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TRINITY COLLEGE

THE EFFECTS OF SOIL COPPER CONTAMINATION ON
EARTHWORM CHOLINERGIC TRANSMISSION,
LOCOMOTION AND MUSCLE PHYSIOLOGY

BY

Tommaso Meregalli

A THESIS SUBMITTED TO
THE FACULTY OF THE NEUROSCIENCE PROGRAM
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The Effects of Soil Copper Contamination on Earthworm Cholinergic
Transmission, Locomotion and Muscle Physiology

BY

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Abstract

In this study I investigated the effects of copper contamination on two earthworm species: *Eisenia foetida* and *Eisenia hortensis*. *Eisenia foetida* and *Eisenia hortensis* are invasive species native to Europe but are commonly found in the soil of many states, such as Connecticut. Earthworms play a key role in maintaining a health soil by aerating the soil and replenishing it with nutrients. Since the state of Connecticut suffers widely from high levels of copper contamination in its soil, I decided to investigate how bioavailable copper chloride (II) may affect earthworms.

In order to do so, I approached the effects that copper chloride may have on earthworms from several perspectives. To look for an effect on muscle function I measured muscle membrane potential but found no effects in either of the two species examined. To look for effects on locomotory behavior I measured the velocity of the escape response using video analysis. For *E. hortensis* there was a significant decrease in velocity of movement compared to earthworms in control environments. However, the experiment for *E. foetida* was inconclusive. To determine if copper chloride altered muscle AChE activity I used a spectrophotometric assay and found a significant decrease in AChE activity when comparing low levels of copper chloride (100mg/kg soil) to high levels of copper chloride (150mg/kg soil). Finally to determine whether the presence of copper chloride altered habitat use I performed a behavior analysis where earthworms were allowed to move between environments containing no copper or 150mg copper/kg soil. Both species strongly preferred soil where there was not copper. Overall I have shown that copper chloride does have significant effects on the physiology and behavior

of these earthworms. Future studies should focus on the effects of copper on earthworms in contaminated environments particularly focusing on acute versus chronic exposure.

Introduction

Humans have waged a battle against invertebrates of ever increasing size and complexity throughout our history. It would not be an exaggeration to say that the battle between humans and invertebrates is 'biblical' as the pestilence of a variety of invertebrate pests are referenced in religious writings from ancient Egyptian hieroglyphs to the Christian bible as well as the Koran of Islam and the pottery of native north American peoples. These texts have numerous references to the insects as vectors of disease and to the destruction of agricultural crops. Even early native American tribes (Mimbres, Hopi and Navajo) recognized the negative and positive roles of a variety of invertebrates which they recorded in their pottery and other art forms (Capinera 1993). To be fair, ethnobiologists have documented how many modern and ancient human cultures used invertebrates in positive ways too, as food sources, pollinators and spiritual entities (Morris 2006). However, it is the negative view of invertebrates that is dominant in modern human culture and threatens to have long-term negative impacts on humans, insects and the environment in general.

Few humans appreciate the essential role of invertebrates in maintaining our shared ecosystem. From the essential roles of detritivores recycling dead organic material, to pollination or even simply serving as a food source in the food chain, invertebrates are indispensable. For example, few creatures inspire as much fear as

spiders, but a recent study has quantified the essential role of spiders as insect predators helping to maintain insect population size and preventing their uncontrolled reproduction (Nyffeler & Birkhofer, 2017). Indeed spiders have long been recognized in agriculture for the beneficial effect they have in slowing down the population growth of hemipteran pests in wheat-, rice-, and cotton-growing areas with no or very low pesticide usage (Sunderland et al. 1986). It is estimated that 400–800 million tons of prey are annually killed by the global spider community (Nyffeler & Birkhofer, 2017). In addition, spiders have been shown to be a food source for an incredibly diverse complex of arthropod-eating carnivores and, given the estimated global spider biomass of 25 million metric tons fresh weight, spiders certainly are a crucial source of nutrition for many predator species (Nyffeler & Birkhofer, 2017).

Pollinators, such as bees, are another example of invertebrate that play a crucial role in maintaining a healthy ecosystem. Pollinators are extremely valuable since, by moving the pollen from one plant to another, facilitate fertilization (Wiemers et al., 2014).

Another class of invertebrates in the food chain, known as shredders and detritivores serve the essential role of breaking down dead organic matter along with the help of microbes, thus recycling this material into new soil. Without this essential function plant growth would soon be significantly limited due to the buildup of dead material and the loss of new soil formation and soil enrichment. Earthworms and ground beetles, are notable members of this group that help maintain healthy soil (Pey et al., 2014). While spiders have the ability to biologically control garden pests, previous studies have shown that earthworms, due to their great potential to enhance soil physical

properties, and thus in bioturbation, are considered as 'soil engineers' (Ojha & Devokta, 2013). Earthworm activity affects both biotic and abiotic soil properties and significantly impacts plant growth (Van Groenigen et al., 2014). By decomposing and breaking down decaying matter, earthworms aerate the soil and replenish it with nutrients. Earthworm burrowing and subterranean movement facilitates soil aeration and root respiration, which could be especially important for plant growth and microbial growth (Sun et al., 2013). In addition, earthworms have also been shown to be instrumental to several ecosystem services that the soil provides, such as nutrient cycling, drainage, and regulating greenhouse gas emissions (Van Groenigen et al., 2014).

Whether it be through direct application of pesticides to agricultural and urban landscapes or inadvertent contamination through industrial pollution; humans have had a significant negative effect on invertebrates in the ecosystem. Of the many classes of chemicals that impact invertebrates it is the heavy metals that are perhaps of most concern due to their long residence in soils and direct effects on the nervous system (Gerhardt, 1993).

