

*L. rubellus* preference for *Quercus* spp. demonstrated by isotopic and density analysis in a northern temperate forest

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**Abstract**

Detritivores by nature, earthworms feed on multiple sources of organic matter. We hypothesized that they exhibit selective foraging habits, which would be reflected in their isotopic composition. We sampled at FASET and Ameriflux at the University of Michigan Biological Station, where we obtained earthworm specimens by using an electro shocking technique. We measured organic soil depth, soil moisture, and mass of leaf litter, and collected leaf and soil samples. We compared the density of worms to the organic soil depths, moisture of soil, and mass of leaf litter and found no significance, although there were definite trends. The composition of the tree species relative to worm densities showed a correlation between oak LAI (leaf area index) and density of *L. rubellus* worms. Our isotopic analysis of the *L. rubellus* worm, leaf litter, and soil samples showed that *L. rubellus* worms have a preference of oak litter.

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## **Introduction**

Originally brought to North America by colonists, European earthworms have long been a favorite of gardeners and farmers, because they aerate and mix nutrients into the soil. However, these exotic earthworms have begun moving into northern forest ecosystems where native earthworm species were wiped out by Wisconsinian-aged glaciations (Lichter 1998). There, the invaders are not so greatly appreciated, with research showing that earthworms negatively affect some plant species, including the protected Trillium (Frelich et al. 2006).

The University of Michigan Biological Station (UMBS) is located in a temperate northern forest where there are four species of exotic earthworms: *L. terrestris*, *L. rubellus*, *Aporrectodea* spp., and *D. octaedra*. These can be divided into three functional groups based on their feeding preferences and burrowing characteristics. *D. octaedra* and *L. rubellus* are both epigeic species, which live near the surface and feed on leaf litter directly. *L. terrestris* also preferentially feeds on leaf litter, but is known as an anecic species because it burrows deep into the mineral horizon. *Aporrectodea* is an endogeic or geophage species, feeding deeper in the soil on organic matter and living in the top 50 cm of soil. Studies have shown that *L. terrestris* species have specific eating habits, while *Aporrectodea* and *L. rubellus* are generalists (Curry and Schmidt 2007).

As a result of their burrowing and feeding habits, invasive earthworms dramatically alter the forest floor by mixing the organic and mineral soil horizons and by removing the litter cover that had once served as a physical barrier to plant establishment. These changes in turn affect nutrient cycling, lowering the equilibrium level of soil carbon (Bohlen et al. 2004). Belote and Jones (2008) found that the loss of more litter mass due to exotic earthworm invasion often

encouraged invasion by other species, and they predicted that human disturbance would further facilitate this effect. One example of a large-scale human disturbance is the Forest Accelerated Succession Experiment (FASET) on the UMBS property. In this experiment aspen and birch, the dominant species of the forest, were girdled to stop their regeneration and presence in the forest. (Replica stands were also created in the Ameriflux site on the UMBS property.) Since dominant tree species are key components in controlling their ecosystem through their litter, earthworms could accelerate the community changes in FASET caused by girdling the aspen and birch.

We put forth two hypotheses regarding the coupled effect of exotic earthworm invasion and the FASET area. Since it is widely known that some species of earthworms prefer certain species of tree litter, we would like to show that this is occurring in the FASET area through the use of stable isotopes and density statistical analysis. While many studies have been done with multiple-choice food experiments (Prince et al. 2004), we think the stable isotope technique will be most beneficial in this case because it observes real trends that are occurring in the field, not in an artificial laboratory setting (Curry and Schmidt 2007). 1) We hope to find correlations in the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  ratios of the different earthworm species and functional groups and the soil and tree leaf detritus, because that is what we expect them to be feeding on. 2) We also expect to see a reduction in the mass of tree litter and the depth of organic layer of soil in areas where there is a higher density of worms, as has been observed in previous studies (Bohlen et al. 2004). Specifically, we predict that the earthworms will show a preference for aspen leaf litter because aspen leaves contain more simple sugars and less lignin. This prediction will be supported if the earthworms have similar isotopic compositions to the aspen leaves and if there are more earthworms in areas with higher aspen density.

## **Materials and Methods**

### *Field Methods*

To collect samples, we selected four plots from the FASET area and one plot from the Ameriflux area. The plots used from FASET were A1, C3, D1 and D2, and the Ameriflux plot sampled was D9 (Fig. 1). These plots were chosen for the purpose of sampling worm species density across a gradient of forest transitioning from predominantly aspen to a mixed deciduous forest. All plots were located in areas where tree girdling had been done the previous year. In each of the selected plots we designated three  $\frac{1}{4}$  m<sup>2</sup> subplots totaling to 15 sampling areas. Mass of subplot leaf litter (g), soil moisture percentage, adult and juvenile worms, six leaves of the three most abundant tree species, a representative sample of pine needles if present, and soil samples were collected. Four soil samples were taken and averaged in each subplot using an Oakfield soil sampler and similar to separating leaves, the soil was separated into organic and inorganic sample bags to obtain isotopic analyses of each.

