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Effect of the endogeic earthworm *Aporrectodea tuberculata* on aggregation and carbon redistribution in uninvaded forest soil columns

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ABSTRACT

The long-term impact of earthworm presence on soil carbon (C) dynamics of previously uninhabited northeastern forests is still largely unknown. Currently, earthworm presence is understood to both enhance soil respiration and create stable microaggregates, processes assumed to have conflicting effects on long term C storage. To date, studies investigating earthworm-created microaggregates and occluded C have rarely been done in undisturbed forest soils. A paired mesocosm study (n = 5) was conducted investigating the impact of the endogeic earthworm species *Aporrectodea tuberculata* on the physical proportion of microaggregates and the associated mineral soil C of a minimally disturbed forest soil. Pairs analyzed after 4 weeks of incubation demonstrated no significant aggregate effects. At 4 months, paired cores with earthworms (WW) showed a 67% increase in large macroaggregates (>2000 μm diameter, lgMA), compared to cores without earthworms (NW). While distribution shifted among various microaggregate pools (free and occluded within macroaggregates), the net proportion of microaggregates in the soil (dry weight basis) was unaltered. After 4 months, the mineral soil of WW cores had an average of 60% more C than the NW cores due to the relocation of the forest floor. The C associated with the microaggregate fractions increased an average of 56%. Of this increase in C, 95% was accounted for by the microaggregates occluded within the lgMA fraction, a fraction that was almost 4 times greater in the WW cores. Over 50% of the C relocated into the mineral soil was associated with the physically protected microaggregate fractions, indicating that though this species of earthworm did not alter the proportion of microaggregates in these soils, they occluded a substantial proportion of C within those physical fractions. In this particular forest soil, the actions of *Aporrectodea tuberculata* increased the physically protected C pool through microaggregate restructuring and C enrichment.

1. Introduction

Earthworms have been termed “ecosystem engineers” due to the far reaching impacts these organisms have on a soil’s physical, chemical and microbial characteristics (Doube and Brown, 1998). Approximately 12,000 years ago the last glaciation event covered most of the northeastern United States (Davis and Jacobson, 1985; Ridge, 2004), eliminating this region’s soil and associated fauna. Due in part to the slow northward expansion of southern species of earthworm, and their inability to adapt to the cold winters of the north, the forests of the northeast have developed without the influence of these soil dwelling organisms (Groffman et al., 2004). Since the introduction of earthworms from Europe and Asia via ship ballast and imported horticultural products (Gates, 1976), various species have slowly moved from agricultural and horticultural settings, where they are considered a beneficial contributor to plant growth, to forests, where their impact is less understood and typically undesirable (Hale et al., 2005). Forest invasions by earthworms are expected to increase in the coming decades, and it is still unclear how earthworm presence in these ecosystems will impact soil stabilization of C. Furthering our understanding of the impact earthworms may offer an opportunity to manage northeastern forests for C retention when earthworms have invaded.

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Earthworms are typically placed into ecological groups (Bouche, 1977), each group occupying a specific ecological niche and influencing soil aggregation and C turnover differently (Doube and Brown, 1998). Endogeic earthworms living and feeding in the mineral soil are the primary group of earthworms that affect aggregation as they are geophagous. Earthworms have a variety of effects on forest soil structure and function. Most studies investigating the *in situ* impact of earthworm invasion into native northern forests have found a reduction of the forest floor as it becomes integrated by earthworm ingestion and egestion into mineral horizons (Alban and Berry, 1994; Bohlen et al., 2004; Lyttle et al., 2011). This action homogenizes soil, bringing bacterial communities into close contact with their food source resulting in increased soil nutrient cycling (Bohlen and Scheu, 2004; Groffman et al., 2004). Microbial communities within the castings of earthworms are greatly altered relative to bulk soil (Brown et al., 2000), favoring populations capable of surviving through the anoxic environment of the earthworm gut (Drake and Horn, 2007) and disrupting mycorrhizal relationships (Dempsey et al., 2011). There is little doubt that through the above processes invading earthworms are increasing the mineralization of C in the short term (Lubbers et al., 2013) however in the long term there is a growing body of evidence suggesting that earthworms may increase C sequestration (Zhang et al., 2013).

There are several ways that C may become stabilized within soil; however the most effective mechanisms of stabilization, as well as the best methods of measurement, are still being debated. One frequently cited stabilization mechanism is the physical segregation of bacterial communities and their enzymes from C occluded within microaggregates (mA, 250-53 μm) (Adu and Oades, 1978; Schmidt et al., 2011; Dungait et al., 2012; Sanchez-de Leon et al., 2014). Presuming that mA-occluded C does represent a pool of stabilized C within the soil, the ability to operationally isolate these structures, as outlined in Six et al. (2002), allows for one mechanism of C stabilization to be accurately analyzed.

Shipitalo and Protz (1989) demonstrated that during passage through the gut of the anecic species *Lumbricus terrestris*, existing mA structures are destroyed by peristalsis and organic debris and clay particles become coated in polysaccharides, providing the nuclei for the formation of new mA. This process of mA structure formation has been observed in both homogenized (Bossuyt et al., 2005; Sanchez-de Leon et al., 2014) and undisturbed agricultural soils (Pulleman et al., 2005), utilizing various earthworm species and methods of measurement. It has been proposed through these studies that earthworm enhancement of stable aggregates is a mechanism by which they may stabilize C in the long term, mitigating their effect on increased soil respiration. There is however limited information on how earthworms will alter aggregate properties and C qualities in undisturbed, earthworm-free, forest soils. Yavitt et al. (2015) recently showed that lumbricid earthworms invading a northern hardwood forest in New York reduced both particulate and mineral-sorbed C within macroaggregates but also reduced the proportion of free microaggregates. Isotope-labeled leaf litter showed that much of the new C was found in microaggregates within macroaggregates.

In a paired mesocosm study we investigated the impact of one common endogeic earthworm species, *Aporrectodea tuberculata*, on the quantity and carbon content of mA in undisturbed soil cores from an earthworm-free, northern hardwood forest. We hypothesized that earthworm presence would increase the pool of mA protected C through enriching this fraction with C relocated from the forest floor and increasing the proportion of total mA (free and occluded within larger aggregate classes) as a proportion of total soil dry mass.

2. Methods

2.1. Site characteristics

The Waterworks Property (WAT) is a 270 ha northern hardwood forest located in the town of Bristol Vermont in the Champlain Valley. Forest composition is primarily *Acer rubrum* (red maple), *Acer saccharum* (sugar maple), and *Fagus grandifolia* (American beech). The cores used in this study were excavated at 73°7′58.68″W 44°9′48.142″N, at an elevation of 237 m with an average slope of 18° on a long west-facing hill-slope. The soils at this location are a coarse-loamy Fullam (Oxyaquic Dystrudept). Previous surveys found a single worm in 2008 (Juillerat, 2011) with no indication of earthworm influence in a 2012 survey (Knowles, 2015). Approximately 500 m from the retrieval location, at the bottom of the hill-slope, several common species, including *Aporrectodea tuberculata*, were observed.

