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Statistical Analysis of Earthworm Populations: Extraction Sampling of Lumbricus terrestris Population in Soil and T-test Analysis

--Manuscript Draft--

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Environmental Science Education Title:
Statistical Analysis of Earthworm Populations: Extraction Sampling of *Lumbricus terrestris* Population in Soil and T-test Analysis

Overview: Using mustard, *Lumbricus terrestris* earthworm populations can be sampled directly from soil depths without landscape disturbance or toxicity. Earthworms can then be counted for data and statistical analysis using a bar graph and student's t-Test.

Monitoring earthworm populations is a vital technique for environmental scientists, as multiple species of earthworms (most notably those from the suborder Lumbricina) have been invasively spreading throughout North America and South America. Exotic earthworms can be found on nearly every land mass and in nearly every ecosystem on the planet, and where and when these species become ~~disruptingly~~ invasive has been a focus of international environmental research.

Ecological invasion typically lowers biodiversity of an ecosystem by directly outcompeting, endangering, or otherwise contributing to the extirpation of native species. As ecosystem engineers, invasive earthworm species alter the cycling of nutrients through decomposition rates of organic matter on the upper horizons of soil, where plant roots mine for nutrients. Invasive Lumbricus species have both extirpated native earthworm species and have been shown to increase the available nitrogen concentration and rates of nitrogen in invaded soils. In a positive feedback loop, accelerated levels of nitrogen in turn make the system more hospitable to invasive plant species that are adapted to high levels of nitrogen compared to native plant species, and will outcompete natives in a phenomenon known as "invasion meltdown." An invasion meltdown relationship has been proposed for invasive earthworm species Lumbricus terrestris (European earthworm) and an invasive plant species Rhamnus cathartica (European Buckthorn).
~~As ecosystem engineers, invasive earthworm species alter the carpet of organic matter on the floors of forests, causing shifts in ecological balances that can have devastating effects for other species.~~

Principles: A solution is prepared by extracting capsaicin from spicy mustard and then poured directly onto the soil within a sampling quadrat on the ground to sample from each collection site. Collection sites are determined in order to compare three random samples from an area that has been invaded by European buckthorn to three random samples from an uninvaded area. Once poured directly on the ground, ~~T~~the mustard solution can penetrate down through the soil matrix to where earthworms reside. The capsaicin in the mustard causes irritation to mucous membranes. Earthworm bodies exposed to the mustard solution react to the capsaicin irritation by moving away from the mustard solution and coming to the soil surface to expose themselves to oxygen, thereby reducing the irritation. After surfacing, earthworms can be collected and population density analyzed for relationships with European buckthorn ~~disturbance~~. The population means of each collection site is compared with a bar graph to

Comment [1]: The authors do a pretty good job here of emphasizing why this experiment is important. They could say a little bit more about the specific nature of the ecological disruption that is a consequence of earthworm invasion. Is there a specific example that can be given?

Comment [2]: I think a sentence is missing that briefly describes the application of the mustard solution to the soil matrix. Up till now, the reader knows that mustard is used to help with earthworm sampling without disturbing the soil, but it's not clear how exactly it's applied to the soil. One sentence should cover that.

determine if areas with other invasive species have more earthworms, thereby supporting the presence of invasion meltdown. A Student's tT-test is used to determine if the two sites are significantly different enough to support the invasional meltdown hypothesis proposed to exist between European earthworm and European buckthorn.

~~Tools:~~
~~- balance and weigh boat~~
~~- approximately 40g of ground oriental mustard~~
~~- 8-ounce HDPE container with cap~~
~~- graduated cylinder~~
~~- 2 8-liter water carriers~~
~~- 3 sample cups~~
~~- quadrat~~
~~- forceps~~

Comment [3]: I'm in favor of the "tools" section going within the procedure or in a materials table at the end of the manuscript. The latter format would be consistent with our articles and might make sense for experiments like this with over a half dozen reagents. Is it important for these authors to discuss the tools within the video? Can they stay within the text?

Comment [DM4]: Moved to the appendix.

Comment [5]: The authors do a pretty good job here of emphasizing why this experiment is important. They could say a little bit more about the specific nature of the ecological disruption that is a consequence of earthworm invasion. Is there a specific example that can be given?

