Endogeic earthworms mediate aggregate formation and carbon storage in an Ultisol[1,2]

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ABSTRACT

The role of soil fauna in the incorporation of carbon into soil aggregates of tropical soils has been understudied. We conducted a field experiment within a secondary forest and a microcosm experiment at the University of Puerto Rico in Mayagüez to test soil aggregate formation and carbon incorporation by earthworm activity. Using ^{13}C natural abundance in vegetation and the difference in $\delta^{13}C$ between C3 and C4 plants, carbon sources in the soil were tracked. Maize leaves (C4 carbon isotopic signal) were used to track the carbon incorporation into soil aggregates (C3 carbon isotopic signal). Earthworms and soil samples (Typic Haplohumults) were collected at 0 to 10 cm soil depth. The treatments for microcosms were: (I) soil, (II) soil + C4 leaves, (III) soil + C4 leaves + two earthworms (low density), and (IV) soil + C4 leaves + three earthworms (high density). Aggregate size classes were separated by the wet sieving method. At the study site, we found two earthworm species belonging to epigeic and endogeic ecological categories. Over six months, our field data suggested that endogeic *P. corethrurus* can reorganize small macroaggregates to form large macroaggregates. The microcosm experiment corroborated that *P. corethrurus* consumes soil and transfers soil-derived carbon from microaggregates to macroaggregates. The treatment with the highest earthworm density did not show higher carbon incorporation (0.23 g $C/kg_{\text{sand-free aggregates}}$) into the soil compared to low earthworm density (0.25 g $C/kg_{\text{sand-free aggregates}}$). Our results suggest that *P. corethrurus* prefers consuming soil-derived carbon and can translocate it from microaggregates to macroaggregates by restructuring soil aggregates.

Keywords: earthworm, aggregate formation, carbon incorporation, Ultisol

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RESUMEN

Formación de agregados y almacenamiento de carbono mediada por lombrices de tierra en un Ultisol

El rol de la fauna del suelo en la incorporación de carbono en los agregados del suelo ha sido poco estudiado en los suelos tropicales. Realizamos un experimento de campo en un bosque secundario y un experimento de microcosmos en la Universidad de Puerto Rico en Mayagüez para probar la formación de agregados del suelo y la incorporación de carbono mediante la actividad de lombrices de tierra. Utilizamos la abundancia natural de ^{13}C en la vegetación y la diferencia de $\delta^{13}C$ entre plantas C3 y C4 para rastrear las fuentes de carbono. Se utilizaron hojas de maíz (señal isotópica de carbono C4) para rastrear la incorporación de carbono en los agregados del suelo (señal isotópica C3). Se recolectaron muestras de lombrices de tierra y suelo (Typic Haplohumults) a una profundidad de 0 a 10 cm. Los tratamientos para los microcosmos fueron: (I) suelo, (II) suelo+hojas C4, (III) suelo+hojas C4+dos lombrices de tierra (baja densidad), y (IV) suelo+hojas C4+tres lombrices de tierra (alta densidad). Las clases de tamaño de los agregados se separaron por el método de tamizado húmedo. En el sitio de estudio se encontraron dos especies de lombrices de tierra que pertenecían a las categorías ecológicas epigeicas y endogeicas. En seis meses, nuestros datos de campo sugieren que la lombriz endogeica *P. corethrurus* puede reorganizar pequeños macroagregados para formar grandes macroagregados. El experimento de microcosmo corroboró que *P. corethrurus* consume suelo y transfiere el carbono derivado del suelo de los microagregados a los macroagregados. El tratamiento con mayor densidad de lombrices de tierra no mostró una mayor incorporación de carbono (0.23 g $C/kg_{\text{agregados libres de arena}}$) en el suelo en comparación con la baja densidad de lombrices de tierra (0.25 g $C/kg_{\text{agregados libres de arena}}$). Nuestros resultados sugieren que *P. corethrurus* muestra una preferencia por consumir carbono derivado del suelo y puede translocarlo desde los microagregados a los macroagregados mediante la reestructuración de los agregados del suelo.

Palabras clave: lombrices de tierra, formación de agregados, incorporación de carbono, Ultisol

INTRODUCTION

Earthworms are among the most abundant and important ecosystem engineers that impact the physical, chemical, and biological characteristics of humid tropical ecosystems (Blanchart et al., 2004; González et al., 2007; Lavelle et al., 1992). Earthworms are known to influence the fluxes of organic matter (OM) decomposition, soil organic carbon (SOC), and direct incorporation of SOC into the soil, changing soil structure and fertility. Moreover, earthworm-formed aggregates provide physical protection to organic carbon (OC) against rapid mineralization by microorganisms, thus contributing to long-term carbon storage in soils (Bossuyt et al., 2005; Pulleman et al., 2005; Sánchez-de León et al., 2014; Six et al., 2002).

Earthworm activities play an important role in aggregate formation by removing part of the plant litter from the soil surface and incorporating it as SOM into soil aggregates (Bossuyt et al., 2005). Earthworms ingest particulate organic matter (POM) and mineral soil, mix the organic material with mineral particles, and produce casts (Bossuyt et al., 2004b, 2005; Sánchez-de León et al., 2014). Through this process, earthworms incorporate organic residues directly and store SOC by forming soil aggregates within their casts (Bossuyt et al., 2005; Fonte et al., 2009; Pulleman et al., 2005; Shipitalo and Protz, 1989). Although several studies show that earthworm species can create new soil macroaggregates (Blanchart et al., 1997; Bossuyt et al., 2004, 2005; Sánchez-de León et al., 2014), it has also been shown that certain earthworm species, like the small Eudrilidae, can fragment existing soil aggregates (Blanchart et al., 1997, 2004; Kamau et al., 2020). Thus, the effect of earthworms in soil aggregation seems to be species-dependent.