Heavy metals can be highly toxic as they induce oxidative stress in cells and subsequent production of oxygen free-radicals which have damaging effects on nucleic acids. For example, inorganic mercury has been shown to induce mutational events in eukaryotic cell lines with doses as low as 0.5 μM (Inoue et al., 2003). These free radicals may also be involved in inducing conformational changes in proteins that are responsible for DNA repair, mitotic spindle, and chromosomal segregation (Valko et al., 2006). In order to combat these effects, cells have antioxidant mechanisms that work to avoid the formation of free radicals in excess by involving low molecular weight compounds, such

as vitamins C and melatonin, that protect the cells by chelating the targeted heavy metal and reducing its oxidative stress potential (Pinheiro et al., 2008). However, if the concentration of free radicals is exceedingly high, the antioxidant mechanisms will eventually become ineffective in combating these adverse effects. A recent study has suggested that heavy metals might be highly toxic also due to the effects that they have on key enzymes such as acetylcholinesterase (Bednarska et al., 2017). The etiology behind the effects of copper on acetylcholinesterase is currently unclear. A recent study suggests that copper ions “may compete with calcium ions for the same absorption sites in cell membranes” (Gioda et al., 2012). Consequently, at the moment that the nervous impulse arrives at a presynaptic neuron, “the cytosolic concentrations of calcium increase due to the opening of voltage dependent calcium channels. The increase of cytosolic calcium ions causes the neurotransmitter acetylcholine to be released on the synaptic gap. Therefore copper competition with calcium ions in the membrane of the endoplasmic reticulum might alter calcium absorption and, consequently, a high concentration of this of this ion in the intracellular mean would lead to a continuous release of acetylcholine, causing an increase in acetylcholinesterase activity” (Gioda et al., 2012).

Acetylcholinesterase (AChE) activity is demonstrable in the central and peripheral nervous system as an excitatory neurotransmitter in many vertebrates and invertebrates. It is especially important in locomotion due to its major role as an excitatory signaling molecule at the neuromuscular junction. AChE is an enzyme that has the function of catalyzing the breakdown of acetylcholine by destroying excess acetylcholine from the synaptic cleft. Since AChE is involved in destroying acetylcholine, a key enzyme involved in ambulation, improper AChE activity can cause

severe adverse effects. In the case that AChE becomes exceedingly high, voluntary movement might be impaired due to low levels of acetylcholine. In addition, a previous study has suggested that, in the case of invertebrates, overly low levels of AChE can result in mortality (Fulton & Key, 2001).

Copper is a trace metal essential for living organisms but when high soil concentrations are reached, copper becomes one of the most toxic heavy metals to small invertebrates (Gerhardt, 1993). When discussing heavy metals as soil contaminants it is important to refer to the concept of bioavailability (John & Leventhal, 1995). Bioavailability is the proportion of total metals that are available for incorporation into the biota (John & Leventhal, 1995, Davis et al., 1994). Most metals in the environment are bound with inorganic molecules such as sulfur compounds and even when ingested are not chemically active in the body. Unfortunately however, heavy metals are often bioavailable and are known to have adverse effects on small invertebrates (Bednarska et al., 2017). Bioavailable copper chloride (II) has previously been found in soils contaminated by several anthropogenic actions, such as industrial areas, mine tailings, disposal of high metal wastes, leaded gasoline and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues and spillage of petrochemicals (Wuana & Okieimen, 2011). In addition, copper chloride (II) may also enter the environment when used intentionally as a pesticide (Fishel, 2014).

Earthworms from soils heavily contaminated with copper can regulate copper more efficiently than cadmium and lead (Morgan and Morgan 1990). However, copper is more toxic to earthworms than lead or zinc in the soil due, in part, to the inability of most

soft tissues to synthesize copper-binding ligands when challenged with copper (Morgan and Morgan 1990). Copper soil contamination has been shown to decrease AChE levels in earthworm *Eisenia foetida* starting from a concentration of 200mg of copper per kg of soil (Bednarska et al., 2017). Such decrease in AChE levels might have adverse effects on several earthworm behaviors such as coordination, locomotion and growth. A previous study has shown that copper can be of acute toxicity, and in some cases lethal, to earthworms starting from 300mg of copper per kg of soil (Song et al., 2002). However, in some cases, such as copper production and handling sites, soil copper levels can reach an alarming 4000mg of copper per kg of soil, well over the lethality threshold of many invertebrates. Copper has also been shown to have adverse effects on earthworms at sub-acute levels such a 200 mg/kg (Bednarska et al., 2017). A previous study has shown that copper contaminated soils prevent successful reproduction in earthworm *Eisenia foetida* (Neaman et al., 2012). Additionally, copper has been shown to adversely effect common stereotyped behaviors, such as startle response, in worm *Lumbriculus variegatus* (O’Gara et al., 2004). A list of general physiological impairments induced by copper exposure includes: disturbances in osmotic balance, metabolic abnormalities, as well as production of reactive oxygen species and lipid peroxidation (O’Gara et al., 2004). Copper has also been shown to alter the physiology of specific organ systems. For example, copper exposure induces a slowing of heart rate in Bivalve mussels (O’Gara et al., 2004).

Connecticut due to a long history of mining has soils fairly contaminated by heavy metals (Hough et al. 2004). Copper has been shown to be present at level such as 50 mg/kg of soil in urban areas (Stilwell et al., 2008). At this level copper is at a sublethal concentration for earthworms. However, the concentration of this heavy metal may easily

be higher near sites for copper production and handling. A previous study has shown that copper, along with other heavy metal contaminants, can be found in Connecticut in high amounts due to the discharge of heavy metals from industries (Gourley & Semrod, 2014). In addition, copper can be quite dangerous since it tends to bind to both inorganic and organic particles that are deposited within fluvial systems (Gourley & Semrod, 2014).

A previous study has shown that acute levels (>200mg/kg soil) of copper have adverse effects on the escape response of the earthworm, *Lumbriculus variegatus* (O’Gara et al., 2004). Because most soil copper exposure of earthworms is much lower than the acute toxicity levels I wanted to test for the effects of sublethal copper concentrations on behavior and physiology of earthworms (<200mg/kg soil). Since a pilot study indicated that earthworms exposed to 200mg copper/kg soil exhibited significant depression of locomotion and noticeably poor muscle tone I decided to focus on movement and muscle physiology. I tested for the ability of the earthworms to sense and avoid sublethal levels of copper as well as the effects of sublethal levels of copper on the speed of movement, muscle membrane potentials, and acetylcholinesterase activity.

