EFFECTS OF EARTHWORMS ON POST-INDUSTRIAL POLLUTED SOIL REMEDIATION

by

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A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Ecology and Evolution

Written under the direction of

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New Brunswick, New Jersey

October, 2017

ABSTRACT OF THE DISSERTATION

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This dissertation consists of a literature review and three main chapters, all of which are central to assessing the impacts of earthworms in polluted metalliferous soils. Chapter One gives a background in earthworm ecology, then summarizes the questions asked for this study, and gives a brief literature review of the work that has been done prior to this research.

Chapter Two describes a biological survey completed through three sampling seasons, which indicate species prevalence and population density in both non-polluted fields and forests, and metal-contaminated brownfields. This survey used a standardized mustard water extraction protocol over 135 sites in Pennsylvania and New Jersey, USA. Although there was a great deal of variation in numbers across sites, both polluted and non-polluted areas contained primarily 3 species– all of which are non-native.

A large community pot experiment is the topic of Chapter Three, where two types of plants and four earthworm species were placed in three different soil controls of low,

mid, and most metal pollution loading and grown together in five-gallon (19 L) buckets for 10 weeks. The primary goals of this experiment were to assess earthworm and plant communities and their potential interactions regarding growth and metal uptake in containers with and without earthworm communities. After all plants were harvested, they were analyzed for metal content including arsenic (As), cadmium (Cd), lead (Pb), copper (Cu), and zinc (Zn).

Chapter Four details a population level experiment, examining individual plants in the pots. This design removes the competition coefficient from having two different plant species together in a community-structured experiment like the previous chapter. This design used 1-gallon tall tree sapling pots with the same soil treatments (low, mid, most pollution levels) as chapter three. This experiment, however, only used one type of plant per pot, and only two species of earthworm; both of which have been very common in all sites studied for the biological survey detailed in Chapter Two. After 60 days of growth, the plants, soil and earthworms were harvested and analyzed for metal content of arsenic, chromium, copper, lead, and zinc.

ACKNOWLEDGEMENTS:

I wish to wholeheartedly thank my committee members: The outstanding Claus Holzapfel, Frank Gallagher, Peter Groffman, and Mark Hodson for all of their mentorship and support. Thanks to the incredible department administrator Marsha Morin for making sure all the paperwork was perfect. Thank you to the wonderful scientists at the Cary Institute of Ecosystem Studies, especially Denise Schmidt and Milada Vomela. None of the analysis would have been possible without you two. Thanks to my fellow worm-wrangler and friend, Kathleen Farley, and a huge acknowledgement to those young researchers that helped along the way: aspiring scientists like Rita Matos, Omar Abukwaik, and Zain Abidin who collected thousands of earthworms from all over New Jersey and Kyle Froehlich, who helped me define exact characteristics of my study plants.

A special thank you to the Rutgers Ecology and Evolution cohort of 2012 (or was it 2013?) for their love and support— When we keep each other up, we can all go higher.

A huge amount of thanks and love to my family, Deb Henshue, Tom Henshue, and Steph Henshue, for the support; whether it be babysitting, moral, or financial, you were always there.

And most importantly, my beloved wife, Jen. She has caught earthworms, given up her space, time, and husband for months at a time, and endured specimens in the refrigerator far more than any person should have. While I may be a Doctor, you will always have more patience. I love you forever.

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CHAPTER 1: INTRODUCTION TO THE ECOLOGY OF EARTHWORMS IN THE NORTHEASTERN UNITED STATES

I. Abstract:

One of the most invasive and transformative organisms in the Northeastern United States are exotic earthworms that have been introduced and re-introduced to North America since the earliest European colonization. Earthworms have been shown to have detrimental effects on forest understory, species composition, and biogeochemical cycling in areas that have adapted over millennia in their absence (Bohlen et al. 2004). However, in agricultural systems plants benefit from earthworms in many ways, such as aeration of soils, increased water flow to root systems, enhanced uptake of nitrogenous compounds, and improved productivity of plant growth. This introduction and subsequent three chapters investigate whether exotic earthworms in polluted areas could improve phytoremediative capabilities through enhancing plant health and overall plant productivity.

There are over 450,000 post-industrial brownfield sites in the United States. These areas range in scale and severity from small, disused gas stations to ecological devastation of several square kilometers from mining, transportation, and manufacturing. Brownfields could have legacy pollutants in the soil such as metal toxicants, persistent hydrocarbons, benzenes or any number of other contaminants. Phytoremediation has been shown to reduce the metalliferous content of contaminated soils significantly. Adding ecosystem

engineers such as burrowing earthworms will homogenize the soil, remove protective arbuscular mycorrhizal fungi, and ameliorate the substrate to increase plant productivity. This may improve cleanup time and reduce costs to stakeholders and land managers. Although earthworms may be non–native or invasive in certain areas, their presence could enhance plant growth in contaminated soils. Earthworm range expansion is inevitable, but including them in plans for remediation on areas laden with metal toxicants could benefit soil cleanup.

II. Introduction to Earthworm Ecology in the Northeastern United States:

In a seminal paper, Marcel Bouché (1977) outlined three functional groups, or lifestyles, of earthworms (Figure 1-1). He created a triangle with epigeic, endogeic, and anecic on each point of what he called his “Trident”. All earthworms can fit somewhere along the continuum of these points. For the ease of description of the ecological research, this dissertation will accept and utilize the "earthworm functional group" nomenclature created by Bouché.

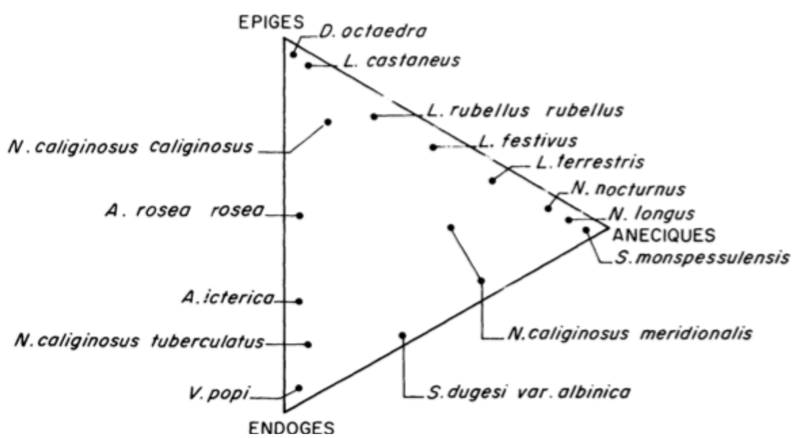

Figure 1-1: Earthworm Ecological Groups from Bouché 1977.

A. Anecic Earthworms:

Anecic earthworms are heavily pigmented, usually large earthworms that live in semi-permanent burrows that are deep and vertical or slightly diagonal (Hale 2013). They sometimes spend their entire lives in the same burrow, coming to the surface to take pieces of leaves or other organic materials into the hole (Darwin 1881). They typically line the walls of the shaft with mucosal discharge and feces to protect their skin from abrasion and aid in movement (Tiunov & Scheu 2000), while excess fecal pellets get deposited on the surface in the form of clumps called castings (Darwin 1881). The lining of the shaft with mucous and feces creates a substantial (1-3 mm thick) microhabitat called the drilosphere, dominated by bacterial species, rather than the fungal species typically dominating the bulk soil (Tiunov & Scheu 2000). The daily vertical movement and geophagy of anecic earthworms lead to a generalized homogenization of soil strata containing nutrient-rich organic compounds mixed with a more abundant mineral soil substrate (Holdsworth et al. 2007). *Lumbricus terrestris* is the most common anecic earthworm in North America (Gates 1976; Stebbings 1962), with *Apporectodea longa*, *Metaphire houlleti,* and *Amynthas indicus* as the only other anecic earthworms occurring on the continent (DriloBase TAXO). *Approectodea longa* does rarely occur in the Northeastern United States, although much better suited to warmer climates (Reynolds &Wetzel 2004, 2008, 2012). *Metaphire houlleti* can be found in the subtropics of Florida and Georgia (Chang et al. 2016; Gates 1982) and *Amyhtnas indicus* is found in rarity throughout New England and the Mid Atlantic (Reynolds &Wetzel 2004, 2008).

More importantly for this research, *Lumbricus terrestris* is the only anecic earthworm regularly occurring in the Northeastern United States and Canada (Stoscheck et al. 2012; Hale 2013). With a life expectancy of up to 9 years (Satchell 1967) and an adult length of up to 38 cm, they are the largest and most long-lived earthworms in the Northeastern United States, and third in North America after *Driloleirus americanus* and *Driloleirus* *macelfreshi,* both of which are exceedingly rare species living in the Pacific Northwest and reaching lengths of over 1 m (James 1995; Reynolds 2016a).

B. Endogeic Earthworms:

Endogeic earthworms are often without pigmentation or have just slight dorsal pigmentation. True endogeics are purely geophagous, and due to the relatively low nutrient quality of the mineral soil in which they reside, hyperdigest everything they eat (Bouché 1977). Endogeics rarely come to the surface. Although they are typically more muscular and robust than earthworms of other functional groups due to the strength required to navigate horizontally through their novel, nonpermanent burrows, they are quite fragile to sun, desiccation, and weather changes (Hale 2013). True subterranean endogeics such as *Perionyx excavatus* are not common in the Northeastern United States, but earthworms that do occasionally surface and subsist on dying root tissue or organic material are more common (Hale 2013).

C. Epigeic Earthworms:

Epigeic earthworms are leaf litter dwellers. They might live in the first few centimeters of the A horizon to evade predators or weather, but spend most of their time in decomposing detritus. These earthworms are typically pigmented as in *Esienia foetida*, and *Dendrodrilus rubidus* (Bouché 1977), but may be devoid of pigmentation as in *Dendrobaena octaedra.* Epigeic earthworms are frequent inhabitants of compost piles, hummock/hollow leaf accumulation in forest blowdowns, firewood piles, ravines, and anywhere else a significant amount of aboveground cellulosic biomass is found (Hale 2013). *Lumbricus rubellus*, a common member of this ecological group, is commonly found in poor quality soils, pine forests, and agricultural fields. Home earthworm composting and large-scale vermicomposting operations use earthworms in this ecological group, and they are highly effective at digesting cellulosic biomass in these situations. However, most epigeic earthworms are from warmer climates, and as such, do poorly in the Northeast if not in the shelter of a warm compost bin for the winter (Reynolds 1977).

D. Epi-endogeic Earthworms:

Although many functional assemblies are created from Bouché's triangle from being somewhere in between the continuum of ecological groups, the most common intermediate assembly in the Northeastern United States bears mentioning. Epi-endogeic earthworms are pigmented earthworms that burrow to 25 cm or more, depending on the time of year and environmental conditions. They may live in non-permanent horizontal burrows or leaf litter, typically in forested habitats or in areas where there is much aboveground organic cover (i.e. flowerbeds, compost heaps, mulch piles). A large part of this dissertation will deal with one of the most significant of these species complexes, *Amynthas*. *Amynthas* (Goto & Hatai 1898) is by far the most common and detrimental to forested areas (Görres 2012). This species complex was the most common earthworm found in the biological survey (Chapter Two) and was used in both plant growth experiments (Chapters Three and Four). All four of the *Amynthas* species complex (*Amynthas hilgendorfi*, *Metaphire hilgendorfi*, *Amynthas agrestis*, *Amynthas tokioensis)* are equally virulent invaders that fulfill the same niche (Callaham et al. 2003, 2016; Chang 2016; Görres 2012) and, as such will have similar environmental impacts across all members of the genus.

E. Native and Non-Native Earthworms:

Many species of earthworms in the northern portions of the United States and Canada are non-native. During the last Pleistocene glacial maximum ca. 19,000 years ago until ca. 10,000 years ago, earthworms that had become naturalized to northern habitats were pushed south (Reynolds 1994). Although various estimates disagree on where the no-earthworm line exactly was (Gates 1976), it is evident that most species of earthworm would not live in glacial areas or the hundreds of miles of tundra and permafrost south of the glacial terminus.

Gordon Gates, the "Dean of Oligochaetology" (1976) outlined the conundrum of the "no earthworm zone" most eloquently:

> "Answers were sought in vain to the following questions: Did not Arctic gales, blowing for millennia across thousands of miles of thousand-foot-thick ice, exterminate earthworms below the southernmost limit of glacial advance? If so, how far from the glacial boundary? Was there permafrost in the soil south of the glacial boundary? If so, to what depths, and when did it finally disappear? How soon after the disappearance of the ice would the deposited rock flour, sand, gravel, and boulders have acquired enough organic matter to support geophagous earthworm populations? Did the Appalachian mountaintops, even shortly, have local glaciers? If so, how many centuries were required for geophagous earthworms to eat their way up to and then down the northern slopes of those mountains to digest their way through Tennessee and Kentucky into Illinois?"

It is important to note that not all of the species we find in the Northeast are non-native. There have been a few species of native earthworms that have recolonized this area, whether moved by their volition or transported by the normal trade and commerce of this country over the last 250 years, in much the same way that non-native earthworms have been human-assisted. Although the numbers vary slightly, there are roughly 183 species of earthworm living in the United States and Canada, and 62 of them are non–native

(Reynolds 1977, 2014; James 1995). In the Northeast, there only 11 native species and 18 non–natives (Görres 2012; Bohlen et al. 2004). Moreover, while native species are in this region, their rarity makes them a much less significant contender for niche competition or displacement of non–native species (Stebbings 1962). In fact, during the biological survey of earthworms reported in Chapter Two of this dissertation, only 6 of the 1,538 earthworms (0.4%) surveyed are pre–European residents of the United States.

The rate of recolonization of a population of earthworms is 6-10 m/yr (James 1995). If it is assumed that habitats were favorable enough for native earthworm movement back to the Northeast within 5,000 years of the last glacial maximum, it is understandable how higher concentrations of native earthworm populations are still south of ≈38° N latitude. At even the most rapid of recolonization speeds, earthworms unassisted by humans would have only theoretically migrated 50 km north. Therefore, it is the widely held theory that any earthworm found in the northern states has moved human–assisted (Gates 1976; Bohlen et al. 2004; Hendrix et al. 2008).

Initial earthworm colonization of more cosmopolitan species (*e.g. Lumbricus rubellus, Lumbricus terrestris,* and *Esienia foetida*) could have happened in North America as early as the Colony of Jamestown (ca. 1607–1770) (Mann 2007). More recent species such as *Amynthas agrestis, Amynthas hillgendorfii,* and *Perionyx excavatus* have invaded within the last 20–30 years (Snyder et al. 2010). Early non-native species of oligochaetes may have found their way to the New World in potted ornamentals or vegetables, in soil ballast of cargo ships, or as small cocoons fastened to shipping containers; and most likely, all of these methods (Smith 1928). Later non–natives were brought by the vermicomposting industry and by wholesale bait trade (Hendrix et al. 2008) as well as by the accidental stowaway in shipping routes (Mann 2007). Interestingly, this has led to waves of earthworm invasion centered around eastern port cities. Even today, we find a higher abundance and diversity of alien earthworm populations based around Baltimore, Philadelphia, New York, and Boston (Reynolds 2015). As the earthworm invasion front has moved north and west, we see higher populations and deleterious ecosystem effects closer to popular fishing lakes, logging roads, and larger Midwestern cities (Bohlen 2004; Hale et al. 2008a, 2008b; Callaham et al. 1997).

Another impact of this widespread invasion are non-native earthworms from Europe and Asia which have been found to extirpate or at least displace native North American earthworms. As early as 1900, there was a concern of an eventual or even possible recolonization in the face of displacement from non-native earthworms (Eisen 1900; Smith 1928; Gates 1976). Stebbings (1962) and Hendrix et al. (2006) demonstrated that native earthworms were being replaced by exotics in highly disturbed forest habitats. Unfortunately, "disturbed forest habitats" applies to an increasingly significant amount of the Nothern United States and Canada. Deforestation, construction, housing, and even logging (Hale 2008a) and fishing (Hendrix et al. 2006) have increased earthworm range expansion.

III. Earthworms Affect Forests:

Darwin (1881) laid the definitive groundwork for the benefits of earthworms, particularly in vermiculture and agricultural systems. However, forested lands that haven't had earthworms for the last several thousand years are being impacted by recent colonization events (Bohlen et al. 2004; Nuzzo et al. 2009). Deleterious impacts on forested ecosystems may include homogenization of organic (O/A) and mineral (B/C) horizons from deep burrowing (anecic) earthworms (Groffman & Bohlen 1999). There is often a net loss of carbon stocks in those same soils (Lubbers et al. 2013), while nitrogen seems to increase in most systems (James 1991). Many of the effects on recently colonized forested areas involve the unseen, yet crucially important, shift from a fungal-dominated O/A layer to a bacterially dominated one (Groffman 2004). This not only creates a trophic disruption from the collapse of mycophage decomposers but effectively changes the entire way in which decomposition occurs and nutrients (particularly C, N, P) cycle. As our understanding of forest mycorrhizal networks and their interspecies communication improve (Simard et al. 1997), it becomes more evident how a major change such as this could severely impact forest health.

Other effects of earthworm colonization on forests are less widespread and much more localized. Areas of high earthworm density have a greater turnover in leaf litter (Bohlen et al. 2004; Hale et al. 2006). As annelids are consuming the detritus on the forest floor, it leaves the exposed soil to desiccate and erode. Removal of the leaf litter also means that seeds have no protection from granivore predators (Fisichelli et al. 2012), and the seeds that do escape notice from rodents like squirrels and chipmunks are more likely to dry out during germination (Loss et al. 2013). Compounding the issue of fewer seedlings is the rapid, anthropogenic increase of whitetail deer in the northeast (Fisichelli et al. 2012), consuming the few remaining saplings and leading to the diminution of the forest understory of the temperate deciduous forest that was known just 30 years ago (Nuzzo et al. 2009). This reduction in leaf litter and understory also leads to a reduction or extirpation of organisms that depend on this habitat. Ground-nesting songbirds such as Ovenbirds (*Seiurus aurocapilla)* and Wood Thrushes (*Hylocichla mustelina*) have seen a reduction in population since earthworm populations have expanded and eaten their nesting material (Loss & Blair 2011, Loss 2012).

IV. Earthworms in Contaminated Soils:

Through years of mining, smelting, shipping, and multiple other industrial processes, humans have polluted the most valuable natural resource in any given habitat; soil. The importance of soil to the health of an ecosystem and all the organisms living there is paramount. Earthworms have been used as an indicator species for contaminated soils (e.g. Ireland 1979). In the majority of terrestrial ecosystems, earthworms are the most abundant animal biomass (Lavelle & Spain 2001), and their presence (or lack thereof) can typically give an indication of the health of the substrate. There are a few situations where earthworms have adapted to metalliferous natural serpentine soils, or contaminated soils near smelters (Sizmur et al. 2011b), or in mine tailings (Morgan & Morgan 1999). Earthworms also make crucial contributions to organic decomposition and nutrient cycling (Sizmur et al. 2011a), including an increase in bioavailability and mobility of metals (Sizmur & Hodson 2009). Studying trace metal accumulation in earthworm tissue versus soil content has been used as a bioindicator of metal availability in the soils (Lanno & McCarty 1997; Morgan & Morgan 1998). They are in constant contact with soils; consume it; live their entire lives in relatively small areas, and reproduce quickly enough to see generational adaptation to pollution (Kille et al. 2013).

Earthworms are so frequently utilized as indicator species of soil contamination that the International Standards Organization has developed a set of defined protocols measuring the toxicity to earthworms in soils treated with chemicals such as pesticides and herbicides (OECD 1984, 2004; ISO 11268). These protocols have become the standard for metal contamination testing as well, but there is conjecture whether or not this is appropriate (e.g. Spurgeon & Hopkin 1995; Davies et al. 2003).

Although the OECD and ISO established protocols work well in controlled laboratory situations, there are some major shortcomings. Typical earthworm testing of soils relies on an established methodology, often using artificial soils. These test soils are comprised of 10% ground sphagnum peat, 20% kaolinite clay, and 70% quartz sand (OECD 1984) and amended pollution levels created by adding metal salts to this substrate. Smolders et al. (2009) showed that soils artificially spiked with fully soluble metal sources do not represent conditions of metal-rich contaminated soils, and Spurgeon and Hopkin (1995) demonstrate that metals in artificial soils are far more bioavailable than in actual field soils.

These earthworm toxicity protocols also rely on the sole use of *Eisenia foetida* for almost all experiments (OECD 2004). *E. foetida* is the standard test organism used in terrestrial ecotoxicology (van Gestel et al. 1991) and is a cosmopolitan epigeic earthworm, used across the globe for composting and vermiculture. It reproduces rapidly and is extremely easy to raise in captivity (Edwards & Bohlen 1992). The benefits to the use of this earthworm are evident in laboratory experiments, but have some drawbacks in applied field trials. As an epigeic species, *E. foetida* does not burrow or have extended contact with the soil itself. Furthermore, it is rare to find them endemically, especially in temperate areas that experience soil freezing over the winter like in the Northeastern United States. *E. foetida* does not thrive in the Northeast climate unless protected by a warm compost pile or deep snow, and so is used as a proxy for "representatives of soil fauna and earthworms in particular" (ISO 11268).

The impacts of metal contamination on earthworms are established and mostly well known (e.g. Römbke 2005; Gish & Christensen 1973; van Hook 1974; Ireland 1979; Martin & Coughtrey 1975; and especially the comprehensive review by Nahmani et al. 2007). There is much evidence that earthworms have the ability to absorb and accumulate metals from their surroundings and other media, and that they can accrue much higher levels of metals than other soil invertebrates (Hodson et al. 2010; Beyer et al. 1982). Interestingly, different organ systems in the annelid absorb certain types of metals preferentially; i.e. cadmium accumulates in the tissues of the posterior digestive system (Stürzenbaum et al. 2004), while lead accumulates in the reproductive organs coelomic fluid and nephridia (Morgan & Morgan 1998). Many of the strong and highly indicative field studies took place around mining sites (e.g. Arnold et al. 2008; Spurgeon & Hopkin 1996, 1999; Weeks 1998; Morgan & Morgan 1999). Others have carefully replicated "worst case scenarios" in the lab– comparing the effects of pristine loam or compost with synthesized contaminated soils or sludge that is far more deleteriously infused than any industrial site found in the real world (Spugeon & Weeks 1998; Sizmur et al. 2011a; Ruiz et al. 2009). There is adequate research on earthworms in brownfields in Europe and China, from Aberystwyth, Wales (Ireland 1975), to Hong Kong, China (Ma et al. 2006). They cover mine tailings (Hankard et al. 2004), smelters (Colgan et al. 2004), and even uranium residues from 'dirty bombs' used in the Kosovo War (Di Lella et al. 2004).

V. Outline of Chapters and Synopsis of Issues:

The Northern United States and Canada have not had endemic earthworm communities since the last ice age. There are scores of papers dealing with trace metals in soils where earthworms have been part of the soil fauna for millennia (e.g. western Europe, Asia), but it needs to be shown that those results are similar where earthworms are non–native. These non–native earthworms could have differential effects on soils and plants in metalliferous or otherwise contaminated areas where native populations have been completely displaced. To this end, Chapter Two describes a biological survey that took place in post–industrial brownfield sites and non-contaminated sites with no history of industrial processes over three sampling seasons. 135 sites at 36 discrete locations were measured for environmental characteristics such as temperature, soil moisture, and pH. Earthworms were then collected using a mustard water extraction method. 61 brownfield sites and 74 nearby or adjacent non-polluted sites were tested for earthworm size, species, and quantity.

The existing earthworm and polluted soil literature deal largely with metal bioaccumulation in earthworms and plants, and their residual improvement of laboratory soils. The next logical step is looking at both native and non-native earthworms' effects on phytoremediation of these brownfields in actual field soils. Research is needed to see if plants can grow larger and accumulate more metals if there are earthworms present in field soils. Chapter Three of this dissertation describes a community–level experiment that addresses some criticisms of the ISO and OECD protocols. Soils used for the experiments were from a brownfield site with high metal contamination, four different ecological groupings of earthworm species, and two different plant species were raised in buckets together. This testing design eliminated the bioavailability issue in amended soils reported by Spurgeon & Hopkin (1995), and the earthworm species-appropriateness issue raised by Spurgeon, Hopkin & Jones (1994).

One of the shortcomings of the current literature is that experiments are typically done only on one type of soil, with just a few targeted pollutants — especially those experiments that are using amended soils in their design. While some analyses are broadly applicable, it would be difficult to transfer all the knowledge germane to polluted sites across the country, and likewise with differing earthworm taxons. Although it reduces variables only to have one pollutant solubilized in the soil, it is less applicable than using actual field soils where there can be more interactions and effects. Chapters Three and Four both take an in–depth look at these interactions, using a gradient of ‘low,’ ‘mid,’ and ‘most’ levels of soil pollution from mixed field soils.

Chapter Four was designed as a population–level experiment, which took the plant ‘community’ variable out of Chapter Three. This experiment removed the competition variable by growing plants separately but in similar soil treatments, and prevented the metals from leaching from excessive rain by containing the pots in a greenhouse with minimal watering. Chapter Four also focused on effects of two earthworms, both of which have constant contact with the substrate, addressing concerns of Spurgeon and Hopkin (1995) by not using *E. foetida.*

Interestingly, it seems scientists and land managers in the United States are focused on earthworms in natural ecosystems and habitats, while European scientists are looking at earthworm uptake and amelioration in polluted zones. Most earthworm populations are not native to the Northeastern United States, but with over 1,300 registered Superfund sites and 450,000 post-industrial brownfields in the United States, the impacts and potential amelioration of earthworms in polluted areas must not be ignored.

