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Soil aggregate formation and carbon storage by endogeic earthworms in an Ultisol

Formación de agregados del suelo y almacenamiento de carbono por lombrices de tierra endogéicas en un Ultisol

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Abstract

The role of soil micro and macro-organism in the incorporation of carbon to soil aggregates of tropical soils has been understudied. To test soil aggregate formation and carbon incorporation by earthworm activity, we conducted a field experiment within a secondary forest and a microcosm experiment at the University of Puerto Rico in Mayagüez. We used 13C natural abundance in vegetation and the difference in δ13C between C3 and C4 plants to track carbon sources in the soil. Maize leaves were utilized to trace the incorporation of C4 carbon within soil aggregates, allowing for a clear distinction between this carbon source and the C3 carbon derived from forest vegetation. Earthworms and soil samples (Typic Haplohumults) were collected at 0-10 cm soil depth. Aggregates size classes were separated by the wet sieving method. In the study site, two earthworm species were found belonging to epigeic and endogeic ecological categories. In a period of 6 months, our field data suggests that endogeic *P. corethrurus* can reorganize small macroaggregates to form large macroaggregates. Our results suggest that *P. corethrurus* shows a preference for consuming soil-derived carbon and can translocate it from microaggregates to macroaggregates by restructuring soil aggregates.

Keywords: Bioturbation, soil organic carbon, organic matter, isotope, tropical soils.

Resumen

El rol de los micro y macroorganismos del suelo en la incorporación de carbono a los agregados del suelo ha sido poco estudiado en los suelos tropicales. Para evaluar la formación de agregados del suelo y la incorporación de carbono mediante la actividad de las lombrices de tierra, realizamos un experimento de campo en un bosque secundario en la Universidad de Puerto Rico en Mayagüez. Utilizamos la abundancia natural de C13 en la vegetación y la diferencia del isótopo de δ13C entre plantas C3 y C4 para rastrear las fuentes de carbono dentro de los agregados del suelo. Se utilizaron hojas de maíz para rastrear la incorporación de carbono C4 en los agregados del suelo, permitiendo una clara distinción entre esta fuente de carbono y el carbono C3 derivado de la vegetación del bosque. Se colectaron muestras de lombrices de tierra y suelo (Typic Haplohumults) a una profundidad de 0-10 cm. 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 pertenecientes a las categorías ecológicas epigéicas y endogéicas. En un período de 6 meses, nuestros resultados sugieren que la especie endogéica *P. corethrurus* puede reorganizar pequeños macroagregados para formar grandes macroagregados. 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: Bioturbación, carbono orgánico del suelo, materia orgánica, isotopo, suelos tropicales.

Introduction

Earthworms are among the most abundant and important "soil engineers" that impact the physical, chemical, and biological characteristics of the humid tropical ecosystems (Blanchart *et al*., 2004; González *et al*., 2007; Lavelle *et al*., 1992). Earthworms are known to affect the fluxes of organic matter (OM) decomposition, soil organic carbon (SOC), and direct incorporation of SOC into soil, thereby altering soil structure and fertility (Aira *et al*., 2008; Blanchart *et al*., 1997; Fonte *et al*., 2009; Le Couteulx *et al*., 2015; Pulleman *et al*., 2005; Sánchez-de León *et al*., 2018; Whalen and Janzen, 2002).

Through their feeding and casting activities, earthworms help to remove part of the plant litter from the soil surface and influence the SOM incorporation into soil aggregates (Bossuyt *et al*., 2005) by ingest particulate organic matter (POM) and mineral soil. This mixing of organic material with mineral particles results in the formation of casts (Bossuyt *et al*., 2004, 2005; Sánchez-de León *et al*., 2014). 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).

Although several studies show that earthworms 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 can fragment existing soil aggregates like small Eudrilidae (Blanchart *et al*., 1997, 2004; Kamau *et al*., 2020) . Thus, the effect of earthworms in soil aggregation seems to be species dependent.