The two species of earthworm used in this experiment were *Eisenia foetida* and *Eisenia hortensis*. *Eisenia foetida* are invasive earthworms native to Europe that can now be commonly found in every continent with the exception of Antarctica. I chose this species of earthworm due to its ubiquity, to the previous literature involving this animal and to the fact that an *Eisenia foetida* colony is easily assembled. *Eisenia hortensis* are earthworm native to Europe that are commonly referred as nightcrawlers and are often sold as fishing bait. Since few previous studies investigated the effects of copper chloride

on *Eisenia hortensis* and since *E. hortensis* earthworms are easily obtained, I chose this species of earthworm.

Methods

The Animals

The two species of earthworms that were used in this experiment were *Eisenia foetida* and *Eisenia hortensis*. The *Eisenia foetida* were obtained from the laboratory of Doctor Smedley at Trinity College, Hartford, Connecticut. The *Eisenia hortensis* were purchased from a local fishing store. All worms were maintained in a colony and fed vegetable based food scraps.

The Experimental Environments

The experimental environments for Experiment 1-3 consisted of three plastic bins with a diameter of 20 cm and a height of 30 cm. Small incisions (3cm in length and 1 mm in width) were made in order to allow airflow. The bins for Experiment 1 were stored in a fridge at 4°C while the bins for Experiment 2 and 3 were stored at room temperature throughout the experiment. Three environments were set up: control, low copper and high copper. In the control environment soil was added to the bin along with spring water and, subsequently, the worms were added. I assumed that little to no copper was present in the soil and in the spring water used for this experiment. The low copper environment was created using a 100mg of copper to 1 kg of soil ratio. The desired copper to soil ratio was obtained by adding copper chloride (II) and spring water to the soil. The high copper

environment was created using a 150mg of copper to 1 kg of soil ratio. The desired copper to soil ratio was obtained by adding copper chloride (II) and spring water to the soil. Once the environments were ready, the earthworms were added with a sample size of n=10 per environment. After seven days in the environments the worms were collected for testing. In the case of Experiment 1 the ratio of copper to soil was reduced from 200mg/kg to 150mg/kg since the earthworms previously placed in the 200mg/kg were not able to respond to chemical stimulation.

Experiment 1: Muscle Membrane Potential

In this experiment *Eisenia foetida* earthworms and *Eisenia hortensis* earthworms were used for each of the three environments (control, 100mg/kg and 200mg/kg). The earthworms were anesthetized using a 10% v/v solution of ethanol and spring water. Following anesthetization the earthworms were placed on a dissection tray and, once the lower quarter of the body was amputated, a ground electrode was placed inside the earthworm. Once the ground electrode had been successfully placed inside the earthworm an incision was made in the upper part of the earthworm in order to easily access the earthworm's muscles. Glass microelectrodes were pulled using a Narishege PC100. Electrodes were tested to verify a minimum resistance of 10 M Ω . Narishege YOU-1 micromanipulators were used to place the microelectrode near the muscle wall. An Iworx 214 DAQ system and LabScribe II software were used to record the data. Once the DC offset was set to zero millivolts, the electrode was advanced into the muscle fiber until the voltage trace dropped and held stable. This was repeated 10 times in different areas of the body wall muscle for each earthworm. An average value for the membrane

potential was calculated for each of the 10 worms. Data was analyzed using an ANOVA with the aid of VassarStats.

Experiment 2: Startle Response

In this experiment *Eisenia foetida* earthworms and *Eisenia hortensis* earthworms were used for each of the three environments (control, 100mg/kg and 150mg/kg). Before placing the earthworms in the experimental apparatus the mass of each earthworm was recorded. The experimental apparatus for this experiment consisted of a circular glass tray (diameter of circa 15 cm) placed on top of a 20 cm ruler. A moist filter paper was then placed in the glass tray. A digital camera was placed with the aid of a stand 12 cm above the glass tray to record the earthworm's startle response. In order to elicit a startle response several approaches were attempted. Chemical stimulation (using glacial acetic acid on a cotton swab) was able to elicit a strong and consistent response for *Eisenia foetida*. Once a cotton swab dipped in glacial acetic acid was placed near the earthworm the response was elicited and the footage was recorded. Subsequently, the earthworms were frozen in order to utilize them in Experiment 3. While chemical stimulation was successful in eliciting a consistent response in *Eisenia foetida*, electrical stimulation (using a 5 mV battery) proved to be the most efficient way of eliciting a strong and consistent response for *Eisenia hortensis*. ImageJ (NIH image) was used to measure various aspects of the behavior. The speed of the movement of the anterior tip of the earthworm was measured. Since the earthworms startle response behavior was to 'wiggle' this resulted in the middle section of the worm essentially staying still and the

front and rear portions of the worm swinging back and forth circumscribing a circle, I decided to measure the number of Arc Vectors represented by the movement of the anterior tip of the worm per second. In order to calculate the length of the wiggle the following formula was used: length of worm/2 x π . Data was analyzed using an ANOVA with the aid of VassarStats.

Experiment 3: Muscle Acetylcholinesterase Activity Level

In this experiment *Eisenia foetida* earthworms and *Eisenia hortensis* earthworms were used for each of the three environments (control, 100mg/kg and 150mg/kg). The earthworms used in this experiment were previously used in Experiment 2 and frozen. In order to avoid the central nervous system of the earthworm, the intestines and the giant nerve cords were removed and a small piece of body wall tissue from the middle section of the earthworm was obtained. Following dissection, the body wall tissue was weighed and, subsequently, sonicated for 30 seconds in 1 mL of phosphate buffer with 1% Triton. The tissue was then centrifuged at 5000 rpm for 3 minutes. Once the centrifugation reached completion the supernatant was obtained and used in the assay. A modification of the Ellman's reaction was used in acetylcholinesterase assay. In a low volume spectrophotometric cuvette the following reagents were mixed; 1ml of phosphate buffer (pH 8.5), 20 μ L of 0.1 M 5-5'-dithiobis-2-nitrobenzoate (DNTB), and 5 μ L of the processed muscle tissue. The reaction was initiated by the addition of 10 μ L of the respective substrate (Propionylthiocholine). Each sample was replicated twice. Absorbance was measured at 412nm. The slope of the linear part of the curve was then calculated and converted to the average change in absorbance/mg of muscle mass/min.