The worms were extracted from the subplots using an electric soil shocking technique. This technique involved eight 1m metal rods placed along two edges of the subplot in lines that ran parallel to each other. We placed the rods 12.5 cm apart within each line in order to spread electric charge uniformly across the subplot. A conductive metal clamp was connected to each rod, one of the parallel lines clamped with all negative charge clamps, the other with all positive charge clamps. We collected the worms as they surfaced and placed them into a jar with small amounts of soil, leaf litter, and water in order to keep them from expiring. During collection the worms were only separated by subplot, not taking into account species or age.

### *Laboratory Methods*

Earthworms were separated and labeled by species and by subplot. All collected samples were brought back to the lab to be separated and submitted for  $^{13}\text{C}$  and  $^{15}\text{N}$  analysis. Juvenile worms were separated out of the collected samples and not used for isotopic analysis due to possible false species identifications. After the samples were separated, the worms, the representative leaf samples and soils samples were ground down into a fine powder and submitted to the mass spectrometry lab at the UMBS.

### *Analytical Methods*

All statistical analyses were produced through SPSS. *L. rubellus* density by plot, mass of subplot leaf litter, average organic soil depth, average soil moisture, LAI (Leaf Area Index) of aspen, LAI of maple and LAI of oak were found to be normally distributed. To test for significant relationships with environmental factors, we ran regression tests. These tests compared the varying densities of *L. rubellus* to the depth of the organic layer and mass of leaf litter. Testing for feeding preference was done through use of the IsoSource isotopic mixing model which can be found at (<http://www.epa.gov/wed/pages/models.htm>).

## **Results**

### *Earthworm Density by Location and environment*

The D9 plot contained the highest density of adult earthworms including six *L. rubellus*, six *Aporrectodea*, eight *L. terrestris*, and nine *D. octeadra*. The C3 plot contained thirteen *L. rubellus*, and three *D. octeadra*. Site D1 contained six *L. rubellus*, D2 contained one *L. rubellus* and two *Aporrectodea*, and A1 contained no adult worms (Fig.2). Juvenile earthworms were abundant in all plots, but not included in this study. The juvenile worms were discounted from isotopic analysis because of the inability to discern species before development of the clitellum.

As the average organic layer depth (cm) increased, the number of earthworms increased by a slight positive correlation ( $R^2 = 0.1173$ ). Organic layer depth ranged from 2.5 cm to 8.25 cm and the number of adult earthworms ranged from 0 to 16 (Fig.3.). Our data showed a negative correlation between increased mass of leaf litter which ranged from 80 to 26 grams, and decreased number of worms ( $R^2 = 0.1251$ ).

### *Leaf Area Index of Oak*

As the leaf area index (total upper leaf surface of oak divided by the oak surface area in FASET & Ameriflux) increased, the number of *L. rubellus* earthworms increased by a slight positive correlation ( $R^2 = 0.2762$ ). The LAI of oak range from 0.14 to 2.1 and the number of *L. rubellus* earthworms range from zero to six (Fig.5). With plot D9 removed, as the leaf area index (total upper leaf surface of oak divided by the oak surface area in FASET only) increases, the number of *L. rubellus* earthworms increases by a strong positive correlation ( $R^2 = 0.6118$ ) (Fig.6, Table 1). D9 was removed to show this correlation because the other four sites were within FASET, and D9 was flooded during the early spring.

### *<sup>13</sup>C and <sup>15</sup>N Isotopic Analysis*

We plotted the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of all adult earthworms analyzed (Fig. 7, Table 2 and 3), tree species collected (Fig. 8), and soil samples (Fig. 9). The program IsoSource calculated the following proportions of the possible sources of  $^{15}\text{N}$  based on the mean values of the *L. rubellus* in each plot, as determined by the UMBS mass spectrometry lab. In C3, 18% of the  $^{15}\text{N}$  in the average *L. rubellus* was attributed to aspen, 17% to maple, 19% to oak, 22% to the inorganic soil, and 24% to the organic soil. In D1, 22% of the  $^{15}\text{N}$  in the average *L. rubellus* was attributed to aspen, 22% to maple, 23% to oak, 16% to inorganic soil, and 17% to organic soil.

In D2, 17% of the  $^{15}\text{N}$  in the average *L. rubellus* was attributed to aspen, 14% to maple, and 18% to oak, 24% to inorganic soil, 27% to organic soil. In D9, 18% of the  $^{15}\text{N}$  in the average *L. rubellus* was attributed to beech, 15% to maple, 18% to oak, 24% to inorganic soil, and 27% to organic soil (Table 4).

## **Discussion**

Worm distribution relative to average soil moisture, average depth of the organic soil depth, and mass of subplot leaf litter proved to be insignificant by regression analyses (Table 1). Since *L. rubellus* was both the species with the highest number of adult earthworms collected and the only normally distributed species, it is likely that more collecting would have yielded more significant results. With additional time, a larger sample size could have been collected, and normality, trends, and significance could have been established.

Although statistically insignificant, there was a general trend showing a decrease in leaf litter mass where there was a higher density of earthworms (Fig. 4). This supports our third hypothesis, but due to the lack of statistical significance, we recommend further study. Previous studies show similar trends. For example, Bohlen (2004) saw significant reductions in the forest floor where earthworms had invaded. Additional earthworm samples could provide a significant correlation between the number of earthworms and the amount of leaf litter. The insignificance in our results could also be a result of human measuring errors, for example, failure to collect the entire mass of leaf litter in a given subplot. Seasonal differences in earthworm feeding habits and developmental stage could also skew our results.