Aggregates formed by earthworm feeding and casting activities can result in the physical storage of soil carbon (Fonte et al., 2007; Sánchez-de León et al., 2014). Bossuyt et al. (2004a) found that soil aggregates formed by earthworm activity contained 3.6 times larger macroaggregates (larger than 2,000 µm) and more total carbon (4.26 g C/kg soil) than treatment without earthworms. Similar results were reported by Sánchez-de León et al. (2014) in an experiment where the macroaggregate mass in treatments with earthworms was two to three times greater than in treatments without earthworms. In addition, Bossuyt et al. (2004a; 2006) found that earthworm activity can increase the formation of water-stable microaggregates inside large macroaggregates four-fold compared to treatments without earthworms.

Few studies have been done on the function that earthworm aggregate production plays in the storage of carbon in Puerto Rico's tropical soils, especially the Oxisols and Ultisols. Ultisols and Oxisols are highly meteorized soils characterized by low-activity clays (1:1 clay minerals) and low fertility (Soil Survey Staff, 2014). Most studies related to earthworms in Puerto Rico have been focused on feeding behavior, density, distribution, diversity, and their influence on soil physical properties (Amador et al., 2013; Dechaine et al., 2005; González et al., 2007; González and Zou, 1999; Hubers et al., 2003; Liu and Zou, 2002; Sánchez-de León et al., 2003). However, studies using stable isotopes and microcosms to understand aggregate formation and the incorporation of carbon into aggregates by earthworms are few (Amador et al., 2013; Hendrix et al., 1999; Lachnicht et al., 2002). The purpose of this experiment was to study the effect of earthworms found in an Ultisol soil in Mayagüez, Puerto Rico, on the formation of soil aggregates and their relationship to carbon incorporation. Our first objective was to measure earthworm abundance and aggregate distribution under natural field conditions in an Ultisol with secondary forest vegetation (C3-vegetation). In addition, we took advantage of the natural ^{13}C isotopic differences between C3 and C4 plants (O'Leary, 1981) to measure the incorporation of maize leaf-derived carbon into aggregates under natural field conditions. Although we did not expect that aggregate distribution would be affected by C4 leaf litter addition, we hypothesized that carbon added through the C4 leaf litter (maize) would be readily incorporated into soil aggregates, and the ^{13}C isotopic difference would allow the tracking of this newly incorporated carbon. Our second objective was to measure earthworm aggregate formation under controlled laboratory conditions and measure the carbon stored within the newly created aggregates in this Ultisol. For the microcosm experiment, we hypothesized that treatments with earthworms would have more soil water-stable aggregates than treatments without earthworms. We also hypothesized that the carbon content within the aggregates will be higher in the earthworm treatments than in treatments without earthworms.

MATERIALS AND METHODS

Study site

The field study was conducted within a secondary forest at the Alzamora Farm at the University of Puerto Rico in Mayagüez (near 18° 13'12.5"N, 67°08'49.0"W). The soil was Consumo clay (fine, mixed, semiactive, isohyperthermic Typic Haplohumults) (Soil Survey Staff, 2014). The area has a tropical climate with a mean annual precipitation of 1020 to 1780 mm and a mean temperature of 26° C (Beinroth et al., 2002; Harmsen et al., 2002; Ravalo et al., 1986). The forest vegetation in the area in the past 45 years has been dominated by C3 plant species such as: *Albizia procera, Swietenia mahagoni*, *Castilla elastica*, *Tilipariti elatum*, *Mangifera indica*, *Guarea guidonia*, *Ceiba petandra*, *Inga fagifolia*, *Delonix regia*, *Peltophorum inerme, and Leucaena leucocephala* (Túa-Ayala, 2023). The field experiment was conducted from June 2017 through December 2017.

In September 2017, the island of Puerto Rico experienced the effects of hurricanes Irma and María. The experimental plots were checked on 11 September 2017, after Hurricane Irma, and on 29 September 2017, after Hurricane María. In both cases, the experimental plots did not show the addition or loss of soil by erosion. Leaf litter and other vegetative debris (e.g., mostly tree trunks) thrust on top of plots were removed after the hurricanes to continue the field experiment.

Plant material and field application

The two treatments were: the control forest floor leaves (C3-leaves) and maize leaves (C4-leaves). In previous studies, C4 plants, such as maize (*Zea mays*), have been used as an isotopic carbon marker to study carbon dynamics in soils (Bossuyt et al., 2004a; Briones et al., 2002; O'Leary, 1981; Phillips and Gregg, 2001). Therefore, maize leaves (C4-leaves) were used to supply the soil with a natural ^{13}C isotope marker in the experimental plots as part of the C4-leaves treatment. The leaves from maize plants were collected from an organic plot also located at the Alzamora Farm. In another treatment, leaves from the forest floor (C3-leaves) at the study site were collected. All leaves were cut, dried and stored in paper bags for 72 hours at 65° C, followed by grinding them to pass through a 2 mm-diameter mesh.

The experimental plots were selected using aerial photography of the study site; 16 sections of 20 × 20 m were digitally delineated with ArcMap v.10.5 (Environmental System Research Institute, Redlands, CA, USA)[5], and four sections were randomly selected. In each of the selected areas of the forest, one experimental plot of 1 × 1 m size was delimited using barrier landscape fabric that did not affect the trees. Each plot was a replicate of the treatment. Geographical information of the plots is included in Table 1. The experimental plots were split in half (sub-plot of 0.5 × 1 m), with each half corresponding to one randomly assigned treatment. Thus, there were four experimental plots and each plot had one replicate of each treatment (n=4). On 5 June 2017, we applied 400 g/m^2 of maize leaves (2.04 g C/kg of soil) to each of the C4-leaf treatment replicates and 400 g/m^2 of forest leaves (2.71 g C/kg of soil) to each of the C3-leaf treatment replicates.