Hinesburg Town Forest is a northern hardwood forest converted from agriculture approximately 80 years ago. The soils are coarse-loamy Marlow/Colonel (Aquic Haplorthods) and have been heavily influenced by the presence of earthworms (approximately 145 worms per m^2). *Aporrectodea tuberculata* adults and juveniles were retrieved from Hinesburg Town Forest (73°2′17.603″W 44°19′45.78″N) on July 11th, 2013. Specimens were placed in soil from WAT for 3 weeks at 15 °C prior to incubation within the undisturbed soil cores.

2.2. Retrieval of soil cores

Segments of 30-cm standard-20 PVC drain pipe were used for collection on July 22nd, 2013. The central retrieval location was chosen near the WAT plot sampled in 2008 and 2012. Retrieval of the cores from the field occurred in six randomly selected locations (A-F), determined by a random number chart for distance and direction from the center point A (Fig. 1). At each of the six locations, five cores were hammered into the ground. The cores were carefully excavated, sealed, and carried back to the lab. If a core was badly damaged during retrieval it was removed from the study. The thick forest floor at location B resulted in inadequate mineral soil for aggregate analysis and all cores from this location were removed from the study. Four cores were included from each of the remaining five locations (20 cores total).

2.3. Experimental design

Soil cores were brought back to the lab and the soil moisture was slowly increased to approximately 25% volumetric moisture. Excluded cores were utilized to determine initial moisture so experimental cores could remain undisturbed for the entirety of the experiment. Based on retrieval location, depth of mineral soil (determined in the field adjacent to core), and weight, cores were paired together resulting in two pairs for each retrieval location, one pair for each opening time. On August 24th, 2014, one juvenile (unclitellated, likely of the same species) and three adult (clitellated) worms were placed in reverse osmosis (RO) water, blotted with filter paper, weighed, and placed randomly in one core from each of the ten pairs. Density of worms added (approximately 510 m^{-2}) was roughly 3.2 times the highest density of endogeic worms found by Knowles (2015) in any of the plots surveyed in the Hinesburg Town Forest (190 m^{-2}) with a total weight ranging from 4.85 g to 6.05 g. The experimental cores were incubated at 15 °C, surrounded by a series of non-experimental cores to account for possible edge effects, and covered with black plastic to reduce light. Cores were kept at a constant weight, with tap water being added every 3–7 days to make up weight lost over that time. On

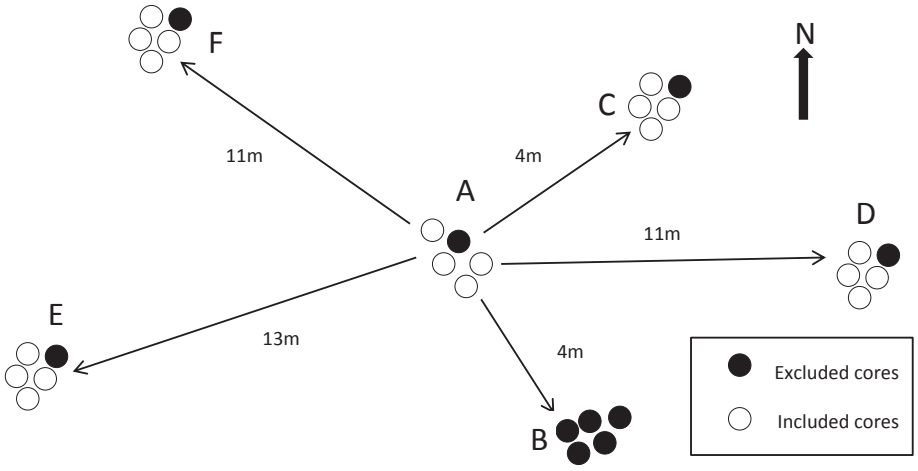

Fig. 1. Representation of core retrieval area. Plot center (A) chosen based on slope and homogeneity of surrounding area. Subsequent locations determined by randomly assigned distance and direction from location A.

September 25th, 2013 (4 weeks after inoculation) and January 8th, 2014 (4 months after inoculation), one pair of cores was randomly chosen from each retrieval location, deconstructed, and analyzed.

2.4. Core deconstruction

Prior to deconstruction, cores were allowed to dry for 3 days at four weeks and 12 days at four months to reduce the possibility of disturbing the soil while it was very moist. Cores were opened by cutting through the PVC on two sides with a table saw, leaving the mineral soil intact. Care was taken not to disturb the mineral soil; however both litter loss and PVC contamination was seen in the Oi horizon. During deconstruction, cores were carefully re-opened and soil was moved away from the core edge with a knife. When worms were encountered they were immediately removed and placed in RO water until the completion of core deconstruction, approximately 30 min. Depth of soil horizons were recorded before the organic layers (Oi and combined Oe/Oa) were removed and placed into aluminum pans to dry. The remaining B-horizon mineral soil was gently passed through an 8 mm sieve and weighed. A small sample was removed for moisture analysis before the remaining soil was laid out to air dry for 48 h. Representative, triplicate 50-g samples were saved for further analyses. Coarse fragments and roots (>8 mm) were separated, washed, and laid out to dry. Worms, if present, were blotted with filter paper and weighed. Dry weights of the empty core, Oi, Oe/Oa, coarse fragments, and roots were recorded after 48 h.

2.5. Aggregate analysis

2.5.1. Water stable aggregate fractionation

A complete synopsis of the following procedures may be found in Fig. 2. Wet sieving was replicated four or three times (4-week and 4-month time points, respectively) for each core according to the methods found in Six et al. (2002), modified from Elliott (1986). This process was done after 8 weeks and 1 week of air drying for the 4 week and 4 month incubations respectively. Briefly, 50 g of air dried soil was submerged in RO water on top of a 2000 μm sieve for 5 min to induce slaking. The sieve was moved in and out of the water manually, in approximate 3 cm circular motions, 50 times over the course of 2 min (synchronized to a metronome), and the material that remained on the sieve was back washed into a clean container with RO water. Any floating particulate organic matter (POM) from the 2000 μm sieve was decanted and discarded. What remained after decanting was the large macroaggregate fraction (lgMA, >2000 μm). The particles that passed through the 2000 μm sieve were transferred to a 250 μm sieve and the sieving procedure was repeated. What was retained on the 250 μm sieve, the small macroaggregate fraction (smMA, 250–2000 μm), was back washed into a clean container with RO water. The particles that passed through the 250 μm sieve were transferred to a 53 μm sieve and the process repeated. What was retained on the 53 μm sieve, the free microaggregate fraction (fmA, 53–250 μm), was back washed into a clean container with RO water, and the silt and clay fraction (<53 μm), was discarded. The lgMA, smMA, and mA fractions were all back washed through coffee filters (modification, *Home 360 Hannaford Brand #2 cone filters*) that were then placed in 65 °C for 18–24 h. Once dry, the fractions were weighed and carefully brushed away from the coffee filters to be stored in plastic bags until further processing.