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Chapter 2: Biological Survey of Brownfield and Non-polluted Soils

I. Abstract:

A biological survey of earthworms was done to establish a baseline of earthworm communities in brownfield soils with an expectation of metalliferous contamination, along with other nearby non-polluted sites. Ground mustard powder in warm water was chosen as a liquid vermifuge to extract the earthworms from 0.25 m^2 of soil. The specimens found at each site were identified, weighed, and cataloged over the spring/summer/fall months at brownfield and non-polluted sites in Eastern Pennsylvania and New Jersey, USA in 2014, 2016, and 2017. A total of 1,538 earthworms were extracted from the 135 sites. Many of the earthworms found (75%) were of three species, all of which are non-native (*Lumbricus rubellus*, *Lumbricus terrestris,* and *Amynthas* species complex). *Amynthas spp.* were found in all site types in later months after the eggs hatched in the spring, making them the most common earthworms found. Contrary to previous assumptions, there were slightly more earthworms located in brownfield areas versus non-polluted areas (12.1 compared with 10.8, p=0.4). and the two had highly significant differences in the overall preserved biomass of the earthworms — in brownfield sites they were 46% smaller overall.

II. Introduction:

Earthworms (Annelida: Oligochaeta) have been well studied in the last few decades for their potentially damaging effects on forests of the Northern United States. During the last ice age (ending ca. 10,000 YBP), earthworms were displaced by the glaciers, tundra, and permafrost to a much lower latitude, (≈35°N) south of the current Smoky Mountains of Tennessee and Georgia (Stebbings 1962; Gates 1976; Reynolds 1978). During European colonization and expansion, earthworms were brought to North America in potted plants and soil ballast in ships, and through many other vectors of transportation (Mann 2007). These non-native earthworms were further spread by westward expansion, agriculture, and the bait industry (Hendrix et al. 2008). Although helpful in farming and composting (Darwin 1881), non-native earthworms prove to be extremely detrimental to forest soils (Hale et al. 2006). Loss of leaf litter and homogenization of soil horizons has led to topsoil erosion (Baker et al. 2006), reduction of crypsis for seeds (Szlavecz et al. 2011), desiccation of seedlings (Mueller 2007), and unfavorable rooting conditions (Bohlen et al. 2004); all of which have led to severe reductions in plant growth and to overall diminution of the forest understory (Blouin et al. 2013).

Phytoremediation is considered to be an environmentally friendly, cheap, and safe way to remove contaminants, in some cases doing the same job as a group of engineers for one tenth of the cost (Gratão et al. 2005). However, such technology cannot necessarily be effective all of the time or be used in all types of contaminated sites. If the contamination runs too deep, or the concentration of toxic compounds is too high, then plants alone cannot efficiently remediate the soil (Cunningham et al. 1993).

Raskin et al. popularized phytoremediation as a bona fide method of soil remediation in 1994, and the search for the most hyperaccumulating plants continues. Studies show that certain plants can absorb disproportionate amounts of metals (Rascioa & Navari-Izzo 2011) and that earthworms have an impact on metals in soils with regard to bioavailability and sequestration, and a few studies have looked at how earthworms can improve growth of hyperaccumulating plants (Aghababaei & Raiesi 2014, 2015; Butt & Grigoropoulou 2009). The possibility of ameliorating contaminated metalliferous soils to improve phytoremediation in brownfields where earthworms are not native requires more study. In order to assess earthworm impacts in brownfield sites, it is important to first survey the earthworms in these locations and compare them to oligochaete populations in non-polluted areas with no history of industry or contamination. This survey created a baseline of earthworm distribution across brownfield and non-polluted soils and throughout different habitats within these areas.

Charles Darwin and Gustav Eisen, both of whom published the first reports of anatomy, physiology, and general classification in oligochaetes completed the earliest earthworm research. However, the most prolific large–scale published earthworm surveys come from Gordon Gates, who was active from 1925–1982, and had some 225 peer–reviewed earthworm–related publications to his credit. In 1949, he published *Miscellanea Megadrilogica* I–VI, and he continued to complete earthworm surveys across North America for the next 30 years. Many of the methods used in early studies of Gates and others (Avel 1929; Stockli 1928; Bornebusch 1930) relied primarily on hand-sorting methods to qualify earthworm species presence in fertile soils, compost, and manure piles. Most of the early literature, and in large part, the current research of earthworm surveys (e.g. Reynolds 2017) aim to determine species biodiversity, which is slightly different than quantifying both organismal presence and abundance. The current biological surveys describe what species are located in certain areas, and do not typically report abundance of the species found. Reports are generally in the form of checklists with no indication of proportion of specimens. One of the earliest and most influential studies was Evans & Guild (1947), in which they surveyed earthworms for eighteen months in permanent old pasture plots from 1945–1946. This paper is believed to be the first that quantifies earthworm temporal distribution and shows dominance and obligatory diapause of different species throughout the year. Satchell (1967) reviewed the relevant papers to date in forest, moor, and pasture land. In this review, he discussed earthworm collection methods including potassium permanganate (Evans & Guild 1947), hand sorting (Svendsen 1955), formaldehyde (Raw 1959), and soil washing (Raw 1960).

Earthworm sampling today is used as not only a soil productivity measurement tool (Eisenhauer et al. 2008), but also a way to rapidly assess soils for contamination (e.g. Vandecasteele 2004) or other anthropogenic damages (e.g. Hankard et al. 2005). Measurements of earthworm densities and species present can be useful tools to measure future site invasibility (Eisenhauer & Scheu 2008; Stoscheck et al. 2012), or important invasive species that are already in place, such as reported by Burtelow et al. (1998) about *Amynthas* spp.

This assessment not only measures biodiversity of earthworm species in soils, but also quantifies species abundance and richness. It is hypothesized that with increasing earthworm density and species richness, soil metal load would be negative and linear.

III. Sampling Methods:

Table 2-1: Sampling method comparison

Method	Success rate	Time	Weight	Expense	Fatal to Non-target	Fatal to earthworms	Damaging to soil	Safety	Public use
Hand Sorting	+	–	+	+	+	+	–	+	+
Electrical Shock	+	+	–	–	+	+	+	–	–
Formaldehyde	+	+	+	±	–	–	–	–	–
AITC	+	+	+	±	±	+	+	+	–
Mustard	+	+	+	+	+	+	+	+	+

There are many earthworm sampling methods, (Table 2-1) including hand-sorting, electrical octet extraction, formalin solution vermifuge, and collection by the delivery of dilute concentrations of allyl isothiocyanate (AITC), which includes mustard and mustard seed powder. Common earthworm sampling techniques are reviewed in Table 2-1 to weigh benefits and drawbacks to each method. 'Success rate' is based on a comparison to hand sorting. 'Time' indicates the time to complete one sample, and while most of the methods can be completed in about 20 minutes per site, hand sorting could take hours or even days, depending on the size of the soil monolith. 'Weight' is the biggest drawback to electroshocking, where the whole apparatus might weigh 50 kg — too heavy for a lone sampler to move to a remote location. 'Expense' is based on concentration units of chemicals given in literature reviewed and was found to be <$0.25 for mustard, and ≤$1.00 for AITC and formaldehyde. Chemicals were priced at Sigma–Aldrich (St. Louis, MO, USA) and the WebRestaurant Store, (Lancaster, PA, USA). In many cases, a severe disturbance to the land is undesirable, and displacing or killing plants and other non-target organisms must be considered. If using undergraduate students or citizen scientists for data collection, 'safety' and appropriateness for 'public use' are also important factors to consider.

A. Hand Sorting:

Hand sorting is the most effective earthworm collection method. As outlined by Raw (1960), Bohlen (1995), and Jiménez et al. (2006), the accuracy and thoroughness of hand sorting is preferred to other methods but comes with a significant time commitment. Hand sorting is ideal for capturing medium-sized earthworms that are larger than 0.2 g, while specimens smaller than this can be difficult to locate (Nelson & Satchell 1962). Likewise, it is not suitable for larger earthworms that can accidentally be fragmented by the digging apparatus (Gunn 1992), darkly pigmented earthworms that blend in with the soil (Nelson & Satchell 1962), or fast moving, deep burrowing anecics (Lawrence & Bowers 2002). Size and depth of the sampled quadrat vary in size across the literature from 20 X 20 cm cylinders (Svendsen 1955), to 0.5 to 1 m^3 as preferred in the United Kingdom, New Zealand and South America (Jiménez et al. 2006), with 30 cm^3 being the most common (Hodson, pers. comm. 2017). As a demonstration of efficacy, in 1962 Nelson and Satchell introduced a known number of earthworms into the soil, and recovered 93% through hand sorting. While hand sorting does recover the highest percentage of earthworms, it is found to be time–consuming, and the physical disturbance it causes is unacceptable on some sites (Zaborski 2003). Most importantly, hand sorting is unsuitable for root-bound, rocky, or high clay and shale content soils like those found in the Northeastern United States (Gunn 1992).

B. Formalin:

Formaldehyde (CH_2O) dissolved in water with methanol stabilizer (commonly: formalin) is frequently used as a chemical vermifuge and has been the most popular method among taxonomists since Raw (1959) first published it in *Nature*. Raw suggested in the original paper where "25 mL of 40 per cent formalin was added to 1 gallon of water and the solution applied to a quadrat of 4 sq. ft. A second application was given when the first ceased to bring earthworms to the surface, usually after about 20 minutes". Arguably the most prolific oligochaetologist of our time, John Reynolds, uses 25 mL of 37% formalin in 4.5 L of water (Reynolds 1977, pers. comm. 2017). This method, much like the AITC method described later, causes irritation in the dermal layers of soil macroinvertebrates including oligochaetes, isopods, and myriapods, which try to escape by coming to the surface of the ground. Many times, as noted by Raw (1959), Singh et al. (2015), and Butt and Grigoropoulou (2009), the top layer of leaf litter is removed and hand-sorted for small epigeic earthworms, and the formalin solution is then poured into the resulting pit to extract anecics and endogeics. Despite the relatively successful track record of formaldehyde solution, it is not without its drawbacks. Formalin is carcinogenic to humans, and long–term exposure can result in a host of chronic problems such as lung cancer, emphysema, and coronary disease, while short–term exposure can lead to skin rashes, asthma, and reduced lung capacity (Sakamoto et al. 1999). In many situations, the use of formaldehyde or formalin is still appropriate in preservation and taxonomic sampling. Users should wear personal protective equipment and take steps to minimize risk. However, since undergraduate students, citizen scientists, and other minimally trained volunteers often do much of the sampling (e.g. The Great Lakes Worm Watch, Wormwatch Canada), the use of formalin with little oversight by qualified personnel is an unnecessary risk (Iannone 2012). Even if dangers to people in the field are mitigated, formalin kills not only the earthworms being sampled, but also soil macroinvertebrates, plants, bacteria, and fungi in the quadrat (Eichinger et al. 2007). This poses an ethical and perhaps legal problem depending on the ownership of the site.

The efficacy of formalin vermifuge is also consistently questioned in the literature. Eisenhauer et al. (2008) found that formalin was superior to mustard and AITC, as did Pelosi (2009) and several others; but that its efficacy was dependent on species being sought (Callaham & Hendrix 1997). Contrary to this, Gunn (1992), Ianonne et al. (2012), and Chan and Munro (2001) among others, found mustard and/or AITC suspension to be slightly better than formalin. Currently, when considering the relative safety and harmlessness of mustard and AITC, formaldehyde is no longer the most widely preferred method of earthworm sampling.

C. Electrical Octet Method:

Electrical extraction of annelids is practiced using an electrical octet machine, an apparatus utilizing a power source, single phase capacitors, and eight stainless steel rods approximately 60 cm in length (Schmidt 2001; Weyers et al. 2008). The eight rods are sunk into the soil in a circular configuration approximately 1 meter in diameter to a depth of at least 40 cm, and voltage is increased stepwise in 5 to 10–minute increments from 120 to 600 volts at <1 amp. The rods are inserted into the ground opposite each other in the sampling area and sequentially charged; thus creating a flow of electrons through the substrate that expels the irritated earthworms. The power source utilized could be 120 or 240 volts AC from a nearby receptacle or generator. In more remote locations, electricity can be derived from a deep cycle marine grade 12 volt battery with an inverter.

Typical costs for the octet electrical sampler can range from a few hundred dollars for a homemade model, to $3,000 or more for a commercially available version (Weyers et al. 2008). The drawbacks to this electrical apparatus are its expense, weight (up to 50 kg depending on battery size), and ability to penetrate the ground to 40 cm. The karst and shale topography of the study region would ensure difficulty with this protocol.

The efficacy of electrical extraction is also a matter of debate. Schmidt (2001) found that while electrical extraction in wheat fields in Ireland yielded numerically higher results, these results were not significantly better than formalin methods of extraction executed at the same time.

D. Pure Allyl Isothiocyanate:

Allyl isothiocyanate (AITC) is an organosulfur compound with the formula CH_2CHCH_2NCS. It is a colorless oil that creates the sharp, spicy, biting taste found in mustard, horseradish, and radishes. AITC irritates the sensitive skin of earthworms, causing them to climb to the surface to escape the burning sensation (Lawrence & Bowers 2002). AITC is best dissolved in isopropyl alcohol, and this mixture is then diluted with water to produce an effective vermifuge (Zaborski 2003). Since AITC is a pure molecule, its concentration strength can be standardized across all sample sites. Naturally occurring AITC levels can vary in the mustard based on many factors, and the use of pure AITC eliminates these variables. Zaborski (2003) reports that because of the low concentrations needed (1 mL/L), it is roughly half the price per sample compared to formalin. Much like formalin, the efficacy of allyl isothiocyanate is dependent on quantity in solution: too little (less than .75 mM), and the earthworms will not react. At concentrations greater than 1.5 mM, the earthworms die in their burrows before they can surface (Čoja et al. 2008).

The drawbacks from AITC include that it is a severe irritant in the concentrated form, which then has to be carefully and safely diluted to the working concentration. AITC also has a brief working time in dilute concentration (Zaborski 2003). It must be ordered from a chemical supply house and requires more expensive shipping protocols. Given these sometimes difficult working parameters, Čoja (2008), Singh et al. (2015) and others report that AITC is only as effective at optimal concentration (1.0 mM) as formalin and mustard. These added layers of complexity make AITC harder to use for school groups, minimally trained undergraduates, and citizen scientists (Valckx et al. 2011).

E. Mustard and Mustard Powder:

Gunn (1992) first suggested the use of prepared mustard (initially bottles of spicy brown mustard), but early studies did not remove the variables of vinegar, sugar, and other flavorings in the preparations, and also could not keep strong mustard solutions in suspension (Gunn 1992; East & Knight 1998). Edible, prepared brand names recipes of mustards are not available everywhere, and some of the strongest formulations may only be available regionally, making it difficult to standardize methods.

A simplifying resolution to standardizing AITC levels in prepared versions of mustard is to use plain mustard powder. Three species of mustard are commercially available and sold as a powder or “mustard flour”: *Brassica nigra,* black mustard; *Brassica* *juncea*, brown mustard; and *Sinapis alba*, white mustard. The amount of allyl isothiocyanate varies slightly between species (Hirasa & Takemasa 1998), and even by year and region of growth (Valckx et al. 2011). There are a few studies that attempt to define a "normalized" AITC concentration in mustard powder. But, while certain brands or years may be slightly higher in AITC levels, efficiency across varieties for earthworm collection is reported to stay about the same (for a review, see Pelosi 2009 and Singh et al. 2015).

As with all the other collection methods, there is literature championing mustard powder (Čoja et al. 2008), as well as doubting its efficacy (Bartlett et al. 2006). Lawrence and Bowers (2002) showed that mustard extraction could account for 98% of total earthworm biomass and 83% of species present when compared to hand sorting.

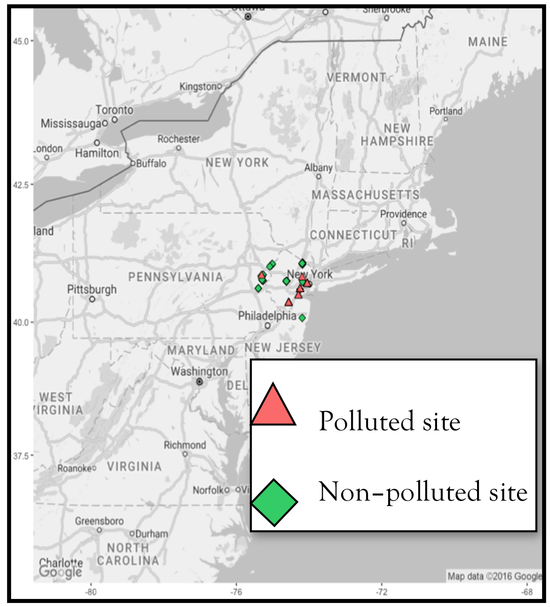
F. Conclusion of Method Review:

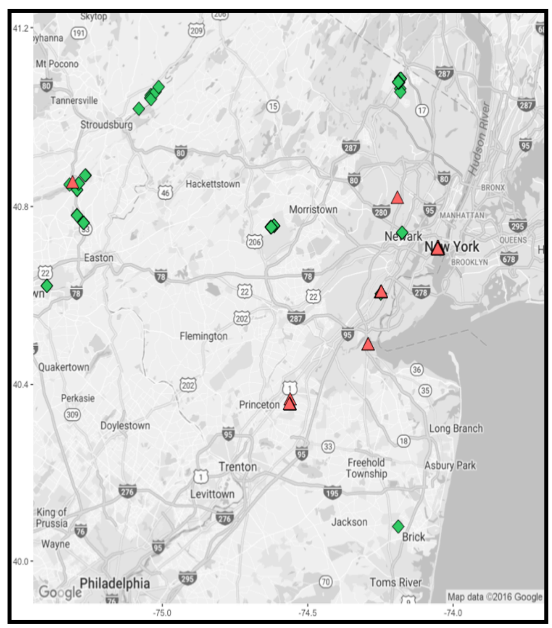
Generally, it has become widely accepted that while some authors can show better earthworm extraction with their protocol of choice, the three (mustard, formaldehyde and electroshocking) methods are comparable (Schmidt 2001). Mustard powder in water was chosen as the collection method in this study because of its safety (Iannone 2012), widespread use (Hale 2004; Hale et al. 2006), efficiency in a variety of soils (Eisenhauer & Scheu 2008), and flexibility in forested, root-bound soils (Nuzzo et al. 2009).

IV. Sampling Locations:

Earthworms were collected throughout Pennsylvania and New Jersey, USA (Figure 2-1) starting from the Pocono Mountains of Pennsylvania (40.849, -75.314) to as far east as New York Harbor, Jersey City, New Jersey (40.702, -74.050), and a span of roughly 110 kilometers. 135 samples were taken from 36 different locations and dates in both brownfield and non-polluted areas (Figure 2-2). Non-polluted sites showed no historical record of industry and little to no contamination, while brownfield sites all have or have had active remediation or restoration programs in place. As noted in Table 2-2, all of the brownfield sites have post-industrial use and the expectation of some type of metal toxicant contamination. However, they are in close ecological proximity to non-polluted sites, and many of the same species inhabit both locations — making them comparable to each other except for the anthropogenic pollution and artifactual texture of the soil. Soils were not tested for contamination at the

Figures 2-1 (top) wide view and 2-2 (bottom) zoom of sampling sites.





time of surveying. Table 2-2 lists the locations, along with city and state. For full details of all 135 samples, see Appendix C in this this chapter.

Table 2-2: Brownfield and Non-polluted locations used in study, noting contaminant and locality.

Brownfield Site Name	Location	State	# Samples
Railroad bed	Wind Gap	PA	1
Railyard 1	Jersey City	NJ	8
Historic dump	Linden	NJ	4
Historic dump	Linden	NJ	5
Railyard 2	Jersey City	NJ	6
Railyard 3	South Amboy	NJ	3
Glass smelter	Bloomfield	NJ	6
Railyard 4	Jersey City	NJ	7
Railyard 5	Jersey City	NJ	9
Railyard 6	Jersey City	NJ	7
Silt collect. basin	Hanover	NJ	4
Power/ gas line	Hanover	NJ	1

Known and expected contaminants:

- Coal, ash, slag
- Metal toxicants (As, Cd, Pb, Zn, etc.)
- Hydrocarbons (petroleum)
- Polynuclear aromatic hydrocarbons
- Polychlorinated biphenyl

Non-Polluted Site Name	Location	State	# Samples
Garden	Easton	PA	3
Backyard	Stockertown	PA	4
Lawn	Wind Gap	PA	1
Gravel	Wind Gap	PA	1
Garden	Pen Argyl	PA	2
Lawn	Pen Argyl	PA	1
Sports field	Pen Argyl	PA	1
Compost pile	Pen Argyl	PA	1
Eco preserve	Saylorsburg	PA	1
Federal park	Marshalls Creek	PA	1
Cornfield	Milford	PA	1
Cemetery	Shawnee	PA	1
Trail access	Milford	PA	1
Boat launch	East Stroudsburg	PA	1
Soy field	East Stroudsburg	PA	1
Eco preserve	Mendham	NJ	10
County park	Mawah	NJ	6
Backyard	Bethlehem	PA	3
Eco preserve	Plainsboro	NJ	3
Eco preserve	Mawah	NJ	10
State park	Nazareth	PA	2
Campus flowerbed	Newark	NJ	2
Eco preserve	Somerville	NJ	9
Eco preserve	Somerville	NJ	8

Once at the location, site sampling was completed using a stratified random sampling technique (Seber 2002). This design specifically targets areas earthworm suitable habitat at a fine scale [m1] . Stratified random sites at each location were chosen loosely based on Loss et al. (2013), in which leaf litter type, detritus age, castings and middens present, soil moisture, soil richness, and plant cover were all factored together to visually target earthworm habitat. Regardless of whether the site was a brownfield or non-polluted site, site selection was always done with the goal of finding earthworm population levels representative of the habitat. In this way, all researchers were able to avoid sampling location bias based between brownfields and non-polluted sites. Individual soils at brownfield sites were not evaluated for metalliferous content, but due to historical records of the site, visible soil texture, and possible previous remedial work, there was a strong expectation of mild to severe contamination.

V. Methods:

Sampling took place in the spring, summer, and fall in 2014, 2016, and 2017 and was completed by the author, fellow graduate students, or undergraduate lab assistants. All personnel were trained in collection protocols several times before collecting alone and a laminated step–by–step instruction sheet was included with all the collection buckets. To organize all the materials, a clean five-gallon bucket with a cloth–pocketed insert was provided to collectors. Inside were all the tools needed (thermometers, shovels, tweezers, weather meters, etc.). This arrangement also provided enough space inside for containers of alcohol and earthworms. The following are the step-by-step instructions from the sheet in the collection buckets and rationales used with all sampling efforts.

1. The liquid vermifuge was prepared with mustard powder as per Gunn (1992) and Iannone (2012). First, 40 g (≈80 mL) mustard powder was soaked in 3.8 L of warm water for more than one hour but for less than a week (the powder begins to degrade, and the smell becomes foul).

2. The mustard water was transported to field sites in 3.8 L milk jugs.
3. The ambient air temperature and general weather conditions recorded from the weather meter were noted in the field notebook, as well as exact latitude and longitude (to 5 digits) using smart phone or GPS.
4. Vegetation and leaf litter were cleared from sample site with the small garden rake and shears. Epigeic earthworms in leaf litter were counted as part of census if they occurred inside where the ring was to be placed.
5. The soil surface temperature was measured using the infrared thermometer.
6. 3-in-1 Moisture Meter was used to determine approximate soil moisture and pH.
7. The plastic ring was placed flat on the soil surface, using the hand spade or rake to get a better seal around the bottom to keep the mustard solution inside the quadrat.
8. The wooden cross was placed on top of the rim, stood on, and checked again for a good seal at soil level.
9. The timer was set for ten minutes.
10. The entire 3.8 L of the mustard solution was slowly poured evenly across the quadrat while standing on the wood cross, which is sealing down the edges of the ring.
11. When the majority of the mustard solution was absorbed, the wooden cross was removed, and collection began.
12. The earthworms were picked up with tweezers or forceps. This minimizes damage to small earthworms and keeps the earthworms more intact for identification later.

Collection Protocol:

a. No earthworms were collected after 10 minutes of sampling effort.

b. No earthworms from outside the ring, no matter how close.

c. Earthworms that surfaced before mustard water were acceptable, as long as they would be in the ring once it was placed.

d. Even small earthworms were counted- since many species experience adulthood in different temporal stages; if only the large earthworms were to be collected, it is possible to ignore a whole species that may be in its non-adult or dormant phase.

13. Detritus and removed leaves were pulled back over sample quadrat to minimize impact.
14. Collected earthworms were placed in a ≈1 L plastic container with about 3 cm of ethanol and water (70% EtOH). When earthworms are exposed to irritant chemicals (like the AITC in the mustard), they can release mucosal slime as a defense. This, along with the mud and soil can be washed off in the alcohol bath before being placed into the specimen jar into which they are stored. The alcohol also euthanizes them in the original container. Many times as they die, they defecate or expel more slime, washing the earthworms prior to storage helps to keep the long-term preservative alcohol clean.
15. The cleaned earthworms were transferred into a labeled specimen jar and covered by more than 0.5 cm with clean 70% ethanol. In a 125 mL jar, no more than 30 earthworm specimens were stored. The preservation capacity of the ethanol becomes too dilute for storage when added to the coelomic and cellular fluid resident in the earthworms.
16. After approximately one week, depending on the quantity of earthworms in the jar, the yellow and sour ethanol was replaced to avoid decomposition. Alcohol was changed

again after two weeks since collection and one month after this, as required to maintain clarity of the preservative fluid.

Quadrat delineation and sampling at each site was done with the top 8 cm of a 55–gallon plastic drum because of its durability and dimensions. The top 8 cm was carefully removed with a table saw; a scroll saw was then used to remove the fused lid, leaving the sturdy rim. The durability was important, as it needs to be stood upon (Figure 2-3). The drum's dimensions were also critical in that the inside surface area of a 55–gallon drum measures exactly 2500 cm^2, or 0.25 m^2. It is enough of an area to be substantial if there are few earthworms, but not unmanageable if there are many. A few sites had over 100 earthworms per 0.25 m^2, and if the area were larger, catching them all before they escaped or time expired would have been a challenge for a single individual to collect.

Figure 2-3: Sampling quadrat with the wooden cross.

The wooden cross was then built to stand on and distribute the weight across the whole ring. The cross was made from lumber scrap and overhung the ring by ≈5 cm on each side. Notches 1 cm deep were cut in the bottoms of the boards to interlock with the ring on all four sides of the wood to avoid sliding. The notches were not part of the initial design, but their necessity became immediately and violently apparent during the first trial run.