Procedure:

1. **Preparing Mustard Concentrate Solution (at least 24 hours prior to experiment)**

1.1. Turn on balance, place a weigh boat on top, and zero the balance.

1.2. Weigh out 38.1 g of ground oriental mustard into weigh boat and transfer to a plastic container with cap.

1.3. Measure 100 mL of tap water in a graduated cylinder and add to the plastic container with mustard.

1.4. Secure cap on container and shake vigorously until all mustard is mixed off the bottom of the plastic container and dissolved into the tap water.

1.5. Let solution sit for 24 hours for maximum capsaicin extraction from mustard.

2. **Dilution of Mustard Solution**

2.1. Fill two 8 L water carriers halfway with tap water (approximately 4 L of water into each carrier).

2.2. Shake mustard concentrate several times to mix then transfer mustard concentrate solution to water carrier.

2.3. Transfer a small amount of the solution from the water carrier back into the concentrate container and shake vigorously. Pour back into the water carrier to transfer all of concentrate into the diluted solution.

2.4. Seal the water carrier cap, ensure cap valve is in "OFF" position, and invert water carrier three times to mix evenly.

3. **Extraction of Earthworms**

3.1. Label three sample cups for each collection site.

3.2. Proceed to sampling site with one quadrat, labeled sampling cups with lids, and water carrier with diluted mustard solution.

3.3. At your site, clear away brush, leaves, or mulch as much as possible to clearly expose the ground.

3.4. Place quadrat randomly on the ground in a cleared spot.

3.5. Invert one water carrier three more times to mix.

3.6. Turn the water carrier cap valve to the "ON" position and pour approximately a third (1.3 L) of the diluted mustard solution within the quadrat, concentrating on the center of the quadrat area. If soil becomes saturated and solution pools, stop pouring and wait until pooled solution infiltrates into soil before continuing to pour.

3.7. Observe quadrat area closely for earthworm appearance for five minutes, including the area directly under sides of quadrat.

3.8. Use forceps to collect all earthworms that appear in quadrate area by waiting for worms to completely emerge from ground before transferring to first sample cup. After five minutes lid the sample cup and proceed to next sampling site.

3.9. Repeat collection steps for all sampling sites, with three replicates per collection site (6 replicates total)

4. **Comparing Earthworm Population Density Between Collection Sites**
    - 4.1. Count the number of earthworms collected for each sample and calculate the mean and standard deviation for each collection site.
    - 4.2. To compare earthworm densities between collection sites, create a bar graph from the means and use the standard deviations to create error bars on the graph.
    - 4.3. Use a Student's tT-test analysis to compare to determine if the difference in earthworm population densities between the two collection sites is enough to be considered statistically significant.

Comment [6]: Up till now, the authors haven't really touched on experimental design. There's been no mention of different samples, variables to be tested, null hypothesis. Without context, this section on performing a T-test seems to be tacked on and is peripheral to the main scope of the video, which is how to carry out the experiment.

Comment [DM7]: Believe they touched on this in the revised Principles section.

8. **Representative Results: Comparison of Sites using Bar Graph and t-Test Results**

8.1. The height of each bar in the graph represents the average number of worms collected at each site and one visual way to compare which site had a higher population density. Because average is a non-robust statistic, standard deviation was used for the error bars on the graph to represent the variation within each collection site.

Figure 1. Bar graph with standard deviation error bars displaying results from each collection site. Site 1 has a higher density of earthworm populations but also has higher variability of sampling, indicating the earthworm population may not be as consistently dense as the average suggests. Error bars for the two collection sites overlap slightly indicating that there is not a statistically significant difference between the two sites due to excessive variability in the samples.

Figure 2: t-Test output table comparing the collection sites. The p (two-tail) >0.05 (alpha) indicating the two sites cannot be considered statistically different.

9. Applications: Invasive species are a major threat to biodiversity. Exotic earthworms (eg: *Lumbricus terrestris*) and European buckthorn (*Rhamnus cathartica*) have been implicated as part of an "invasional meltdown" occurring in mid-western United States wooded communities. An invasional meltdown is the process where one invasion of a species facilitates the invasion of others (Belote and Jones 2009). Thus, the rate of loss of ecological health can greatly accelerate as one invasive species makes way for additional ones. As undesired *Rhamnus* populations currently account for over 90% of vegetative cover in Illinois, the role of *Lumbricus* populations in landscape management has become critical to understanding and predicting *Rhamnus* invasion on managed land. Landscape disturbance tends to facilitate *Lumbricus* invasion and sampling for *Lumbricus* populations can be an indicator of vulnerability of land areas to likely invasion. Comparing samples of *Lumbricus* populations can help land management to know where more intensive methods are needed to maintain intended plant diversity and prevent invasion of *Rhamnus*.