2.5.2. Microaggregate isolation

Microaggregate fraction, mA, occluded in the lgMA and smMA fractions were released from the larger aggregate fractions by the method of Six et al. (2000). From the above fractionation method duplicates, each macroaggregate fraction was combined into 8 g samples. These samples were slaked in RO water on top of a 250 μm sieve for 20 min. The submerged 250 μm sieve was then shaken vigorously by hand with 50 stainless steel bearings (4 mm diameter) while a continuous flow of RO water passed over the apparatus. This was done in order to wash the smaller material through the sieve quickly, and avoid the further breakup of the mA. After 4 min of shaking, the larger aggregates remaining on the sieve were gently prodded with a soft rubber stopper. The prodding, combined with shaking and water flow, continued until all but coarse sand and POM (lgPOM, >250 μm or smPOM, 250–2000 μm, dependent on starting fraction) had passed through the sieve. Material that passed through the 250 μm sieve was collected on a 53 μm sieve and wet sieved for 2 min, as described above, resulting in the stable mA occluded within the lgMA (mAlg, 250-53 μm) or smMA (mAsm, 250-53 μm), depending on the starting material. The material that passed through the 53 μm sieve was operationally defined as the silt and clay fraction and was discarded. All retained fractions were back washed through coffee filters before being dried and weighed.

2.5.3. Density fractionation of light fraction (LF)

The light organic matter fraction (LF) is composed of non-complexed decomposing plant and animal tissues, believed to be

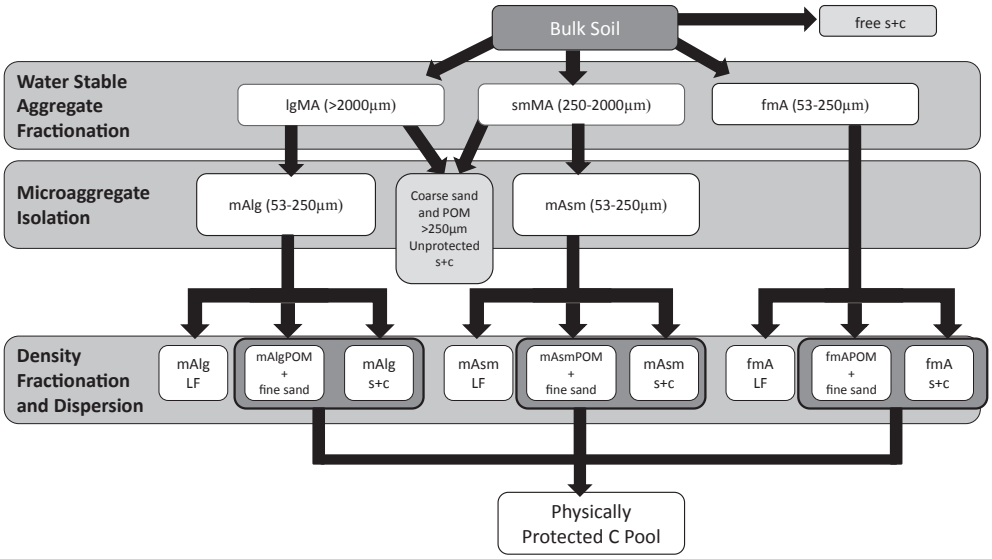

Fig. 2. Diagram representing processing steps and functional soil fractions obtained from each. lgMA: large macroaggregate, smMA: small macroaggregates, fmA: free microaggregates, mAlg: microaggregates occluded in large macroaggregates, mAsm microaggregates occluded within small macroaggregates, s + c: silt and clay fraction (<53 μm), LF: organics (<1.85 g cm^{-3}) between mA fractions, POM: particulate organic matter within mA fractions.

more labile and non-protected (Evans et al., 2001). It is defined as any fraction that is lighter than the mineral fraction of the soil because the more recalcitrant SOM becomes intimately associated with mineral portions of the soil during the humification process (Barrios et al., 1996). Conversely, any fraction having a density less than the mineral fraction and that is not occluded within a mA, is assumed to be free LF, and more bio-available. In order to get a proper assessment of the amount of protected C found within the mA (fmA, mAsm, mAlg), the between-mA LF must be removed prior to C analysis. The method for this process is outlined in Six et al. (1998), which was modified from Elliott and Cambardella (1991).

The mA fractions were oven dried at 70 °C for 18–24 h. After cooling to room temperature in a desiccator, the samples were weighed and added to a 50-mL graduated conical centrifuge tube already filled with 25 mL of 1.85 g cm^{-3} (±0.01 g cm^{-3}) sodium polytungstate (SPT). This mixture was gently inverted 10 times bringing the sample into suspension without disruption of the mA structure, the goal being to remove only the LF outside of any mA. The material remaining on the cap and sides of the centrifuge tube was rinsed into the suspension with an additional 10 mL SPT, and after 20 min at equilibrium the samples were centrifuged at 1174*g* RCF for 60 min. The samples sat at room temperature for 18–24 h in order to allow materials to settle completely before the floating material (free LF), as well as most of the SPT, was aspirated onto a 10-μm nylon mesh, rinsed thoroughly with RO water to remove any remaining SPT, and transferred to a small aluminum pan. Samples were dried at 60 °C for 18–24 h, cooled to room temperature in a desiccator, and weighed.

2.5.4. Dispersion

The heavy fraction (HF) remaining on the bottom of the conical tube after aspiration was rinsed twice with 50 mL of RO water in order to clean away any remaining SPT. The sample was mixed with 35 mL of 0.5% hexametaphosphate and dispersed by shaking on a reciprocal shaker for 18 h. The dispersed HF was then passed through a 53-μm sieve, rinsed with RO water, and wet sieved for 2 min. The material remaining on the sieve was quantified as the intra-microaggregate POM (fmAPOM, mAlgPOM or mAsmPOM), and fine sand. This fraction was transferred to a small aluminum pan and dried 18–24 h at 60 °C. The material passing through the sieve (fmAs + c, mAlgs + c, mAsms + c) was discarded.

2.5.5. Calculations

Due to our assessment of lgMA (>2000 μm), the non-soil fraction (defined as coarse fragments and free POM > 2000 μm) was calculated and subtracted from the total soil starting weight for all calculations. Weights for all aggregate sizes were corrected for sand content of the same size class (Six et al., 2000). All silt and clay sized fractions were discarded and values for these fractions were calculated by mass balance.

2.6. Lab and statistical analysis

2.6.1. Nutrient analysis

Basic soil nutrient analysis was carried out on the B horizons of all cores following the procedures of the University of Maine Soil Testing Service and the University of Vermont Agricultural and Environmental Testing Laboratory. Soil samples were dried at 45 °C, crushed to pass a 2 mm sieve, and extracted with Modified Morgan's solution (0.62 *M* NH_4OH + 1.25 *M* CH_3COOH; 4 g, 20 mL, shake 15 min). After filtering through 2-μm medium-speed paper, they were analyzed for phosphorus (molybdate blue procedure) and macro and micronutrients (inductively coupled plasma spectroscopy). Soil pH was determined in 0.01 M $CaCl_2$ 2:1 *v*:*v*; water pH was estimated by adding 0.6 pH units to the salt value. Organic matter was determined by loss on ignition at 375 °C (Wolf and Beegle, 2011).