VI. Results:

Figures 2-4 and 2-5 are violin plots of results of specimens collected per site in non-polluted (green) and brownfield(red) locations. As an orientation, note that violin plots have all the same features as a Tukey's original box plot, with the added benefit of a swarm plot to describe the shape of density distribution (Hintze & Nelson 1998). The dark central line demonstrates the first to the third quartile, showing interquartile range. The white dot represents the median of the population, the thin black line shows the upper and lower adjacent values ([first or third] quartile value ±1.5 • interquartile range), while the outliers fall into the colored peaks on the top and bottom. The width of the plot shows population density at a particular point on the Y-axis, effectively acting as an unbinned horizontal histogram. Significance codes are given between each comparable pair of data sets, and are based on a converted t–score from a Student's two–way paired distribution test.

Figure 2-4: Count of Specimens per m^2

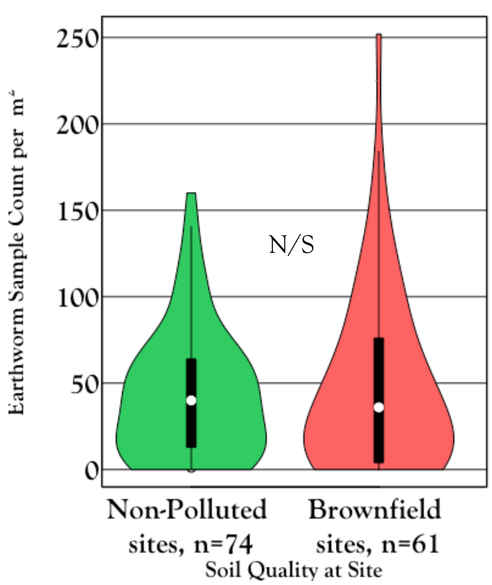
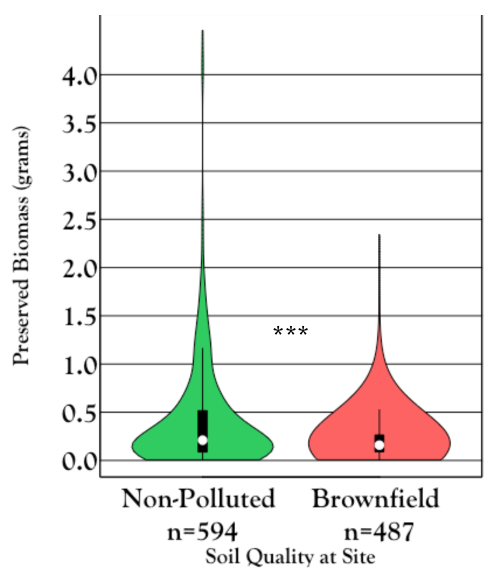


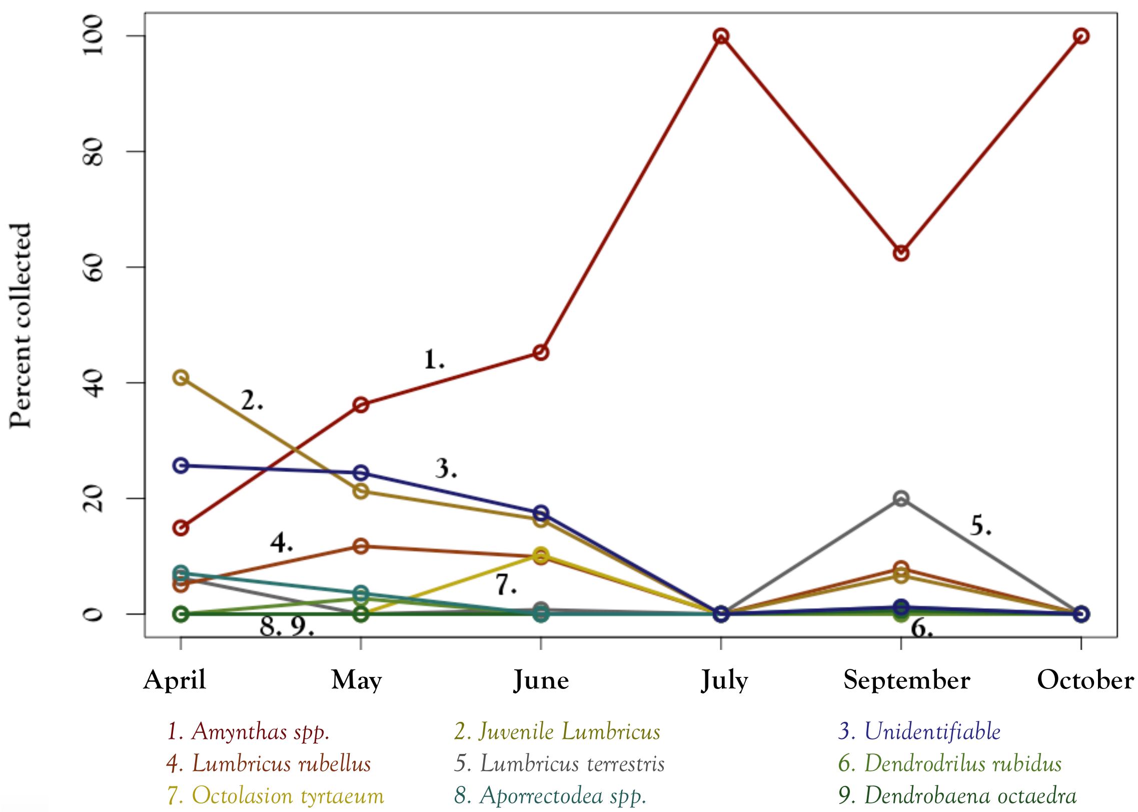
Figure 2-5: Comparison of Specimen Weight by Site



A. Temporal and Biomass Distribution Throughout the Sampling Season:

In order to quantify when particular species were captured, all sampling efforts were divided into their respective months and placed in Figure 2-6, below.

Figure 2-6: Earthworm Species Percentage by Month, 2014, 2016, 2017

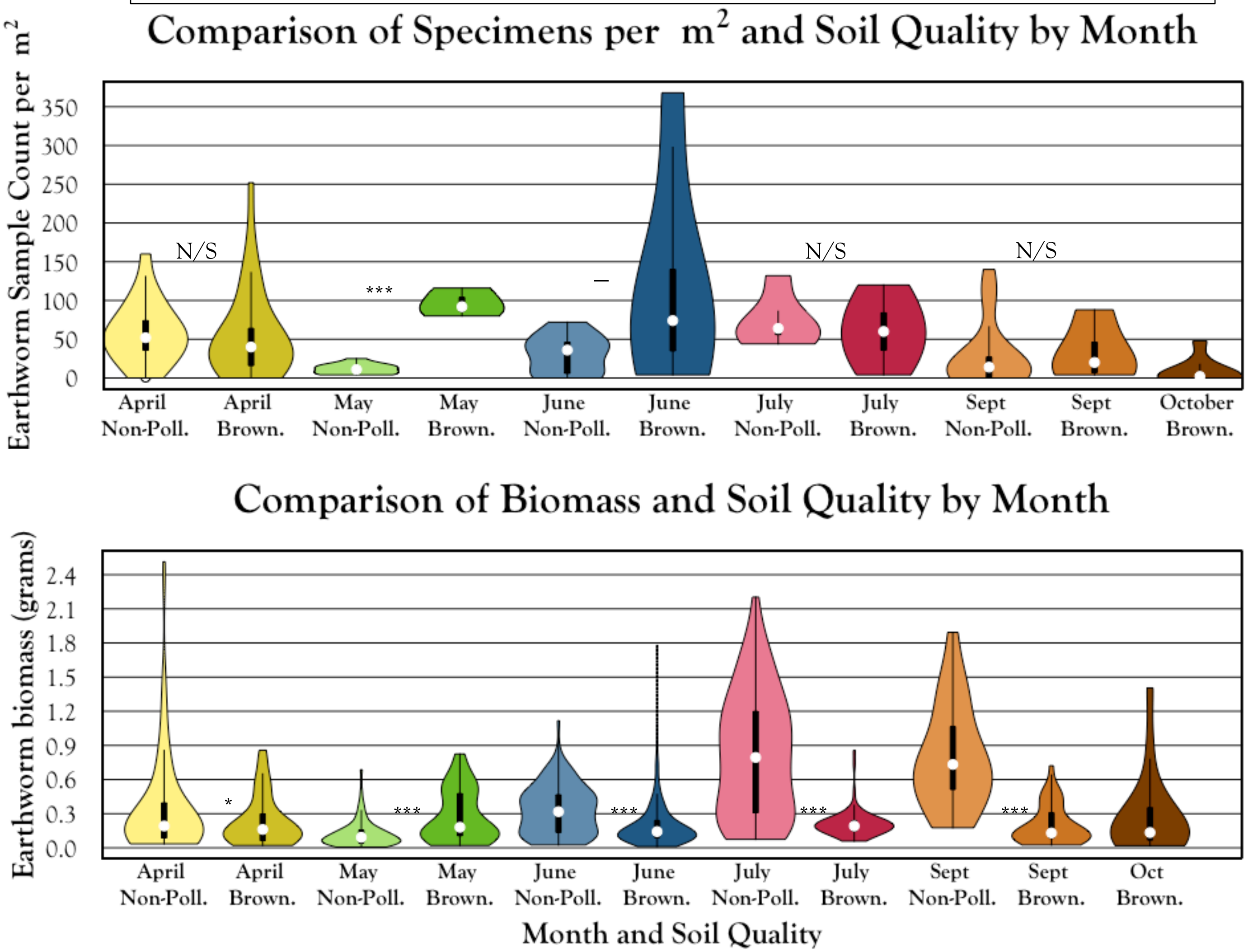


The temporal patterns shown in Figure 2-6 are exactly concurrent with previous research. *Amynthas* population density (Figure 2–6:1) is lower in the spring when the eggs are first hatching, and eventually becomes the primary earthworm in most habitats. This agrees with Stebbings (1962) and Hendrix et al. (2006) when they posited that more invasive earthworms would outcompete native and less aggressive non-native species in the same habitats. The population density of *Aporrectodea* disappears (Figure 2–6:8) in the hottest months of the summer, and they are found again in the fall after their "obligatory diapause". This obligatory diapause could be due to lack of moisture in the soil, as Evans & Guild (1947) noted with *Aporrectodea* spp., or as Callaham et al. (2003, 2006, 2016) and Görres (2012) described with *Amynthas* as their annual breeding cycle.

Juvenile Lumbricids are common in the spring, but as they have a chance to mature, they become identifiable as either *L. rubellus* or *L. terrestris*, which is why the curve for juveniles (Figure 2–6:2) drops off in the early summer. July weather was remarkably hot during the execution of this survey, and although there were 28 samples collected over the three years, only *Amynthas* were present in any of them. Weather also seems to affect *L. terrestris*, as populations during the spring dispersal (2–6:5) vanish during the summer and re–emerge in cooler fall temperatures (Butt & Grigoropoulou 2009).

Figure 2-7 on the next page shows overall earthworms at each sample site in brownfield and non-polluted soils separated by the month the samples were taken.

Figures 2-7 & 2-8: Month by month comparison of soil quality and biomass



B. Specimen Counts in Non-polluted and Brownfield Sites:

Non–polluted sites contained an average of 43 earthworms per m^2 (site n=74, SE=4.32, CI=7.16, SD=36.9), while brownfield sites yielded on average 49 specimens per m^2 (site n=61, SE=6.67, CI=11.06, SD=51.73). There were slightly more earthworms found in brownfield sites overall, but this quantity was insignificant with a P-value=0.45 based on a Welch Two Sample T-Test.

Table 2-3: Summarized Results of Biomass

Month	Soil quality	Average quantity	p–value quantity	Average biomass	p–value biomass
April	Non-polluted	59.3684	0.94	0.3780	0.0128
April	Brownfield	58.2069		0.2358	
May	Non-polluted	11.4615	0.0000	0.1222	0.0000
May	Brownfield	96		0.2813	
June	Non-polluted	30.5	0.0639	0.3344	0.0000
June	Brownfield	88.3333		0.1961	
July	Non-polluted	72.8	0.6454	0.8220	0.0000
July	Brownfield	60.8		0.2115	
September	Non-polluted	29.3333	0.8760	0.7905	0.0000
September	Brownfield	33		0.2012	
October	Brownfield	9		0.2791	N/A

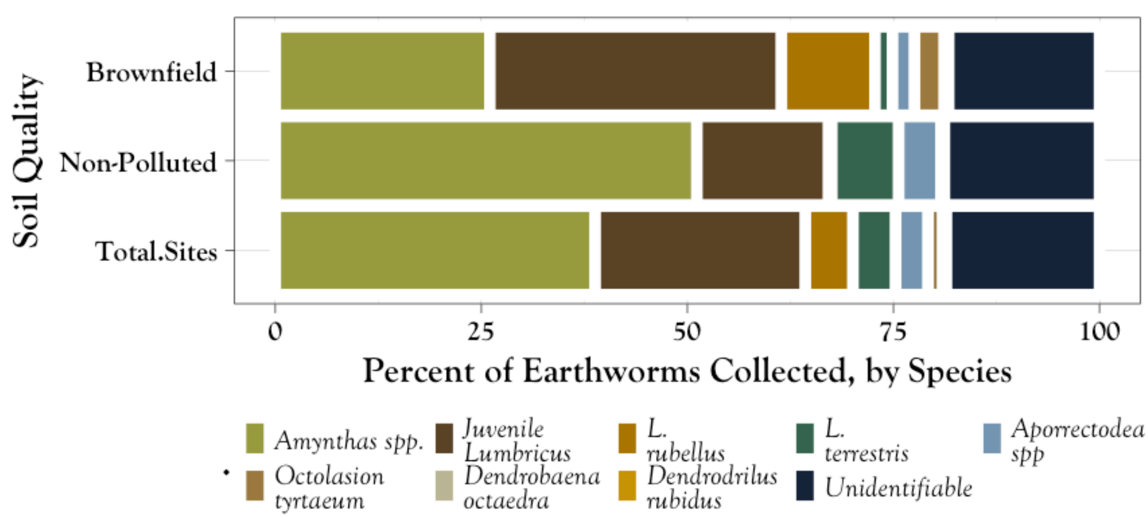
Table 2-3 details 1,538 earthworm specimens collected from 135 sites at 31 locations, 74 of which were non-polluted (with no history of industrial use) and 61 that were located in post-industrial brownfield sites.

The biomass of earthworms collected in brownfield sites that were weighed (n=594, average=0.22 g, SD= 0.2, SE=0.008, CI=0.015) was, on average, 46% lower than earthworms collected in non-polluted areas (n=487, average=0.38 g, SD=0.43, SE=0.019, CI=0.038). This disparity is highly significant (<0.0001) when compared using a Welch's two-tailed unpaired T-Test.

C. Identification and Classification of Species:

All collected samples and their corresponding data were analyzed after returning from the site. Earthworms were identified according to Hale (2013), Reynolds (1978) and occasionally by consulting the online dichotomous key at discoverlife.org at (http://www.discoverlife.org/mp/20q?guide=Earthworms). For a full chart of all sites and their quantities collected, see appendix 2C. Figure 2-9 below plots total proportions of all earthworms collected. Many of the specimens (25% over all sites) were classified as "juvenile Lumbricids". It is important to note that setal patterns, pigmentation, and general morphology can identify lumbricids to genus, but the main difference between *Lumbricus rubellus* and *Lumbricus terrestris* is the beginning of the clitellum on XXVII or XXXII. In aclitellate juveniles, a positive identification was impossible. For purposes of this survey, those data have been recorded in the "juvenile" column for maximum accuracy. As with the juvenile Lumbricids, "unidentifiable" earthworms were aclitellate, non-sexually

Figure 2-9: Earthworms Sampled in Brownfield versus Non-Polluted Soils.



mature specimens, without identifying pigmentation, unique setal patterns, or other characteristic traits. They ranged in morphology from newly hatched young that could be described as segmented threads to older, physically stunted samples from heavily polluted sites. In all cases, even if there was only one other species identified in the quadrat, these specimens were considered unidentifiable.

Genus *Aporrectodea* has three possible species regionally. *A. calignosa, A. longa,* and *A. rosea* are relatively easy to discern when adults (Reynolds 1978), but are much harder without molecular analysis as juveniles (Hale 2013). Since they serve similar ecological duties, they are just referred to in this survey as *Aporrectodea spp*.

In 2016, Chang et al. published a manuscript that greatly enhances the identification of the *Amynthas* complex. This identification had previously been based mainly on Goto and Hatai (1898) and identification with "outdated taxonomic information and based primarily on internal morphology". They then go on to state "that many recent claims of invasion of *A. agrestis* need to be re-evaluated for potential misidentification" when it is more likely the entire complex is present.

Likewise, based on Chang (2016), all earthworms that fit the *Amynthas agrestis* identification have been put into *Amynthas spp.,* pending future identification based on novel taxonomy characteristics.

D. Analysis of Diversity Indices:

Based on the richness and evenness of earthworms captured during the survey, the following table (Table 2-4) lists both Simpson's Index of Diversity and Shannon's Diversity Index for A.) all sites, B.) non-polluted sites, and C.) brownfield sites.

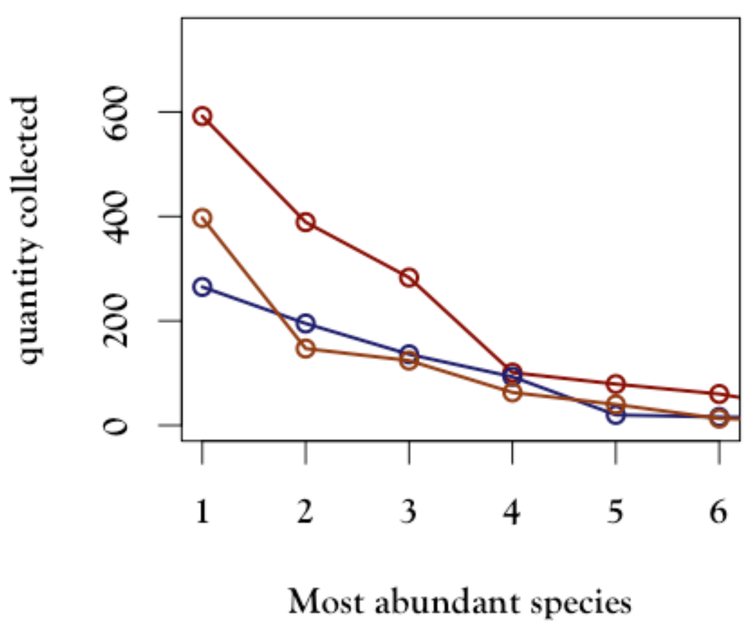
Table 2-4: Simpson's and Shannon's Diversity Indices

Location type:	Simpson's	Shannon's
A. All sites	D=0.337	*H*=0.346
B. Non–polluted sites	D=0.358	*H*=0.326
C. Brownfield sites	D=0.312	*H*=0.37

Both measures in Table 2-4 (Shannon's and Simpson's Indices) are fairly even, as are the diversity measurements between differing sites. Therefore, it is concluded that the brownfield sites and non–polluted sites contain similar diversity of earthworm communities.

Figure 2-10 shows quantity of each of the species ordered from largest to smallest. The red (top line) is total earthworms collected, orange (second line down) is all non-polluted sites, and blue line (third down) is brownfield sites.

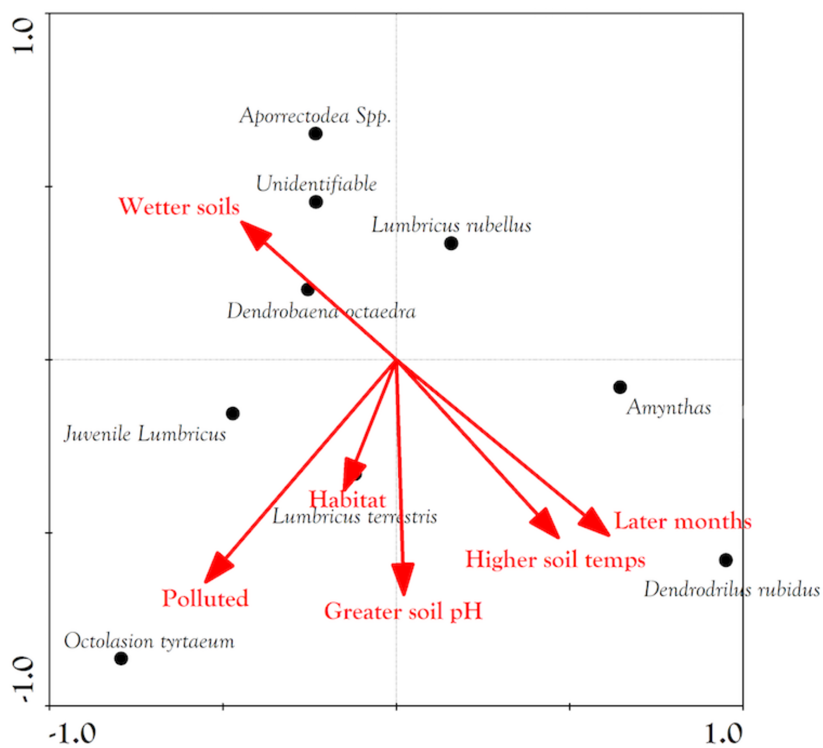
Figure 2-10: Species abundance curves by site type and proportion.



E. Canonical Correspondence Analysis of Assessment Results:

In order to more completely explain species found and some of the factors that influenced their quantities and locations, all specimens have been plotted onto a canonical correspondence analysis matrix (CCA) (Figure 2-11). Plotting multivariate analyses can be explanatory, but is not indicative of location per se. All CCA multivariate analyses were completed with Canoco for Windows V. 4.5 (Microcomputer Power, Ithaca, NY). The longer the arrow appears, the more important of a factor of earthworm locations and quantities. In Figure 2-11, note the overall importance of "later month" and insignificance of "habitat", based on arrow length. For a larger version, see Appendix B of this Chapter. The relative length that an arrow is drawn represents the overall importance to the location or quantity of the species located during the survey.

Figure 2-11: Multivariate Analysis of Earthworm Survey



For instance, "habitat" was relatively unimportant for determining where earthworms would be located, or how many would be there. CCA can also indicate relationships – in this case, later months are nearer to higher soil temperatures and directly opposite of soil moisture.

Figure 2-12: CCA of earthworm survey with LOESS plot of moisture.

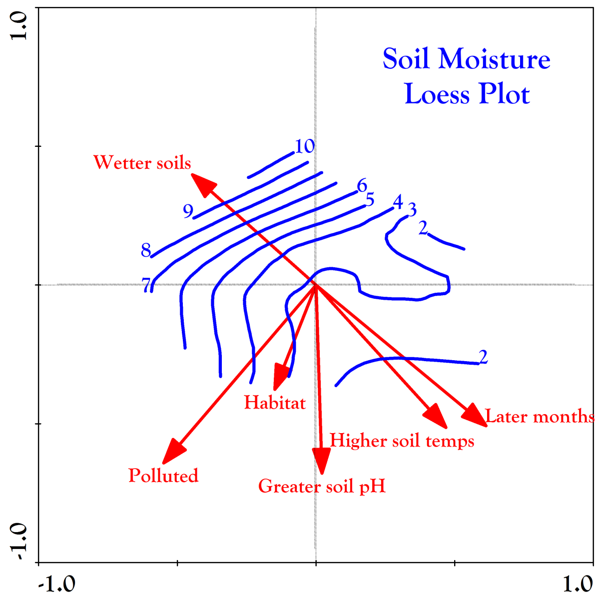
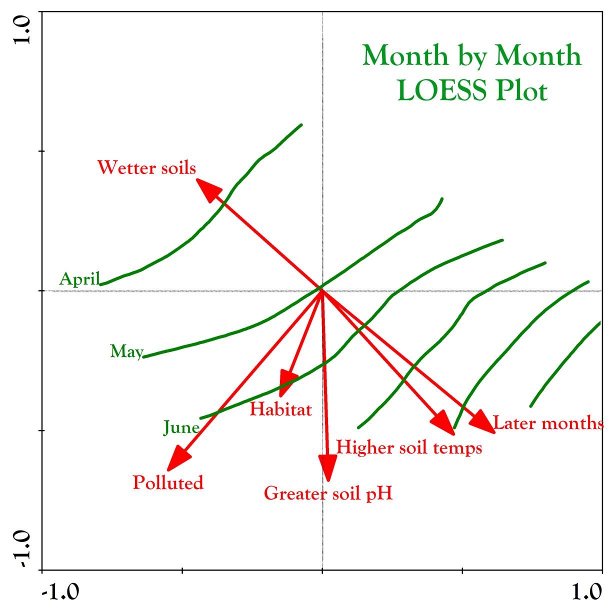
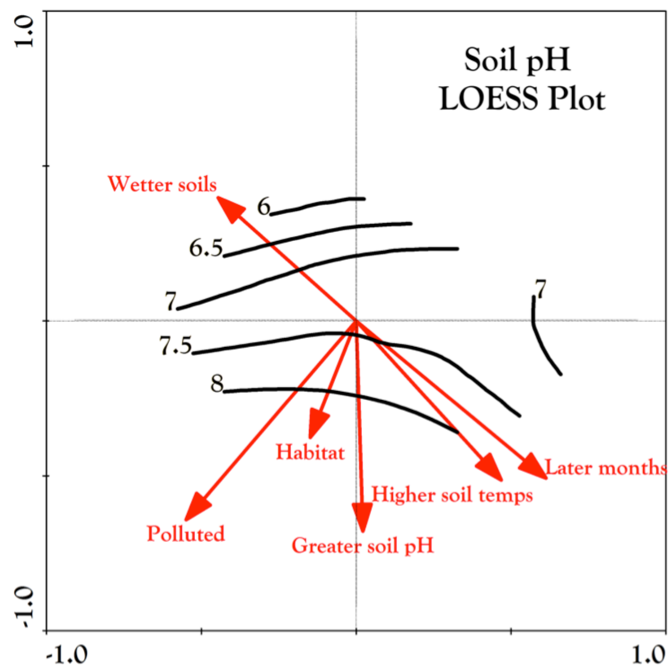


Figure 2-13: CCA and LOESS plot of monthly distribution.