Soil 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*. (2004) found that soil aggregates formed by earthworm's activity contained 3,6 times larger macroaggregates (larger than 2 000 μm) and more total carbon (4,26 g C kg-1 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 treatments without earthworms. In addition, Bossuyt *et al*. (2004, 2006) found that earthworm activity can increase the formation of water-stable microaggregates inside large macroaggregates four-fold compared to treatments without earthworms.

Despite these findings, the role of earthworm aggregate formation and their influence on carbon storage in tropical soils of Puerto Rico (i.e., Ultisols and Oxisols) has been little studied. Ultisols and Oxisols are highly meteorized soils characterized by low activity clays (1:1 clay minerals), and low fertility (e.g., Soil Survey Staff, 2014). Many 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 to understand the aggregate formation, and incorporation of carbon into aggregates by earthworms are few (Amador et al., 2013; Hendrix *et al*., 1999; Lachnicht et al., 2002).

The aim of the experiment was to study the impact of earthworms on an Ultisol found from the secondary forest at Finca Alzamora in Mayaguez, Puerto Rico, focusing on soil aggregates formation and its relationship with carbon incorporation. Our objective was to measure the earthworm abundance and aggregate distribution under natural field conditions in an Ultisol within a 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 within 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 will allow the tracking of this newly incorporated carbon.

Materials and methods

The field study was conducted within the secondary forest at the Alzamora Farm, located in 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 1.020 to 1.780 mm and 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: *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 Maria. The experimental plots were checked on September 11, 2018, after the Irma hurricane and on September 29, 2018 after the Maria hurricane. In both cases, the experimental plots did not show addition or loss of soil by erosion. Leaf litter and other vegetation debris (e.g., mostly tree trunks) on top of plots were removed to continue the field experiment.

Table 1. Latitude, longitude, aspect and gradient of experimental plots of Consumo soil series at Finca Alzamora secondary forest

Plot	Latitude	Longitude	Aspect (º)	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

Plant material and field application

Leaves from maize plants were collected from an organic orchard located at the Alzamora Farm. leaves from the forest floor (C3-leaves) at the study site were collected as other treatment. All leaves were cut and dried in paper bags for 72 hours at 65 °C in an oven, followed by grinding to < 2 mm. For selecting the experimental plots, we used an 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) and of these, four sections were randomly selected. In each of these selected areas of the forest, one experimental plot of 1 × 1 m size was delimited using barrier landscape fabric without altering the trees. Each plot was a replicate of the experiment. 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. The two treatments were: control forest floor leaves (C3-leaves) and maize leaves (C4-leaves) with four repetitions (n=4). On 5 June 2017, we applied 400 g m^{-2} of maize leaves (2,04 g C kg^{-1} of soil) on each of the C4-leaves treatment replicates and 400 g m^{-2} of forest leaves (2,71 g C kg^{-1} of soil) on each of the C3-leaves treatment replicates.

Earthworm sampling

During November 2 017; six months after treatment addition; 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, during the same day of collection.

Earthworms from each sub-plot were measured by their abundance (individuals m^{-2}), and fresh weight (grams of fresh weight m^{-2}). 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 1:10 dilution of formaldehyde 37% for taxonomic identification. Taxonomic identification was performed by earthworm taxonomist Dr. Sonia Borges using (Borges, 1996) 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 10 cm from soil surface from each sub-plot. Soil samples were collected between November 27 to December 15, 2017. Each soil core was broken along its natural breaking points (Fonte et al., 2009; Jastrow *et al.*, 2005). The soil was gently passed 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 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.

Three sieves were used to obtain four size fractions: 1) large than 2.000 µm (large macroaggregates); 2) 250-2.000 µm (small macroaggregates); 3) 53-250 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 by 10 minutes at 4.500 rpm and 20 °C.