2.6.2. Carbon analysis

Total carbon analysis was conducted on a Flash EA 1112 NC Analyzer (CE Elantech, Lakewood, NJ). The bulk soil and the lgMA fraction were ground by hand to pass through a 250 μm sieve, with coarse rocks and twigs >2000 μm removed. All fractions were oven dried to a constant weight at 60 °C prior to analysis. Sub-samples of 20–80 mg from the mineral fractions, and 2–5 mg of the organic fractions were weighed into tin capsules in duplicate. Analyzer calibration and quality control was conducted using soils obtained from the North American Proficiency Testing program.

Any quality control sample with greater than 10% error had

samples immediately preceding and following it re-run, along with any samples in which duplication had greater than 10% error. A quality control run was included at the end of all sample processing for which 10% of all samples were randomly chosen and re-run.

2.6.3. Statistical analysis

All comparisons between the control and earthworm treatments were within an aggregate class and based on the pairing outlined in section 2.3. We used the Matched Pairs paired *t*-test (JMP 9.0, SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Earthworm survival and core measurements

All specimens survived and were active at the end of 4 weeks, though average fresh weight was reduced by approximately 4%. At the end of 4 months, all worms were recovered, with approximately 30% found in diapause, as indicated by specimens being curled into tight balls. All WW cores at 4 months had new juveniles present, accounting for an average fresh weight of 0.48 g per core. Even with this added juvenile weight, average fresh weight decreased by approximately 32%. This reduction in fresh weight, as well as the noted diapause behavior, was possibly due to the length of time cores were allowed to dry prior to deconstruction, which was 12 days at 4 months as compared to 3 days at 4 weeks. Length of drying was increased in the 4-month cores to lessen the likelihood of disturbance during core opening.

3.2. Earthworm effect on physical proportion of aggregates

At the end of 4 weeks of incubation no significant differences in aggregate properties were seen. After 4 months of incubation the main effect of earthworm activity was an increase in the lgMA (>2000 μm) fraction (Fig. 3). Cores with earthworms had 67% more lgMA than paired cores without earthworms. Through this action, the smMA (250–2000 μm) fraction was reduced by 10% (Table 2).

At 4 months little effect was seen on the proportion of total soil dry mass comprised of the mA fractions (Fig. 4b). The mAlg and the mAsm proportions were significantly higher and lower respectively due to the shifts in the lgMA and smMA fractions (Fig. 3). These shifts resulted in no impact on the soil's total mA (Table 2).

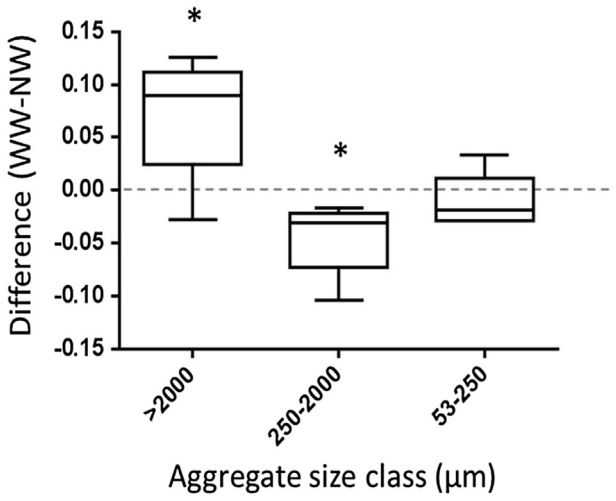

Fig. 3. Mean difference in aggregate size proportion of soil dry mass in paired cores after 4-months incubation. lgMA: >2000 μm, smMA: 250–2000 μm, fmA: 53–250 μm n = 5, (*) statistically significant at P < 0.05, bars represent minimum and maximum difference from core pairs.

3.3. Earthworm effect on mA associated C

There were no significant earthworm effects on soil aggregate C after 4 weeks of incubation. While it was apparent that the earthworms were active within the soil, only a small proportion of the total mineral soil volume seemed to have been ingested by the earthworms over the 4 weeks. Any effect the earthworms had within the drilosphere was overshadowed by the bulk soil properties.

At 4 months the A horizon in the WW cores was more prominent as the result of earthworm activity, presumably through incorporation of the Oa horizon (Table 1). The mineral portion of the WW cores contained, on average, 26.01 g C $kg_{bulksoil}^{-1}$ (±1.98 SE) while NW cores contained 16.22 gC $kg_{bulksoil}^{-1}$ (±0.55 SE), an earthworm effect on total mineral soil C of 60%. The protected pool of C, defined as the within-mA POM and mA associated silt and clay-sized fraction (s + c), increased by an average of 5.16 g C $kg_{bulksoil}^{-1}$ (±0.23 SE), or 55%. Of this increased protected C pool, 95% was due to changes in the mA occluded within the lgMA fraction (mAlgs + c and mAlgPOM), which increased 2.75 g $C_{fraction}$ $kg_{bulksoil}^{-1}$ (±0.27 SE), and 2.16 g $C_{fraction}$ $kg_{bulksoil}^{-1}$ (±0.31 SE), respectively. The mA protected pool explained 53% of the difference in total mineral soil C between the cores, while the mA associated LF, an unprotected pool, explained another 33%. The increase in the mA protected C pool was seen despite there being no difference in the soil physical proportion of mA (Fig. 4).

3.3.1. C of the within-mA associated silt and clay (s + c) and POM

The protected C of the mA associated silt and clay size fraction (s + c) and mA associated POM was increased in the mAlg fraction by 3 fold and 9 fold respectively (Fig. 5). This increase, along with changes of the distribution in the fmA and mAsm fractions (Fig. 4) resulted in an average increase of 3.01 g $C_{fraction}$ $kg_{bulksoil}^{-1}$ (±0.54 SE) for the total mA associated s + c, an increase of approximately 40%, and 2.14 $gC_{fraction}$ $kg_{bulksoil}^{-1}$ (±0.45 SE) for the total protected mA associated POM, an increase of almost 80%. The increase in the mA s + c and mA protected POM fractions account for 60% and 40% respectively of the increase in total mA protected C.

3.3.2. Between-mA associated light fraction (LF)

The total between-mA associated POM (POM occluded within lgMA or smMA that is not occluded within mA), quantified by the light fraction (LF) obtained from the mA, mAlg, and mAsm fractions prior to dispersion, was 3 fold greater in the WW cores (0.98 ± 0.09 $gC_{fraction}$ $kg_{bulksoil}^{-1}$ (NW), 4.21 ± 0.05 $gC_{fraction}$ $kg_{bulksoil}^{-1}$ (WW) p = 0.001). This difference was due primarily to the mAlg LF (Table 3) which increased from 0.16 ± 0.03 $gC_{fraction}$ $kg_{bulksoil}^{-1}$ to 3.07 ± 0.45 $gC_{fraction}$ $kg_{bulksoil}^{-1}$, an almost 20-fold increase accounting for 90% of the total LF C difference. As a function of total mineral soil C, the total LF accounted for 16% of the total mineral soil C in the WW cores and 6% in the NW.