TABLE 1.—*Latitude, longitude, aspect, slope and bulk density (0 to 10 cm) of experimental plots of Consumo soil series at Finca Alzamora secondary forest.*

Plot	Latitude	Longitude	Aspect (°)	Slope (%)	Bulk density (g/cm^3)
1	18° 13' 4.89" N	67° 08' 38.46" W	227	1	1.02
2	18° 13' 14.89" N	67° 08' 37.55" W	295	1	1.09
3	18° 13' 14.31" N	67° 08' 38.42" W	320	2	1.21
4	18° 13' 15.40" N	67' 08' 36.34" W	298	18	1.13

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[5] Company or trade names in this publication are used only to provide specific information. Mention of a company or trade name does not constitute an endorsement by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

Earthworm sampling

In November 2017, six months after treatment addition in the sub-plots, earthworms and cocoons were collected from a soil area of 25 × 25 cm to a depth of 10 cm by hand sorting and gently breaking the soil. Earthworm samples were placed in plastic bags with a moistened paper towel and transported in a cooler to the Soil Chemistry Laboratory at the University of Puerto Rico, Mayagüez, on the same day of collection.

Earthworms from each sub-plot were measured by their abundance (individuals per square meter), and fresh weight (grams of fresh weight per square meter). Earthworms were placed in petri dishes with fiber glass filter paper for 72 hours to let earthworms empty their gut content (Schmidt, 1999; Whalen and Janzen, 2002). A sub-sample of adult earthworms was selected to be preserved in a 1:10 dilution of 37% formaldehyde for taxonomic identification. Taxonomic identification was performed by earthworm taxonomist Dr. Sonia Borges using Borges (1996a) taxonomic key.

Water stable aggregates

A soil core sampler (AMS Inc., USA) with 4.5 cm diameter was used to collect the soil sample at a depth of 0 to 10 cm from each sub-plot. Soil samples were collected between 27 November 2017 to 15 December 2017. Each soil core was broken along its natural breaking points (Fonte et al., 2009; Jastrow et al., 2005). The soil was passed gently through an 8-mm sieve and roots were removed (Jastrow et al., 1996, 2005; Six et al., 1998). Afterwards, soil samples were air dried at room temperature. The soil samples were fractionated in three 50 g soil sub-samples to be separated into four aggregate size fractions by the wet sieving method (Bossuyt et al., 2005; Elliott, 1986; Sánchez-de León et al., 2014). For this experiment, 12 sub-samples were analyzed per treatment, for a total of 24 sifted sub-samples (4 plots × 2 treatments × 3 soil sub-samples).

Three sieves were used to obtain four fraction sizes: 1) larger than 2,000 µm (large macroaggregates); 2) 250 to 2,000 µm (small macroaggregates); 3) 53 to 250 µm (microaggregates); and 4) less than 53 µm (silt+clay fraction). Aggregates were manually sieved by vertical oscillation, moving the sieve up and down 50 times by 3 cm, during a two-minute period. Particles belonging to less than 53 µm (silt+clay fractions) were released in the rinse water and collected via centrifugation for 10 minutes at 4,500 rpm and 20° C.

All fractions were placed in an oven to dry at 65° C for 24 hours and then weighed. The soil aggregate fraction was corrected and expressed as a sand-free aggregate fraction, as shown in Equation 1 (Elliott, 1986; Sánchez-de León et al., 2014; Six et al., 2000). To express carbon concentration of aggregates on a sand-free basis, we used Equation 2 (Six et al., 1998).

$$Sand - free\ aggregate_{fraction} = (aggregate + sand)_{fraction} \times [1 - (sand\ proportion)_{fraction}] \quad (1)$$

$$Sandfree\ (C)_{fraction} = \frac{(C\ )_{fraction}}{1 - (sand\ proportion)_{fraction}} \quad (2)$$

Where C represents the carbon concentration within each aggregate fraction, the sand content of macroaggregate and microaggregate size fractions was determined using the modified particle size analysis method described by Menrha and Jackson (1960).

Microcosm experiment

The microcosm experiment was conducted from 30 June to 19 July 2018. To conduct the microcosm experiment, we used soil (0 to 10 cm) and earthworms, both sourced from the field study site. Soil samples were air-dried, ground and passed through a 250 µm-mesh sieve to guarantee that no macroaggregates were present at the beginning of the experiment. We evaluated four treatments: (I) 150 g soil; (II) 150 g soil + 1.2 g C4-leaves; (III) 150 g soil + 1.2 g C4-leaves + two earthworms; and (IV) 150 g soil + 1.2 g C4-leaves + three earthworms. Each treatment had four repetitions for a total of 16 experimental units. The experimental unit was a mason jar of one liter. Ground maize leaves were used as the carbon source. Maize leaves exhibited a $\delta^{13}C$ signal of -13.8‰ (±0.08), while the soil showed a $\delta^{13}C$ signal of -27.0‰ (±0.15). All treatments were wetted to 30% (w/w) moisture before and during incubation to maintain gravimetric soil moisture (Sánchez-de León et al., 2014).

Treatments III and IV represented different earthworm abundance according to the soil volume and depth within the mason jars. Treatment III had an equivalent earthworm abundance of ~ 394 individuals per square meter (low abundance), and treatment IV had an equivalent earthworm abundance of ~ 591 individuals per square meter (high abundance) with mean soil bulk density of ~0.92 (± 0.01) g/cm^3 and mean soil depth of ~3.22 (± 0.01) cm in each mason jar.

We chose endogeic earthworms from areas near our field plots based on the morphological characteristics of the ecological category (Borges, 1996b; Coleman et al., 2004; Curry and Schmidt, 2007; Fragoso and Lavelle, 1992). After visual inspection, we concluded that earthworms belonged to the *Pontoscolex* genus, due to their morphological features and dominance at this site. Earthworms were added to treatments eight days after incubation (Bossuyt et al., 2004b; Sánchez-de León et al., 2014). The microcosm incubation process was conducted under dark conditions, with a mean temperature of 22° C and a mean humidity of 72% for 20 days in closed mason jars.