Data was analyzed using an ANOVA with the aid of VassarStats.

Experiment 4: Ability to Sense Copper and Soil Preference

The experimental environment for this experiment was created using ten rectangular plastic boxes (height of 7 cm, length of 30 cm and width of 20 cm). Each box had two distinct halves. One half of the box was filled with control soil and the other half was filled with soil containing 150mg of copper per kilogram of soil. Once the environment was appropriately prepared one earthworm was placed in the middle of each box and was allowed to move for a period of 24-hours. After the 24-h period had elapsed the half in which the earthworm was found was recorded. Data was analyzed using a χ^2 test with the aid of VassarStats.

Results

Experiment 1. Muscle Membrane Potential

When testing *Eisenia hortensis* no significant difference was found between the muscle membrane potential readings of earthworms in each of the experimental environments ($p > 0.05$, $p = 0.990053$, Figure 1, Table 1). The membrane potentials were remarkably stable with very low variation and a mean of 34.2 mV.

When testing *Eisenia foetida* no significant difference was found between the muscle membrane potential readings of earthworms in each of the experimental environments ($p > 0.05$, $p = 0.811896$, Figure 2, Table 2). With an overall average of 46.1 mV the muscle membrane potential of *E. foetida* was also remarkably consistent.

A post-hoc comparison of the baseline membrane potential between the two species with an alpha adjusted to .025 was highly significant ($T = -16.16$, $df = 58$, $P < 0.0001$).

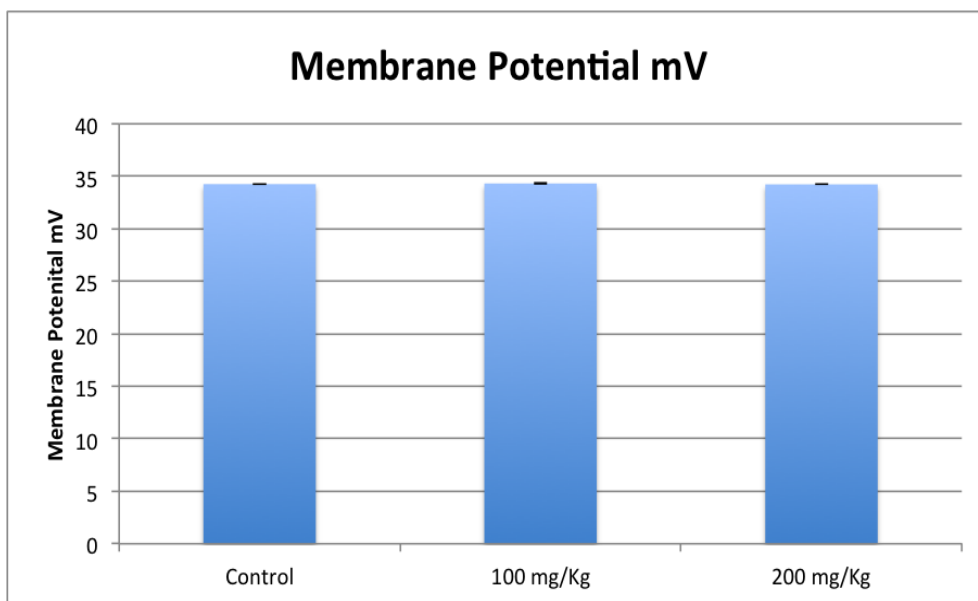


Figure 1. Graph displaying the membrane potentials readings (mV) for *Eisenia hortensis* in each environment (control, 100 mg/kg, 200 mg/kg).

Table 1. ANOVA summary of the data gathered by testing the membrane potentials of *Eisenia hortensis*.

Source	SS	df	MS	F	P
Treatment (between groups)	0	2	0	0.01	0.990053
Error	0.000076	27	0.000003		

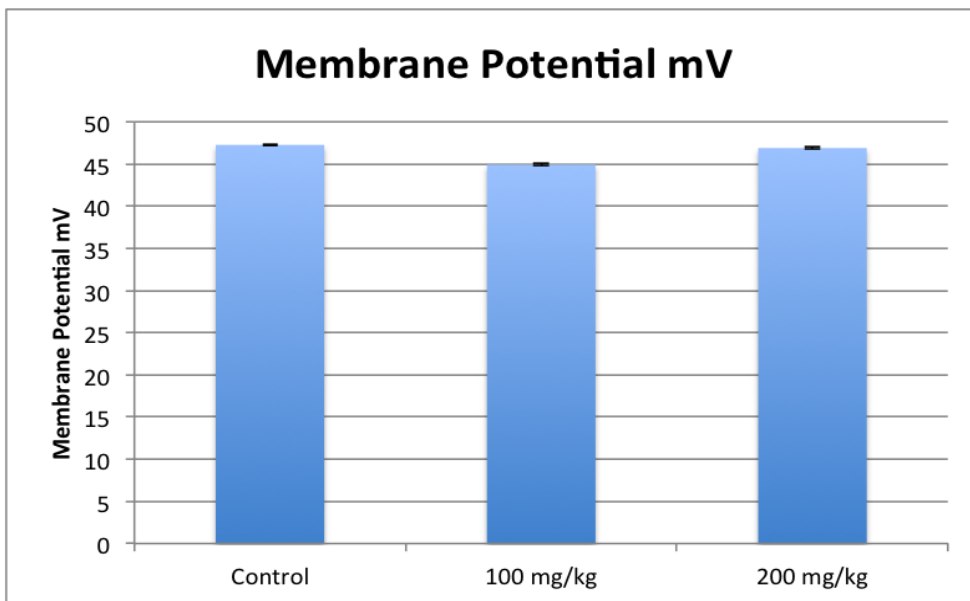


Figure 2. Graph displaying the membrane potentials readings (mV) for *Eisenia foetida* in each environment (control, 100 mg/kg, 200 mg/kg).

Table 2. ANOVA summary of the data gathered by testing the membrane potentials of *Eisenia foetida*.