4. Discussion

4.1. Aggregate proportions

At 4 months, the presence of *A. tuberculata* significantly increased the proportion of lgMA. No change was noted after 4 weeks. While many researchers have found that endogeic earthworms increase proportions of larger aggregates after as little as 3 weeks (Bossuyt et al., 2005; Mummey et al., 2006) these studies almost always utilize an earthworm density much higher than what is found in nature, exaggerating the noted effects (Sanchez-de Leon et al., 2014). Mummey et al. (2006) used 2 adult earthworms

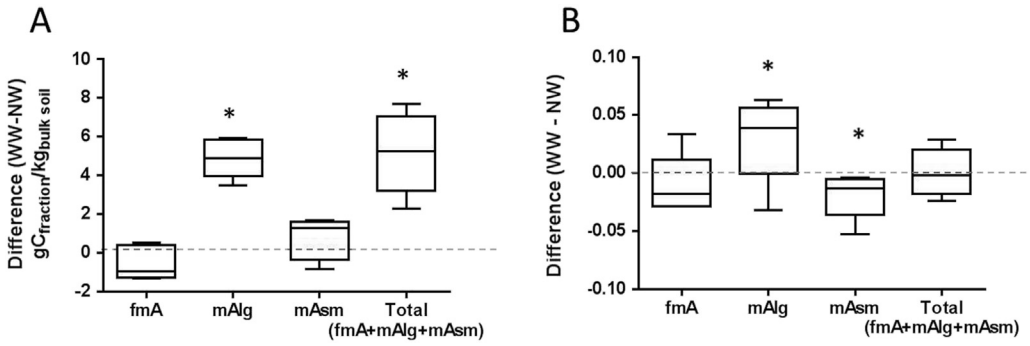

Fig. 4. A.) Difference in paired cores (WW-NW) of $gC_{fraction}\ kg_{bulk\ soil}^{-1}$. B.) Difference in paired cores (WW-NW) in the proportion of total soil dry mass $g_{fraction}\ kg_{bulk\ soil}^{-1}$. n = 5, (*) statistically significant a $P < 0.05$, bars represent minimum and maximum differences from core pairs.

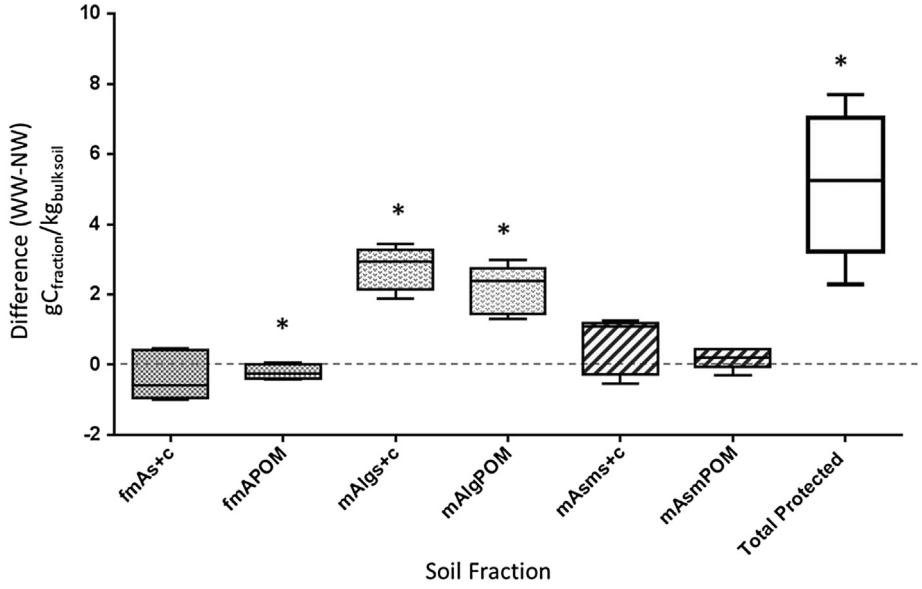

Fig. 5. Difference in paired cores (WW-NW) of all quantified protected C pools., fmA: free microaggregates, mAlg: microaggregates occluded in large macroaggregates, mAsm microaggregates occluded within small macroaggregates, s + c: silt and clay fraction (<53 μm), POM: particulate organic matter within mA fractions. n = 5, (*) statistically significant a $P < 0.05$, bars represent minimum and maximum differences from core pairs.

Table 1
Conditions of 4-month core soil properties at the time of deconstruction. Soil moisture measured gravimetrically.

Plot ID	Experimental group	Soil dry weight (g)	Moisture (%)	Worm density (Worms m^{-3})	CF (>8 mm) weight (g)	Horizon depth (cm)			
						Oi	Oe	Oa	A
A	NW	1172.8	20.2		85.6	1.5	2.5	3.0	1.0
A	WW	1189.2	22.9	2400	103.4	1.5	2.0	0.0	4.0
C	NW	1117.9	18.6		356.4	2.5	1.0	1.5	5.0
C	WW	1066.8	20.0	2550	434.4	2.0	1.5	0.0	7.0
D	NW	922.8	20.9		82.8	2.5	1.0	2.0	2.5
D	WW	1041.1	22.6	2940	110.4	2.5	1.5	0.0	7.0
E	NW	1369.3	21.3		83.4	1.5	1.0	3.0	4.0
E	WW	1463.9	23.1	1820	64.2	2.0	1.5	0.5	6.0
F	NW	1238.3	17.1		142.1	3.5	1.0	3.0	2.5
F	WW	1222.9	17.2	2120	241.6	3.0	1.0	0.0	3.5

in just 100 g of mineral soil while Bossuyt et al. (2005) used 6 adult earthworms in just 150 g of mineral soil. In our paired study we placed 4 worms in approximately 1–1.5 kg mineral soil, which while three times the highest density seen in an extensive survey recently conducted in Knowles (2015), is about 10x less than the above mentioned studies, possibly explaining why we did not see the significant effects expected at 4 weeks. An alternative explanation for the lack of effect after 4 weeks is that, even though endogeic, the worms could have initially been more active in the organic surface horizons of the intact cores, moving into the mineral soil later in the study. Another factor related to worm density is that even though an equal number of worms were placed in each experimental core (3 adult, 1 juvenile), the volume of soil was somewhat variable between pairs, and the level of earthworm

Table 2
Average difference between paired cores with worms (WW) and without worms (NW) as a proportion of total soil dry weight. $n = 5$, bold represents statistical significance at $P < 0.05$.