All fractions were placed into an oven to dry at 65 °C, by 24 hours, and then weighed. Soil aggregate fraction was corrected and expressed as 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 in aggregates as a sand free basis, we used Equation 2 (Six *et al.*, 1998).

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

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

Where C represents the carbon concentration within each aggregate fraction. Sand content of macroaggregates and microaggregates sizes were determined using the modified method of particle size analysis described by Jackson (2005).

Isotope analysis

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 at Chicago, at the Geochemistry Stable Isotope Laboratory. The result was expressed in delta notation using part per thousand (δ ‰) as follows in Equation 3.

$$\delta^{13}\mathrm{C} = \left[\left(\frac{{}^{13}\mathrm{R}_{\mathrm{sample}} - {}^{13}\mathrm{R}_{\mathrm{standard}}}{{}^{13}\mathrm{R}_{\mathrm{standard}}}\right)\right] \times 1000 \qquad (3)$$

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*., 2004; O'Leary, 1981). Equipment also provided data for percent of soil carbon ($\%C = g_C/100\ g_{soil}$ and $\%N = g_N/100\ g_{soil}$). To determine the amount of the 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 experimental plots were established in the field using a 2×4 factorial design, incorporating two types of leaf inputs (C4-leaves and C3-leaves) and four aggregate size classes (large macroaggregates, small macroaggregates, microaggregates and silt+clay). We implemented a posteriori contrast analysis to evaluate the effects of soil-derived carbon and leaf-derived carbon on the aggregate size classes.The design includes five specific contrasts, each designed to evaluate particular hypothesis or comparison among the treatments group. The results were subjected to statistical analysis using a two-way ANOVA. The relationship between earthworm abundance and across different aggregate size classes was analyzed using a linear regression model PROC REC analysis. 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 were tested using Tukey's honestly significant difference at a level of $p < 0,05$.

Results and discussion

In 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 2 adult earthworms of the total earthworms taxonomically classified. Overall, the earthworm abundance ranged from 64 individuals m^{-2} to 336 individuals m^{-2} and the biomass was from 9,46 to 53,1 grams of fresh weight m^{-2}. The mean earthworm abundance in treatments with C3-leaves was 200 (±62,82) individuals m^{-2} and in treatment with C4-leaves was 192 (±50,6) individuals m^{-2}. The mean biomass in plots with C3 leaves was 23,59 (±5,96) grams of fresh weight m^{-2} and in plots with C4-leaves was 32,63 (±8,53) grams of fresh weight m^{-2}.

As expected, there was no significant difference for the factor leaves additions (Table 2). For the aggregate size class factor, the proportion of aggregate mass was significantly higher for small macroaggregates (250-2.000 µm) than large macroaggregates (> 2.000 µm), microaggregates (53-250 µm) and silt+clay (< 53 µm) (Figura. 1, Table 2). Additionally, the soil aggregates showed significantly higher values of $\delta^{13}C$ for C4-leaves [-26,95 (±0,16) ‰] than C3-leaves [-27,58 (±0,16) ‰] (Table 2).

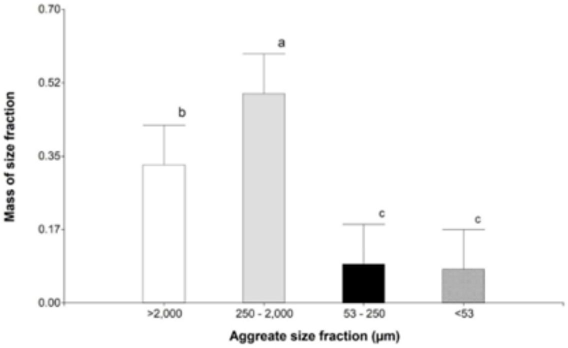

Figure 1. Aggregate size distribution in the field experiment in the Consumo soil. Letters with different lowercase are significantly different among aggregate size classes within each treatment. Means (n=4) are significantly different as determined with Tukey's Least Significance Difference test (two-way ANOVA; *p*<0,05). Bars represent standard error