Source	SS	df	MS	F	P
Treatment (between groups)	0.000006	2	0.000003	0.21	0.811896
Error	0.000397	27	0.000015		

Experiment 2: Startle Response

In testing for an effect of copper on locomotion I decided to measure the speed of the stereotyped startle response common to earthworms. Unfortunately, in the first experiment on *Eisenia foetida* an error was made where the animals in the control and 100mg/kg copper treatment were larger than those in the high copper treatment. It wasn't until the data was analyzed that I realized that body size could have a significant effect on movement. Using an ANCOVA to check for an effect of body size revealed the error and thus our analysis indicates that larger worms moved faster than smaller worms and any effect of the copper treatment was not discernable (Figure 3, Table 3).

When testing the startle response of *Eisenia hortensis*, the earthworms in the high copper (150mg/kg) environment had a significantly slower startle response when compared to earthworms in the low copper (100mg/kg) environment ($p < 0.05$, $p = 0.00042$, Figure 3, Table 3). No significant difference was found when comparing the earthworms in the control environment to the earthworms in the low copper environment and to the earthworms in the high copper environment ($p > 0.05$). In addition, an ANCOVA analysis was ran in order to test if body weight had a significant effect on the results. The ANCOVA shows that body weight doesn't have a significant effect ($p > 0.05$, $p = 0.503070$).

Because *Eisenia hortensis* is larger than *E. foetida* one would predict that in general they would move faster, but unfortunately this data is not adequate to make that comparison.

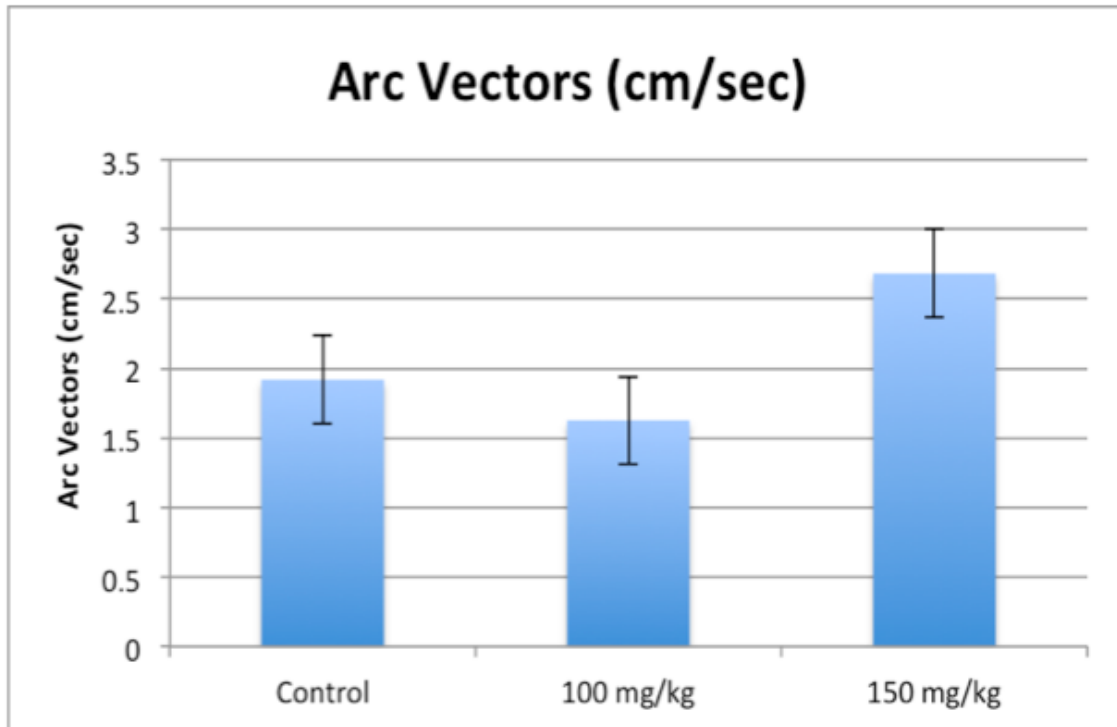


Figure 3. Graph displaying the startle response, measured in Arc Vectors (cm/sec), for *Eisenia foetida* in each environment (control, 100 mg/kg, 150 mg/kg).

Table 3. ANCOVA summary of the data gathered by testing the startle response of *Eisenia foetida*.

Source	SS	df	MS	F	P
Treatment (between groups)	3.0628	2	1.51314	20.28	0.00042
Error	2.0387	27	0.0755		