As a measure of checking for accuracy of the CCA model, obvious relationships like higher soil temperatures in later months, with the opposite effect of wetter soils can be beneficial. The CCA depicts multiple levels or categorical environmental factors across a gradient. For example, Figure 2-12 has highlighted soil moisture levels in a LOESS plot (from LOWESS, or LOcally WEighted Scatterplot Smoothing) showing the gradient of readings from the soil moisture meter in the field. Figure 2-13 indicates months of the survey, and along with moist soils, are the two key factors for locating *Aporrectodea* and *Denrobaena octaedra.* Earlier months are also the most likely times to find earthworms that are "unidentifiable", since newly hatched earthworms are present in the spring. *Octolasion tyrtaeum*, *Lumbricus terrestris*, and *Dendrodrilus rubidius* were the only earthworms located in basic soils, (Figure 2-14) while the other species seem to prefer slightly acidic habitats.

Figure 2-14: CCA and LOESS plot of soil pH



Although the pollution level was factored as bimodal (Y or N), earthworms found in both types of sites lie between the contour lines (Figure 2-15) e.g. *Dendrodrilus rubidius* was only found in brownfield sites, but juvenile *Lumbricus*, *Dendrobaena octaedra*, and *Amynthas spp*. were found in both.

Figure 2-15: CCA and LOESS plot of pollution in soils

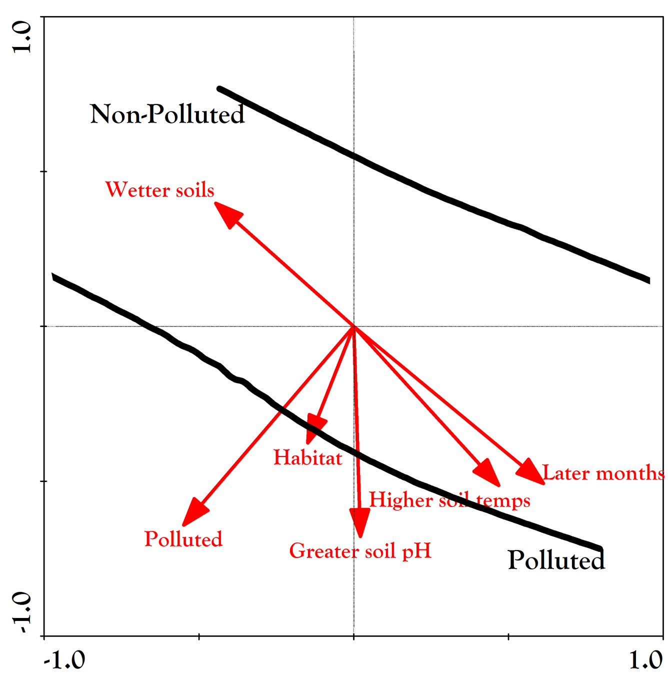


Figure 2-16 is an example of how site locations were calculated. The small numbers are all 135 sampling sites plotted onto the plane, based on the relationships to the environmental factors (computed by the square of the Euclidean distance in a linear regression model). Species locations (e.g. Figure 2-17) are derived from the sites where the earthworms were located and weighted by the quantity found at the site in a scatterplot, as indicated by the size of the circles. Sites not containing that particular species are indicated with a tiny +. In Figure 2-16, *Amynthas* were found in many sites, especially later in the summer and early fall. The proximity to the center of the plot indicates that they are more generalist, and are not impacted by habitat, soil pH, or pollution level. Overall counts, along with the gradients dictate where the species is plotted (demonstrated here by the gold star).

Figure 2-16: All sites plotted on the CCA matrix based on environmental factors.

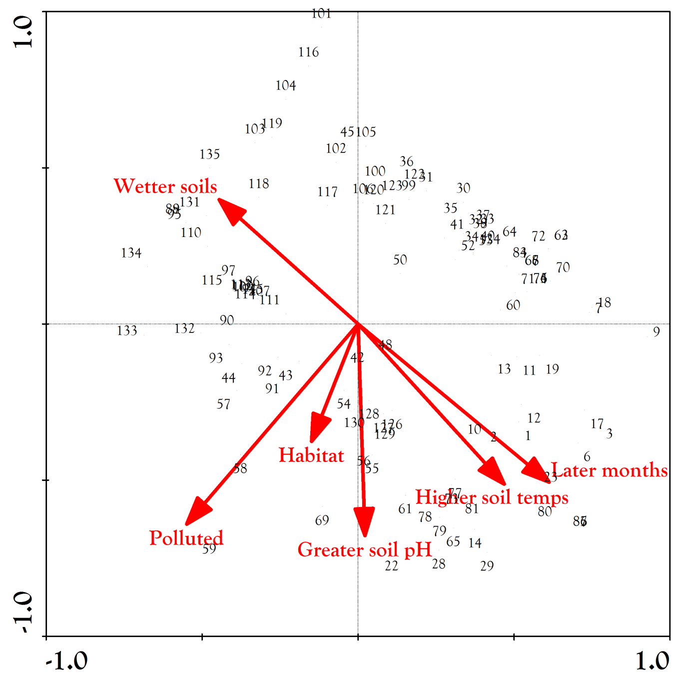
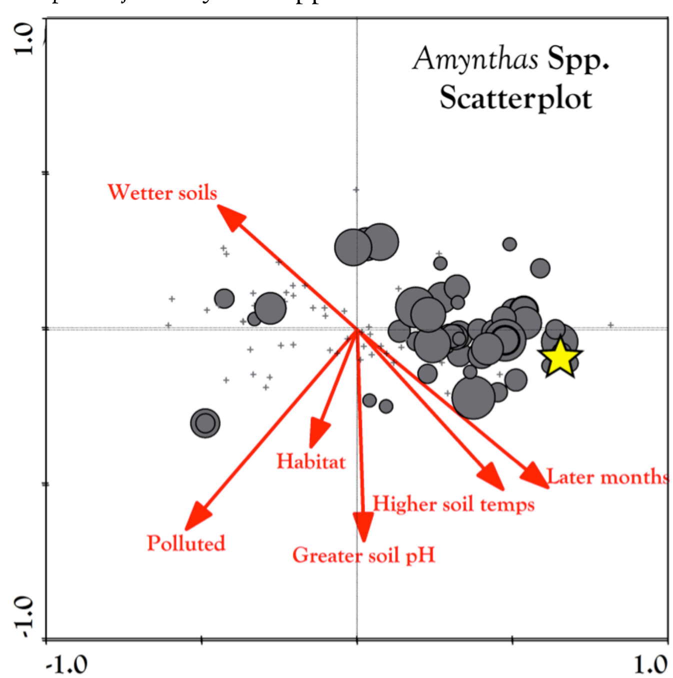


Figure 2-17: Scatterplot overlaid on the CCA matrix, demonstrating location on the coordinate plane for Amynthas spp.



VII. Discussion:

Many earthworms (and invertebrates, in general) have a seasonal or temporal distribution (Rözen 1988). *Amynthas* especially demonstrate this annual life cycle (Callaham et al. 2003; Snyder et al. 2010; Chang 2016) although it has been noted in Evans and Guild (1947) that many other species — especially the *Aporrectodea* complex — must undergo an annual or obligatory diapause. This earthworm species survey was indicative of the types of earthworms living in different areas but as diverse as the sites were, the quantities of earthworms in each quadrat and the species identified did not change a great deal. There were eight different species found during the survey, but three species were the most common, often by several orders of magnitude: *Lumbricus terrestris* (Linnaeus 1758), *Lumbricus rubellus* (Hoffmeister 1843), and *Amynthas spp*. (Goto & Hatai 1898; Chang 2016). Commonly, these earthworms are referred to as nightcrawlers, red worms, and crazy or jumping worms, respectively, and none of them are native to North America.

Niche apportionment models (based on MacArthur 1957, 1961) hypothesize that the most common species will be multiple times more prevalent than the next most common species. Although that was not found to always be mathematically valid in this survey (Figure 2-10), each subsequent most common species was on average 51% less prevalent than the one before it. While these numbers do fit into hypothesized diversity models, greater quantities of species were initially expected.

Burtelow et al. (1998) report their initial finding of *Amynthas* species complex (*A. agrestis, A. corticis, A. gracilis*, and *Metaphire hilgendorfi*), in New York State, USA and notes the patchy distribution of extremely dense populations present, and their detrimental consumption of leaf litter and granularization of topsoil — all effects echoed in the current study. They also studied the deleterious impact of *Amynthas* on forest soils, including this species removal of carbon stocks and rapid biogeochemical cycling. Görres et al. concurs with these assessments (2012, 2014) on the significant impacts from *Amynthas* in the forests of Vermont, and they note that *Amynthas* can mature in 90 days from hatching in the spring. This could have serious implications for the spread of this earthworm into the Northern United States and Canada as temperatures rise.

Callaham et al. (2003) created pitfall traps for earthworms in the Appalachian Mountains of northern Georgia. The majority of the captured organisms were *Amynthas spp.*, which displayed a temporal distribution pattern. *Amynthas* adults do not survive the winter by burrowing below frost level and suspending animation like most other earthworms in the northern latitudes. Instead, they lay many eggs in the fall and die as temperatures begin to drop (Callaham et al. 2003; Gorres et al. 2014). Eggs hatch in mid-spring, and adult prevalence increases throughout the summer, while mature *L. rubellus* and *L. terrestris* were present throughout the entire sampling season. It was more likely, however, to find juvenile Lumbricids and unidentifiable earthworms in the spring when the newly hatched young had not matured yet. This finding is also heavily supported by the CCA biplot result (Figure 2-11), which demonstrates the prevalence of unidentifiable juveniles early in the spring.

Later in the sampling season (September, October), *Amynthas* were the most common earthworm found; it seems that they are actually outcompeting other non-native earthworms in this region either directly or through habitat suitability throughout the summer. This has been mentioned in Görres (2014) and Callaham et al. (2003) and was noticeably true at some sites, but would benefit from more study. Another revelation was that in the brownfield sites, many of the earthworms demonstrated developmental stunting. Although many *Amynthas* and *L. rubellus* in severely polluted metalliferous soils in Liberty State Park were indeed reproductively mature and (by the presence of cocoons) viable, some were remarkably shorter and thicker than conspecifics living in clean soils. This is more clearly shown by their biomass, Figure 2-7. While this is not a novel finding in earthworms (as reviewed in Nahmani et al. 2007) or in other organisms living in metal polluted soils (Everhart et al. 2006), it certainly warrants more research for discerning whether this is a nutrient availability, toxicity reaction, or a physiological response to potential reproductive stress. Earthworms located in the brownfield sites weighed 46% less overall than their non-polluted conspecific counterparts. While it would be reasonable that there would be a slight difference in overall biomass, almost half the size was quite unexpected. Other authors (including Arnold et al. 2008; Na et al. 2011; Spurgeon et al. 1996, 2000) have noticed this change in biomass as well, but it is notable here because many of the other completed studies involved amended soils, whereas these surveys were on field soils located in 61 different post-industrial brownfield sites with expectations of high metal loading in the soil.

VIII. Conclusion:

The results of this biological survey yielded both interesting and insipid results. The outcomes are unexpected because earthworm sampling in metalliferous brownfield areas yielded slightly more than the number of specimens per quadrat in non-polluted soils. Intuitively, this should not be the case. It would make sense, given that earthworms are frequently used as bioindicators of healthy soils (Giulia 2012; Peijnenburg & Vijver 2009), that there should be less of them in post-industrial sites than in clean locations; even higher amounts are unexpected.

It was also surprising that the biomass of earthworms was 46% lower in contaminated areas. Other authors (including Ireland, 1979; Marino & Morgan 1999) have extensively looked at bioaccumulation in earthworm tissues, and many have also noted stunted plant productivity in metalliferous sites (including Gallagher 2008). This finding is exciting and novel as it is thought to be the first of this magnitude in both transect size and comparison of non-polluted versus brownfield soils in areas without native earthworm populations. Further study is needed, as noted earlier, as to whether the stunting is a self-preservation tactic, a reproductive strategy, or both.

Another remarkable finding across all sites was the overwhelming presence of *Amynthas spp.* This is a more recent earthworm invader to the Northeast (Görres et al. 2014), but its impacts such as loss of ground cover and pelletizing of topsoil are evident in almost all habitats surveyed. In clean habitats, Amynthas outnumbered all other specimens three to one.

The results of this study also inform the methods used in the following experiments found in Chapters Three and Four. It was clear that *Lumbricidae* and *Amynthas* had to be present in each of the future mesocosm experiments since they were the most commonly sampled earthworms. Unfortunately, this was also the less attractive part of the results. Although each earthworm was carefully and individually identified, it became a routine matter of choosing *Amynthas, L. rubellus, L. terrestris,* juvenile Lumbricid, or unidentifiable. More diversity in sampling was expected, since there are over 180 species of earthworm in North America, with 60 considered non-native (Blakemore 2006). Pennsylvania has 28 species, 20 of which are non-native (Reynolds 2014b), and New Jersey contains 22 detected species with 19 non-native to North America (Reynolds 2014b).

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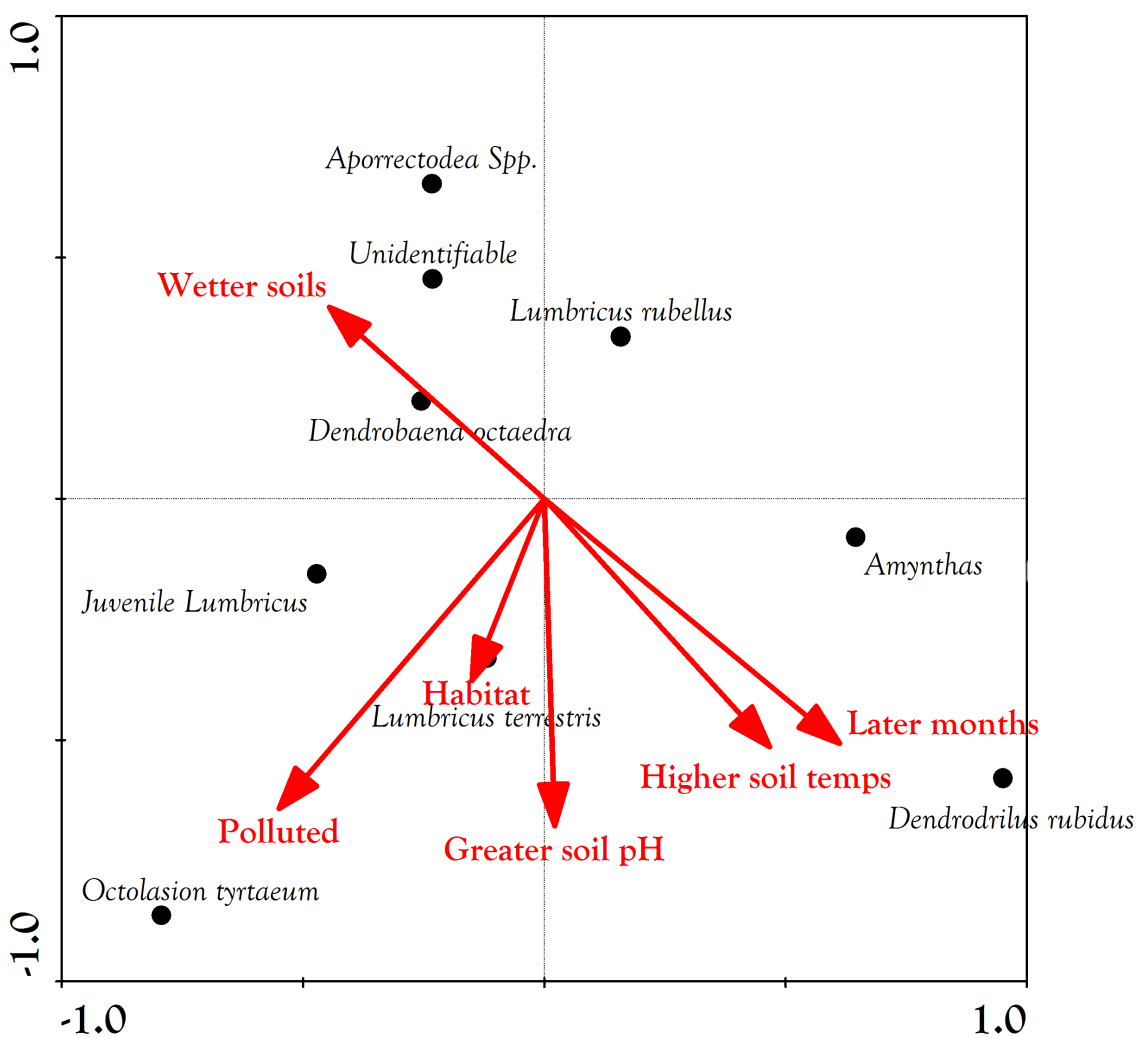
X. Appendix:

A. Map of All Biosurvey Sampling Sites (Large):

B. Large CCA Biplot Results:



C. Full Earthworm Survey Results:

Sample	Date	Latitude	Longitude	Weather	Air Temp	Soil Temp	Soil Moisture	Soil pH	Polluted	Habitat	Total Earthworms	Earthworms per m^2
1	9/11/14	40.76387	-75.27061	Cloudy	22.8	22	5.5	7.5	No	Lawn	7	28
2	9/11/14	40.763851	-75.27060	Cloudy	22.8	22	7	7.5	No	Lawn	10	40
3	9/11/14	40.763758	-75.27055	Cloudy	22.8	22.2	2	7.5	No	Lawn	3	12
4	9/11/14	40.76362	-75.27051	Cloudy	22	22	2	8	No	Forest	0	0
5	9/11/14	40.763429	-75.27063	Cloudy	22	22	1	8	No	Lawn	0	0
6	9/11/14	40.763282	-75.27051	Cloudy	23	25	4	7.5	No	Lawn	3	12
7	9/11/14	40.763394	-75.27051	Cloudy	23	23.4	7	6.5	No	Forest	28	112
8	9/19/14	40.837215	-75.29283	Sunny	21.7	20	1	6.5	No	Lawn	0	0
9	9/19/14	40.837613	-75.29291	Sunny	21	20	1	6.5	No	Lawn	1	4
10	9/19/14	40.851661	-75.28742	Sunny	22.5	25	10	7	No	Lawn	4	16
11	9/19/14	40.851706	-75.28753	Sunny	22.5	17.8	5	7	No	Lawn	2	8
12	9/19/14	40.869968	-75.26333	Sunny	23	23.9	7	7	No	Lawn	25	100
13	9/19/14	40.869691	-75.26522	Sunny	23	17.5	6	7	No	Lawn	35	140
14	9/19/14	40.850778	-75.30856	Overcast	21	19	1	8.5	Yes	Forest	8	32
15	9/19/14	40.849314	-75.31880	Overcast	21	17.5	2	8	No	Forest	0	0
16	9/26/14	41.049407	-75.03940	Sunny	18	16.2	2	6	No	Forest	0	0
17	9/26/14	41.043522	-75.04145	Sunny	18	29	7	6.5	No	Lawn	4	16
18	9/26/14	41.040498	-75.03873	Sunny	18	16	2	6.5	No	Lawn	4	16
19	9/26/14	41.056938	-75.02280	Sunny	19	17.8	4	7	No	Lawn	6	24
20	9/26/14	41.067624	-75.01255	Sunny	19	19.2	1	7	No	Wetland	0	0
21	9/26/14	41.018834	-75.08060	Sunny	20	19.4	2	6.5	No	Lawn	0	0
22	10/17/14	40.701009	-74.05334	Sunny	15	18.5	6	8.5	Yes	Forest	1	4
23	10/17/14	40.701808	-74.05517	Sunny	15	20.2	3	7	Yes	Forest	12	48
24	10/17/14	40.705117	-74.05380	Sunny	15	20.4	1	8	Yes	Forest	0	0
25	10/17/14	40.705477	-74.05246	Sunny	16	20	4	7	Yes	Field	0	0
26	10/17/14	40.70074	-74.05320	Sunny	16	20.2	4	7	Yes	Field	0	0
27	10/17/14	40.701586	-74.05469	Sunny	16	23.5	2	8	Yes	Field	0	0
28	10/17/14	40.70068	-74.05358	Sunny	17	19.2	4	8	Yes	Field	4	16
29	10/17/14	40.706092	-74.05160	Sunny	17	19	2	8.5	Yes	Forest	1	4
30	5/19/16	40.756370	-74.61629	Cloudy	21	15	2	7.2	No	Forest	15	60
31	5/19/16	40.756300	-74.61500	Cloudy	21	15	4	7	No	Forest	4	16

Sample	Date	Latitude	Longitude	Weather	Air Temp	Soil Temp	Soil Moisture	Soil pH	Polluted	Habitat	Total Earthworms	Earthworms per m^2
32	5/19/16	40.756900	-74.61625	Cloudy	21	16.5	2	7.5	No	Forest	4	16
33	5/19/16	40.757150	-74.61600	Cloudy	21	16.5	1.5	7.5	No	Forest	11	44
34	5/19/16	40.754160	-74.62643	Cloudy	22	17	2	7.7	No	Forest	4	16
35	5/19/16	40.754000	-74.62688	Cloudy	22	15	2	7.5	No	Forest	6	24
36	5/19/16	40.754260	-74.62633	Cloudy	21.5	17	6.5	6.5	No	Forest	7	28
37	5/19/16	40.753980	-74.62681	Cloudy	22	16	1.5	7.5	No	Forest	9	36
38	5/19/16	40.753710	-74.62592	Cloudy	22	17	2	7.5	No	Forest	15	60
39	5/19/16	40.753580	-74.62582	Cloudy	22	17	2	7.5	No	Forest	18	72
40	5/19/16	40.753720	-74.62541	Cloudy	22	17	1.3	7.7	No	Forest	15	60
41	5/19/16	40.753670	-74.62542	Cloudy	22	17	3	7.5	No	Forest	25	100
42	5/26/16	40.603650	-74.24920	Cloudy	30	32	10	5	Yes	Field	29	116
43	5/26/16	40.604000	-74.25000	Cloudy	30	21	6	7	Yes	Forest	20	80
44	5/26/16	40.604300	-74.24868	Cloudy	30	21	8.5	7	Yes	Forest	23	92
45	5/26/16	40.604380	-74.24866	Cloudy	29	17	10	6	No	Forest	16	64
46	6/9/16	41.063900	-74.18196	Cloudy	22	24.1	7	7	No	Field	0	0
47	6/9/16	41.086530	-74.18155	Cloudy	21	18.4	7	6	No	Field	0	0
48	6/9/16	41.056520	-74.18146	Cloudy	21	18	6	8	No	Field	2	8
49	6/9/16	41.086070	-74.18106	Sunny	21	19	8	7	No	Forest	0	0
50	6/9/16	41.086350	-74.18018	Sunny	20	19	7	7	No	Field	3	12
51	6/9/16	41.086010	-74.18056	Sunny	21	18	8	6	No	Forest	0	0
52	6/10/16	40.605030	-74.24732	Sunny	21	16.8	4	7.5	Yes	Forest	7	28
53	6/10/16	40.605020	-74.24752	Sunny	22.2	16.2	3	7.5	Yes	Forest	1	4
54	6/10/16	40.604650	-74.24753	Sunny	22.2	17.8	3.5	7.5	Yes	Forest	23	92
55	6/10/16	40.604670	-74.24716	Overcast	22.7	18	0.5	8.5	Yes	Forest	14	56
56	6/10/16	40.605120	-74.24709	Overcast	23.8	17	0.5	8.5	Yes	Forest	39	156
57	6/2/16	40.702169	-74.05142	Sunny	23.8	16	8	7.5	Yes	Forest	92	368
58	6/2/16	40.702048	-74.05122	Sunny	23.8	15	6	8.5	Yes	Forest	64	256
59	6/2/16	40.701957	-74.05134	Sunny	23.8	14	4	8.5	No	Wetland	103	412
60	6/17/16	40.07851	-74.18870	Sunny	27	19.3	2	8	No	Forest	11	44
61	6/29/16	40.486470	-74.29240	Sunny	29	24	1	8	Yes	Field	3	12
62	6/17/16	41.078595	-74.18858	Sunny	26	20	2	7	No	Forest	13	52