The relationship between organic soil layer depth and the density of worms was found to be insignificant, possibly due to the age of the soil. The organic soil layer around the UMBS is

thin and has had little time to accumulate because of recent glaciation in the area. There were also difficulties in distinguishing the organic and sub-organic layers from each other. Some mixing seemed to have occurred at the boundary between the layers, possibly due to earthworm activity.

The regression analyses relating earthworm and tree densities found only the relationship between oak density and *L. rubellus* density to be significant (Fig. 5, Table 1). Again, this likely occurred because we collected more *L. rubellus* than other species. We suggest that this relationship shows a feeding preference in the *L. rubellus* for the oak leaf litter. This correlation is strengthened when we isolated the FASET sites (C3, D1, D2) from the single Ameriflux plot (D9), which had abnormal amounts of flooding in the early spring (Fig. 6).

Further evidence of the *L. rubellus* preference for oak leaves comes from our isotopic analyses. We used the program IsoSource and our isotopic data to determine the proportions in which *L. rubellus* consumed each of the possible food sources (Grant and Kopple 2009). Of the food sources, oak leaf litter was preferred in all sample locations above the other types of leaf litter. *L. rubellus* also consumed soil in variable amounts, which is expected from a generalist species. However, we assert that because there is an abundance of leaf litter (reducing foraging time), they can be more selective in that respect by choosing oak leaf litter.

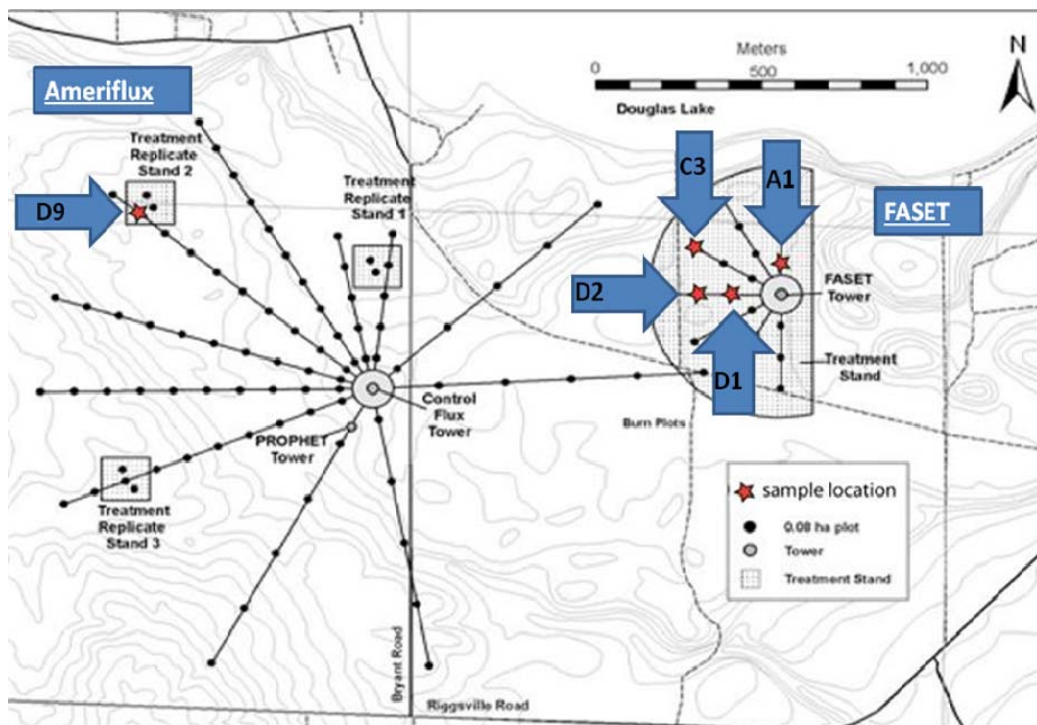
We plotted our  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for each earthworm in each subplot in order to look for general trends both within species and within plot locations (Fig. 7). There appear to be trends within the species. For example, the *L. terrestris* data points are fairly clumped, even at different sites, as is expected from a dietary specialist. On the other hand, *Aporrectodea* data points are widely distributed, indicating little feeding preference. We also saw strong