Fraction	Mean proportion of total soil dry mass (±SE)					
	lgMA	smMA	fmA	mAlg	mAsm	Total mA
WW	0.183 ± 0.012	0.395 ± 0.010	0.087 ± 0.006	0.081 ± 0.007	0.165 ± 0.004	0.333 ± 0.007
NW	0.110 ± 0.017	0.439 ± 0.034	0.098 ± 0.022	0.051 ± 0.012	0.184 ± 0.006	0.332 ± 0.007
WW-NW	(+) **0.072 ± 0.026**	(−) **0.044 ± 0.016**	(−) 0.010 ± 0.011	(+) **0.030 ± 0.016**	(−) **0.020 ± 0.009**	(+) 0.0004 ± 0.009

Table 3
Mean difference of earthworm effect on $gC_{fraction}\ kg^{-1}_{bulk\ soil}$ and C/N ratio for all quantified mA fractions. fmA: mA not occluded within macroaggregation, mAlg: mA occluded within the lgMA fraction, mAsm: mA occluded within the smMA fraction, s + c: protected silt and clay component of mA, POM: protected particulate organic matter occluded within mA, LF: unprotected organics found between mA, TP: Sum of protected fractions (s + c and POM). n= 5, bold represents statistical significance at $P < 0.05$.

Fraction	C ($g_{fraction}\ kg^{-1}_{bulk\ soil}$)			C/N Ratio	
	Mean ± SE		Mean difference	Mean ± SE	
	WW	NW	(WW-NW)	WW	NW
Total Soil	26.01 ± 1.98	16.22 ± 0.55	(+) **9.79 ± 2.15**	16.87 ± 0.75	16.13 ± 0.65
fmA s + c	1.79 ± 0.07	2.11 ± 0.32	(−) 0.32 ± 0.31	14.53 ± 0.63	14.28 ± 0.86
fmA POM	0.35 ± 0.05	0.54 ± 0.12	(−) **0.19 ± 0.09**	21.93 ± 2.69	22.08 ± 1.91
fmA LF	0.20 ± 0.01	0.22 ± 0.04	(−) 0.01 ± 0.04	26.00 ± 2.12	25.51 ± 2.07
fmA TP	2.14 ± 0.12	2.66 ± 0.42	(−) 0.52 ± 0.39	15.19 ± 0.59	15.27 ± 0.85
mAlg s + c	4.17 ± 0.15	1.42 ± 0.23	(+) **2.75 ± 0.27**	16.62 ± 0.93	16.54 ± 0.89
mAlg POM	2.46 ± 0.30	0.29 ± 0.06	(+) **2.16 ± 0.31**	17.61 ± 0.48	17.75 ± 1.39
mAlg LF	3.07 ± 0.45	0.16 ± 0.03	(+) **2.92 ± 0.45**	**21.54 ± 0.42**	**28.72 ± 1.85**
mAlg TP	6.62 ± 0.32	1.71 ± 0.28	(+) **4.91 ± 0.45**	16.99 ± 0.70	16.70 ± 0.97
mAsm s + c	4.76 ± 0.37	4.18 ± 0.22	(+) 0.58 ± 0.36	15.28 ± 0.62	14.54 ± 0.65
mAsm POM	1.04 ± 0.09	0.86 ± 0.09	(+) 0.19 ± 0.13	19.19 ± 0.80	17.77 ± 0.93
mAsm LF	0.93 ± 0.06	0.61 ± 0.07	(+) **0.32 ± 0.07**	26.56 ± 1.29	27.39 ± 1.22
mAsm TP	5.81 ± 0.46	5.04 ± 0.26	(+) 0.77 ± 0.48	15.80 ± 0.63	15.00 ± 0.70
Total s + c	10.72 ± 0.42	7.71 ± 0.26	(+) **3.01 ± 0.54**	15.65 ± 0.68	14.81 ± 0.69
Total POM	3.85 ± 0.32	1.69 ± 0.17	(+) **2.14 ± 0.45**	18.25 ± 0.48	18.53 ± 1.02
Total Protected	14.57 ± 0.68	9.41 ± 0.33	(+) **5.16 ± 0.23**	16.26 ± 0.60	15.35 ± 0.74
Total LF	4.21 ± 0.50	0.98 ± 0.09	(+) **3.22 ± 0.45**	**22.61 ± 0.55**	**27.11 ± 1.21**

effect appeared to vary with this earthworm density. The pair of cores from sampling plot E contained the lowest earthworm density by soil volume (1820 worms m^{-3}) and also showed a minor decrease in lgMA, an opposite effect as was seen in all other pairs. This contrary effect, likely due to low earthworm density and soil property variation, was not an outlier and was therefore included in all statistical analysis; however its inverse effect influenced several other fractions. Had the incubation time been longer, or the earthworm density higher in the E pair, we speculate that the mean effects on mA proportions at 4 months would have been more pronounced.

Contrary to our hypothesis, earthworm presence did not increase the total proportion of mA in the soil, even after 4 months. In this particular soil the impact on the total mA pool was undetectable, though C data suggest that much of the soil's mA within the experimental cores originated from earthworm ingestion. The mA-stabilized C pool could not have increased in the WW treatment without the breakdown and subsequent reformation of mA. The addition of organic binding agents, in the form of earthworm mucus polysaccharides and microbial exudate, along with peristaltic pressure along the earthworm alimentary canal, has been shown to increase the proportion of stable mA (Bossuyt et al., 2005; McCarthy et al., 2008; Pulleman et al., 2005; Sanchez-de Leon et al., 2014). For a related study (Knowles, 2015), WAT was included with eight other Vermont forest sites that underwent the aggregate analysis outlined above, and WAT contained almost 60% more mA than the average of the other sites (0.261 ± 0.015 kg kg^{-1} (WAT), 0.169 ± 0.004 kg kg^{-1} (Remaining sites, $n = 8$), $P < 0.001$). All soils were relatively low in clay content and similar in texture (loams and sandy loams), and no differential influence of texture on aggregation was likely. There are not many data on the total mA proportions (free and occluded) in forest soils and a typical value is unknown. It is possible that the mA proportion present in the WAT soils was already high enough that the net effect of earthworm ingestion and reformation of mA was slight.

While the net effect was negligible, the mA proportions shifted among fractions. The largest change was an increase in mA occluded with lgMA, coincident with the overall lgMA increase. The proportion of total soil composed of mA occluded within the smMA fraction (mAsm) was significantly decreased ($P < 0.05$) due primarily to the significant reduction in the smMA fraction. The fmA fraction trended slightly lower, possibly as these structures became occluded within the macroaggregate fractions, however this effect was not significant. Yavitt et al. (2015) found a significant decrease in the fmA fraction in an earthworm-invaded northern hardwood forest, similar to ours, and a concomittant increase in mA within macroaggregates.