Sample	Date	Latitude	Longitude	Weather	Air Temp	Soil Temp	Soil Moisture	Soil pH	Polluted	Habitat	Total Earthworms	Earthworms per m^2
63	6/17/16	41.07859	-74.18857	Sunny	26	20	2	7	No	Forest	11	44
64	6/17/16	41.07785	-74.18692	Sunny	25	19.3	4	7	No	Forest	6	24
65	6/17/16	40.81621	-74.19166	Sunny	25	28.4	0.5	8	Yes	Field	1	4
66	6/17/16	41.0774	-74.18730	Sunny	27	19	2	7.5	No	Forest	10	40
67	6/17/16	41.07745	-74.18735	Sunny	27	19	2	7.5	No	Forest	13	52
68	6/17/16	41.07747	-74.18737	Sunny	27	19	2	7.5	No	Forest	18	72
69	6/29/16	40.4863	-74.29260	Cloudy	28	25	5	8	Yes	Field	1	4
70	6/17/16	41.07856	-74.18624	Sunny	24	20	1	7.5	No	Forest	17	68
71	6/17/16	41.07851	-74.18833	Sunny	25	21.3	3	7.5	No	Forest	10	40
72	6/17/16	41.07831	-74.18876	Sunny	27	20	3	7	No	Forest	8	32
73	6/29/16	40.4871	-74.29238	Sunny	28	27	1	7.5	Yes	Field	1	4
74	7/6/16	40.621953	-75.39674	Sunny	25	19	3	7	No	Field	33	132
75	7/6/16	40.621955	-75.39673	Sunny	25	19	3	7	No	Field	14	56
76	7/6/16	40.62195	-75.39674	Sunny	25	19	3	7	No	Field	11	44
77	7/1/16	40.35191	-74.56052	Cloudy	27	22.6	1	8	Yes	Forest	15	60
78	7/1/16	40.3629	-74.56065	Cloudy	27	22.1	1	8	Yes	Field	30	120
79	7/1/16	40.3524	-74.56074	Cloudy	27	23.3	1	8.5	Yes	Forest	21	84
80	7/1/16	40.35211	-74.56143	Cloudy	27	29.4	1	7.5	Yes	Forest	1	4
81	7/1/16	40.35219	-74.56155	Cloudy	27	24.7	1	8	Yes	Forest	9	36
82	7/1/16	40.3526	-74.56294	Cloudy	27	40	9	8.5	Yes	Wetland	0	0
83	7/9/16	40.780895	-75.29347	Sunny	25	19	5	7	No	Forest	16	64
84	7/9/16	40.780028	-75.29313	Sunny	25	19	5	7	No	Forest	17	68
85	9/18/16	40.701977	-74.05184	Cloudy	28.5	25.6	1	7	Yes	Field	2	8
86	9/18/16	40.701775	-74.05226	Cloudy	28.5	25.6	1	7	Yes	Field	1	4
87	9/18/16	40.70187	-74.05339	Cloudy	28.5	25.6	1	7	Yes	Field	22	88
88	4/30/16	40.741026	-74.17513	Sunny	12	10	8	7.5	No	Lawn	18	72
89	4/30/16	40.741603	-74.17561	Sunny	12	10	8	7.5	No	Lawn	16	64
90	4/7/17	40.70605	-74.05681	Sunny	13	15.7	4	7	Yes	Field	46	184
91	4/7/17	40.70572	-74.05719	Sunny	11	18.2	2	7.5	Yes	Field	27	108
92	4/7/17	40.70653	-74.05614	Sunny	11	15.9	1.5	7.5	Yes	Field	25	100
93	4/7/17	40.70627	-74.05579	Sunny	11	12.4	2	7.7	Yes	Field	13	52

Sample	Date	Latitude	Longitude	Weather	Air Temp	Soil Temp	Soil Moisture	Soil pH	Polluted	Habitat	Total Earthworms	Earthworms per m^2
94	4/7/17	40.70106	-74.05311	Sunny	12	12.3	2	7.5	Yes	Forest	15	60
95	4/7/17	40.70093	-74.05368	Sunny	12	10.6	6.5	6.5	Yes	Forest	13	52
96	4/7/17	40.70067	-74.05403	Sunny	12	11.3	1.5	7.5	Yes	Forest	12	48
97	4/7/17	40.70077	-74.05398	Sunny	12	9.8	2	7.5	Yes	Forest	4	16
98	4/12/17	40.547463	-74.62522	Sunny	21	14.3	2	7.5	No	Forest	0	0
99	4/12/17	40.547468	-74.62523	Sunny	21	13.3	1.3	7.7	No	Forest	5	20
100	4/12/17	40.549502	-74.62631	Sunny	21	12.9	3	7.5	No	Forest	9	36
101	4/12/17	40.550183	-74.62667	Sunny	21	13	10	5	No	Forest	18	72
102	4/12/17	40.543199	-74.61938	Sunny	22	13.9	6	7	No	Forest	4	16
103	4/12/17	40.545819	-74.62149	Sunny	20	11	8.5	7	No	Forest	25	100
104	4/12/17	40.542958	-74.62980	Sunny	18	13.8	10	6	No	Forest	40	160
105	4/12/17	40.542958	74.69282	Sunny	17	12	4	7	No	Forest	25	100
106	4/12/17	40.544464	-74.63220	Sunny	17	11.7	2	7.5	No	Field	12	48
107	4/19/17	40.70395	-74.05793	Cloudy	11	12.6	1.5	7.5	Yes	Forest	1	4
108	4/19/17	40.70393	-74.05802	Cloudy	10	16	2	7.7	Yes	Field	0	0
109	4/19/17	40.70457	-74.05283	Cloudy	9	12	2	7.5	Yes	Forest	16	64
110	4/19/17	40.7034	-74.05203	Cloudy	9	13	6.5	6.5	Yes	Forest	4	16
111	4/19/17	40.70331	-74.05204	Cloudy	9	13.8	1.5	7.5	Yes	Forest	1	4
112	4/19/17	40.70064	-74.05438	Cloudy	9	11.8	2	7.5	Yes	Forest	3	12
113	4/19/17	40.70079	-74.05448	Cloudy	9	11.7	2	7.5	Yes	Forest	14	56
114	4/19/17	40.70056	-74.05386	Cloudy	9	11.3	1.3	7.7	Yes	Forest	3	12
115	4/19/17	40.70053	-74.05363	Cloudy	9	11	3	7.5	Yes	Forest	9	36
116	4/24/17	40.5532	-74.63223	Cloudy	14	14.5	10	5	No	Field	12	48
117	4/24/17	40.5536	-74.63267	Rain	14	16	6	7	No	Field	14	56
118	4/24/17	40.55446	-74.63279	Cloudy	14	14.6	8.5	7	No	Field	29	116
119	4/24/17	40.55512	-74.63745	Cloudy	14	15.2	10	6	No	Field	13	52
120	4/24/17	40.55507	-74.63707	Cloudy	14	16	4	7	No	Field	9	36
121	4/24/17	40.55485	-74.63675	Cloudy	13	14.5	2	7.5	No	Field	3	12
122	4/24/17	40.55293	-74.62811	Rain	13	13.5	1.5	7.5	No	Forest	11	44
123	4/24/17	40.55289	-74.62803	Rain	12	13.2	2	7.7	No	Forest	19	76
124	4/28/17	40.702318	-74.05176	Sunny	27	22	2	7.5	Yes	Forest	11	44

Sample	Date	Latitude	Longitude	Weather	Air Temp	Soil Temp	Soil Moisture	Soil pH	Polluted	Habitat	Total Earthworms	Earthworms per m^2
125	4/28/17	40.702112	-74.05175	Sunny	27	20.5	6.5	6.5	Yes	Forest	10	40
126	4/28/17	40.702213	-74.05188	Sunny	27	26.6	1.5	7.5	Yes	Forest	4	16
127	4/28/17	40.702048	-74.05181	Sunny	27	27	2	7.5	Yes	Forest	5	20
128	4/28/17	40.703633	-74.05190	Sunny	27	25.2	2	7.5	Yes	Forest	34	136
129	4/28/17	40.703565	-74.05176	Sunny	26	26.2	1.3	7.7	Yes	Forest	35	140
130	4/28/17	40.703655	-74.05173	Sunny	27	26.2	3	7.5	Yes	Forest	26	104
131	4/24/17	40.835313	-74.38997	Cloudy	13	18	10	5	Yes	Field	7	28
132	4/24/17	40.833517	-74.39134	Cloudy	16	16.5	6	7	Yes	Field	10	40
133	4/24/17	40.833578	-74.38897	Cloudy	16	16.5	8.5	7	Yes	Field	9	36
134	4/24/17	40.83353	-74.38878	Cloudy	15	16	10	6	Yes	Field	2	8
135	4/24/17	40.849336	-74.38456	Cloudy	15	15.3	10	5	Yes	Forest	63	252

Chapter 3: The Impact of an Earthworm Community on the Phytoextraction of a Metalliferous Soil by Two Different Plants

I. Abstract:

Perhaps the most undesirable and damaging effect of the industrial revolution since the mid-1800's is the widespread pollution of many locations that served as factories, rail yards, smelters, and other potentially environmentally damaging industries. One of the simplest and most cost-effective methods of cleaning up these areas is through phytoextraction. Metal hyperaccumulating plants are placed in damaged soils, where they collect and biologically sequester disproportionately large amounts of metal toxicants.

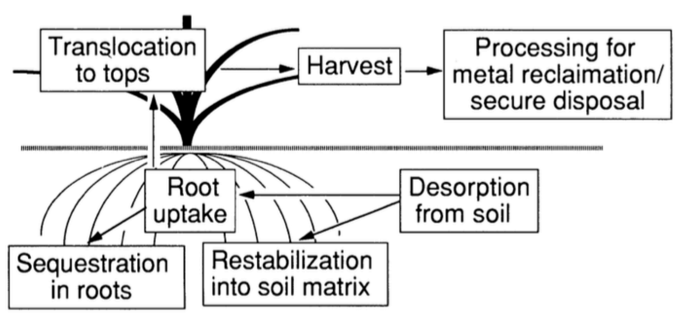
Earthworms have been used successfully in agriculture to increase plant growth through soil aeration, root abrasion, increased subsurface water absorption, and more complete nutrient availability. Soil amelioration by earthworms in post-industrial polluted brownfields was tested to measure the enhanced phytoremediative effect of hyperaccumulating plants. Three levels of polluted soil treatments (low, mid, and most) were planted with *Secale cereale* (rye) and *Fagopyrum esculentum* (buckwheat) in one hundred twenty 19 L (five gallon) buckets. Earthworm communities were added to half of the pots. After 2 months, the plants were harvested and dried. The plants and soils were digested and analyzed in an Inductively Coupled Plasmography — Optical Emission Spectroscopy (ICP–OES) for content of five metals: As, Cd, Cu, Pb, and Zn. Results show two threshold effects whereby increasingly polluted soils showed greater growth of non–mycorrhizal forbs in the presence of earthworms. However graminiod mycorrhizal dependednt plants

increased metal sequestration and grew smaller when earthworms were introduced. Phytosequestration levels were relatively unchanged with *Fagopyrum* when earthworms were present, but *Secale* uptake of metal contaminants improved up to four fold when earthworms were present. It is believed that earthworm consumption of mycorrhizae reduce capabilities of protection and filtration for these contaminants, thereby allowing this mycorrhizal plant to hyperaccumulate greater toxicants in their presence.

II. Introduction:

Over the last 175 years, booming industry, lack of materials knowledge, deep rooted beliefs that we would never run out of space (Leopold 1949), and sometimes accidents or ignorance have led to areas that will continue to be an environmental hazard without human intervention. The United States has over 1,300 locations on the National Priority List of the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA, commonly called Superfund Sites) (epa.gov/superfund), and an estimated 450,000 registered post-industrial brownfield sites (epa.gov/brownfields). Intervention and restoration in these areas can be very slow and subject to political considerations, and most common remediation methods of contaminated soil such as excavation, landfilling, incineration, and capping are often prohibitively expensive (Cunningham & Berti 1993).

Figure 3-1: Phytoremediation of metal contaminated soils, from Cunningham et al. 1993.



It is possible that natural processes could help with these cleanups. Phytoextraction is the process by which plants are introduced into an environment and allowed to assimilate hugely disproportionate amounts of certain contaminants into their roots and leaves (Chaney et al. 1997). Although phytoremediation was recognized and documented by humans more than 300 years ago (Hartman 1975), the scientific study of hyperaccumulating plants was not conducted until the early 1980's (Lasat 2002). This process is used to clean up metals, pesticides, organic compounds (Newman & Reynolds 2004), toxic aromatic pollutants (Singh & Jain 2003) and acid mine drainage (Archer & Caldwell 2004).

Phytoremediation is considered to be an environmentally friendly, cheap, and safe way to remove contaminants, in some cases doing the same job as a group of engineers for one tenth of the cost (Gratão et al. 2005). However, such technology cannot necessarily be effective all of the time or be used in all types of contaminated sites. If the contamination runs too deep, or the concentration of toxic compounds is too high, then plants alone cannot efficiently remediate the soil (Cunningham & Berti 1993) or be used to extract the toxicants.

Earthworms living in and around polluted soils have been a well-studied topic for nearly forty years. Gish and Christiansen (1973) and Ireland (1975) are largely credited with the first published studies characterizing earthworm response with metal toxicants. Gish and Christiansen tested earthworms along busy highways for Cd, Ni, Pb, and Zn. Ireland tested earthworms in mine tailings in Wales and England. Others have followed, with experiments in artificial soils with different ameliorants such as solubilized Pb, As, Cd, and Cu (Scaps et al. 1997; Conder et al. 2002). Studies also expanded to river sediments and sludge (Bleeker & van Gestel 2007; Beyer et al. 1982), copper and zinc smelters and mining operations (e.g. Nannoni et al. 2011; Morgan & Morgan 1999; Arnold & Hodson 2007), and even the effect of uranium from 'dirty bombs' on earthworms after the Kosovo War in 1999 (Di Lella et al. 2005). For a comprehensive review of metalliferous soils on earthworm health, see Sizmur and Hodson (2009) and Nahmani et al. (2007).

Raskin et al. popularized phytoremediation broadly and phytoextraction particularly as bona fide methods of soil remediation in 1994, and the search for the most hyperaccumulating plants continues. Studies show that certain plants can absorb disproportionate amounts of metals (Rascioa & Navari-Izzo 2011) and that earthworms have an impact on metals in soils with regard to bioavailability and sequestration, and a few studies have looked at how earthworms can improve growth of hyperaccumulating plants. (Aghababaei et al. 2014, 2015; Butt & Grigoropoulou 2009).

The main goal of this experiment was to both quantify habitat amelioration with earthworms and hyperaccumulating phytoremediative plants, and address effects of earthworm communities (ecological groupings) living in field soils in areas that have not adapted to earthworm presence since the last glaciation when native earthworm species were believed to be pushed far to the south.

III. Site History:

Soils used for this study were from Liberty State Park in Jersey City, NJ USA (40.704, –74.052). Originally, the area known today as Liberty State Park was just a protected cove of Communipaw Marsh on the western side of New York Harbor outside Jersey City, New Jersey, USA. Communipaw's unique position in New York Harbor ensured that it would continue to be a major trade and travel location for several hundred years.

Starting in the mid 1840's, the salt marshes of the area were filled with dredge and

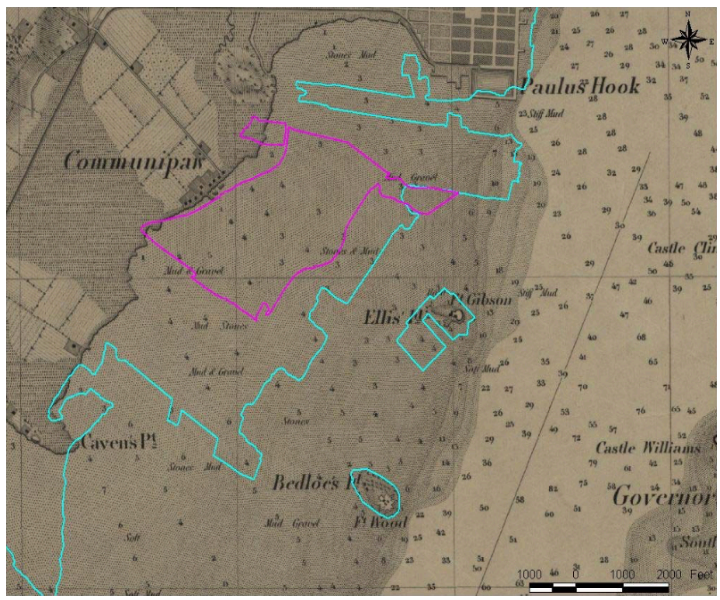
Figure 3-2: Communipaw Marsh, 1854 (left) and today (right). Note almost the entirety of the green area in the satellite image is fill. For a larger map, see appendix. Maps courtesy NY Public Library (left) and Google (right).

unwanted soil from building projects in and around New York City. Coal ash, mining slag, glass and metal refuse, and construction debris were also used to fill and extend the shoreline further (Figure 3-2) into the Hudson River (Gallagher 2002; Brown 1878). Throughout the later part of the 19th century, the filling continued until we see the shoreline as it is now, with approximately 15.2 million cubic meters (20 million cubic yards) of fill added to the area (USACE 2004).

Figure 3-3: Central New Jersey Rail Terminal, ca. 1949. Note that almost everything in the photo is manmade. shoreline. (Public Domain)

Upon this manmade coastal plain grew the most important railroad terminal station in the greater New York area (Figure 3-3). Many European immigrants that were admitted to the USA through Ellis Island disembarked at the terminal, and then both people and goods were loaded on trains for points further west into the United States. The Communipaw (and later called) The Central New Jersey Railroad Terminal saw as many as 10.5 million immigrants enter the United States during its 103-year reign (NJ Parks). The Central New Jersey and Lehigh Valley Railroads began running on these tracks in 1864.

Figure 3-4: Communipaw Cove before fill, 1845. The blue lines represent the modern coastline, and the purple line represents the fenced-in project area. For a larger map, see appendix. From USACE 2004.



Upon the closing of the terminal in 1967, the tracks were removed for scrap, and soon after the New Jersey Department of Environmental Protection realized how contaminated some of the soils were (Texas Instruments 1976). When the park was officially opened July 4, 1976, as part of the American Bicentennial celebrations, a ten-foot-high chain link fence had been built around the interior portion of the area (the purple line in Figure 3-4). In 1990, NJ Department of Environmental Protection collected 57 soil samples from 28 different sites and found that many of them exceeded acceptable levels for polynuclear aromatic hydrocarbons, metals, and pesticides (Gallagher 2002). Today, the outer park area is what most people know as Liberty State Park, including over 380 hectares of running paths, parking lots, open green space, and recreational facilities, all of which are open to the public. The most contaminated interior portion of 102 hectares remains inaccessible to recreation (USACE 2004). Although there have been remediation efforts and ongoing discussions of cleanup, the high costs and differing public opinions have almost certainly ensured the contamination will stay in place for a long time. The benefit of this, however, is that many state agencies, universities, and outreach groups use the property for many experiments and as a sort of "test site" proxy for contaminated site succession throughout the northeastern United States (e.g. Gallagher et al. 2011).

IV. Soil Characterization:

The soils of the park's 102 ha restoration area are classified as Ladyliberty fine sandy loam (Lad-A, National Map Unit Symbol 2qjwj), showing 0–3% slopes and 0–3 m elevation. Ladyliberty soils are defined as having a primary component of "sandy-skeletal human-transported material comprised of mostly homogenized layers of sandy loam, and extremely artifactual loamy sand." Soils contained less than 2% calcium carbonate. (USDA National Resource Conservation Service 2017; Gallagher 2008; USACE 2004; Brown 1878). Soils used for this experiment are from areas of known high concentrations of metal toxicants (Gallagher 2008a). The soil had elevated levels of coal cinders and

Figure 3-5: Polluted soil from Liberty State Park. Note extreme color differences indicating differing pollution levels (size 12 shoeprint for scale).

unburned slag material, creating a matrix that was approximately 30% combustible carbon when the loss on ignition was measured in a vented muffle furnace at 450˚C for 24 hours and matches the results that Gallagher et al. (2008) reported. Colorization varies by area and deposited fill (Figure 3-5), but holds nearest to a 2/3 5YR on the Munsell Color Scale (Munsell 2010).

V. Plant Selection:

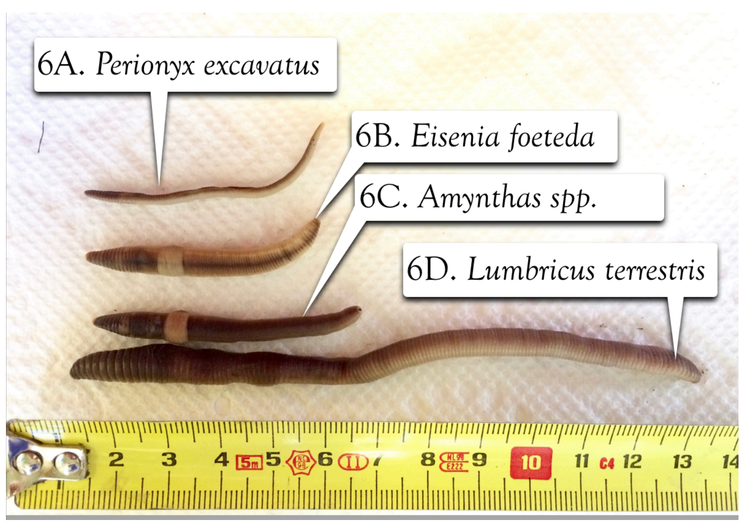
Plants used in the buckets were *Fagopyrum esculentum*, (Moench 1794) (buckwheat) and *Secale cereale (*Linneaus 1753) (rye). Both have been used extensively in phytoextraction studies as noted below, but have some key differences that made them appropriate to use together in a community level mesocosm experiment. *Fagopyrum esculentum* is a short season crop that is classified as a forb, dicot, and is non-mycorrhizal dependent. *Secale cereale* is a longer season crop that is a graminoid monocot and is mycorrhizal dependent. Together, these plants cover many characteristics of the majority of herbaceous plants found in the northeastern United States, but each has been found to have their own niche in hyperaccumulation. *Fagopyrum* is a relatively recent addition to phytoextraction, especially for high amounts of Pb in soils (Honda et al. 2007; Tamura et al. 2005). It has also proven very helpful in removing toxic amounts of Al from soils (Ma et al. 1997, 2000; Shen 2003).

Secale cereale improves soils, in no small part due to their extensive root network (Dittmer 1937). Many toxic metals (As, Cd, Cu, Pb, Zn) have been shown to bioaccumulate into *Secale* well, but the addition of a chelating agent improves their capabilities significantly (Quartacci et al. 2007). In many cases, *Secale* is utilized to clean up activated human sludge (Lagerwerff et al.1977) and areas that had excessive hydrocarbon contamination (Muratova et al. 2008).

VI. Earthworm Selection:

This community level experiment was designed to simulate a more complete functioning ecosystem in that there were two species of plants with different characteristics and four different species of earthworm used in the design. Based on Bouché (1977), earthworms are loosely gathered into four ecological groups. Epigeic earthworms live in the surface litter, commonly called composting earthworms. *Eisenia foetida* (Savigny 1826) fulfilled this role (Figure 3-6B). Endogeic earthworms are found in and around the root zone. They live in semi-permanent burrows that are mostly horizontal but occasionally come to the surface to feed (Reynolds 1977). *Perionyx excavatus* (Perrier 1872) is a small (<5 cm) earthworm (Figure 3-6A), originally from Vietnam, Malaysia, and some parts of the Indonesian islands (Blakemore 2003). Part of the reason Bouché's ecological groupings have worked so well is that there are multiple gradients between the, as he called them, "tridents". One of the most influential of these mid-groupings are epi-endogeic earthworms. Epi-endogeic earthworms both burrow and consume the leaf litter, leading to severe forest understory modification

Figure 3-6: Preserved samples of earthworms used in experiment.



(Reynolds 1977). In the northeastern United States, this leaf litter loss is due most notably to *Amynthas spp.* (Goto & Hatai 1899). *Amynthas* is an invasive from Asia (Snyder et al. 2010) and is an easily collected specimen for this study (Figure 3-6C). The fourth ecological grouping is referred to as anecic. The most common, and in many parts of North America, the only anecic earthworm (Figure 3-6D) is *Lumbricus terrestris* (Linneaus 1758). They build vertical burrows that they may inhabit for their entire lifespan — typically two, but as long as eight or nine years (Satchell 1967).

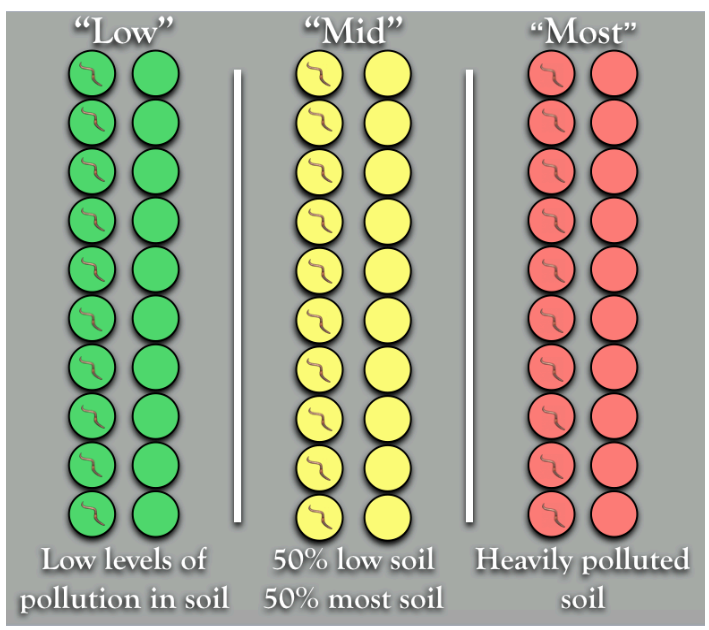
VII. Methods:

Before the experiment, all earthworms were acclimated in species–specific 38 L plastic totes (breeder bins) containing by weight 45% topsoil, 45% composted cow manure, and 10% peat moss, based on OECD guidelines (1984). This mixture was homogenized in a Gleason MB-20 industrial soil mixer. Totes were then filled, moistened to 70% water holding capacity, and allowed to settle for one week to encourage bacteriological response (as noted in Jager et al. 2003). Breeder bins were located in a basement, which held at 18.6° C (±1.2°C) and 79 % RH (±10.2%) over the eight-week period until the bucket experiment was underway. All earthworms were purchased or collected 6-7 weeks before commencement of the experiment. Utilization of breeder bins was based off Mariño and Morgan (1999) and meant to remove any sick or unhealthy earthworms before the experiment and to minimize any variability of the substrate in which they were packaged or shipped. It also served to equilibrate soil chemical change in buckets from previously consumed substrate in their digestive tract and equalize gut microbiomes across all individuals of a species.

Then, thirty buckets were used to collect soils from various locations in Liberty State Park in May 2015. The experiment used a total of 60 5–gallon (19 L) buckets that were prepared with three 2.5 cm holes drilled in the bottom and covered with a piece of fiberglass window screen that would allow water drainage. All soil was sifted through 7 mm mesh to remove stones and large organic detritus. Because of very small-scale pollution variability (i.e. spills and poor housekeeping creating a fine patchwork gradient of contamination), all thirty collected buckets were homogenized together before filling the buckets for the experiment.