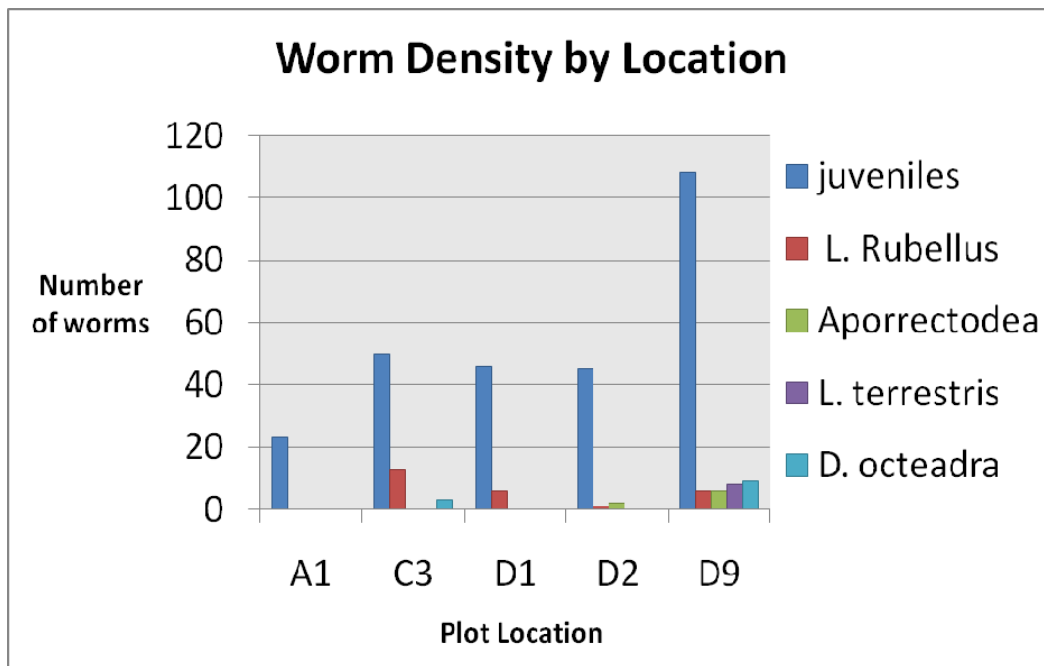
differentiation in earthworm isotopic composition between plots, especially between D9 in Ameriflux and the three FASET sites. This differentiation was mimicked when we plotted tree isotopic composition (Fig. 8) and soil isotopic composition (Fig. 9). This suggests that earthworms have similar isotopic compositions to the area where they are found and the tree litter and soil which they feed upon. This supports our second hypothesis that there will be a reduction in the mass of tree litter and the depth of organic layer of soil in areas where there is a higher density of worms.

In conclusion, our data on leaf litter and organic soil layer depth were insignificant, but mass of leaf litter relative to the number of earthworms showed a trend that further research might find significant. Our leaf litter and earthworm density comparisons and our isotopic results support our hypothesis that earthworms prefer certain types of litter, specifically; *L. rubellus* prefers oak leaf litter. The stable isotope analysis technique proved useful and we believe it should be utilized more extensively in the future. The FASET and Ameriflux sites provided a unique opportunity to study the interactions between invasive earthworms and forest succession. This experiment was a snapshot in time, and if continued in the future and in different seasons, more trends and greater sample sizes should produce more interesting and conclusive results.

**Figures & Tables**



*Fig.1: FASET & Ameriflux sample locations.*



*Fig.2. Earthworm density by subplot in FASET (A1, C3, D1 D2) and Ameriflux (D9).*

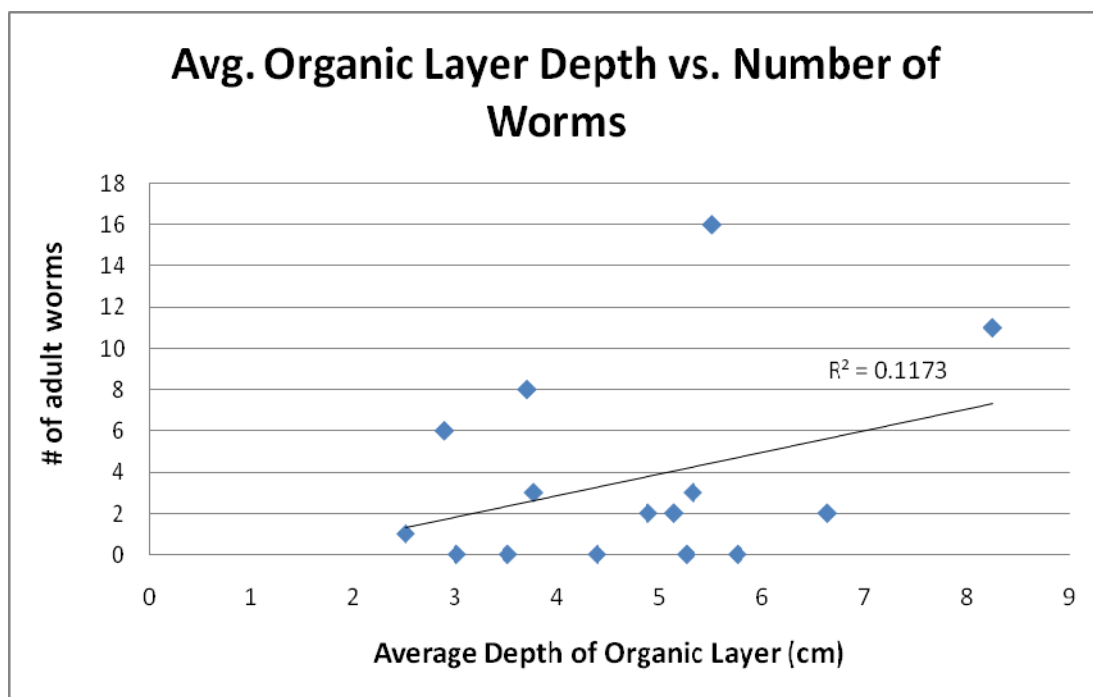


Fig.3. Average organic layer depth (cm) of soil compared to the number of adult earthworms collected within the subplot.