Legend:

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Materials Appendix:

Tools:

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- approximately 40g of ground oriental mustard
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- graduated cylinder
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- quadrat
- forceps

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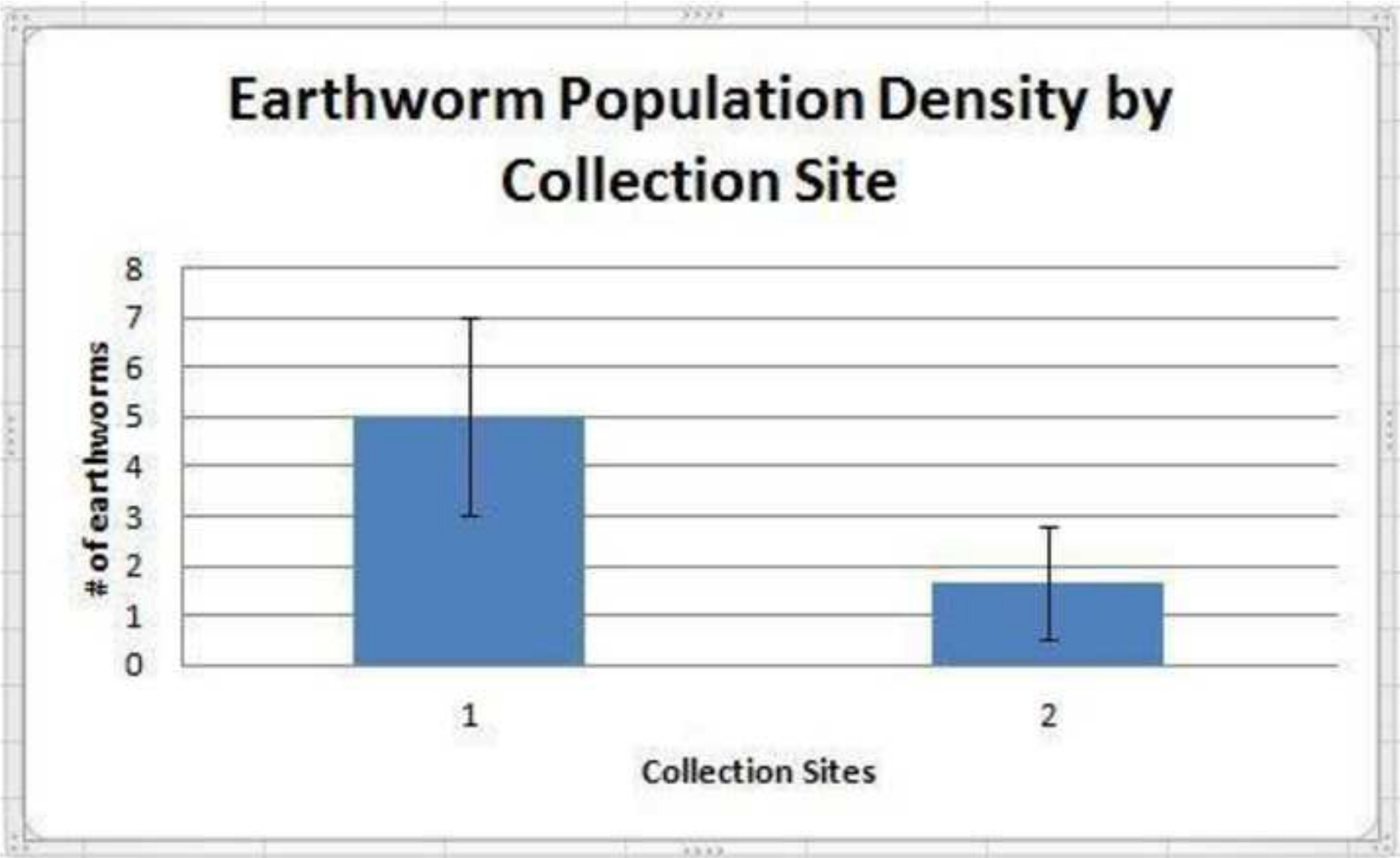

t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	*Variable 2*
Mean	5	1.666667
Variance	4	1.333333
Observations	3	3
Pooled Variance	2.666667	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.5	
P(T<=t) one-tail	0.033383	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.066767	
t Critical two-tail	2.776445	