Isotope analysis

For field and microcosm experiments, samples of soil aggregates, maize leaves and leaf litter were analyzed for $\delta^{13}C$, with an isotope ratio mass spectrometer (Finnegan Delta Plus XL, Bremen, Germany) coupled with an elemental analyzer (Costech Elemental Analyzer, California, USA). The isotope analysis was performed at the University of Illinois, Chicago, at the Geochemistry Stable Isotope Laboratory. The result was expressed in delta notation using part per thousand (δ ‰) as follows in Equation 3:

$$\delta^{13}C = \left[\left(\frac{^{13}R_{sample} - {}^{13}R_{standard}}{^{13}R_{standard}} \right) \right] \times 1000 \tag{3}$$

Where $^{13}R_{\text{sample}}$ represents the ratio $^{13}C/^{12}C$ in the sample and $^{13}R_{\text{standard}}$ is the ratio of the two isotopes in the standard PDB (belemnite from Pee Dee Formation) (Bossuyt et al., 2004b; O'Leary, 1981). The equipment also provided data for the percentage of soil carbon ($\%C = g_C / 100\ g_{soil}$ and $\%N = g_N/100\ g_{soil}$). To determine the amount of soil carbon and labeled maize carbon that was incorporated into each soil aggregate size class, we used the mixing model ISOERROR 1.04 spreadsheet (Phillips and Gregg, 2001).

Data analysis

The field experiment was conducted and analyzed as a two-way ANOVA with leaves (C4-leaves vs. C3-leaves) and aggregate size classes (large macroaggregates, small macroaggregates, microaggregates and silt+clay) as class variables. The relationship between earthworm abundance, earthworm biomass and the aggregate size classes was analyzed using a linear regression model PROC REG analysis. For the microcosm experiment, two-way ANOVA was performed to evaluate the interaction between treatments (I, II, III, and IV) and aggregate size classes. Normality and homogeneity of variances were tested using Shapiro-Wilks and Levene's tests. Transformation attempts of non-normal data were unsuccessful for the mass of size fraction and carbon concentration. Therefore, we used the PROC GLIMMIX of SAS University Edition version 9.4 (SAS Institute Inc., 2015) for analysis of variance. Separation of means was tested using Tukey's honestly significant difference at a level of $P < 0.05$.

RESULTS

Field experiment

At the study site, we found two exotic earthworm species: *Pontoscolex corethrurus* (Müller, 1856) from South America with endogeic behavior and *Amynthas hawayanus* (Rosa, 1981) from Asia with epigeic behavior. The most common species was *Pontoscolex* sp., with 15 immature earthworms, compared to *A. hawayanus* with two adult earthworms of the total earthworms taxonomically classified. Overall, the earthworm abundance ranged from 64 to 336 individuals per square meter and the biomass was from 9.46 to 53.1 g fresh weight/m^2. The mean earthworm abundance in treatments with C3-leaves was 200 (±62.82) individuals per square meter and in treatment with C4-leaves, 192 (±50.6) individuals per square meter. The mean biomass in plots with C3 leaves was 23.6 (±5.96) g fresh weight/m^2, and in plots with C4-leaves, 32.6 (±8.53) g fresh weight/m^2.

The mass proportion of water-stable aggregates and total carbon concentration were not significantly different between the C4-leaves and C3-leaves added (Table 2). In general, there were more small macroaggregates (250 to 2,000 µm) than large macroaggregates (> 2,000 µm), microaggregates (53 to 250 µm) and silt+clay (< 53 µm) (Figure 1; Table 2). However, the mean δ^{13}C of soil aggregates were significantly higher for C4-leaves treatment [-26.95 (±0.16) ‰] than C3-leaves treatment [-27.58 (±0.16) ‰] (Table 2).

The differences detected in the leaf litter isotopic signal allowed for the detection of leaf litter-derived carbon incorporated into soil aggregate fractions (Table 2). Soil-derived-carbon concentrations were not significantly different for the aggregate size classes and their interaction as shown in Table 2. We detected differences in leaf derived-carbon concentrations in soil large macroaggregates and microaggregate fractions only (Tables 3 and 4). In terms of the amount of carbon in the aggregates, the contribution of carbon from C3-leaves was higher [4.24 (±0.70) g C/kg $_{\text{sand-free aggregates}}$] than from C4-leaves [0.32 (±0.70) g C/kg $_{\text{sand-free aggregates}}$] (Table 4). Among the aggregate sizes compared, only the large macroaggregates+C3 were significantly higher in carbon than large macroaggregates+C4, and the microaggregates+C3 were significantly higher than microaggregates+C4 (Table 4).

We found a relationship between earthworm abundance and macroaggregates, but it was different for large and small macroaggregates (Figure 2). The aggregate mass proportion had a positive quadratic relationship between earthworm abundance and large macroaggregates+C4-leaves (R^2=0.95; P=0.001), as shown in Table 5

TABLE 2.—*Summary of ANOVA to evaluate the aggregate proportion, carbon concentration, and δ^{13}C signal at the field study sites. Variables tested were treatments (C4- vs. C3-leaf litter), aggregate size classes (large macroaggregates, small macroaggregates, microaggregates and silt+clay), and the interaction Treatments × Aggregate size classes.*

	Leaves (C4 vs. C3)				Aggregate size classes				Leaves (C4 vs. C3) × Aggregate size classes			
	numDF†	denDF‡	F-value	P-value	numDF	denDF	F-value	P-value	numDF	denDF	F-value	P-value
Aggregate mass proportion	1	24	1.00	0.7	3	24	16.37	**<0.0001**	3	24	0.30	0.82
Carbon Concentration	1	18	2.18	0.16	2	18	0.73	0.50	2	18	1.01	0.38
δ^{13}C signal	1	18	7.72	**0.01***	2	18	2.33	0.13	2	18	0.10	0.90

* Significant at the 0.05 probability level.
†Degrees of freedom for numerator (numDF).
‡Degrees of freedom for denominator (denDF).