The distinct isotopic signal of the leaf litter enabled the detection of carbon derived from the leaf litter that was incorporated in the 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. In our study, we observed differences in leaf-derived carbon concentrations, particularly within the soil's large macroaggregate and microaggregate fractions (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 $kg^{-1}_{\text{sand-free aggregates}}$] than C4-leaves [0,32 (±0,70) g C $kg^{-1}_{\text{sand free aggregates}}$] (Table 4). From the aggregate sizes compared, only the large macroaggregates+C3 were significantly higher than large macroaggregates+C4, and the microaggregates+C3 were significantly higher than microaggregates+C4 (Table 4).

Table 2. Summary of ANOVA to evaluate the aggregate proportion, carbon concentration, and δ13C signal in the field study sites. Variables tested were treatments (C4- vs. C3 leaf litter), aggregate size classes (large macroaggregates, small aggregates, 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	0,00	0,97	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
$\delta^{13}C$ 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).

Table 3. Statistics of two-way ANOVA for soil derived-carbon and leaf derived-carbon sources in samples of Consumo soil under field conditions. Variables tested were leaves (C4 vs. C3), aggregate size classes [large macroaggregates (LM), small aggregates (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-1 sand-free aggregates) incorporated from C4-and C3-leaves sources in samples of Consumo soil under field conditions. Letters with different lowercase among aggregate size class within each carbon sources, are significantly different as determined with Tukey's Least Significance Difference test (two-way ANOVA; *p*<0,05). Values are means (n=4) with standard error in parentheses

	C4-leaves source	C3-leaves source
	--- g C kg^{-1} sand-free aggregates ----	
Aggregate size class		
Large macroaggregates	**0,00 a*** (1,21)	4,76 a (1,21)
Small macroaggregates	0,08 a (1,21)	3,10 a (1,21)
Microaggregates	0,89 a (1,21)	4,85 a (1,21)

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

Or study uncovered a relationship between earthworm abundance and macroaggregates, showing distinct patterns based on the size of these aggregates (Figura 2). The aggregate mass proportion demonstrated a positive quadratic relationship between earthworm abundance with large macroaggregates+C4-leaves (R2=0,95; P=0,001), as detailed in Table 5 and illustrated in Figura 2A. The proportion of large macroaggregates proportion peaked at an earthworm abundance of 262 individuals m-2 (Figura 2A), beyond which a slight decline was observed. While the relationship between small macroaggregates+C4 leaves, and earthworm abundance was negative (R2=0,80; P=0,001) as shown in Table 5 and Figure 2B. For small macroaggregates+C4-leaves the lowest proportion was observed at an earthworm abundance of 217 individuals m-2 (Figura 2B), after which there was a slight increase.

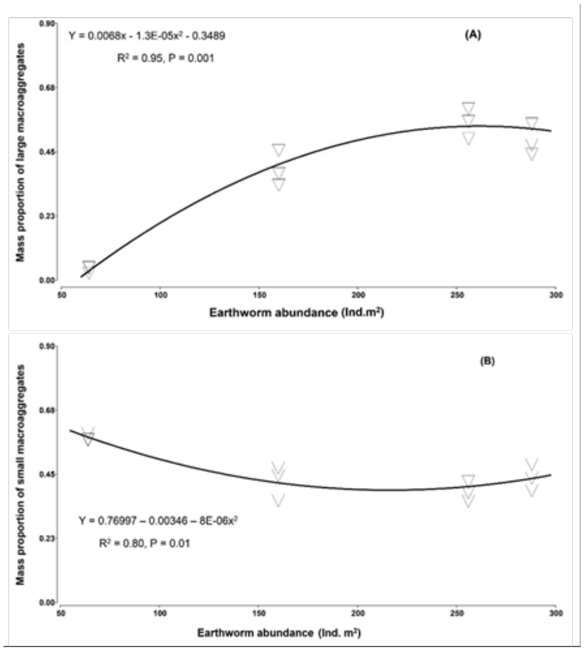

Figure 2. Relationship between earthworm abundance with mass proportion of site fraction. (A) large macroaggregates+C4 and (B) small macroaggregates+C4 of Consumo soil. Each x-axis value corresponds to one replicate for earthworm abundance and values in y-axis correspond to three soil sub-samples per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