Twenty of the buckets were used at full concentration, hereafter referred to as 100% or "most" level pollution (red circles on Figure 3-7), while ten were then homogenized with an equivalent amount of non-polluted soils purchased at the local home improvement center. This mixing created twenty buckets of 50%, or "mid" level pollution (yellow circles in Figure 3-7). The last twenty buckets were only non-polluted, homogenized topsoil purchased from the local home improvement center, creating a 0%, or "low" level pollution (green circles in Figure 3-7). These differing levels of pollution in soil are

Figure 3-7: Experimental design. Note color codes for gradient levels will be used throughout this manuscript.



loosely based on the work of Jusselme et al. (2012, 2013), where they created pollution gradients in pots by amending solubilized lead and cadmium, as well as Mariño et al. (1998) when he had three different locations in Wales creating a "high", "medium", and "low" scale.

Half of the sixty total buckets (thirty; ten of each soil treatment) received an earthworm community of ten each of *Eisenia foetida, Perionyx excavatus, Amynthas spp.*, and *Lumbricus terrestris,* as indicated by the earthworm icons in Figure 3-7. *Amynthas spp.* were the only earthworms not available for purchase and were hand–collected near Easton, Pennsylvania USA. The others came from wholesale bait dealers or earthworm farms.

All of the containers were seeded with 4 g (≈80 seeds each) of *Fagopyrum esculentum* and *Secale cereale*. 50 g of dry leaf litter was added to cover the seeds and soil to prevent seed desiccation and predation, and to provide habitat for the eipgeic and epi-endogeic earthworms. Buckets were arranged outside in rows in the open sun and rotated weekly throughout a grid pattern from front to back and side to side, ensuring even sunlight and open space. They were watered only naturally with rain, except for the driest three weeks in August when each bucket received an additional 6 L of water each per week.

After 67 days, *Fagopyrum*, *Secale*, all rootstocks, soil samples, and any living earthworms were destructively harvested. After all sampling had been completed, all contaminated soils were returned to original excavation sites in Liberty State Park.

The samples of plants were separated by species and then split into two groups — the tallest and largest twenty plants from each bucket, and the "leftovers", as well as twenty seeds of *Fagopyrum* from each bucket (The *Secale* did not develop seeds). All aboveground biomass from both plants and *Fagopyrum* seeds were placed into brown paper bags and desiccated to constant weight in a drying oven at 60° C for 24 – 48 hours. The largest twenty plants of both species from each bucket were then weighed to calculate dry biomass across all six soil/earthworm treatment combinations to the nearest 0.01 g.

The second part of this experiment was to assess the metal toxicant content of all plants and soils from the six different treatments in order to evaluate earthworm impacts on metal absorption. After plants had been weighed, they were then sealed in plastic bags for transport to the Cary Institute of Ecosystem Studies, Millbrook, New York, USA.

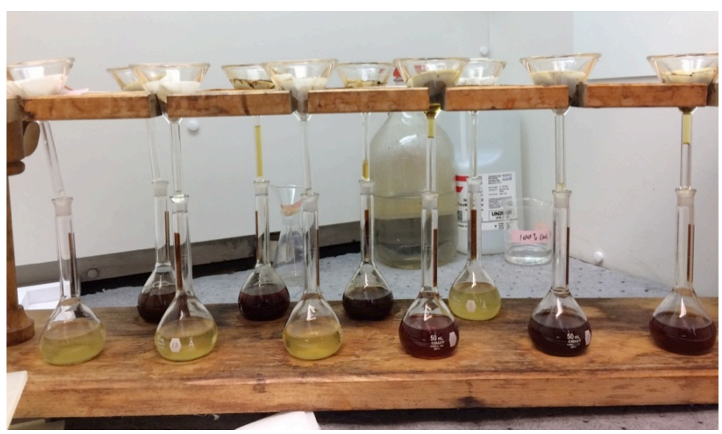
Dried cuttings of *Secale cereale* and *Fagopyrum esculentum* were each randomly pulled from all 120 bags to get a mix of leaves and stems, and weighed to 0.500 g (±0.02 g). These very small quantity samples were then individually digested in nitric acid (HNO_3, 70 % w/W) using a microwave digester.

Figure 3-8: The Milestone Ethos microwave digester

The microwave digester (Milestone Ethos EZ) contained ten Teflon digestion vessels, which were then sealed in a larger ceramic, explosion–proof sleeve. The ten vessels with sleeves were arranged in a carousel that rotated consistently to ensure even heating (Figure 3-8). The 0.5 g samples were placed in the digestion vessels along with 10 mL of trace metal grade nitric acid. The Ethos was programmed to run at 750 watts while the closed vessels reached 180° C. Upon reaching this plateau, microwave power was thermostatically controlled to maintain this temperature for 25 minutes. When the heating cycle was complete, microwave power was stopped, but the carousel continued to rotate for an additional 10-minute “cool down” period. Pressures inside the vessels averaged 12 bar (9000 mmHg) and, along with temperatures far above the boiling point of nitric acid (180° C compared to 83° C at sea level), ensured almost complete dissolution of all samples. This method was based on US EPA Method 3052, “Microwave Assisted Acid Digestion of Siliceous and Organically Based Matrices” (EPA 1995) and is the standard protocol followed at the Cary Institute of Ecosystem Studies for digestion of organic materials.

After digestion and an additional 20–30 minutes for cooling, each vessel was opened and allowed to vent excess pressure. The now dissolved samples were filtered through Whatman GF/A glass microfiber filters into volumetric flasks. Teflon digestion vessels were rinsed with nanopure water (6X distilled meeting ATSM Type I regulations, and having 17.4 megohms ionic purity) water, and filters were washed with the same; all rinseate was funneled into the volumetric flasks (Figure 3-9). Nanopure water was then added to the flask to bring the entire sample to 50 mL, when samples were transferred to trace metal grade 50 mL skirted “falcon type” centrifuge tubes for storage. After each digestion batch, a cleaning cycle was run through

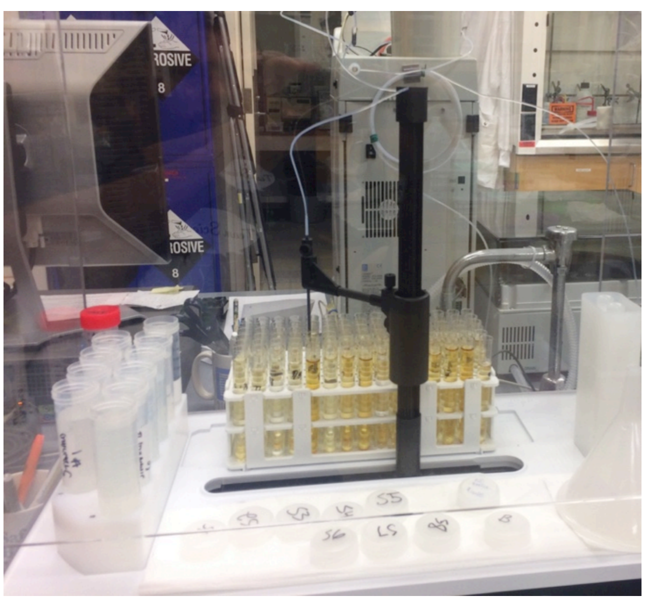
Figure 3-9: Volumetric flasks and filtering apparatus for processed analytes.



the microwave digester with each Teflon vessel containing 5 mL of HNO_3 and 5 mL of nanopure water. The full microwave cycle was rerun in order to remove any latent traces not rinsed from the containers. Each time the sample type changed (e.g. medium pollution to high pollution soil, or every 20 digestion samples), a blank was run containing only 10 mL of HNO_3 to be compared to the analyte. This blank was treated to exactly the same process and was analyzed alongside the actual samples. Every five digestion cycles (fifty samples and blanks), all Teflon vessels, ceramic sleeves, lids, and pressure caps were run through an acid wash automatic dishwasher cycle to maintain cleanliness.

Metals analysis was completed using a Perkin-Elmer Optima 8000 ICP—OES (Inductively Coupled Plasma — Optical Emission Spectroscopy) also located at the Cary Institute of Ecosystem Studies. Samples in 50 mL centrifuge tubes were first shaken, and using a clean pipette, 7 mL was removed and placed into a trace metal polystyrene disposable test tube. Ninety tubes at a time were loaded into the autosampler with ten standards to create a standard curve (Figure 3-10). Trace metal standards were run concurrently to test the calibration of the instrument. Standards used were National Institute of Standards and Technology # 2704 "Buffalo River

Figure 3-10: Auto sampling apparatus for ICP-OES



Sediment". The analyte from this certified reference sample was found to be within 5% of certified values, which are included in the appendix (Epstein 1989). Frequency output for all samples was then aligned to a quadratic best-fit curve in mg/kg and multiplied by 100 to account for the dilution factor of acid and water additives (increasing total volume to 50 mL).

VIII. Results:

After 7 weeks of growth, there was a noticeable difference between buckets of different pollution levels (Figure 3-11). The three separate soil treatments demonstrated discernable growth differences.

Figure 3-11: Soil treatments of low, mid and most polluted soils with earthworms show indicative growth patterns– photo at 49d.

Buckets that had similar soil treatments visually showed improvement when earthworms were present as well (Figure 3-12). The full table of all comparable results are listed in Appendix C. Plants listed are *Fagopyrum esculentum* (buckwheat), *Fagopyrum* seeds (buckwheat seed), and *Secale cereale* (rye). Pollution

Figure 3-12: Earthworm treatment (right) can notably improve plant growth compared to no earthworms in (left). Mid pollution concentration, 49 d growth.

levels are indicated by "Low," "Mid," and "High," as noted earlier. Plants were weighed to the nearest 0.01 g, while the much smaller seeds were weighed to 0.0001 g. Standard deviations given are for the entire population of 20 plants per bucket, times ten replicates each. The difference (Δ) between plants and seeds grown with and without earthworms is demonstrating the potential improvement of annelid presence, and p-values based on earthworm amelioration is a two-tailed, paired T-test, converted to p-values.

Figures 3-13 through 3-15 are violin plots of results of plant and seed biomass. As an orientation, note that violin plots have all the same features as a Tukey's original box plot, with the added benefit of a swarm plot to describe the shape of density distribution (Hintze & Nelson 1998). The dark central line demonstrates the first to the third quartile, showing interquartile range. The white dot represents the median of the population, the thin black line shows the adjacent upper and lower values, while the outliers fall into the colored peaks on the top and bottom. The width of the plot shows population density at a particular point on the Y-axis, effectively acting as an unbinned horizontally oriented histogram. Significance codes are given between each comparable pair of data sets, and are based on a converted T–Score from a Student's two–way paired distribution test.

Figure 3-13: Aboveground biomass of Fagopyrum esculentum

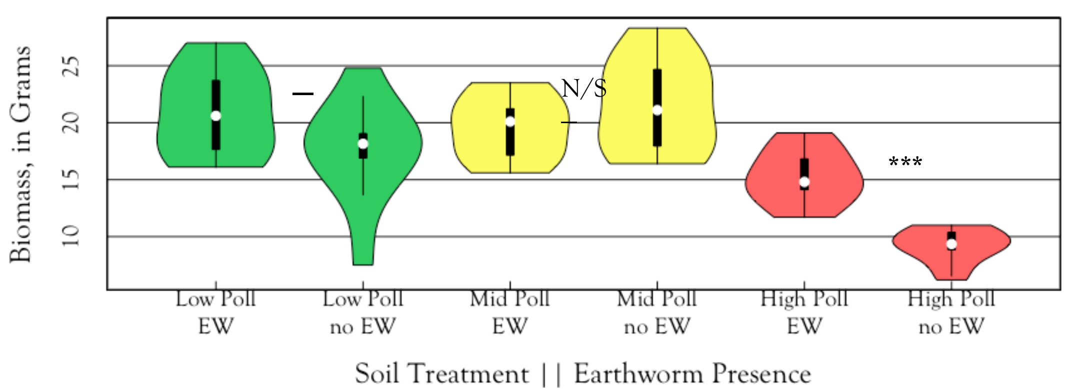


Figure 3-14: *Aboveground biomass of* Fagopyrum esculentum *seeds*

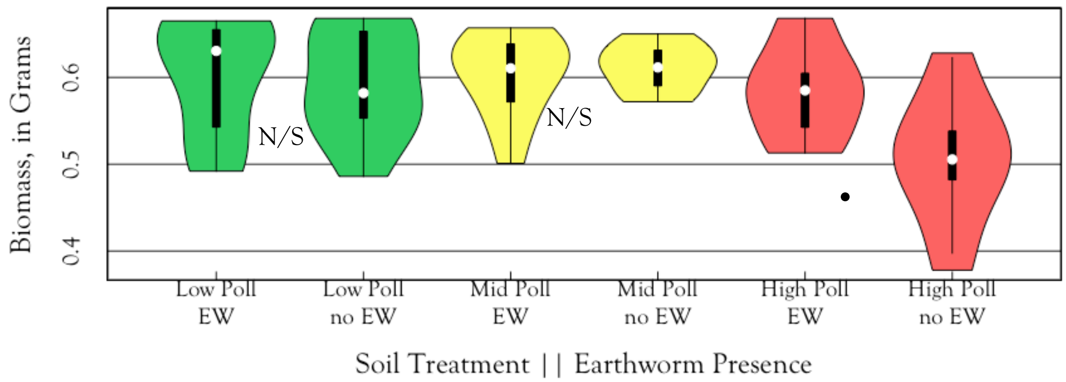
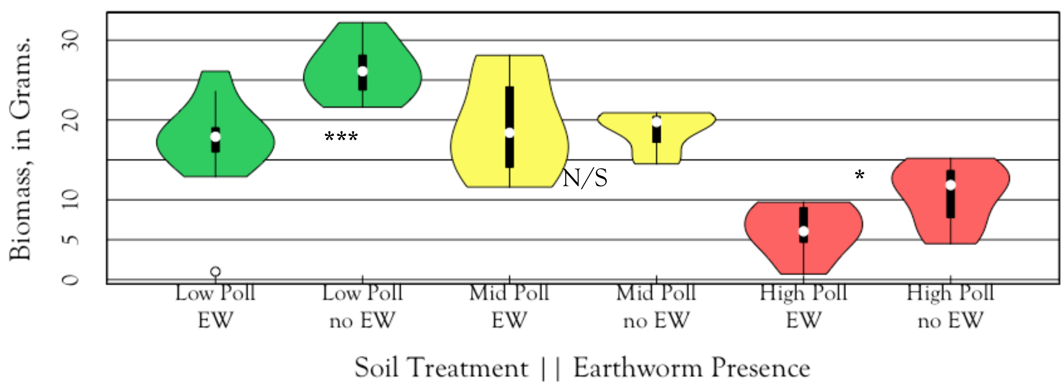


Figure 3-15: *Aboveground biomass of* Secale cereale



Significance codes: $p \leq 0.0001$ '***' | $p \leq 0.001$ '**' | $p \leq 0.01$ '*' | $p \leq 0.05$ '•' | $p \leq 0.1$ '–' | $p \leq 1$

The following charts and subsequent graphs are the results of testing the digested biomass in the ICP—OES. Biomass is given in g, and all metals are shown as mg/kg^{-1} (ppm). Lowercase sigma (σ) is the notation used for standard deviation, and all p–values are the results of a Student's two–tailed T-test of equal variance.

Table 3-1: Fagopyrum esculentum (Seeds)		Biomass	σ Biomass	p–value Biomass	As (mg/kg)	σ As	p–value As	Cd (mg/kg)	σ Cd	p–value Cd	Cu (mg/kg)	σ Cu	p–value Cu	Pb (mg/kg)	σ Pb	p–value Pb	Zn (mg/kg)	σ Zn	p–value Zn
Low	EW	0.60	0.06	0.758	1.21	0.61	0.622	0.70	0.04	0.197	8.1	0.6	0.246	2.7	0.6	0.000	19.2	9.2	0.267
Low	No EW	0.59	0.06		1.11	0.05		0.72	0.02		9.3	1.2		1.6	0.1		22.8	2.1	
Mid	Ew	0.60	0.05	0.576	1.17	0.11	0.449	0.73	0.02	0.070	9.1	3.0	0.034	2.3	0.6	0.501	52.5	8.2	0.658
Mid	No EW	0.61	0.03		1.05	0.48		0.60	0.20		11.6	1.4		2.1	0.7		55.7	19.6	
Most	EW	0.58	0.05	0.013	0.50	0.19	0.016	0.39	0.03	0.505	14.8	1.8	0.660	2.9	1.0	0.394	74.2	10.6	0.498
Most	No EW	0.51	0.07		0.88	0.39		0.40	0.03		16.1	8.6		7.3	15.2		84.8	44.8	

Table 3-2: Fagopyrum esculentum (Buckwheat)

		Biomass	σ Biomass	p–value Biomass	As (mg/kg)	σ As	p–value As	Cd (mg/kg)	σ Cd	p–value Cd	Cu (mg/kg)	σ Cu	p–value Cu	Pb (mg/kg)	σ Pb	p–value Pb	Zn (mg/kg)	σ Zn	p–value Zn
Low	EW	20.83	3.56	0.096	0.84	0.11	0.789	0.40	0.02	0.000	7.4	0.1	0.071	2.2	0.1	0.217	37.2	25.8	0.084
Low	No EW	17.56	4.31		0.85	0.07		0.43	0.01		4.3	0.6		2.1	0.1		21.3	4.1	
Mid	Ew	19.63	2.65	0.242	0.78	0.14	0.027	0.40	0.03	0.000	6.0	2.1	0.240	4.4	2.1	0.242	30.1	8.0	0.047
Mid	No EW	21.52	3.87		0.56	0.23		0.62	0.02		4.9	1.7		3.3	1.4		41.4	13.7	
Most	EW	15.28	2.25	0.000	0.38	0.16	0.996	0.13	0.09	0.280	14.9	4.8	0.830	26.8	11.1	0.039	262.3	42.4	0.002
Most	No EW	9.21	1.42		0.38	0.21		0.09	0.06		14.5	3.5		37.8	10.0		337.6	43.5	

Table 3-3: Secale cereale (Rye)

		Biomass	σ Biomass	p–value Biomass	As (mg/kg)	σ As	p–value As	Cd (mg/kg)	σ Cd	p–value Cd	Cu (mg/kg)	σ Cu	p–value Cu	Pb (mg/kg)	σ Pb	p–value Pb	Zn (mg/kg)	σ Zn	p–value Zn
Low	EW	18.16	3.67	0.000	0.71	0.17	0.675	0.75	0.10	0.016	20.0	0.3	0.001	1.0	0.3	0.005	109.5	28.5	0.539
Low	No EW	26.17	3.31		0.75	0.27		0.61	0.12		8.6	1.6		2.4	0.5		102.5	17.5	
Mid	Ew	19.25	5.81	0.792	2.95	3.64	0.360	0.65	0.03	0.089	25.2	15.5	0.526	42.9	42.5	0.289	156.9	76.9	0.011
Mid	No EW	18.69	2.34		1.80	0.47		0.62	0.02		21.7	5.0		27.4	4.5		78.5	32.7	
Most	EW	6.13	2.93	0.00	5.37	5.37	0.078	0.41	0.13	0.003	50.8	32.3	0.051	77.7	67.7	0.089	533.8	237.6	0.072
Most	No EW	10.72	3.50		1.84	1.81		0.56	0.05		27.0	10.9		35.6	19.0		339.5	191.8	

Significance codes: $p \leq 0.0001$ '***' \| $p \leq 0.001$ '**' \| $p \leq 0.01$ '*' \| $p \leq 0.05$ '•' \| $p \leq 0.1$ '–' \| $p \leq 1$ 'N/S'

A. Analysis of Variance:

The full analysis of variance tables can be found in Appendix D of this chapter. Two-way ANOVAS were run on each sample type (*Fagopyrum* plants and seeds, and *Secale* plant material). ANOVAS compared relative explanation of variance of metal content of biomass due to: A.) *Pollution*, B.) *Earthworm presence*, and C.) *Pollution • Earthworm presence*. These full results are summarized here in Figure 3-20.

Table 3-4: Analysis of Variance Table Summary

Metal content P–values		Arsenic	Cadmium	Copper	Lead	Zinc
Fagopyrum (buckwheat) seeds	Pollution level	***	***	***	n/s	***
	Earthworms	n/s	n/s	n/s	n/s	n/s
	EW and Pollution	–	•	n/s	n/s	n/s
Fagopyrum (buckwheat) leaves and stems	Pollution level	***	***	***	***	***
	Earthworms	n/s	***	–	–	*
	EW and Pollution	–	***	n/s	*	***
Secale (rye) leaves	Pollution level	•	***	***	***	***
	Earthworms	•	n/s	*	•	•
	EW and Pollution	n/s	***	n/s	n/s	n/s

Significance codes: $p \leq 0.0001$ '***' | $p \leq 0.001$ '**' | $p \leq 0.01$ '*' | $p \leq 0.05$ '•' | $p \leq 0.1$ '–' | $p \leq 1$ 'N/S'

Analysis of variance measures demonstrate the variability that can be explained by pollution level, earthworm presence, or both. When interpreting the p-values given (Table 3-4), it is expected that the pollution levels account for the majority of the variation in metalliferous content. Earthworm presence or absence can account for little to no variation in *Fagopyrum* seeds and explains >95% of the variation in *Fagopyrum* shoots and leaves. However, these are less significant than earthworm effects on *Secale*. While earthworms do have little effect on the phytoremediative capabilities of *Fagopyrum*, the ANOVA shows they play a much bigger role in the same abilities of Secale.

B. Metals in Soils:

Figures 3-16 through 3-21 depict soil contamination of metal toxicants in low, mid, and most polluted soils. Samples taken from the beginning soils were bulk material from the buckets while ending values were taken from rhizospheric soils shaken from the root mass. Figure 3-16 shows soil metal concentrations of arsenic levels before the experiment and at the end of the 67 days for both earthworm present and earthworm absent soils.

Figure 3-16: Concentration of Arsenic in Soils

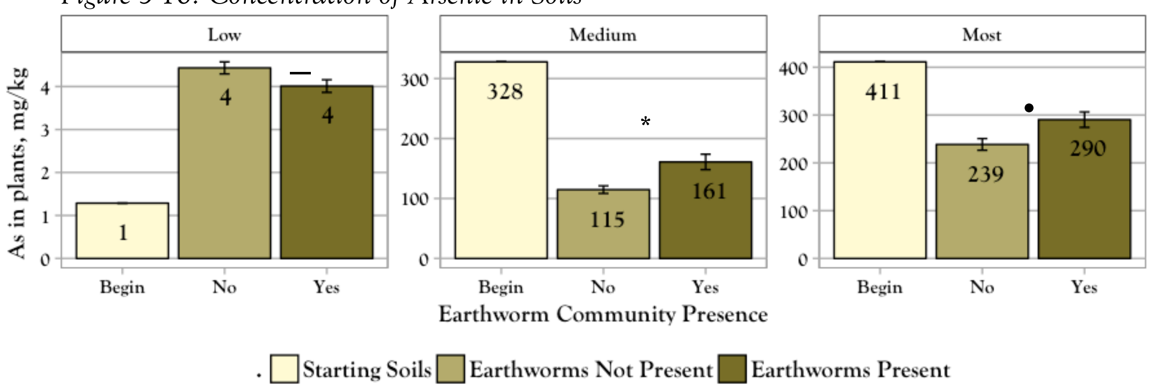


Figure 3-17: Concentration of Cadmium in Soils

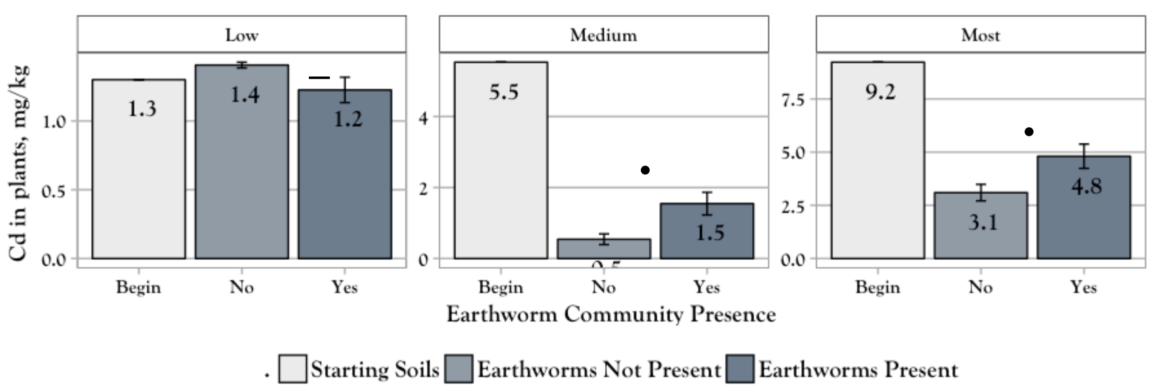


Figure 3-18: Concentration of Copper in Soils

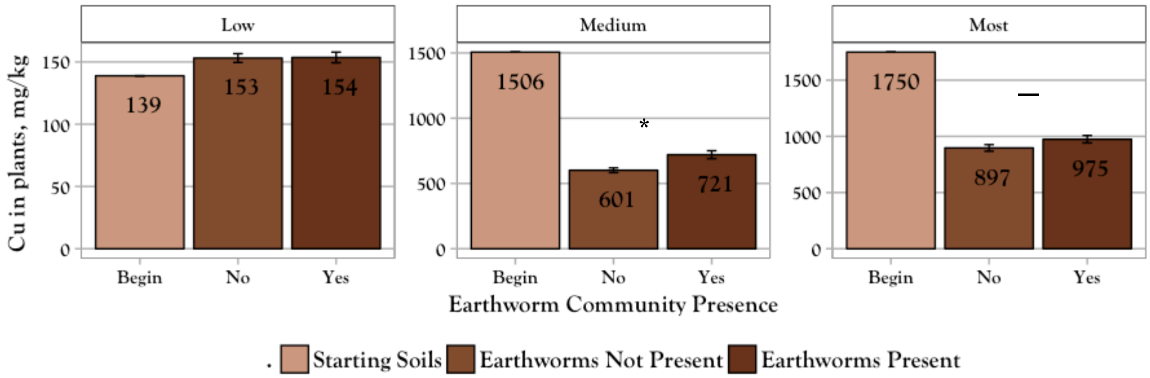


Figure 3-19: Concentration of Lead in Soils

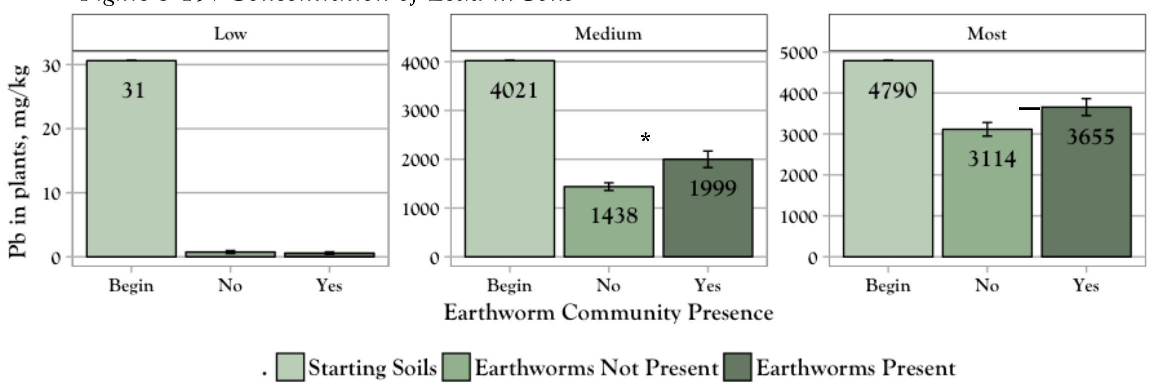
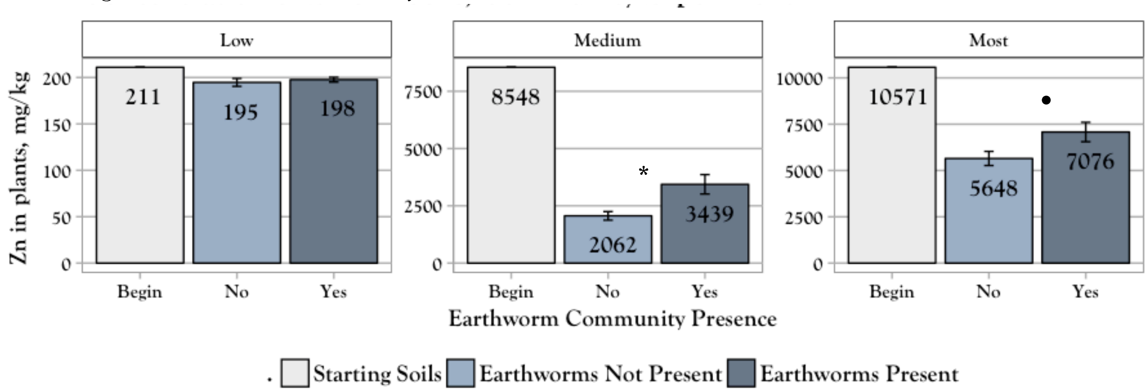


Figure 3-20: Concentration of Zinc in Soils



Significance codes: $p \leq 0.0001$ '***' | $p \leq 0.001$ '**' | $p \leq 0.01$ '*' | $p \leq 0.05$ '•' | $p \leq 0.1$ '–' | $p \leq 1$ 'N/S'

C. Results of Metals in Plants with and without Earthworms:

Figures 3-21 through 3-25 graphically show the concentrations of individual metals in each plant, soil treatment, and earthworm presence or absence.