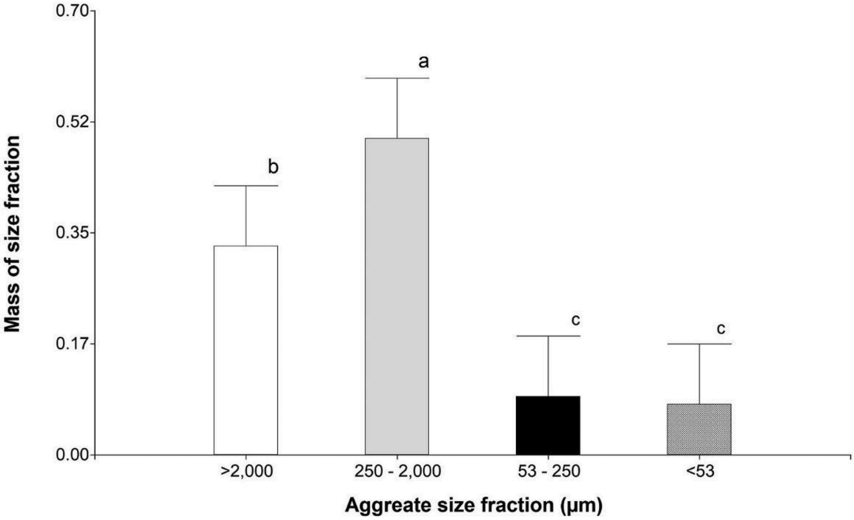

FIGURE 1. Aggregate size distribution in the field experiment in a Consumo soil. Different letters indicate significant differences among aggregate size classes within each treatment. Means (n=4) are significantly different as determined with Tukey's Least Significance Difference test (two-way ANOVA; P<0.05). Bars represent standard error.

and Figure 2A. The maximum of large macroaggregates proportion occurs at earthworm abundance of 262 individuals per square meter (Figure 2A). With a higher value of earthworm abundance, the large macroaggregates decreased slightly; while the relationship between small macroaggregates+C4 leaves and earthworm abundance was negative (R^2=0.80; P=0.001) as shown in Table 5 and Figure 2B. For small macroaggregates+C4-leaves, the minimum of small macroaggregates proportion occurs at earthworm abundance of 217 individuals per square meter (Figure 2B). A larger value of earthworm abundance increased the proportion of small macroaggregates slightly.

Microcosm experiment

After 20 days, most earthworms survived the microcosm experiment. However, one earthworm exhibited low activity within one of the experimental units of treatment III (soil, maize leaves, earthworms-low density). In one experimental unit of treatments III and IV (soil, maize leaves, earthworms-high density), all earthworms died during the experiment. Earthworms did not show diapause behavior (indicating water-stress) during the experiment.

Earthworms did not affect the aggregate mass proportion of water stable aggregates test (Table 6). However, aggregate size distribu-

TABLE 3.—*Statistics of two-way ANOVA for soil-derived-carbon and leaf-derived-carbon sources in samples of a Consumo soil under field conditions. Variables tested were leaves (C4 vs. C3), aggregate size classes [large macroaggregates (LM), small macroaggregates (SM), and microaggregates (M)], and the interaction Treatments × Aggregate size classes.*

			Soil derived-carbon		Leaf derived-carbon	
	numDF[†]	denDF[‡]	F-value	P-value	F-value	P-value
Aggregate size classes	2	18	0.63	0.55	0.57	0.57
Leaves (C4 vs. C3) × Aggregate size classes	2	18	0.55	0.58	0.26	0.77
LM+C4 vs. LM+C3	1	18	N/A[#]	N/A	7.80	**0.01***
SM+C4 vs. SM+C3	1	18	N/A	N/A	3.14	0.09
M+C4 vs. M+C3	1	18	N/A	N/A	5.39	**0.03***
(LM vs. SM vs. M)+C4	2	18	N/A	N/A	0.17	0.84
(LM vs. SM vs. M)+C3	2	18	N/A	N/A	0.67	0.53

* Significant at the 0.05 probability level.

[†] Degrees of freedom for numerator (numDF).

[‡] Degrees of freedom for denominator (denDF).

[#] Not applicable.

TABLE 4.—*Carbon concentrations (g C/kg $_{\text{sand-free aggregates}}$) incorporated from C4-and C3-leaf sources in samples of Consumo soil under field conditions. Different lowercase letters among aggregate size classes within each carbon source indicate significant differences as determined with Tukey's Least Significance Difference test (two-way ANOVA; P<0.05). Values are means (n=4).*

	C4-leaves source	C3-leaves source
	--------- g C/kg sand-free aggregates ----------	
Aggregate size class		
Large macroaggregates	**0.00 a***	4.76 a
Small macroaggregates	0.08 a	3.10 a
Microaggregates	0.89 a*	4.85 a

*Indicates a significant difference between carbon sources (C4- and C3-leaves) within each aggregate size class.

tion showed significant differences within aggregate sizes and across treatments (Table 7). Aggregate size classes were significantly higher for microaggregates (0.34 ± 0.02) than large macroaggregates (0.23 ± 0.018), small macroaggregates (0.23 ± 0.018) and silt+clay fraction (0.18 ± 0.016) (Table 6). Within treatments, no clear pattern emerged in aggregate size distribution from treatments with earthworms (treatments III and IV) or without earthworms (treatments I and II) (Table 7).

The total carbon concentration in the soil was not significantly different among microcosm treatments; however, carbon concentrations were different among aggregate size-classes (Table 6). The interaction microcosm × aggregate size classes was also significant (Table 6). Total carbon concentration was higher in the microaggregate fractions across treatments, except for treatment IV (high earthworm density). In treatment IV, the addition of earthworms led to a reduction in the proportion (Table 7) and carbon concentration (Table 8) of microaggregates, resulting in an increased proportion and carbon concentration in the small macroaggregates. In terms of carbon distribution within treatments, there was no pattern (Table 8); however, carbon concentration follows the mass distribution shown in the field experiment (Table 2). In all aggregate size classes, the source of organic carbon was derived mostly from soil (100 to 95%) rather than maize leaves (5 to 0%) (Table 9). Across treatments, most of the soil-derived carbon was allocated in the microaggregates and in the small macroaggregate fractions (Table 10). Within treatments, treatment IV allocated most of the soil-derived carbon from microaggregates to small macroaggregates (Table 10). For treatments I and III, soil-derived carbon allocation did not follow a pattern across aggregate sizes.