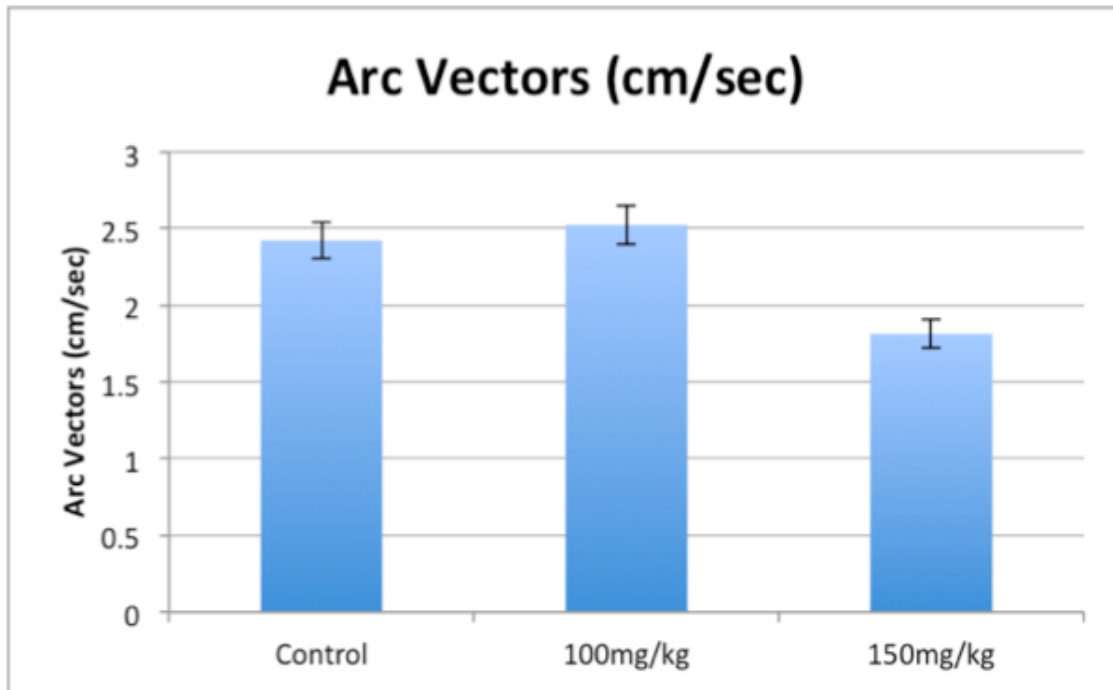


Figure 4. Graph displaying the startle response, measured in Arc Vectors (cm/sec), for *Eisenia hortensis* in each environment (control, 100 mg/kg, 150 mg/kg).

Table 4. ANCOVA summary of the data gathered by testing the startle response of *Eisenia hortensis*. The overall model is significant ($F = 11.1$, $P < 0.001$) and body size measured as weight in grams (Weight) does not differ between treatments ($F = 0.917$, $P = 0.348$).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power ^b
Corrected Model	2.843 ^a	3	0.948	11.119	0.000	0.592	33.356	0.997
Intercept	6.517	1	6.517	76.455	0.000	0.769	76.455	1.000
Weight	0.078	1	0.078	0.917	0.348	0.038	0.917	0.151
Treatment	2.510	2	1.255	14.721	0.000	0.561	29.442	0.997
Error	1.960	23	0.085					

Table 5. Pairwise comparisons for effect of copper treatments on speed of the startle response. The control and low copper treatment were not different but both were slower than the high copper treatment. (Alpha set at 0.05 and Bonferroni adjustments made for repeated samples)

Treatment		Mean Difference	Std. Error	Sig. ^b	95% Confidence Interval for Difference ^b	
					Lower Bound	Upper Bound
100mg/kg	150 mg/kg	.699*	0.139	0.000	0.340	1.058
	Control	0.123	0.144	1.000	-0.248	0.494
150 mg/kg	100mg/kg	-.699*	0.139	0.000	-1.058	-0.340
	Control	-.576*	0.139	0.001	-0.934	-0.218
Control	100mg/kg	-0.123	0.144	1.000	-0.494	0.248
	150 mg/kg	.576*	0.139	0.001	0.218	0.934

Experiment 3: Muscle Acetylcholinesterase Activity Level

When measuring acetylcholinesterase activity of *Eisenia hortensis*, the earthworms in the high copper (150mg/kg) environment had significant lower AChE activity when compared to earthworms in the low copper (100mg/kg) environment ($p < 0.05$, $p = 0.034334$). No significant difference was found when comparing the earthworms in the control environment to the earthworms in the low copper environment or to the earthworms in the high copper environment ($p > 0.05$).

When measuring acetylcholinesterase activity of *Eisenia fetida*, the earthworms in the high copper (150mg/kg) environment had significant lower AChE activity when compared to earthworms in the low copper (100mg/kg) environment ($p < 0.05$, $p =$

0.008195). No significant difference was found when comparing the earthworms in the control environment to the earthworms in the low copper environment or to the earthworms in the high copper environment ($p > 0.05$).

Although unusual the consistent finding that there is a statistically significant difference between the low copper and high copper treatments but not between the control and high copper treatment suggests that there is an increase in AChE levels between the control and low copper treatment (although it does not reach the level of significance). See the discussion for comments on this observation.

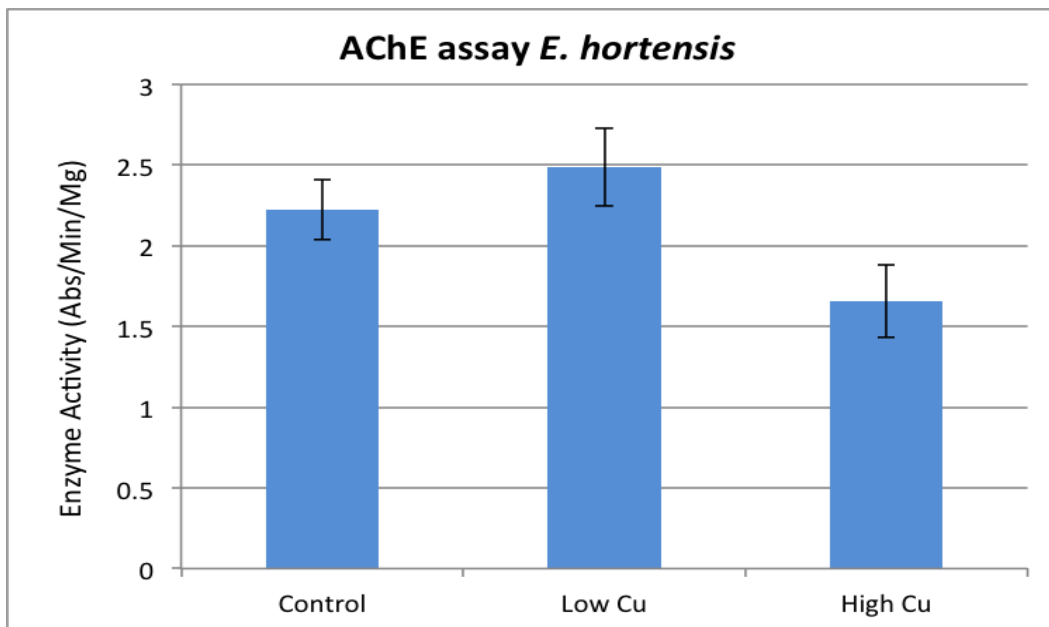


Figure 5. Graph displaying acetylcholinesterase activity, expressed in Abs/Min/mg, for *Eisenia hortensis* in each environment (control, 100 mg/kg, 150 mg/kg).

Table 6. ANOVA summary of the data gathered when measuring acetylcholinesterase activity of *Eisenia hortensis*.