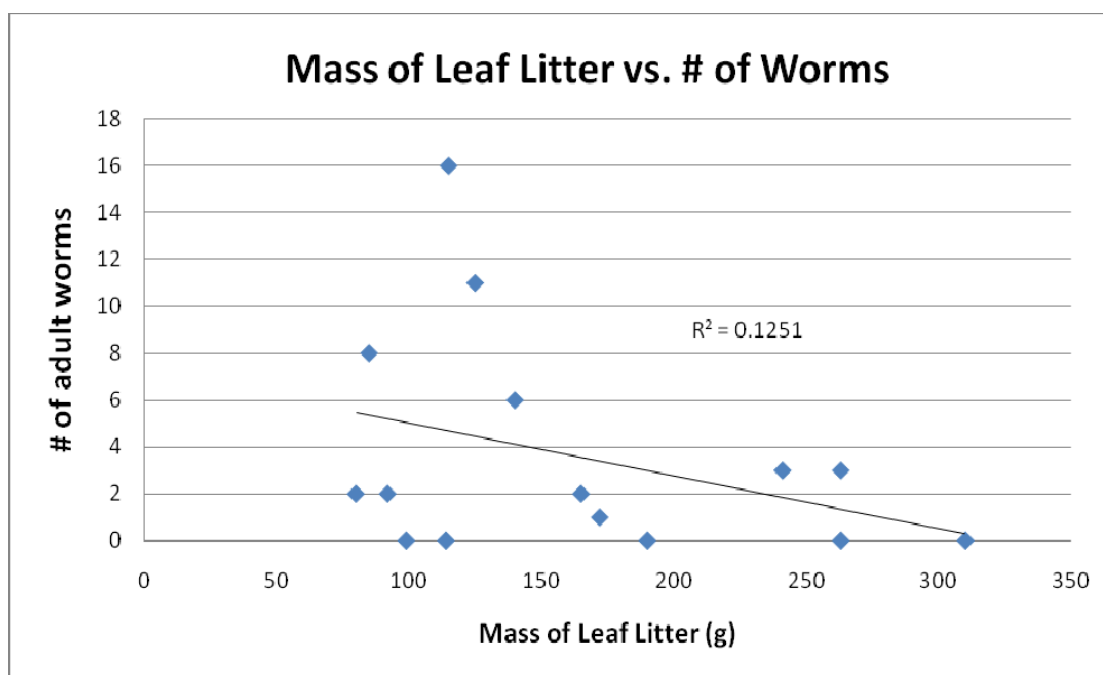


Fig.4. Mass of leaf litter (g) collected within the  $\frac{1}{4} m^2$  subplot compared to the number of adult earthworms collected within the subplot.

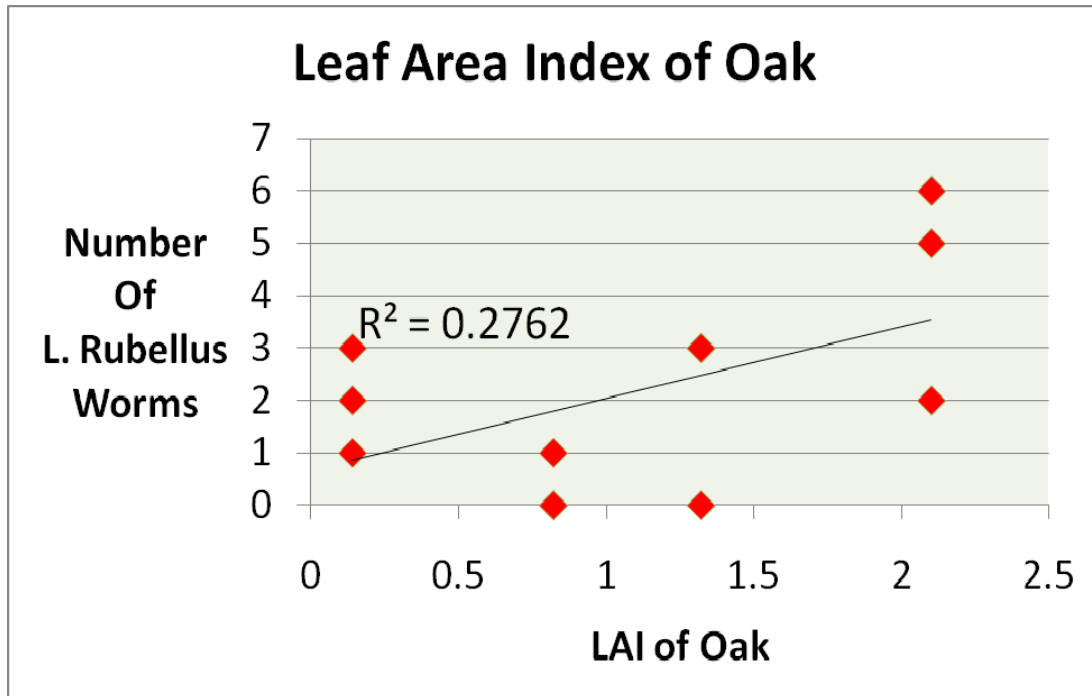


Fig.5. Oak leaf area index compared to the number of adult *L. Rubellus* collected at each subplot. Note: 2 data points are located at (0.82, 0) and (1.32, 3).