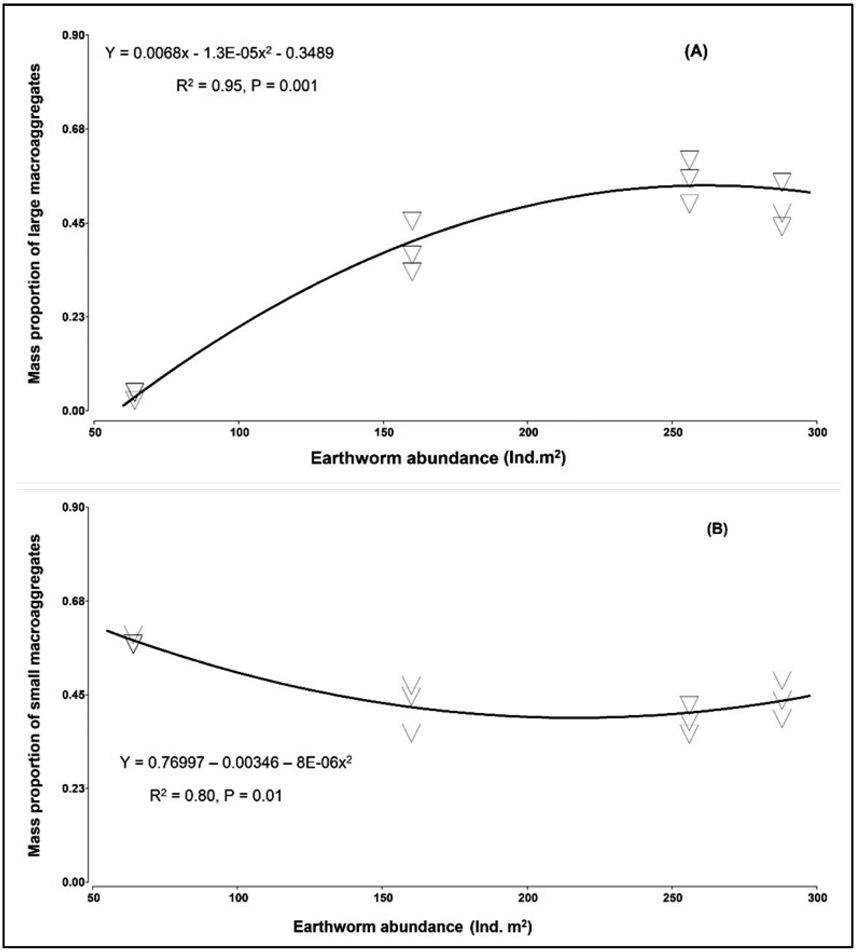

FIGURE 2. Relationship between earthworm abundance and mass proportion of site fraction. (A) large macroaggregates+C4 and (B) small macroaggregates+C4 in a Consumo soil. Each x-axis value corresponds to one replicate for earthworm abundance, and values in y-axis correspond to three soil sub-samples per micro-plot.

DISCUSSION

Field experiment

Our results partially support the hypothesis that differences in isotopic composition between C4- and C3-leaves allow the tracking and measurement of carbon incorporation into aggregate size classes under field conditions. Six months after applying the maize leaves, the change in $\delta^{13}C$ signal was found only for mean values between C4-

TABLE 5.—*Relationship of aggregate size classes to abundance and biomass of earthworms of Consumo soil series in field conditions.*

	Earthworms abundance (individuals per m^2)			Earthworms biomass (grams of fresh weight per m^2)		
Aggregates	R^2	F	P-value	R^2	F	P-value
Large macroaggregates+C4	0.95	21.31	**0.001**[§]	0.68	7.29	0.02
Large macroaggregates+C3	0.91	5.61	0.04	0.34	0.10	0.76
Small macroaggregates+C4	0.80	11.64	**0.01**[§]	0.68	11.45	0.01
Small macroaggregates+C3	0.95	53.8	**<0.0001**[§]	0.67	0.13	0.72
Microaggregates+C4	0.98	35.57	0.0002	0.66	5.27	0.047
Microaggregates+C3	0.51	5.22	0.048	0.40	5.19	0.049

(§) Indicates no significant lack of fit test.

TABLE 6.—*Summary of ANOVA to evaluate the aggregate proportion, carbon concentration, $\delta^{13}C$ signal, soil-derived carbon, and leaf- derived carbon in the microcosm experiment. Variables tested were microcosm treatments (I, II, III, and IV), aggregate size classes (large macroaggregates, small aggregates, microaggregates and silt+clay), and interaction microcosm Treatments × Aggregate size classes.*

	Microcosm treatments				Aggregate size classes				Microcosms × Aggregate size classes			
	numDF[†]	denDF[‡]	F-value	P-value	numDF	denDF	F-value	P-value	numDF	denDF	F-value	P-value
Aggregate mass proportion	3	12	0.06	0.98	3	36	13.17	**<0.0001**	9	36	4.08	**0.0010**
Carbon Concentration	3	12	0.07	0.97	3	36	15.04	**<0.0001**	9	36	4.26	**0.0008**
$\delta^{13}C$ signal	3	12	7.99	**0.003***	3	36	19.15	**<0.0001**	9	36	9.35	**<0.0001**
Soil-derived carbon	2	9	55.38	**<0.0001**	3	27	77.24	**<0.0001**	6	27	21.94	**<0.0001**
Leaf-derived carbon	2	9	4193.56	**<0.0001**	3	27	24834.50	**<0.0001**	6	27	3953.41	**<0.0001**

* Significant at the 0.05 probability level.

[†] Degrees of freedom for numerator (numDF).

[‡] Degrees of freedom for denominator (denDF).

