matter and soil microbial biomass). Higher δ^{13}C signature in both endogeic earthworms could be explained by their utilization on soil microbial populations (i.e., bacteria and fungi) as food resources. Fungal species (such as mycorrhizal and saprotrophic fungi) have been reported to have a higher ^{13}C enrichment than plant foliage, fine roots, and soils because of fungal biochemical synthesis and transport between plant parts [36]. Microbial activity releases the lighter ^{12}C in respiration and gradually results in an increase of ^{13}C concentration in humified residues and its own population [37,38]. As a result, endogeic earthworms (active in rhizosphere and the mineral soils), *P. corethrurus* and *O. borincana* in this study, showed higher δ^{13}C signature and tissue ^{13}C (%)

than anecic *Estherella* spp. due to their preferential consumption of ^{13}C-enriched decayed/humified debris in the mineral soil layer, to a significant portion of ^{13}C-enriched microbial (higher microbial δ^{13}C observed in P, O + P and E + O + P earthworm treatments; Table 5) and fungal populations, or to both [6,36,37]. The possibility that both endogeic *O. borincana* and *P. corethrurus* consumed the microbial populations in the mineral soil, the rhizosphere, or both is also confirmed by their heavier δ^{15}N signatures (0.6‰ and 2.7‰ δ^{15}N heavier, respectively) compared with the soil δ^{15}N (Tables 2–4).

We found that soil microbial-δ^{15}N was on average 6.1‰ heavier than *Estherella* spp., 5.8‰ heavier than *P. corethrurus*, and 2.6‰ heavier than *O. borincana* (Tables 3, 4 and 6). The stronger ^{15}N enrichment in endogeic *O. borincana* could be derived from its utilization of ^{15}N-labeled rhizosphere (plant roots, root exudates, and rhizosphere-related microbes). Even though no study has yet investigated the feeding behavior of *O. borincana*, some endogeic earthworms (e.g., *P. corethrurus*) are often found aggregated in the root zones utilizing living root fragments and dead root cells, or as response to enhanced microbial activities in the rhizosphere [35,39]. In this study, the presence of *O. borincana* seemed to relate to higher microbial biomass ^{15}N and δ^{15}N (in the E + O earthworm mesocosms) and higher DIN and higher ^{15}N-DIN (%) in the O + P treatment (although not statistically significant), as compared with other earthworm treatments (Table 6). The potential effect of endogeic *O. borincana* on rhizospheric microbial populations and activities is a topic of interest, yet in need for further research.

Pontoscolex corethrurus showed a prolific reproduction (a total of eight juvenile *P. corethrurus*) within the 22-day soil mesocosm experiment. The stronger δ^{15}N signal observed in juvenile *P. corethrurus*, as compared with the adults, might be explained by (1) the possibility that adult *P. corethrurus* allocated its assimilated ^{15}N into cocoon reproduction, which later integrated into the tissue of juvenile *P. corethrurus*, and (2) a higher soil consumption and biomass increase in relation to overall biomass by juvenile worms than the adult worms [6]. *Pontoscolex corethrurus* has been described as one of the cosmopolitan earthworm species that has aggressively invaded many regions in the tropics, including Puerto Rico, Central Amazonian, and Peruvian soils [40–43]. Exceptional reproductive strategies of *P. corethrurus*, such as a high rate of cocoon production and hatching success, a short development time, and the ability of parthenogenesis, critically influence the local native earthworm community in the invaded soils [2]. The rapid population growth of *P. corethrurus* may increase competition pressure on food resources to the local native earthworm community [22]. The relevance of resource availability to the population growth of *P. corethrurus* and its significance in a *P. corethrurus* invasion is certainly a topic in need of future research.