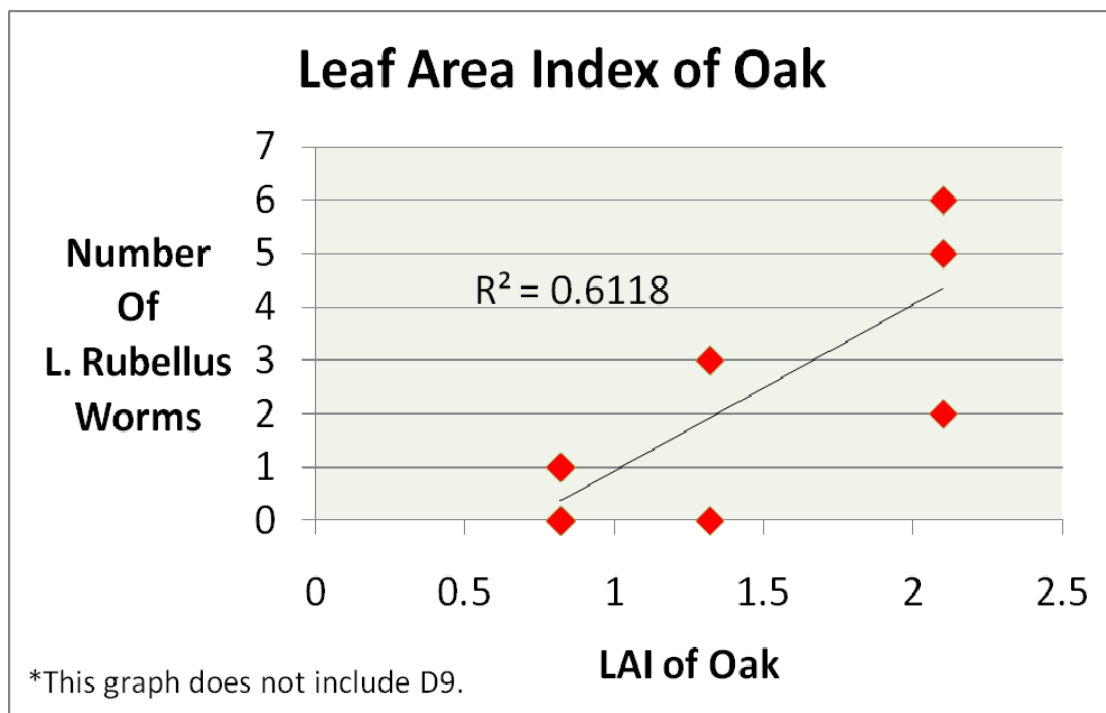


Fig.6. Oak leaf area index compared to the number of adult *L. Rubellus* collected at C3, D1, and D2. Note: 2 data points are located at (0.82, 0) and (1.32, 3).