TABLE 7.—*Aggregate size distribution in microcosm experiment in Consumo soil. Treatments are: I (soil-only), II (soil + C4-leaves), III (soil + C4-leaves + two earthworms), and IV (soil + C4-leaves + three earthworms). Uppercase letters that are different among treatments within an aggregate size class (horizontal rows) indicate significant differences. Lowercase letters that are different among aggregate size classes within each treatment (vertical columns) indicate significant differences. Means (n=4) are significantly different as determined with Tukey's Least Significance Difference test (two-way ANOVA; P<0.05)*

	Treatments			
	I	II	III	IV
	- - - - - - - - - - - Mass proportion of size fraction - - - - - - - - - - -			
Aggregate size class				
Large macroaggregates	0.12 B c	0.29 A ab	0.27 A ab	0.28 A a
Small macroaggregates	0.26 AB b	0.15 C c	0.20 BC bc	0.31 A a
Microaggregates	0.42 A a	0.34 AB a	0.36 AB a	0.25 B a
Silt and clay	0.20 A bc	0.22 A bc	0.17 A c	0.15 B b

and C3-leaf treatments. Only a small carbon fraction from maize leaf-derived carbon (C4 leaves) was incorporated and stabilized into soil aggregates. Most C4 leaf-derived carbon may have been incorporated into the silt + clay fraction or microorganism, plant roots, and soil fauna breakdown of organic matter and released CO_2 as a byproduct of respiration.

It was found that carbon incorporation follows the hierarchy of soil aggregate formation (Oades and Waters, 1991; Six and Paustian, 2014). This is because carbon from the C4-leaf litter source was incorporated first into the microaggregates and small macroaggregates, but not into large macroaggregates (Table 7). The first state of hierarchy of aggregate formation is clay flocculation with fresh plant material and microbial products to form stable microaggregates (Jarvis et al., 2012; Oades, 1993; Oades and Waters, 1991). We observed a significantly higher C4 leaf litter-derived carbon concentration for microaggregates+C4 and small macroaggregates+C4 than large macroaggregates. Our results show evidence of the first stages of aggregate formation and pathway to carbon incorporation across aggregate size classes, where microaggregates are formed by C4 leaf litter-derived carbon encrusted in clay particles to form macroaggregates. Perhaps a longer incubation would have permitted re-allocation of C4-leaf litter into macroaggregates. While large macroaggregates+C3 showed a higher C3 leaf litter-derived carbon concentration than small macroaggregates+C3 and microaggregates+C3, they exemplify the last level of the hierarchy

Table 8.—*Total carbon concentration for aggregate size classes in microcosm experiment in Consumo soil. Microcosm treatments are I (soil-only), II (soil + C4-leaves), III (soil + C4-leaves + two earthworms), and IV (soil + C4-leaves + three earthworms). Uppercase letters that are different among treatments within an aggregate size class (horizontal rows) indicate significant differences. Lowercase letters that are different among aggregate size classes within each treatment (vertical columns) indicate significant differences. Means (n=4) with standard error in parentheses are significantly different as determined with Tukey's Least Significance Difference test (two-way ANOVA; P<0.05).*

	Treatments			
	I	II	III	IV
	- - - - - - - - - - - - - - - g C/kg whole soil - - - - - - - - - - - - - - -			
Aggregate size class				
Large macroaggregates	5.15 B c	13.50 A a	12.51 A ab	12.20 A a
Small macroaggregates	11.36 AB b	7.22 AB b	9.48 B bc	13.78 A a
Microaggregates	19.10 A a	14.54 AB a	16.21 A a	10.86 B a
Silt and clay	7.84 A bc	8.71 A b	6.71 A c	5.95 A b
Whole soil¶	43.45	43.96	44.91	42.79

¶Calculated as the sum of all aggregate fractions.

of carbon incorporation in the soil aggregates as shown in Table 7 (Oades and Waters, 1991; Six et al., 2000; Tisdall and Oades, 1982). Our results suggest that carbon from maize leaves residue requires a longer period for carbon stabilization and development of soil aggregate structures in each aggregate size class (Elliott, 1986; Oades and Waters, 1991; Six et al., 2002).

This study found that earthworm abundance with endogeic dominance had a relationship with large macroaggregates+C4 leaves, and small macroaggregates+C4 leaves. These trends also suggest that the large macroaggregates were formed at the expense and reorganization of small macroaggregates during burrowing and casting activities. Our results are consistent with previous studies. For example, Barois et al. (1993) observed with a transmission electron microscopy that *P. corethrurus* destroyed microaggregates during the gut transit and new microaggregates were formed. Sánchez-de León et al. (2014) observed that microaggregates and silt+clay fractions were reduced in quantity in the presence of *Diplocardia* spp. under microcosm conditions. Barois et al. (1993) found that aggregates were restructured during the transit by the gut of *P. corethrurus*.

Our results indicate that earthworm abundance can impact the proportion of both large and small macroaggregates. Specifically, earthworms facilitate the restructuring of small macroaggregates into large macroaggregates. In addition, interactions between earthworms and

TABLE 9.—*Soil and C4-leaf litter proportion sources for each aggregate size class in the microcosm experiment. Microcosm treatments are II (soil + C4-leaf litter), III (soil + C4-leaf litter + two earthworms), and IV (soil + C4-leaf litter + three earthworms).*

	Treatments		
	II	III	IV
	(%)		
Aggregate size classes			
Large macroaggregate			
Soil	96	96	95
Maize leaves	4	4	5
Small macroaggregates			
Soil	96	96	97
Maize leaves	4	4	3
Microaggregates			
Soil	98	98	99
Maize leaves	2	2	1
Silt and clay			
Soil	96	100	100
Maize leaves	4	0	0

aggregate fractions may vary based on the earthworms' feeding behavior and size. Blanchart et al. (1997) reported that earthworm *Milsonia anomala* may ingest microaggregates and create new macroaggregates larger than 5 mm.