Source	SS	df	MS	F	P
Treatment (between groups)	3.588	2	1.794	3.83	0.034334
Error	12.6469	27	0.4684		

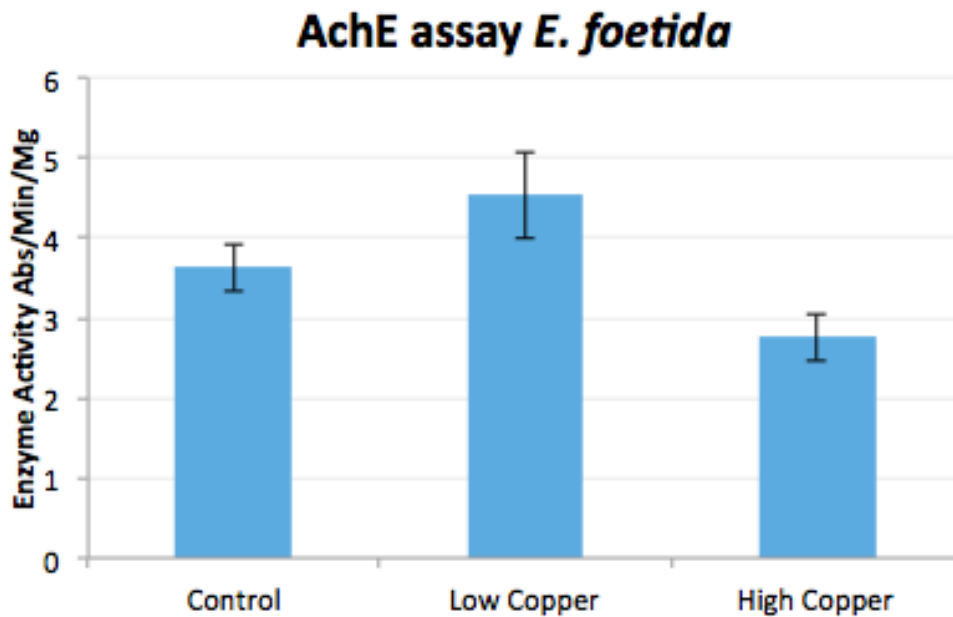


Figure 6. Graph displaying acetylcholinesterase activity, expressed in Abs/Min/mg, for *Eisenia foetida* in each environment (control, 100 mg/kg, 150 mg/kg).

Table 7. ANOVA summary of the data gathered when measuring acetylcholinesterase activity of *Eisenia foetida*.

Source	SS	df	MS	F	P
Treatment (between groups)	47.8717	2	23.9359	5.07	0.008195
Error	425.1169	90	0.47235		

Experiment 4: Ability to Sense Copper and Soil Preference

Several pilot studies were done to come up with the final experimental design. A longitudinal study where earthworm movement within the chamber was observed every 30 minutes did not yield consistent results after 8 hours of observation so the final design was an endpoint observation at 24 hours.

When placed in the chamber with soil on one half that was copper free and the other half contained copper at a concentration of 150mg/Kg soil *Eisenia hortensis* did avoid the copper soil and prefer the copper free soil ($\chi^2 = 5.934$, p -value= 0.014851, table 8). The analysis assumed that worms exhibiting no choice would be found equally in either side of the chamber.

The same experiment conducted on *Eisenia foetida* shows exactly the same result with most animals on the copper free side ($\chi^2 = 5.934$, p -value= 0.014851, Table 9).

Table 8. Summary of the data recorded when testing *Eisenia hortensis* for copper ability to sense copper and soil preference.

Treatment	Observed	Expected
Control	24	15
Copper	6	15

Table 9. Summary of the data recorded when testing *Eisenia foetida* for ability to sense copper and soil preference.

Treatment	Observed	Expected
Control	24	15
Copper	6	15

Discussion

In pilot studies observations of earthworms from copper containing treatments in the lab clearly indicate a change in muscle tone and movement. The animals move very little and slowly and the tone of the body wall appears relaxed and slack compared to the firm appearance of normal worms. These observations prompted the series of experiments conducted here. The results of several experiments show that copper at sublethal levels has significant effects on both *Eisenia foetida* and *Eisenia hortensis*. When analyzing the muscle membrane potential (RMP) readings of both species of earthworms no significant difference was found between the three experimental environments (Table 1 and Table 2). Several studies have been published on annelid body wall muscle (Drewes and Pax 1974). However most studies have been on a single species of annelid, *Lumbricus terrestris* (Muscle RMP = approx. 48mV) and knowledge of the variation in RMP across the phylum is lacking. One major limiting factor identified by Drewes and Pax (1974) was that studies previous to theirs had not been using a standardized saline solution when conducting their experiments. RMP is clearly effected by the extracellular saline composition and studies using different formulations are not comparable and there is concern that different species of earthworm may have different extracellular fluid ion composition. This is especially true when comparing marine, freshwater and terrestrial species. The saline formula used in this experiment is that of Drewes and Pax (1974) although it may not be equivalent to the extracellular ion composition of the 2 species used. Indeed this study seems to indicate that although the copper treatment did not alter RMP, these two species may differ in either RMP or

extracellular ion concentration or both variables. Much work has been done on vertebrate and insect muscle fibers and variation between different types of muscles and between different animal species is significant (Fraser et al. 2006, Mullins and Koda 1963, Wareham et al 1974). I am not aware of any other studies that measured muscle RMP in response to copper exposure. The factors controlling muscle membrane potentials are numerous and beyond the scope of this experiment.

Unfortunately, there was an error in the design of the experiment to measure locomotion in *E. foetida* and while the experiment does clearly show that larger worms move faster than smaller worms, the effect of copper was not detectable. When this error was discovered it was too late to repeat the experiment in time for this thesis. Given the clear results from the experiment with *E. hortensis* a follow up with *E. foetida* is warranted. However, the lesson about experimental design was valuable and the knowledge that body size often affects physiological and behavioral variables will remain with me in my future. It was a valuable lesson learned.