Figure 3-21: Concentration of Arsenic in Plants, Community Experiment

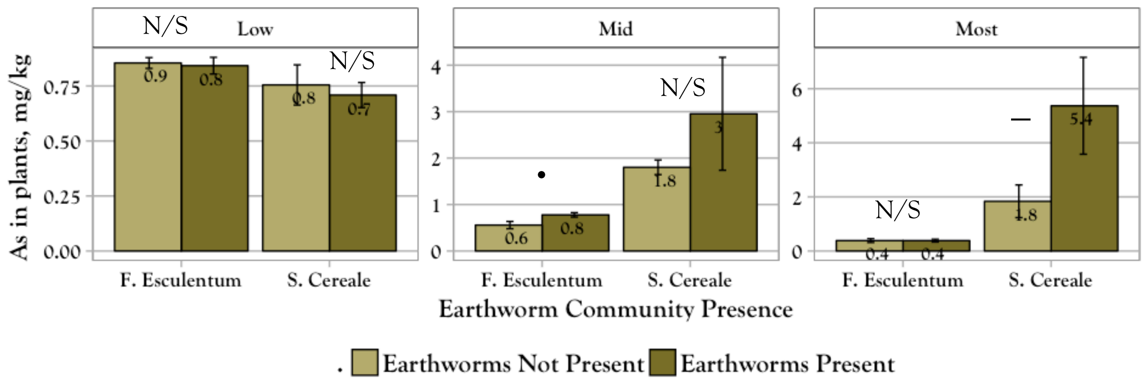
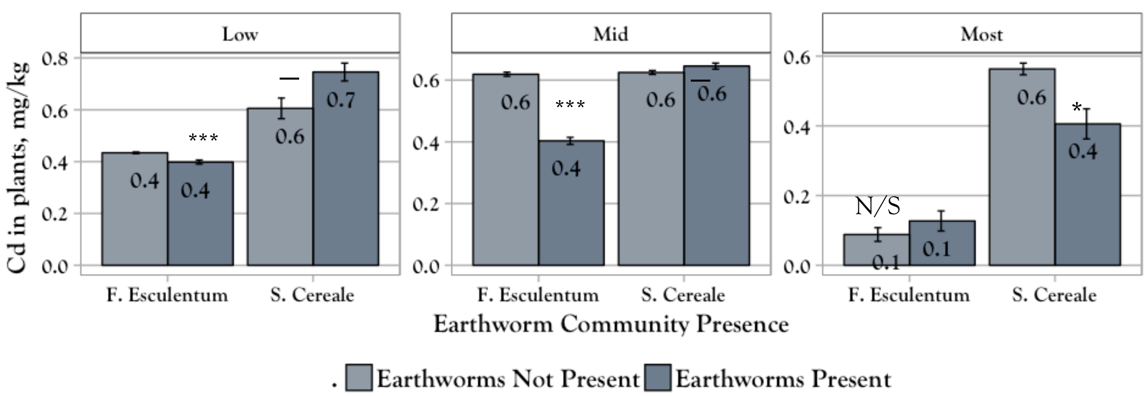


Figure 3-22: Concentration of Cadmium in Plants, Community Experiment



Significance codes: p ≤ 0.0001 '***' | p ≤ 0.001 '**' | p ≤ 0.01 '*' | p ≤ 0.05 '•' | p ≤ 0.1 '–' | p ≤ 1 'N/S'

Figure 3-23: Concentration of Copper in Plants, Community Experiment

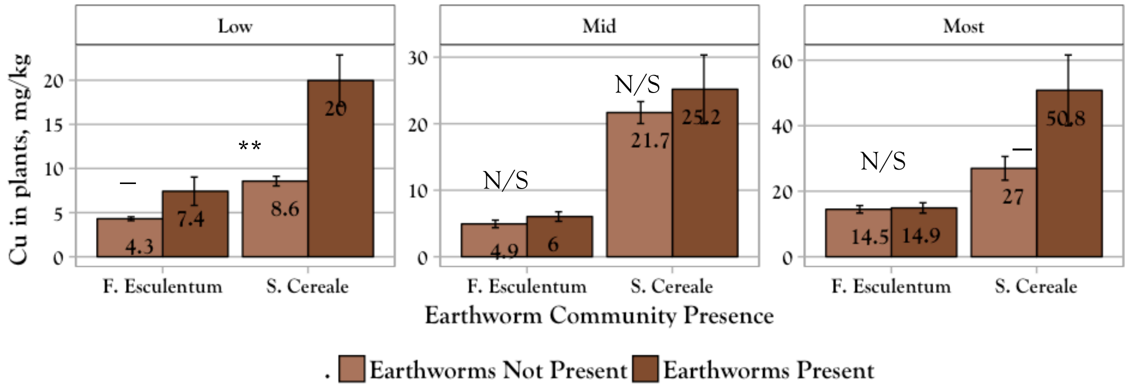


Figure 3-24: Concentration of Lead in Plants, Community Experiment

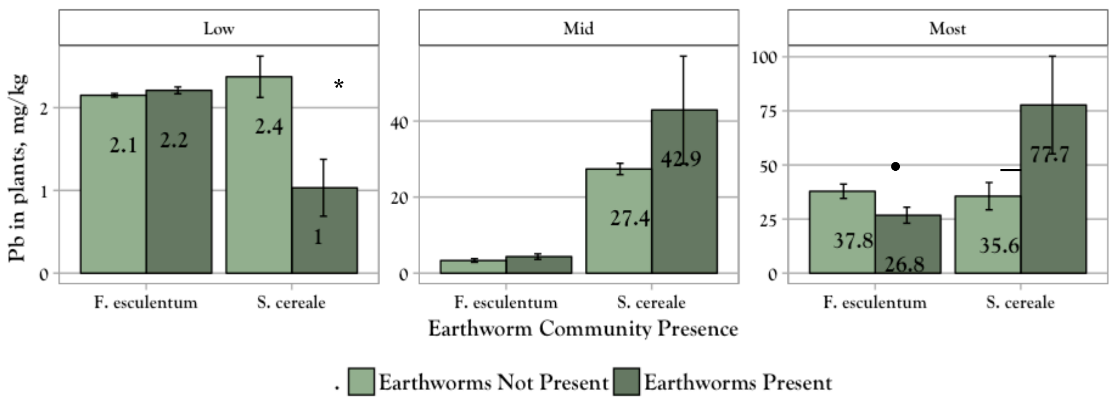
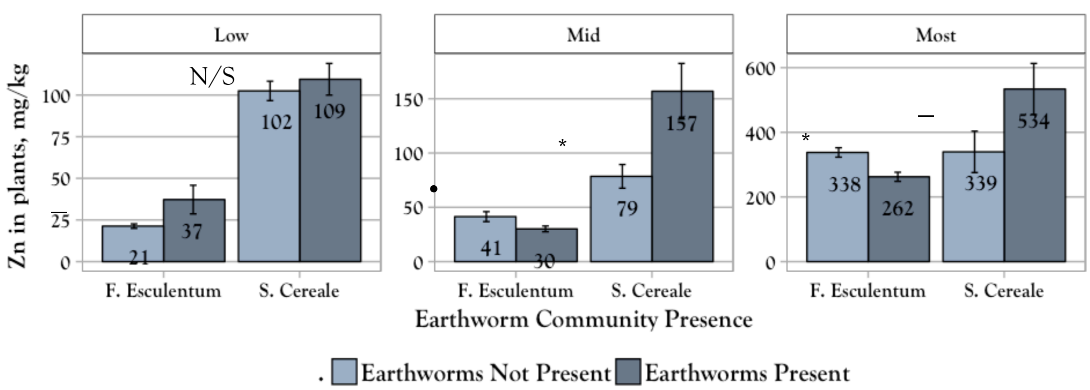


Figure 3-25: Concentration of Zinc in Plants, Community Experiment



Significance codes: $p \leq 0.0001$ '***' | $p \leq 0.001$ '**' | $p \leq 0.01$ '*' | $p \leq 0.05$ '•' | $p \leq 0.1$ '—' | $p \leq 1$ 'N/S'

IX. Discussion

As evidenced by the measurements, earthworms do have an impact on overall plant growth. In all soil and earthworm treatments, there was a difference in biomass of both of the plants tested in some but not all of the treatments (Figures 3-10, 3-12, 3-13). Seeds did not show any meaningful difference except in the most polluted soils (Figure 3-11). The largest percentage of improvement of plant growth linked to earthworm presence was in the most polluted soils. Low levels of metalliferous pollution in soil allowed the plants to successfully grow whether earthworm communities were present or not. In more polluted soils, there was a greater influence from earthworm amelioration. This is consistent with the findings of Ruiz et al. (2009), Dandan et al. (2007), and Yu et al. (2005). It appears that plants are more influenced or benefitted from earthworm presence as metal loads become higher (Figure 3-13). — up to a point when the earthworms and/or plants die (Aghababaei et al. 2014)

More research is needed in creating a pollution gradient and measuring whether there is a linear effect of improvement on overall biomass in plants with earthworms. This has been done previously with artificially amended soils (e.g. Lukkari et al. 2004, 2005; Spurgeon et al. 2004) as well as non–laboratory amended “real” soils (e.g. Vandecasteele 2004; Spurgeon & Hopkin 1996; Morgan & Morgan 1990), but the study of earthworm effects in soils that contain metal toxicants in areas where those earthworms are non–native is less well published.

Total metal concentrations in *Fagopyrum esculentum, Fagopyrum esculentum* seeds, and *Secale cereale* did vary slightly with the presence of earthworms, especially as pollution levels got higher. Seeds were tested for biomass and metal content separately from the *Fagopyrum* shoots and leaves because there was a concern that large, lipid–rich seeds can create a bioaccumulation hazard to higher trophic levels (F. Gallagher, pers comm. 10/15/15). The seeds seemed to not vary in size significantly with their soil treatment, nor did they accumulate nearly the amount of metal toxicants that the shoots and leaves did. In fact, on average they only contained about 25% of what the other biomass of *Fagopyrum* absorbed. *Secale cereale* took up more metals in the presence of earthworms than *Fagopyrum* did. This is believed to be due to the absence of arbuscular mycorrhizal fungi (AMF) in buckets containing the earthworms. *Secale* is a graminoid mycorrhizal dependent species, and as such, relies on AMF to 'protect' the roots and act as a filter for toxicant uptake. Lawrence et al. (2003) found that earthworm burrowing disrupts plant mycorrhizae to the detriment of the plant species that depend on them. AMF, while increasing the surface area and nutrient exchange capacity of roots (Meharg & Cairney 2000), also serve as toxicant sinks, effectively immobilizing metals and reducing availability to the plants (Audet & Charest 2007a).

The addition of earthworms to this experiment and assumption that the anecic earthworm, *Lumbricus terrestris,* was consuming the mycorrhizae in the bucket (as they do outside captivity (Lawrence et al. 2003)) supports the metal binding hypothesis of Audet and Charest (2007a). *Secale cereale* is mycorrhizal dependent with significant fine root growth (Dittmer 1937). This is further supported by independent collaboration of a helpful undergraduate student who grew 20 small pots of *Secale* and successfully located AMF in presence and absence of earthworms (Froehlich, pers. comm. 2017). Dittmer (1937) measured the incredibly dense root network of *Secale* and found that without mycorrhizae (which he did not quantify), an average 4-month-old plant had a root surface area of over 237 m^2, and root fibers collectively achieving 622.8 km^{-1} in length.

Results of Phase I (biomass and growth) of *Secale* show an advantage of the absence of earthworms on rye. Overall dry biomass differences between earthworm and non-earthworm buckets were highly significant in low pollution, not significant in mid, and significant in the most polluted soils (Figures 3-13:3-15). When addressing the difference between metal uptake, however, the improvement in absorption with the presence of earthworms is clearly demonstrated. At the highest levels of soil contamination, the *Secale* in buckets containing earthworms took in significantly (all p-values ≤ 0.1) more pollution in all five of the toxicants tested. It is therefore hypothesized that earthworms do eat mycorrhizal fungi, and those same fungi are acting as sinks or filters for metal contamination preventing the metals from entering the root fibers of the plant.

The soils in each of the bucket mesocosms also demonstrate some contamination variability. All of the contaminants tested had some significant differences (all p-values $\leq$ 0.1) in the mid and most polluted soils between earthworm presence and absence. Unexpectedly, however, the trend of change in metal contamination is opposite of the expected result. Testing showed that anecic earthworm presence actually kept more metal contaminants in the soil. It has been reported that after earthworm presence, the distribution of bioavailable metals in soil was significantly changed (Devliegher & Verstraete 1995; Ma et al. 2003; Wen et al. 2004; Udovic & Lestan 2007). It is then logical that these newly bioavailable contaminants would be easier to be sequestered into growing plants. While results of the testing of plants for metal contaminants support this, the fact that there are more metals retained in soils that had earthworm populations does not.

The best explanation for this difference in metal content of the soil is earthworm-based sequestration. It has been demonstrated that some earthworms can survive in contaminated soils and can even accumulate metals, such as Cd, Cu, Zn, and Pb, in their tissues (Morgan & Morgan 1990; Lanno et al. 2004; and reviewed in Sizmur & Hodson 2009). Earthworms can hyperaccumulate (Nannoni et al. 2011) and sequester (Kennette 2002) metals into their bodies, more metal remains in the soil while rainwater would have been able to leach more contaminants that were not sequestered, away.

i. **Arsenic:** Concentrations of arsenic in plants are relatively similar despite earthworm presence, with two notable exceptions (Figure 3-21). In mid-level pollution (starting soil 328 mg/kg As), *Fagopyrum* sequestered more As in the presence of earthworms ($p= \leq 0.05$). In the most polluted soils (starting value 411 mg/kg), *Secale* concentrated more in the presence of earthworms ($p=\leq 0.1$). Mains et al. (2007) report a similar result on gold mine tailings with *Secale*.

ii. **Cadmium:** Cd concentrations in plants with and without earthworms overall achieved varying levels of significant differences, but the result in Figure 3-22 shows that the plants in the low, mid, and most polluted soil treatments all finished with similar measures of Cd, while the soils themselves were contaminated at 1.3, 6, and 9 ppm, respectively.

iii. **Copper:** Phytoextraction of copper, shown in Figure 3-23 is clearly better with *Secale cereale*, as the *Fagopyrum* only absorbed 41% of what the *Secale* did over all treatments — without earthworms present. With earthworms, the *Fagopyrum* only absorbed 38% as much of the copper that *Secale* did. Quartacci et al. (2007) report a similar result and further demonstrate that the use of chelating agents improved the overall result.

iv. **Lead:** Pb accumulations as shown in Figure 3-24 were unexpected. Honda et al. (2007) report that *Fagopyrum* is an excellent hyperaccumulator of Pb, and so the results of this experiment were less than expected. *Secale*, however, did accumulate considerable quantities of lead, especially in the presence of earthworms. Although the sequestration in the most polluted soils (which started out at 4,790 ppm Pb) is over twice as much in the presence of earthworms, it remains at $p = \leq 0.1$ because of the wide standard deviation noted in testing.

v. **Zinc:** Figure 3-25 shows that the quantities of Zn that have accumulated in *Fagopyrum* are opposite of what would be expected. In the presence of earthworms, the plants concentrated less zinc than in their absence. *Fagopyrum* was not expected to absorb as much as *Secale* however; it is well documented for Al and Pb, while *Secale* is reported for zinc remediation in much of the existing literature (Quartacci et al. 2007; Mains et al. 2007). Zinc was also the highest contaminant in the experiment, with levels at 211, 8,548, and 10,571 mg/kg in low, mid, and most, respectively. Soil amelioration from anecic earthworms does have an impact on *Secale*, and although the significance is ≤ 0.05 and ≤ 0.1 in mid and most polluted soils, it was still 56% on average more effective to have earthworms present.

X. Conclusion:

The relationship between earthworms and mycorrhizal fungi seems to be critical when dealing with phytoextraction of metalliferous sites. Just because some earthworm presence has been shown to improve plant growth as pollution levels increase does not mean that earthworm presence in all contaminated brownfields would be appropriate. It is also important to consider the long-term goals for the particular location. If it were desirable to sequester metals *in situ*, earthworms, especially anecic species, combined with mycorrhizal dependent hyperaccumulating plants would be detrimental. For example, if terrain makes harvesting of hyperaccumulating plants difficult, land managers and stakeholders may choose to sequester the metals in place and allow an organic cap to develop over the contaminated soils. This is the case in Lehigh Gap Nature Center, a restored superfund site near a defunct zinc smelting operation in Palmerton, Pennsylvania, USA (40.793, -75.562). The terrain in the nature center is so steep that reseeding had to be completed by aircraft (Sopper 1989). In a scenario like this, it would be beneficial to not plant mycorrhizal dependent grasses if anecic earthworm populations are abundant and it was desirable to keep the metals in place.

Conversely, if it were easy to harvest and dispose of the plant material, anecic earthworms would improve phytoextraction in the presence of mycorrhizal plants. About 450 angiosperm species have been identified so far as hyperaccumulators (Rascio & Navari-Izzo 2011), and an estimated 74% of plants require AMF symbiotic relationships (Brundrett 2009). *Secale cereale*, although it worked quite well for this experiment, is certainly not the only option when considering phytoremediative and phytoextractive species for restoration projects. Plant options in polluted soils must look at specific metals targeted, but also presence of anecic earthworms and other environmental engineers at the site.

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XVIII. Appendix:

A. Map of Communipaw Cove, 1854:

B. Satellite Image of Liberty State Park:

C. Table of Biomass Between Pollution Levels and Earthworm Presence:

Plants listed are *Fagopyrun esculentum* (Buckwheat), *Fagopyrum* seeds (Seed), and *Secale cereale* (Rye). Pollution levels are indicated by “Low”, “Mid” and “High”, as noted earlier. Plants were weighed to the nearest 0.01g, while seeds were weighed to 0.0001g. Standard deviations given are for the entire population of 20 plants per bucket, times ten replicates each. The difference (Δ) between plants and seeds grown with and without earthworms is demonstrating the potential improvement of annelids, and p-values based on earthworm presence is a two-tailed, paired T-Test, converted to p-values.

Plant	Pollution Level	Earthworms	20 plant weight average (g), 10 buckets	Standard Deviation among buckets (g), n=10	Δ between earthworm treatments (g)	P-values between earthworm presence
Buckwheat	Low	Yes	20.83	3.6	3.27	0.09634
Buckwheat	Low	No	17.56	4.3		
Buckwheat	Mid	Yes	19.63	2.6	-1.89	0.24182
Buckwheat	Mid	No	21.520	3.9		
Buckwheat	Most	Yes	15.28	2.3	6.07	0.000002
Buckwheat	Most	No	9.21	1.4		
Seed	Low	Yes	0.6018	0.064	0.009	0.758464
Seed	Low	No	0.5928	0.059		
Seed	Mid	Yes	0.6016	0.046	-0.01	0.576473
Seed	Mid	No	0.6116	0.026		
Seed	Most	Yes	0.5846	0.051	0.0794	0.01273
Seed	Most	No	0.5052	0.069		
Rye	Low	Yes	18.16	3.7	-8.01	0.000125
Rye	Low	No	26.17	3.3		
Rye	Mid	Yes	19.25	5.8	0.56	0.79168
Rye	Mid	No	18.69	2.3		
Rye	Most	Yes	6.13	2.9	-4.59	0.007395
Rye	Most	No	10.72	3.5		

D. Analysis of Variance for Biomass of Plants:

Scores below are based on a two-way analysis of variance, assessing the relative importance or explanatory power of three different factors:

1. The impact that earthworms had on overall plant growth
2. The impact that soil quality or pollution level had on overall plant growth
3. The impact that BOTH earthworms AND soil pollution had on overall plant growth, versus just normal variability in consistent treatments (H_0)

Thus far, results have only been to compare one variable (earthworm presence) against the same soil treatment without earthworms, ten buckets against ten buckets. The ANOVA takes into account all sixty buckets at the same time, comparing both earthworm and pollution levels.

Fagopyrum esculentum Biomass Growth

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Pollution Level	1	797.3	398.7	35.702	1.32e-10 ***
Earthworms	2	92.5	92.5	8.284	0.00572 **
EW and Poll	2	163.0	81.5	7.301	0.00156 **
Residuals	54	603.0	11.2		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Fagopyrum esculentum Seeds- Biomass of 20 Seeds

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Pollution Level	1	0.04426	0.022130	6.761	0.0024 **
Earthworms	2	0.01024	0.010244	3.130	0.0825 .
EW and Poll	2	0.02218	0.011091	3.388	0.0411 *
Residuals	54	0.17676	0.003273		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Secale cereale Biomass Growth

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Pollution Level	1	2068.0	1034.0	66.066	3.09e-15 ***
Earthworms	2	241.6	241.6	15.437	0.000244 ***
EW and Poll	2	186.1	93.1	5.946	0.004637 **
Residuals	54	845.1	15.7		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

E. Analysis of Variance for Metal Concentration in Plants:

This measures relative probability of $H_0 \neq 0$ given both pollution level and earthworm presence. ANOVA formulae were all conducted as ***a*** (pollution level); ***b*** (earthworm presence); and ***a • b*** (pollution level multiplied by earthworm presence).