We anticipated that the proportion of total mA would increase primarily through an increase in the proportion of mA occluded within macroaggregates. Earthworms have been shown to facilitate the creation of mA within macroaggregates in the field (Jongmans et al., 2001; Pulleman et al., 2005) as well as in the lab (Bossuyt et al., 2005; Mummey et al., 2006) and mA formed within macroaggregates are thought to be the primary mechanism by which overall mA are increased in soils (Oades, 1984; Six et al., 2000). We saw no effect of earthworms on the proportion of mA within macroaggregates, however; in general the lgMA fraction mass was composed of 6% more mA mass than the smMA fraction, regardless

of earthworm presence (0.347 ± 0.015 kg_{mAlg} kg^{-1}_{lgMA}, 0.285 ± 0.005 kg_{mAsm} kg^{-1}_{smMA}, n = 40, P < 0.001). Perhaps if the study had been allowed to continue for a longer period of time the increase in lgMA alone may have had influence on the proportion of mA in the soil, even if these structures did not themselves have a higher proportion of occluded mA.

4.2. Protected C

Analysis was not done for the forest floor horizons or C mineralization and so no balance of total core C was calculated and the amount of C lost to mineralization is unknown. Earthworms are known to relocate C downward into the mineral soil, and the objective of the study was to determine where within the soil structure this species of earthworm would allocate the relocated C. We observed that 53% of C mixed into the mineral soil by earthworms was directed into the stabilized pools within mA structure, while only 33% was found as unprotected POM. Of this unprotected POM, 90% was occluded within the lgMA fraction and could be assumed to exhibit minor protection relative to bulk soil C. As this fraction continues to decompose it will likely become nucleating sites for future mA creation (Baldock, 2002; Six et al., 2000). Only 14% of the difference in total mineral soil C between the WW and NW cores was unaccounted for by the pools measured in this study. This remaining pool contains the unprotected POM (250–2000 μm) occluded within the lgMA and smMA fractions, which would have been included in the total mineral soil C measurement, but was removed during the mA isolation procedure (see Fig. 2).

The mineral soil from WAT used in this study inherently contains very low amounts of SOC (approximately 16 g kg^{-1}), and with only 46 Mg C ha^{-1} its mineral soil is substantially lower than the United States northeastern forest mineral soil average of approximately 90 Mg C ha^{-1} (Birdsey, 1992). The reason for the low amount of C at WAT could be influenced by prior land use. Local records suggest that it was near the site of a surface iron ore mine active in the very early 1800's (Charles Cogbill, personal communication). Disturbance from this industry may have had a long-lasting effect on C stores. Another possible explanation is that the relatively high proportion of stable microaggregates in the WAT soils inhibits C accumulation. In ecosystems where aggregate turnover is slow, incoming organic materials may be degraded before becoming occluded and protected within aggregates (Plante and McGill, 2002; Six et al., 2004). Freeze thaw cycles, wind throw, and bioturbation are the primary modes of soil mixing in temperate hardwood forests, with mineral soil C originating primarily from dissolved organic carbon (DOC), decomposing root tissues, and microbial biomass (Currie et al., 2002). Although lower rates of aggregate turnover often lead to C accumulation (e.g. Six et al., 2000), it is the balance between C inputs and aggregate turnover that determines the steady-state C pools (Plante and McGill, 2002). Limited C inputs along with limited soil mixing and a high proportion of physically stable C-deficient mA could be a partial explanation for the low concentration of SOC at the WAT site.

The C occluded within mA is not protected indefinitely. The binding agents maintaining soil structure are not inert and are therefore subject to decomposition (Frey, 2005). As the structural stability of aggregates becomes compromised, occluded C may become available for microbial degradation (Baldock, 2002). Theoretically, for every system there exists an ideal rate of aggregate turnover that would allow for organic matter occlusion and protection, while still limiting the re-exposure of previously occluded C (Plante and McGill, 2002). Due to the high concentration of mA and low level of turnover present in many forests, it may be aggregate turnover, rather than aggregate creation, that will enhance C stabilization in forests showing the potential for C sequestration. Earthworm invasion, with its preferential occlusion of C within castings, may potentially be able to accomplish that. However, it is still unknown how the continuous ingestion of castings, which would occur in highly invaded forests over long periods, will alter the C residence time within mA. Yavitt et al. (2015) found that isotope-labeled litter was incorporated by invasive earthworms into macroaggregates but that much of the C was mineralized within three years. Although, most of the C loss appeared to be from POM and the increase in mA within the macroaggregates may still promote long-term C stability.

4.3. Conclusions

We found that *Aporrectodea tuberculata* significantly increased total mineral soil C, primarily through the relocation of the Oa horizon. The majority of this relocated C was allocated into newly formed mA, and was therefore considered protected with an increased residence time. We found an increase in the proportion of macroaggregates with no change in the proportion of total mA, though C data suggested that much of the mA fraction underwent earthworm ingestion. We suggest that for soils similar to the one studied here, which are C limited and have a high proportion of soil mass composed of stable aggregates, any increased aggregate turnover mediated by endogeic earthworms may increase the pool of sequestered C in the long term, though initially a C loss is likely.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.06.016.

References

Adu, J., Oades, J., 1978. Physical factors influencing decomposition of organic materials in soil aggregates. Soil Biol. Biochem. 10, 109–115.
Alban, D., Berry, E., 1994. Effects of earthworm invasion on morphology, carbon, and nitrogen of a forest soil. Appl. Soil Ecol. 1, 243–249.
Baldock, J.A., 2002. Interactions of organic materials and microorganisms with minerals in the stabilization of soil structure. In: Huang, P.M., Bollag, J.M., Senesi, N. (Eds.), Interactions between Soil Particles and Microorganisms. John Wiley & Sons, pp. 85–131.
Barrios, E., Buresh, R., Sprent, J., 1996. Organic matter in soil particle size and density fractions from maize and legume cropping systems. Soil Biol. Biochem. 28, 185–193.
Birdsey, R., 1992. Carbon Storage and Accumulation in United States Forest Ecosystems. Gen. Tech. Rep. WO-59. U.S. Department of Agriculture, Forest Service, Washington Office, Washington D.C.
Bohlen, P., Pelletier, D., Groffman, P., Fahey, T., Fisk, M., 2004. Influence of earthworm invasion on redistribution and retention of soil carbon and nitrogen in northern temperate forests. Ecosystems 7, 13–27.
Bohlen, P., Scheu, S., 2004. Non-native invasive earthworms as agents of change in northern temperate forests. Front. Ecol. Environ. 2, 427–435.
Bossuyt, H., Six, J., Hendrix, P., 2005. Protection of soil carbon by microaggregates within earthworm casts. Soil Biol. Biochem. 37, 251–258.
Bouche, M.B., 1977. Strategies lombriciennes. In: Lohm, U., Persson, T. (Eds.), Soil Organisms as Components of Ecosystems. Ecological Bulletin, Stockholm, pp. 122–132.
Brown, G., Barois, I., Lavelle, P., 2000. Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. Eur. J. Soil Biol. 36, 177–198.
Currie, W., Yanai, R., Piatek, K., Prescott, C., Goodale, C., 2002. Processes affecting