Table 5. Relationship between aggregate size classes with abundance and biomass of earthworms of Consumo soil series in field conditions

Aggregates	Earthworms abundance (individuals m^{-2})			Earthworms biomass (grams of fresh weight m^{-2})		
	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	0,1	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	0,4	5,19	0,049

Our results partially support the hypothesis that differences in isotopic composition between C4- and C3-leaves allow the tracking and measure the 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- and C3-leaves treatments. Only a small carbon fraction from maize leaves-derived carbon (C4 leaves) was incorporated and stabilized into soil aggregates. Most C4 leaves-derived carbon may have been incorporated into silt + clay fraction or respired as CO_2.

The lignin and aromatic compounds from plant debris can be easily associated with silt and clay fractions or lost from soil as respired CO_2 (Bossuyt *et al*., 2006; Oades and Waters, 1991; Paul *et al*., 1999). While the C3 leaf litter-derived carbon has been accumulated over a long time and in higher amounts, showing a higher carbon concentration than C4 leaf litter treatment. According to (Paul *et al*., 1999), 6% from total carbon can be lost in the form of CO_2 from aboveground residues in conventional tillage treatments, 11% in no-tillage treatments, 11% in low-chemical inputs, and 20% in zero-chemical inputs. In a study of mean residence time conducted by Paul *et al*. (2008), in soils derived from volcanic ash, the researchers could not detect the pasture-derived carbon cultivated a few decades before that site changed to a secondary forest. In another study conducted by (Sánchez-de León *et al*., 2018) using free-air CO_2 enrichment in a sweetgum (*Liquidambar styraciflua* L.) plantation, the researchers did not find a change in the soil (Ultisol order) carbon isotopic composition two years after CO_2 fumigation ended.

We found that carbon incorporation follows the hierarchy of soil aggregate formation (Oades and Waters, 1991; Six and Paustian, 2014). This is because carbon from C4-leaf litter source was incorporated first into the microaggregates and small macroaggregates, but not into large macroaggregates (Table 4). 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 can be showing the first stages of aggregate formation and pathway of 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 indicate the last level of the hierarchy of carbon incorporation in the soil aggregates as shown in Table 4 (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 aggregates structures in each aggregate size classes (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 show that earthworm abundance could affect the proportion of large macroaggregates and small macroaggregates as well as earthworm abundance influences the restructuration of small macroaggregates to form large macroaggregates. In addition, it is possible to find other interactions between earthworms with aggregate fractions according their feeding behavior and size. Blanchart *et al.* (1997) reported that earthworm *Milsonia anomala* may ingest microaggregates and create new macroaggregates larger than 5 mm. Differences in earthworm abundance and earthworm biological stages observed across the study site could also influence the dynamics of aggregate formation and could create the observed variations.

Conclusion

The field experiment showed that leaf-derived carbon follows the aggregate hierarchy process. Carbon from C4-leaf source was incorporated first into the microaggregates and small macroaggregates, but not in the large macroaggregates. The relationship between earthworm abundance and aggregate proportion indicates that earthworms (with dominance of *P. corethrurus*) consumed small macroaggregates and created large 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. The endogeic *P. corethrurus* plays a crucial role in soil bioturbation by restructuring soil aggregates, nutrient cycling, and organic soil carbon. Its widespread presence and adaptability make it vital for soil ecology research, providing insights into soil health and future studies on ecosystem resilience.

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