Microcosm experiment

Higher δ^{13}C values in the aggregate fractions indicate higher carbon incorporation from C4-leaf litter. As expected, the treatments with earthworms (treatments III and IV) showed a shift in δ^{13}C compared with the control (treatment I, soil only) for the large macroaggregate and small macroaggregate size classes. However, this was not the case with treatment II (soil + C4-leaf litter). These results show that treatments with earthworms did not incorporate more C4-leaf carbon into aggregates through other processes, such as organo-mineral interactions or microbially-driven aggregate formation (Lavelle et al., 1998; Sánchez-de León et al., 2014; Topoliantz and Ponge, 2003). Our results differ from Bossuyt et al. (2004b), who found differences in δ^{13}C signal in large macroaggregates and no differences in small macroaggregates with endogeic *Aporroctodea caliginosa* earthworms. Bossuyt et al. (2004b), in their microcosm experiment, reported a high concentration of ^{13}C in large macroaggregates, within and between microag-

TABLE 10.—*Carbon derived from soil and C4-leaf litter after incubation in microcosm experiments in Consumo soil. Microcosm treatments are II (soil + C4-leaves), III (soil + C4-leaves + two earthworms), and IV (soil + C4-leaves + three earthworms). Uppercase letters that are different among treatments within an aggregate size class (horizontal rows) indicate significant differences. Lowercase letters that are different among aggregate size classes within each treatment (vertical columns) indicate significant differences. Means (n=4) are significantly different as determined with Tukey's Least Significance Difference test (two-way ANOVA; $P<0.05$).*

	Soil-derived carbon source			C4 leaf litter-derived carbon source		
	II	III	IV	II	III	IV
	-------------------------------- g C/kg whole soil --------------------------------					
Aggregate size classes						
Large macroaggregates	11.51 A ab	12.09 A ab	11.29 A a	0.43 A a	0.45 A a	0.54 A a
Small macroaggregates	6.80 B c	8.99 AB bc	12.72 A a	0.23 A b	0.30 A b	0.34 A b
Microaggregates	14.55 A a	15.10 A a	10.51 A ab	0.24 A b	0.25 A b	0.05 B c
Silt and clay	8.43 A bc	6.71 A c	5.95 A b	0.28 A b	0.00 B c	0.00 B c

gregates in treatments with earthworms than in treatments without earthworms.

The microcosm experiment showed a shift in $\delta^{13}C$ compared to the field experiment. The homogenization of the ground maize leaves with soil in the microcosm experiment allowed a higher carbon incorporation within the aggregates and identified the variation of $\delta^{13}C$ signal. By contrast, in the field experiment the ground maize leaves were added to the soil surface from the sub-plot that supposes the Bossuyt et al. (2004b) lowest carbon incorporation into the soil. When organic residues are placed on the surface, these are in lesser contact with the mineral soil (Bossuyt et al., 2006). Kuang et al. (2014), reported that 29.1% of maize straw decomposed when incorporated into the soil, and 20.8% of maize straw decomposed when placed at the soil surface.

Only treatment IV (high density earthworms) presented a high proportion of small macroaggregates but did not show differences with treatment I (soil only). These results contrast with Sánchez de Leon et al. (2014), in which the highest proportion of macroaggregates were measured in the treatments with earthworms *Diplocardia* sp. (endogeic) and *Lumbricus rubellus* (epi-endogeic). Similarly, Bossuyt et al. (2004) observed that the treatments with *Aporrectodea caliginosa* (endogeic) contained 3.6 times more large macroaggregates than treatments without earthworms.

Earthworms play an important role in soil structure and organic matter dynamics through casting activities that influence particle size distribution of soil aggregates (Blanchart et al., 1997, 2004; Fragoso and Lavelle, 1992). Our results showed a weak trend that earthworms used microaggregate fractions to form macroaggregates. It is possible that the death of earthworms in one repetition of treatments III and IV reduced the power of statistical analysis. Barois et al. (1993) found that *P. corethrurus* ingests and destroys old microaggregates during the transit by the earthworm gut and, through excretion, created new microaggregates. Likewise, Sánchez-de León et al. (2014) mentioned that new aggregates are formed by the rearrangement of differing-size classes of soil fractions.

The microcosm experiment demonstrated that endogeic earthworms mostly redistribute the soil-derived carbon rather than incorporating the C4 leaf-derived carbon into the macroaggregates. Bossuyt et al. (2004) reported a higher incorporation of carbon in large macroaggregates in treatments with endogeic *Aporrectodea caliginosa* than in treatments without earthworms. The treatments with earthworms presented a reduction of carbon concentration in the microaggregate size class compared to treatment I (only soil). Therefore, there was a reduction in the amount of microaggregate fractions after earthworms were added. Bossuyt et al. (2005) mentioned that earthworms induced redistribution of carbon and residue–derived carbon through casting activities.

A trend was observed in the flux of soil carbon among aggregate size classes, where endogeic earthworms ingested selected microaggregates with high soil-derived carbon and allocated them into new macroaggregates. Most earthworms in the tropics ingest selectively organic particles according to their feeding behavior (Blanchart et al., 2004). In another study conducted with endogeic and anecic species, it was observed that endogeic earthworms had a preference for casts from anecic earthworms that had higher quality organic matter and mineralized nutrients (Keith et al., 2017; Lavelle et al., 1998). Earthworm casts contain more organic matter than surrounding bulk soil and have a direct influence on the mineralization and decomposition of soil organic matter (Bossuyt et al., 2005, 2006; Schon et al., 2015). Thus, the new macroaggregates formed from earthworm casts increased the amount of carbon in the macroaggregate fraction.

CONCLUSION

The field experiment showed that leaf-derived carbon follows the aggregate hierarchy process because carbon from the C4-leaf source was incorporated first into the microaggregates and small macroaggregates, but not into large macroaggregates. In addition, the relationship between earthworm abundance and aggregate proportion indicates that earthworms (with dominance of *P. corethrurus*) consumed small macroaggregates and created large macroaggregates. The microcosm experiment showed that the incorporation of carbon within soil aggregates by endogeic earthworms was not always consistent. The comparison between treatments with different earthworm densities showed that high earthworm abundance did not incorporate more carbon than lower earthworm abundance. The microcosm experiment also showed that endogeic earthworms could transfer soil-derived carbon through selective ingestion of soil particles and microaggregates into new macroaggregates. In conclusion, our results suggest that *P. corethrurus* shows a preference for consuming soil-derived carbon and may translocate it from microaggregates to macroaggregates by restructuring soil aggregates.

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