carbon storage in the forest floor and in downed woody debris. In: Kimble, J. (Ed.), The Potential for US Forest Soils to Sequester Carbon and Mitigate the Greenhouse Effect. Lewis Publishers, Boca Raton FL, pp. 135–157.
Davis, R.B., Jacobson, G.L., 1985. Late glacial and early Holocene Landscapes in northern New England and adjacent areas of Canada. Quat. Res. 23, 341–368.
Dempsey, M., Fisk, M., Fahey, T., 2011. Earthworms increase the ratio of bacteria to fungi in northern hardwood forest soils, primarily by eliminating the organic horizon. Soil Biol. Biochem. 43, 2135–2141.
Doube, B., Brown, G., 1998. Life in a Complex Community: functional interactions between earthworms, organic matter, microorganisms, and plants. In: Edwards, C. (Ed.), Earthworm Ecology, pp. 179–211.
Drake, H., Horn, M., 2007. As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. Annu. Rev. Microbiol. 61, 169–189.
Dungait, J., Hopkins, D., Gregory, A., Whitmore, A., 2012. Soil organic matter turnover is governed by accessibility not recalcitrance. Glob. Change Biol. 18, 1781–1796.
Elliott, E., 1986. Aggregate structure and carbon, nitrogen, and phosphorus in native and cultivated soils. Soil Sci. Soc. Am. J. 50, 627–633.
Elliott, E.T., Cambardella, C.A., 1991. Physical separation of soil organic matter. Agric. Ecosyst. Environ. 34, 407–419.
Evans, J., Fernandez, I., Rustad, L., Norton, S., 2001. Methods for Evaluating Carbon Fractions in Forest Soils: a Review (Orono, ME).
Frey, S., 2005. Soil aggregation: microbial aspects. In: Hillel, D., Rosenzweig, D., Powlson, K., Scow, K., Singer, M. (Eds.), Encyclopedia of Soils in the Environment. Academic Press, London, pp. 22–28.
Gates, G., 1976. More on Oligochaeta distribution in north America. Megadrilogica 2, 1–6.
Groffman, P., Bohlen, P., Fisk, M., Fahey, T., 2004. Exotic earthworm invasion and microbial biomass in temperate forest soils. Ecosystems 7, 45–54.
Hale, C., Frelich, L., Reich, P., 2005. Exotic European earthworm invasion dynamics in northern hardwood forests of Minnesota, USA. Ecol. Appl. 15, 848–860.
Jongmans, A.G., Pulleman, M.M., Marinissen, J.C.Y., 2001. Soil structure and earthworm activity in a marine silt loam under pasture versus arable land. Biol. Fertil. Soils 33, 279–285.
Juillerat, J., 2011. Influence of Forest Composition on Mercury Deposition in Litterfall and Subsequent Accumulation in Soils (M.S. thesis). University of Vermont, 145 pages.
Knowles, M.E., 2015. Earthworm Presence in Northern Forests: Impact on Distribution of Soil Carbon Within Aggregate Fractions (M.S. thesis). University of Vermont, 125 pages.
Lubbers, I., van Groenigen, K., Fonte, S., Six, J., Brussaard, L., van Groenigen, J., 2013. Greenhouse-gas emissions from soils increased by earthworms. Nat. Clim. Change 3, 187–194.
Lyttle, A., Yoo, K., Hale, C., Aufdenkampe, A., Sebestyen, S., 2011. Carbon mineral interactions along an earthworm invasion gradient at a Sugar Maple Forest in Northern Minnesota. Appl. Geochem. 26, S85–S88.
McCarthy, J., Ilavsky, J., Jastrow, J., Mayer, L., Perfect, E., Zhuang, J., 2008. Protection of organic carbon in soil microaggregates via restructuring of aggregate porosity and filling of pores with accumulating organic matter. Geochim. Cosmochim. Acta 72, 4725–4744.
Mummey, D., Rillig, M., Six, J., 2006. Endogeic earthworms differentially influence bacterial communities associated with different soil aggregate size fractions. Soil Biol. Biochem. 38, 1608–1614.
Oades, J., 1984. Soil organic matter and structural stability: mechanisms and implications for management. Plant Soil 76, 319–337.
Plante, A.F., McGill, W.B., 2002. Soil aggregate dynamics and the retention of organic matter in laboratory-incubated soil with differing simulated tillage frequencies. Soil Tillage Res. 66, 79–92.
Pulleman, M., Six, J., Uyl, A., Marinissen, J.C.Y., Jongmans, A.G., 2005. Earthworms and management affect organic matter incorporation and microaggregate formation in agricultural soils. Appl. Soil Ecol. 29, 1–15.
Ridge, J.C., 2004. Quaternary Glaciations-extent and Chronology - Part II: North America, Developments in Quaternary Sciences, Developments in Quaternary Sciences. Elsevier.
Sanchez-de Leon, Y., Lugo-Perez, J., Wise, D., Jastrow, J., Gonzalez-Meler, M., 2014. Aggregate formation and carbon sequestration by earthworms in soil from a temperate forest exposed to elevated atmospheric CO2: a microcosm experiment. Soil Biol. Biochem. 68, 223–230.
Schmidt, M.W., Torn, M., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I. a, Kleber, M., Kögel-Knabner, I., Lehmann, J., Manning, D., Nannipieri, P., Rasse, D., Weiner, S., Trumbore, S., 2011. Persistence of soil organic matter as an ecosystem property. Nature 478, 49–56.
Shipitalo, M., Protz, R., 1989. Chemistry and micromorphology of aggregation in earthworm casts. Geoderma 45, 357–374.
Six, J., Bossuyt, H., Degryze, S., Denef, K., 2004. A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. Soil Tillage Res. 79, 7–31.
Six, J., Callewart, P., Lenders, S., Morris, S., Paul, E., 2002. Measuring and understanding carbon storage in afforested soils by physical fractionation. Soil Sci. Soc. Am. J. 66, 1981–1987.
Six, J., Elliott, E., Paustian, K., 2000. Soil macroaggregate turnover and microaggregate formation: a mechanism for C sequestration under no-tillage agriculture. Soil Biol. Biochem. 32, 2099–2103.
Six, J., Elliott, E., Paustian, K., Doran, J., 1998. Aggregation and soil organic matter accumulation in cultivated and native grassland soils. Soil Sci. Soc. Am. J. 62, 1367.
Wolf, A., Beegle, D., 2011. Recommended Soil Testing Procedures for the Northeastern United States.
Yavitt, J.B., Fahey, T.J., Sherman, R.E., Groffman, P.M., 2015. Lumbricid earthworm effects on incorporation of root and leaf litter into aggregates in a forest soil, New York State. Biogeochemistry 125, 261–273.
Zhang, W., Hendrix, P., Dame, L., Burke, R., Wu, J., Neher, D., Li, J., Shao, Y., Fu, S., 2013. Earthworms facilitate carbon sequestration through unequal amplification of carbon stabilization compared with mineralization. Nat. Commun. 4, 1–9.