Earthworms showed differential effects on soil mineralization processes in this study. All earthworm treatments along with the control (no worms) had higher soil respiration C-CO_2 at Day 21, especially in the P, E + O, and O + P treatments, as compared with other control treatments (Soil Only, Grass, and Litter mesocosms). There were higher ^{13}C-CO_2 (%) and δ^{13}C from the P mesocosms (Tukey's HSD, $p < 0.001$) and the mixed E + O mesocosms (marginally significant; $p = 0.06$) compared with those from the O + P treatments. The effects on soil microbial activities by earthworms could be explained by earthworms' direct grazing behavior on soil microbial community or indirect burrowing and casting activities [11,14]. Whether the higher soil respiration C-CO_2 from the control (no worms) mesocosms was due to the release from earthworms' grazing activity is uncertain. However, the significantly higher soil respiration ^{13}C-CO_2 (%) and δ^{13}C from *P. corethrurus* (includes P and E + P) were an indicator of facilitating effects of earthworms on the enriched soil microbial biomass δ^{13}C from the same mesocosms. *Pontoscolex corethrurus* might cause an increase in soil respiration via its simulation on the activity of the ^{13}C-labeled microbial population. However, the lower soil respiration ^{13}C-CO_2 (%) and δ^{13}C in the mixed *P. corethrurus* and *O. borincana* treatments (i.e., O + P) suggested that the presence of *O. borincana* and its interaction with *P. corethrurus* might have a negative effect on the ^{13}C-labeled microbial community and facilitate the ^{15}N-labeled microbial communities in the rhizosphere. Such a possibility is supported by the observation of the slightly increased ^{15}N (%) in the soil DIN from the increased microbial activity related to the ^{15}N-labeled rhizosphere in the O + P treatment (Table 6).

The individual stimulation on soil N mineralization by *Estherella* spp. and *O. borincana* was slightly reduced when they were incubated with other earthworm species (mixed-species earthworm treatments; Table 6). No significant change was observed in microbial biomass (C and N) between treatments, thus the changes shown in soil respiration δ^{13}C and DIN could be explained by the changed activities from the microbial population or possibly alternation of microbial community induced by the inter-specific earthworm interactions from the mixed earthworm treatments. Studies have suggested that the preference of earthworms on utilizing different food resources can reshape microbial communities in the detritusphere and the rhizosphere [44,45]. Native *Estherella* spp. and *O. borincana* may individually sustain a microbial community that specialized on N mineralization in the rhizopshere, yet the microbes switched to those which utilized a labile, newly added ^{13}C-labeled resource when sharing resources with the other species. Earthworm effect on either microbial activities or microbial community by individual species is confounded when inter-specific interactions are considered, and the individual effect on microbial activities and communities was not additive. Furthermore, changes in microbial activities and alterations to the microbial community by earthworms could gradually alter soil nutrient dynamics and availability of labile C and N over time [46], which later has an effect on habitat suitability for other species. For example, invasive *Amynthas agrestis* (Goto and Hatai, 1899) was documented to change soil microbial communities, which positively affected the habitat invasibility for another invasive species, *Lumbricus rubellus* (Hoffmeister, 1843) [47]. Many studies have focused on the earthworm effects on soil microbial biomass and soil mineralization [11,47–53]; however, research investigating the effects of earthworms with different feeding strategies (i.e., epigeic, anecic, and endogeic) on soil microbial activities and communities in terms of functional groups is still limited.

5. Conclusions

In this study, anecic *Estherella* spp. was observed to reduce its utilization on ^{13}C-labeled litter or ^{13}C-related microbial community when cultivated with *P. corethrurus* or both *P. corethrurus* and *O. borincana.* Resource utilization by different earthworms changed the activities and composition of soil microbial community and further affected soil respiration and nitrogen mineralization processes. However, the individual species effect on soil C and N dynamics was altered with mixed earthworm assemblages. *Pontoscolex corethrurus* was found to stimulate soil respiration by facilitating the activity of the ^{13}C-labeled microbial activity; however, the positive effect was suppressed when it coexisted with *O. borincana.* The stimulated N mineralization process by native *Estherella* spp. and *O. borincana* individually were reduced when each of them cultivated with other earthworm species. We concluded that the earthworm effect on soil microbial community and activity varies by species, and the individual species effect is not additive when considering multiple earthworm species assemblages. Regulation on soil nutrient dynamics by native *Estherella* sp. and *O. borincana* may potentially affect habitat suitability (e.g., resource availability) to invasive *P. corethrurus* during colonization. However, the rapid population growth of *P. corethrurus* may increase competition pressure on food resources to the local earthworm community. The relevance of resource availability to the population growth of *P. corethrurus* and its significance as an invasive species is a topic in need of future research.

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