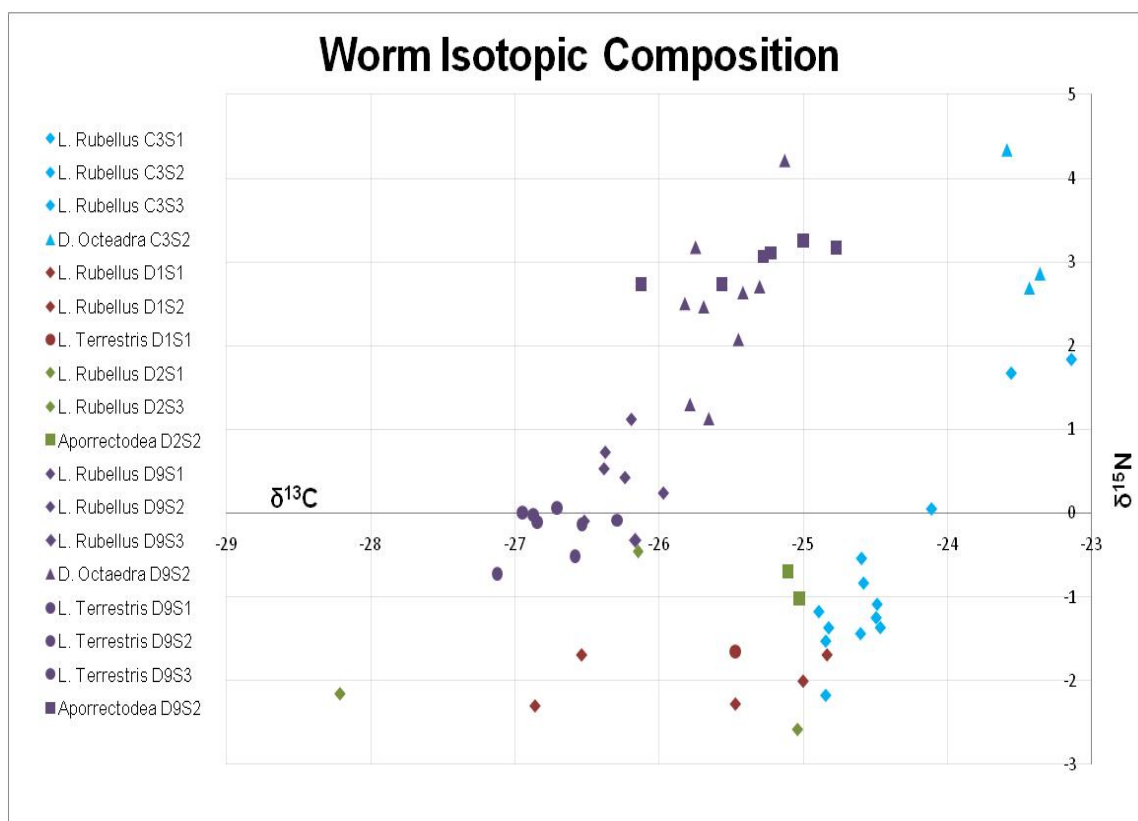
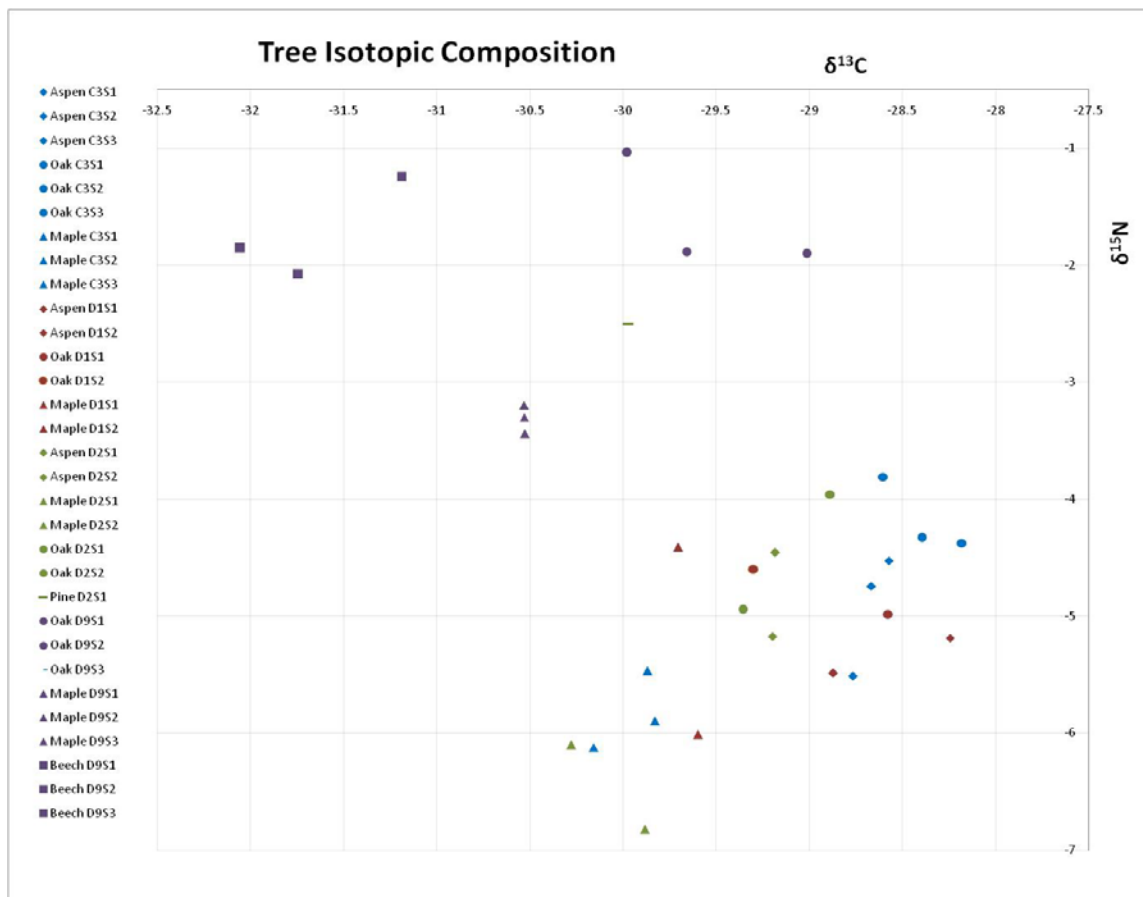


Fig.7.  $^{13}\text{C}$  and  $^{15}\text{N}$  isotopic analysis of earthworm composition. Different colors were used to symbolize different plots: blue for C3, red for D1, green for D2, and purple for D9. On the earthworm plot, *L. rubellus* was symbolized by a rhombus, *L. terrestris* by a circle, *Aporrectodea* by a square, and *D. octaedra* by a triangle.