When conducting the startle response testing of *Eisenia hortensis*, earthworms of similar weight were distributed across the three experimental environments. When analyzing the data for startle response of *Eisenia hortensis* I saw a significant effect of copper on escape response velocity (Table 3). The data showed that earthworms in a high copper environment have a significantly slower startle response behavior when compared to earthworms that were placed in low copper environment (Table 3). A similar finding was reported for an aquatic, freshwater annelid, *Lumbriculus variegatus* (O'Gara et al., 2004). The exact relationship between copper and altered locomotion could be through effects on acetylcholinesterase or through generalized perturbation on metabolism or

cellular physiology. Several other studies and I show a decrease in AChE levels in various species of earthworms (Howcroft et al. 2011, Langdon et al. 2001) and Bundy et al. (2008) characterize numerous metabolic markers that are disturbed by copper exposure in earthworms. Again as with the muscle membrane data, the only study I found documenting an effect on locomotion (O’Gara et al. 2004) is on a species that is distinctly different from the soil dwelling *Eisenia* species of our study. *Lumbriculus sp.* are small and aquatic, and are filter feeders rather than substrate feeders. Their locomotion and other lifestyle factors are significantly different from *Eisenia* species and thus comparisons are limited.

Since movement is largely mediated by acetylcholinesterase I hypothesized that perhaps earthworms in high copper environments had a significantly slower escape response due to the effects of copper on this specific enzyme. Interestingly enough, when analyzing the data for AChE activity in *Eisenia foetida* and *Eisenia hortensis* I saw a significant effect of copper on enzyme activity (Table 5 and Table 6). The results show that earthworms placed in a high copper environment had significantly lower AChE activity (Table 5 and Table 6). This finding agrees with the results from the escape response testing since lower AChE activity can cause less efficient movement. The reason why copper caused lower AChE activity is unclear. Previous studies have produced contrasting results, some suggesting that copper increases AChE activity (Gioda et al., 2012) and some suggesting that copper decreases AChE activity (Bednarska et al., 2017). A possible explanation regarding how copper affects AChE activity suggests that copper could interact with the acetylcholine receptors affecting its binding efficiency (Dias Bainy et al., 2006). Affecting the binding efficiency would cause an

increase in AChE synthesis in order to decompose the higher levels of receptor activity. A previous study suggests, “copper ions might compete with calcium ions for the same absorption sites in cell membranes” (Gioda et al., 2012). On the other hand, a recent study has shown that copper decreases AChE activity in earthworms, thus agreeing with our findings (Bednarska et al., 2017). Our hypothesis towards how copper causes a decrease in AChE activity is linked to free radicals. A previous study has shown that copper can be involved in inducing conformational changes in proteins that are responsible for DNA repair, mitotic spindle, and chromosomal segregation (Valko et al., 2006). Thus, I believe that perhaps the mutations that copper causes could lead to decreased levels of AChE activity.

Furthermore, the studies looking at copper effects on AChE levels have differed in whether they are testing acute high doses or acute low doses on animals that have or have not been exposed to copper frequently. There is some indication that animals in the wild, living in contaminated soil have developed a tolerance to significant levels of copper. Langon et al. 2001 show that animals collected from sites with soil copper levels of 300mg/kg soil or higher were able to withstand copper levels in the lab up to 600mg/kg soil whereas these levels were quickly lethal to copper ‘naïve’ worms. Studies showing that copper increases AChE levels used very low levels of copper 0.1 to 0.5ppm (Romani et al. 2003). Thus, like many neuroactive chemicals, the level of copper may be significant when predicting effects on the nervous system. Further work is necessary to understand how copper affects nervous tissue and again studies in different taxa are confusing the issues.

When analyzing the data for the ability to sense soil copper and soil preference of both species I saw that copper chloride had a significant effect on the earthworms (Table 7 and Table 8). The data shows that both *Eisenia foetida* and *Eisenia hortensis* were able to sense copper contaminated soil and significantly preferred a copper-free environment (Table 7 and Table 8). Since, to our knowledge, no previous study has investigated the ability of earthworms to sense copper in the soil I believe that these results are of valuable importance. Earthworms may be able to avoid toxic levels of copper by migrating to uncontaminated soil and apparently they may be able to adapt to some level of contamination although it may reduce their reproductive capacity (Langdon et al. 2001, Bednarska et al., 2017, Neaman et al., 2012). Some work has been done on the diversity of sensory structures in the large marine group Polychaeta but the Oligochaeta, to which the earthworms belong, have not received very much attention (Santer and Laverak, 1971, Muller and Horrage, 2005). Some work has been done to describe the external anatomy and speculate about the function of various surface structures that seem to be sensory in nature (Muller and Horrage, 2005). The different types of sensory structures seen in polychaetes comprehend antennas, oral filaments and buccal tentacles (Muller and Horrage, 2005). However little physiology has been done to verify their function. In addition, *E. Foetida* and *E. Hortensis* don't share the said anatomical regions with polychaetes (Hama, 1959). I am unaware of any papers describing the ability of annelids or invertebrates in general to sense copper within the digestive tract, but it would seem that this would be a likely place to search for sensory structures of this nature.

A previous study has shown that when analyzing the copper concentration of soils in the vicinity of industries an unusually high copper concentration can be found (Wuana & Okieimen, 2011). Since earthworms have been shown to aid plant growth and to play a key role in maintaining a healthy ecosystem, industrial waste policies should perhaps be more strictly enforced (Pey et al., 2014). In addition, copper is commonly found in several pesticides used for agricultural purposes (Fishel, 2014). Our findings suggest that farmers should opt, if possible, for a copper-free pesticide in order to have a soil with a high presence of earthworms, thus increasing the quality of their crops.

In the future I would like to replicate the experiments using a higher sample size since the sample size used for most of our experimental procedures was quite small ($n \approx 10$). In addition, it would be interesting to investigate if a period of more than 7 days would exacerbate the adverse effects that copper has on earthworms. A particular focus would be on understanding the ability of earthworms in field sites in Connecticut to sense copper and either behaviorally or physiologically adapt to local copper or other heavy metal contamination.

In conclusion, the data shows that copper at sublethal concentrations has adverse effects on earthworm acetylcholinesterase activity and startle response. In addition, the data also shows that earthworms are able to sense copper contaminated soils and significantly prefer copper-free soils.

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