*Fig.8.  $^{13}\text{C}$  and  $^{15}\text{N}$  isotopic analysis of tree composition. On the tree species plot, aspen was symbolized by rhombus, oak by a circle, maple by a triangle, pine by a bar, and beech by a square.*

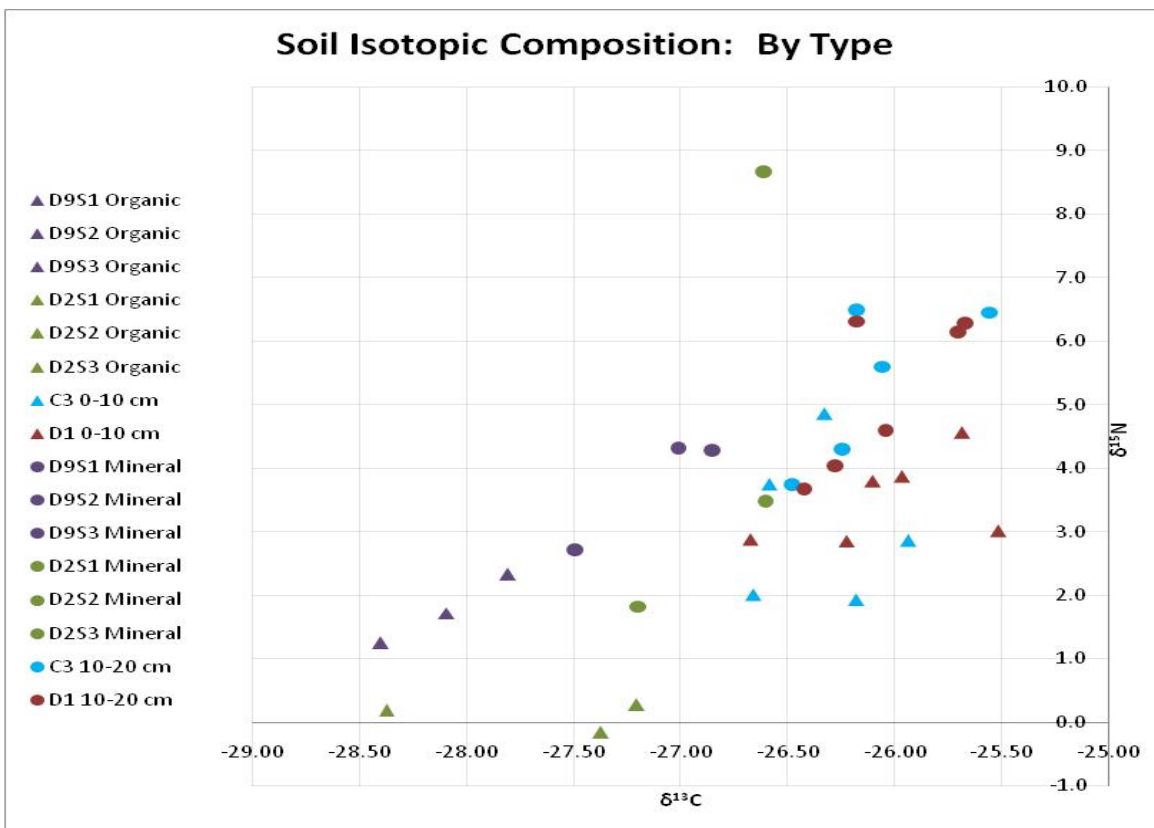


Fig.8. Organic and mineral soil isotopic compositions. On the soil plot, organic soil was symbolized by a triangle and inorganic by a circle.

Table 1. Regression analyses

Number of Rubellus Worms Relative to:	P-Value
Mass of Leaf Litter	0.067
Average Organic Depth	0.128
Average Soil Moisture	0.32
LAI of Aspen & Birch	0.224
LAI of Maple	0.072
LAI of Oak	0.016

Table 2. Results of the spectrometry analysis.

<b>Species</b>	$\delta$ N15 vs. Air Average	% N Average	$\delta$ 13C vs. VPDB Average	%C Average	C:N (molar) Average
Rubellus	-1.0	8.53	-25.68	42.23	5.83
Oct	2.9	9.24	-24.51	44.89	5.72
terres	-1.0	7.57	-26.09	40.46	6.28
appo	1.46	8.12	-25.20	41.14	5.95

Table 3. Standard mean error of the spectrometry analysis.

<b>Species</b>	$\delta$ 15N vs. Air SME	%N SME	$\delta$ 13C vs. VPDB SME	%C SME	C:N (molar) SME
Rubellus	0.09	0.17	0.21	0.75	0.07
Octa	0.28	0.23	0.28	0.71	0.14
Terr	0.18	0.33	0.16	1.84	0.13
Appo	0.64	0.31	0.15	1.06	0.18

Table 4. Feeding preferences of *L. rubellus* as determined by the IsoSource mixing model

Plot	aspen	maple	oak	beech	organic soil	inorganic soil
C3	18.2%	17.0%	19.3%	N/A	24.0%	21.5%
D1	22.1%	22.2%	22.7%	N/A	17.5%	15.5%
D2	17.0%	14.4%	17.7%	N/A	27.4%	23.5%
D9	0.0%	14.7%	18.9%	18.6%	25.8%	22.1%



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