Arsenic in *Fagopyrum esculentum* Seeds

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	2.633	1.3165	8.898	0.000457 ***
Earthworms	1	0.039	0.0390	0.264	0.609674
EW and Poll	2	0.821	0.4107	2.776	0.071202 .
Residuals	54	7.989	0.1479		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Arsenic in *Fagopyrum esculentum*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	2.1861	1.0930	36.517	9.31e-11 ***
Earthworms	1	0.0724	0.0724	2.420	0.1256
EW and Poll	2	0.1709	0.0855	2.855	0.0663 .
Residuals	54	1.6164	0.0299		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Arsenic in *Secale cereale*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	83.0	41.51	4.900	0.0111 *
Earthworms	1	35.8	35.83	4.229	0.0446 *
EW and Poll	2	33.1	16.57	1.956	0.1513
Residuals	54	457.5	8.47		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Cadmium in *Fagopyrum esculentum* Seeds

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	1.1595	0.5797	72.913	4.54e-16 ***
Earthworms	1	0.0162	0.0162	2.034	0.1596
EW and Poll	2	0.0677	0.0338	4.254	0.0192 *
Residuals	54	0.4294	0.0080		

Cadmium in *Fagopyrum esculentum*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	1.7791	0.8896	364.75	< 2e-16 ***
Earthworms	1	0.0755	0.0755	30.94	8.49e-07 ***
EW and Poll	2	0.1715	0.0858	35.16	1.66e-10 ***
Residuals	54	0.1317	0.0024		

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Cadmium in *Secale cereale*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	0.4062	0.20309	24.072	3.36e-08 ***
Earthworms	1	0.0000	0.00002	0.002	0.964
EW and Poll	2	0.2246	0.11230	13.311	2.00e-05 ***
Residuals	54	0.4556	0.00844		

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Copper in *Fagopyrum esculentum* Seeds

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	498.3	249.13	14.072	1.21e-05 ***
Earthworms	1	40.8	40.82	2.306	0.135
EW and Poll	2	5.6	2.78	0.157	0.855
Residuals	54	956.0	17.70		

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Copper in *Fagopyrum esculentum*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	1083.5	541.7	44.483	3.83e-12 ***
Earthworms	1	36.1	36.1	2.963	0.0909 .
EW and Poll	2	19.5	9.8	0.802	0.4536
Residuals	54	657.6	12.2		

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Copper in *Secale cereale*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	6203	3101.3	11.118	9.04e-05 ***
Earthworms	1	2503	2502.5	8.971	0.00413 **
EW and Poll	2	1051	525.3	1.883	0.16199
Residuals	54	15063	279.0		

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

CHAPTER 4: EARTHWORM IMPACTS ON PHYTOSEQUESTRATION BY PLANTS GROWN IN METALLIFEROUS SOIL

I. Abstract:

Phytoextraction is a well–studied field of restoration ecology, and demonstrates significant potential to hundreds of thousands of post-industrial brownfield sites. This study was designed to investigate improving the capabilities of phytoremediative plants *Fagopyrum esculentum* (buckwheat) and *Secale cereale* (rye) with the addition of two ecosystem–modifying earthworm populations, *Lumbricus terrestris* and *Amynthas spp.* in order to increase their metal accumulation behavior. In this population–level experiment, *Fagopyrum* and *Secale* were grown separately, in contrast to the previous community–level study in which they grew in the same container. Forty replicates of three soil treatments of low, mid, and most metalliferous soil pollution were planted with either buckwheat or rye and earthworms were added to half of the pots. Plants were allowed to grow in a greenhouse for 60 days, after which they were dried, weighed and digested into analytes assessed for metal content in an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES), along with the digested analytes of soils and earthworms. Trials mostly indicated a significant increase of metal uptake in plants in the presence of earthworms, especially in *Secale cereale's* absorption of arsenic (43% increase in uptake), chromium (59% increase), copper (23% increase), and lead (12% increase). *Fagopyrum esculentum* showed increases in metal absorption a full order of magnitude less in the presence of earthworms.

II. Introduction:

The severe and long–lasting pollution effects of environmentally damaging industries is something that land managers and stakeholders will be dealing with for generations to come. Of these legacy pollutants, metal toxicants are considered to be the longest–lasting. For example, Pb, which is regarded as one of the more persistent metals, is estimated to have legacy effects in soil for 150–5,000 years (Kumar et al. 1995). Other metals that may be deleterious to organismal health include (but are not limited to) As, Ag, Cd, Cr, Cu, Ni, and Ti (Ferńandez et al. 2006). One of the many ways land managers have to remove metals (as well as breaking down hydrocarbons, PCB's and similar environmentally-degrading compounds) is through the technique of phytoremediation. Phytoremediation, including phytoextraction, phytosequestration, phytodegradation, and phytovolatilization (Ruiz et al. 2011; Chaney et al. 1997; Tangahu et al. 2011) provides a cost effective (Jusselme et al. 2015a) and environmentally friendly way to remediate sites.

Plants that are used in phytoextraction are referred to as hyperaccumulators, as they are capable of concentrating metals up to 100 or 1,000-times those taken up by nonaccumulator plants (Tangahu et al. 2011). There have been about 450 angiosperm species identified as hyperaccumulators, which accounts for less than 0.2% of all known species (Rascio & Navari–Izzo 2011). The most commonly used is *Brassica juncea*, which is a broad uptake generalist (Lasat 2002), but there are many others. Most of the hyperaccumulating plants have a specific cadre of metals they will accumulate, for instance, *Fagopyrum esculentum* for Pb and Al (Honda et al. 2005) and *Secale cereale* for As (Mains et al. 2007); Cu, Pb, and Zn (Quartacci et al. 2007).

Many of the studies in phytoextraction use artificially amended soils to measure the total amount of metals concentrated in the roots, stems, and leaves of the plant. While this method can show which plants can accumulate mass quantities of pollutants, it is not always applicable to the real world. Bioavailability in soils has been a hurdle to quantifying phytoremediative properties in actual practice (Raskin et al. 1997). Smoulders et al. (2009) showed that soils artificially spiked with fully soluble metal sources do not represent conditions of metal-rich contaminated soils, and Spurgeon and Hopkin (1995) demonstrate that metals in artificial soils are far more bioavailable than in actual field soils.

Earthworms (Annelida: Oligochaeta) are well documented to improve plant productivity through increased soil aeration, root abrasion, and slowed water infiltration. Plants grown in the presence of earthworms have been shown to grow larger and faster than plants do in their absence. A very comprehensive review by Blouin et al. (2013) summarizes many of these growth and biomass experiments.

Earthworms have been shown to change bioavailability and mobilization pathways of metals in soils (e.g. Sizmur & Hodson 2009; Stürzenbaum et al. 1998; Andre et al. 2010), but could also prove to be a complicating factor in remediation due to biosequestration and concentration in the tissues (e.g. Stürzenbaum et al. 2004; Marino & Morgan 1999b; Morgan & Morgan 1998). Earthworms alone, while changing some of the properties of the metals in soils, may not have a lasting effect since they are not being removed and the sequestered compounds just return to the matrix upon decomposition. To create lasting remedial change, metals must be eliminated somehow from the soils.

Harvesting and removing plants from contaminated areas is the typically accepted method. It is worth noting that many of the studies on plants with hyperaccumulation abilities have been done on amended laboratory soil, while many of the earthworm experiments used field soils. It is, therefore, worth studying what happens when the two are combined: i.e. earthworms having a positive impact on plant productivity and remedial properties when present in metalliferous field soils. Several manuscripts demonstrate the benefits of the earthworm/metal accumulating plant relationship (Rascioa & Navari-Izzo 2011; Aghababaei & Raiesi 2014a, b, 2015; Butt & Grigoropoulou 2009). These studies, however, were completed in areas where earthworms have been naturalized or native for millennia. In the Northeastern United States during the last Pleistocene glacial maximum ca. 19,000 years ago until ca. 10,000 years ago, earthworms that had become naturalized to northern habitats were pushed south (Reynolds 1994). Although various estimates disagree on where the no-worm line exactly was (Gates 1976), there is agreement that the line was at least near the 35°N latitude parallel — nearly 800 km from the area in this study. While it is fairly well documented what effects can be expected in metalliferous soils within native ranges of earthworms, there could be a different result in areas that have not acclimated to their presence over millennia. This experiment was designed to test improvements in phytoextraction of two plants — a non–mycorrhizal dependent dicot forb and a mycorrhizal dependent monocot grass — with and without the presence of two locally common introduced earthworms. The soils that were used for this experiment were taken from the interior portion of Liberty State Park, Jersey City, New Jersey, USA, and are more completely described in Chapter Three.

Plants used for this experiment were *Fagopyrum esculentum* and *Secale cereale*, and were also used and more completely described in Chapter Three. *Fagopyrum esculentum* (Moench.), commonly buckwheat, is a non-mycorrhizal dependent dicot forb that has been noted for its uptake of Al (Ma & Hiradate 2000) and especially Pb (Honda et al. 2007). Many other hyperaccumulating plants have difficulty with Pb due to limitations from solubility and root contact (Rascioa & Navari-Izzo 2011).

Secale cereale (*L.*) referred to as rye or winter rye, is a mycorrhizal–dependent monocot grass that is used successfully for multiple metal toxicants, notably As (Mains 2007); Cu, Pb, and Zn (Quartacci et al. 2007); and hydrocarbons (Muratova et al. 2008).

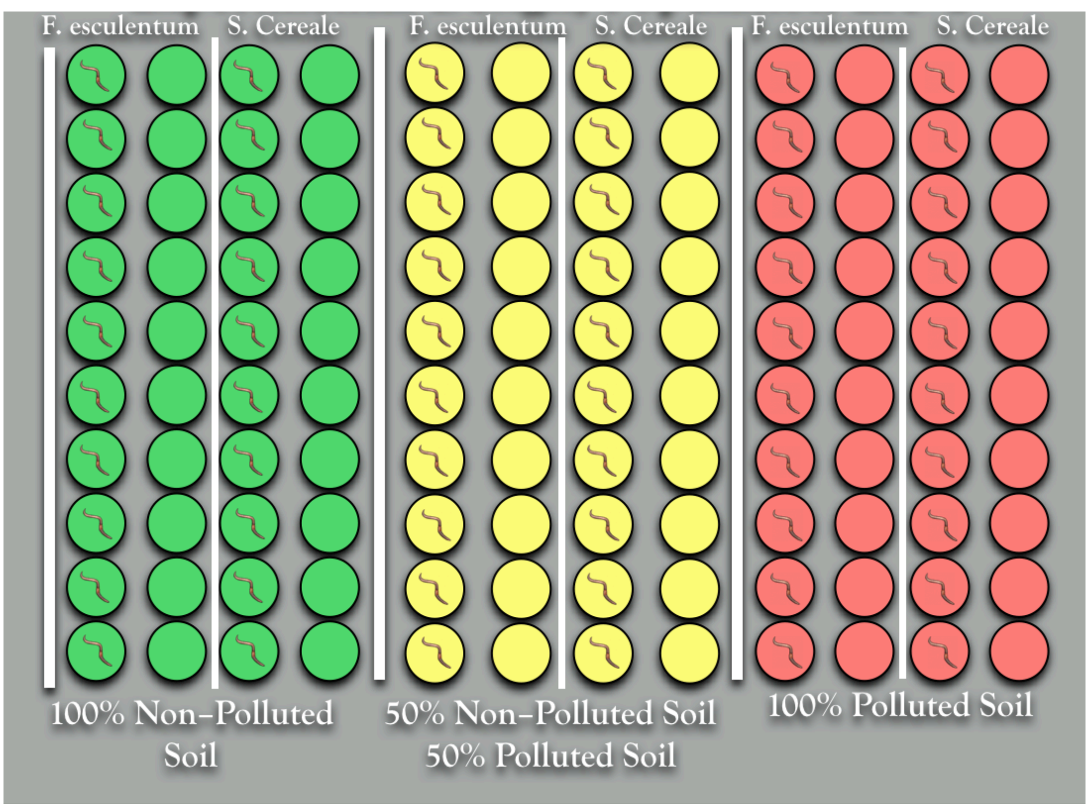
Earthworms chosen for this study were *Amynthas spp.* (Goto & Hatai) and *Lumbricus terrestris* (*L.*), species commonly found in the biological survey conducted for Chapter Two in both brownfield and non-polluted soils.

III. Methods:

This experiment was designed to test the individual properties of a small earthworm community living in field–polluted soils with one specific plant species. This is in contrast to the experiment described in Chapter Three when the two plants (*Secale* and *Fagopyrum*) were raised together. This population level trial was designed to eliminate the variables of competition between plants, such as space, light, water, or allelopathy.

This study used contaminated field soils and compared plant productivity and metal sequestration among three pollution thresholds. This design allowed for a more realistic analysis of phytoextraction in post-industrial brownfield soils than artificially amended laboratory analyses. 100 L of soil from Liberty State Park's interior portion was screened through 7 mm mesh to remove large rocks and detritus and homogenized to minimize localized concentration of pollution effects in the legacy fill from the defunct rail yard. Forty, 4-L sapling pots (Model TP414 "Tall One"; Stuewe and Sons, Tangent, Oregon, USA) were filled with the polluted soil from Liberty State Park, creating a "most" soil pollution level (red circles on Figure 4-1). 40 more pots were homogenized 50:50 by volume with "low" pollution concentration topsoil from the local home improvement

Figure 4-1: Experimental Design for Population Experiment



center and “most” polluted soil — creating a "mid" level of soil contamination (yellow circles on Figure 4-1). Last, 40 pots were filled completely with "low" level polluted soil (green circles on Figure 4-1).

Sixty of these 120 pots were planted with seeds of *Fagopyrum esculentum* (buckwheat), and the other sixty were planted with seeds of *Secale cereale* (rye). Half of the pots in each seed and soil treatment (ten each, times six) received five *Lumbricus terrestris* and three *Amynthas spp.* to compare earthworm effects in differing treatments. 2 g of pine shavings were added to each pot as a protectant layer for the seeds and earthworms. Pots were randomly arranged in plastic milk crates and housed in Moravian College’s greenhouse (Bethlehem, PA, USA). Plants were watered weekly and rotated throughout the growth tables for even sunlight.

Growth was terminated after 60 days, and all pots were destructively harvested. Aboveground biomass of plants was separated from roots, placed in paper bags and dried for 24 hours at 60° C. The tallest and largest ten plants from each replicate were weighed immediately after drying. Unexpurgated earthworms were euthanized in 70% ethanol and placed in different containers for each replicate. Finally, soil samples were taken from each pot and placed in 75 mL containers for later analysis.

Plants that had been weighed for biomass were then analyzed for metalliferous content, along with earthworms and soils. All measurements were made in the Analytical Lab of the Cary Institute of Ecosystem Studies, Millbrook, NY, USA, and methods were precisely the same as written in Chapter Three.

IV. Results:

A. Biomass:

The following charts and subsequent graphs are the results of weighing biomass after drying. Biomass is given in g, and all metals are shown as mg/kg^{-1} (ppm). Error bars note standard deviation, and all p–values are the results of a Student's two–tailed t-test of equal variance.

Figure 4-2: Individual violin plots of biomass for Fagopyrum

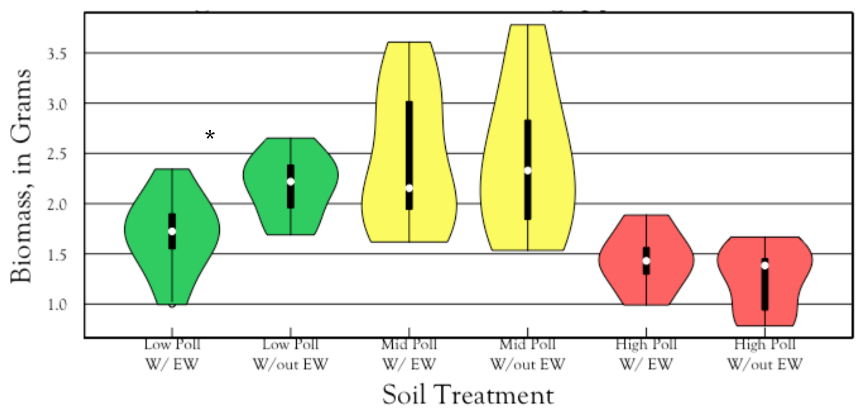
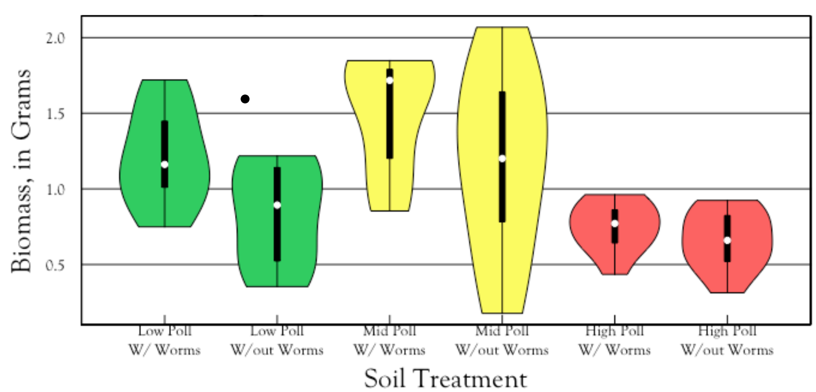


Figure 4-3: Individual violin plots of biomass for Secale



Significance codes: p ≤ 0.0001 '***' | p ≤ 0.001 '**' | p ≤ 0.01 '*' |
p ≤ 0.05 '•' | p ≤ 0.1 '–' | p ≤ 1 'N/S'

B. Metals in Plants:

The following charts and subsequent graphs are the results of testing the digested biomass in the ICP—OES. Figures 4–4 to 4–8 graphically show the results of the metals analysis which is included in its entirety in the appendix. All measurements are given in mg/kg. Note that each pollution level has its own scale.

Figure 4-4: Concentration of Arsenic in Plants, Population Experiment

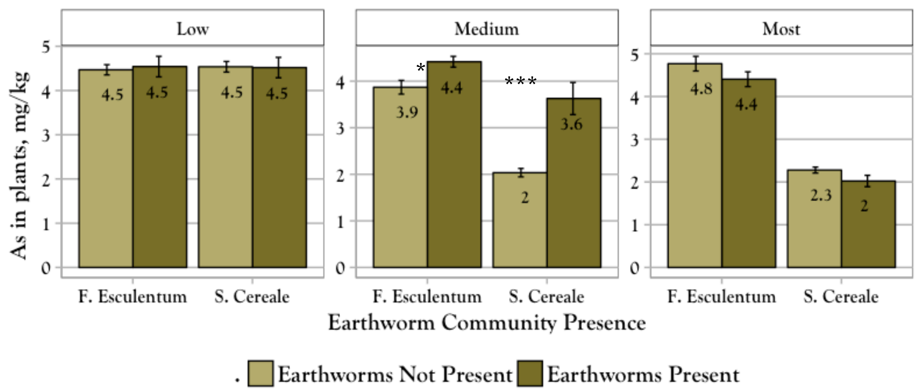
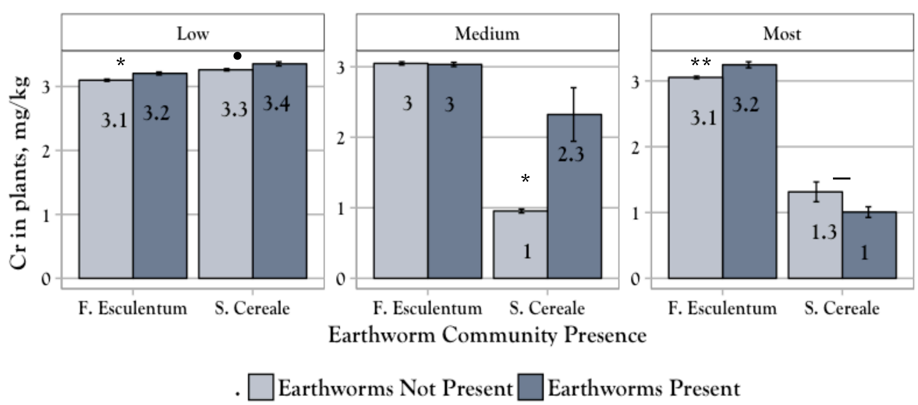


Figure 4-5: Concentration of Chromium in Plants, Population Experiment



Significance codes: p ≤ 0.0001 '***' | p ≤ 0.001 '**' | p ≤ 0.01 '*' | p ≤ 0.05 '•' | p ≤ 0.1 '–' | p ≤ 1 'N/S

Figure 4-6: Concentration of Copper in Plants, Population Experiment

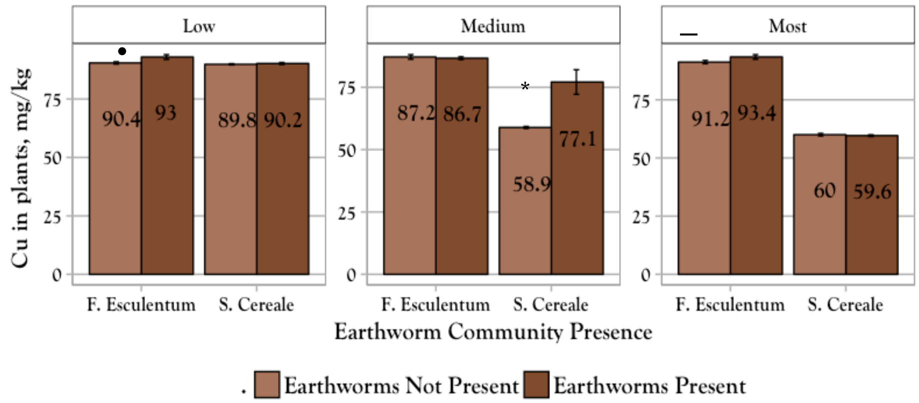


Figure 4-7: Concentration of Lead in Plants, Population Experiment

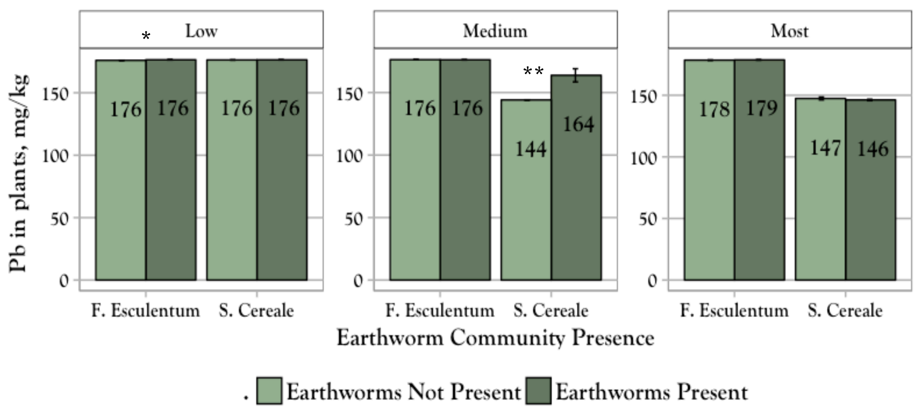
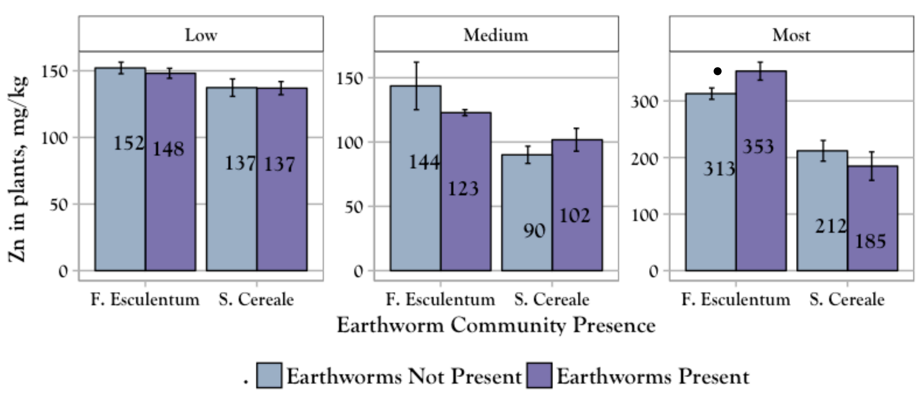


Figure 4-8: Concentration of Zinc in Plants, Population Experiment



Significance codes: p ≤ 0.0001 '***' | p ≤ 0.001 '**' | p ≤ 0.01 '*' | p ≤ 0.05 '•' | p ≤ 0.1 '–' | p ≤ 1 'N/S

C. Metals in Soils:

Figures 4-9 through 4-11 depict soil contamination of metal toxicants in low, mid, and most polluted soils, respectively. Figure 4-9 shows metal concentrations in low pollution levels before the experiment and at the end of the 60 days for both earthworm-present and earthworm-absent soils. All measurements are in mg/kg, and standard deviation is shown in error bars for all plots. Each graph has the beginning value of the soil (bar 1), then soils at the termination of the experiment for *Fagopyrum* without earthworms (bar 2) and with (bar 3), followed by *Secale* without earthworms (bar 4) and with them (bar 5).

Figure 4-9: Values of Metals in Ending Soils – Low Pollution

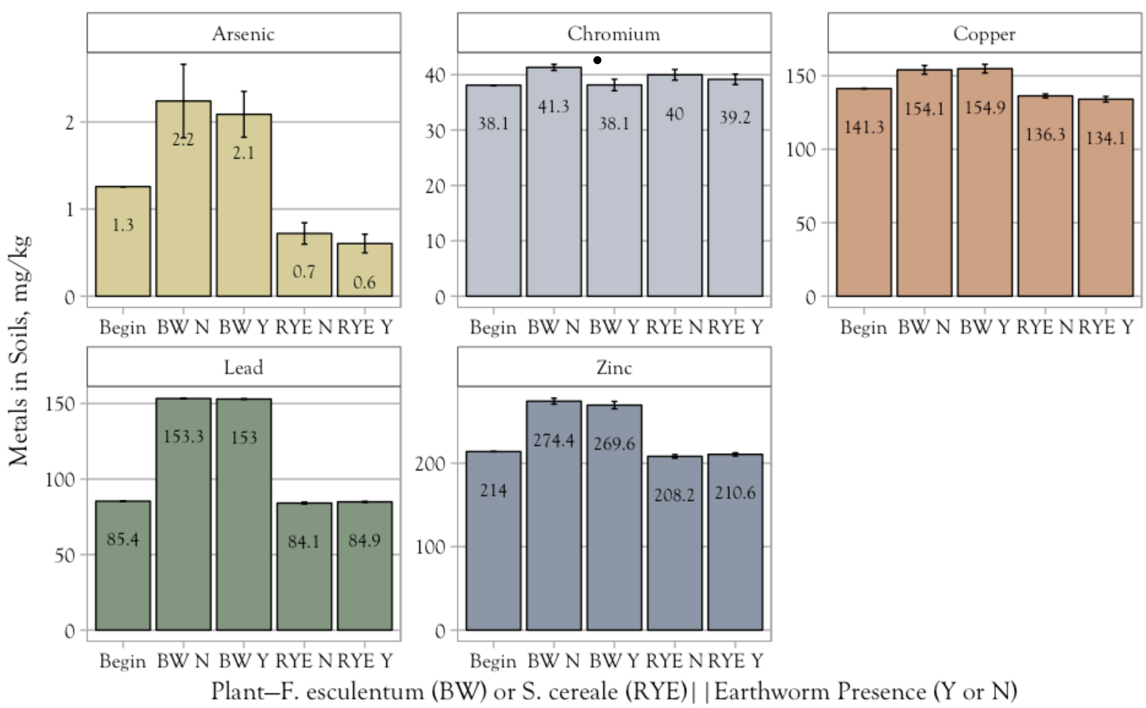


Figure 4-10: Values of Metals in Ending Soils – Mid Pollution

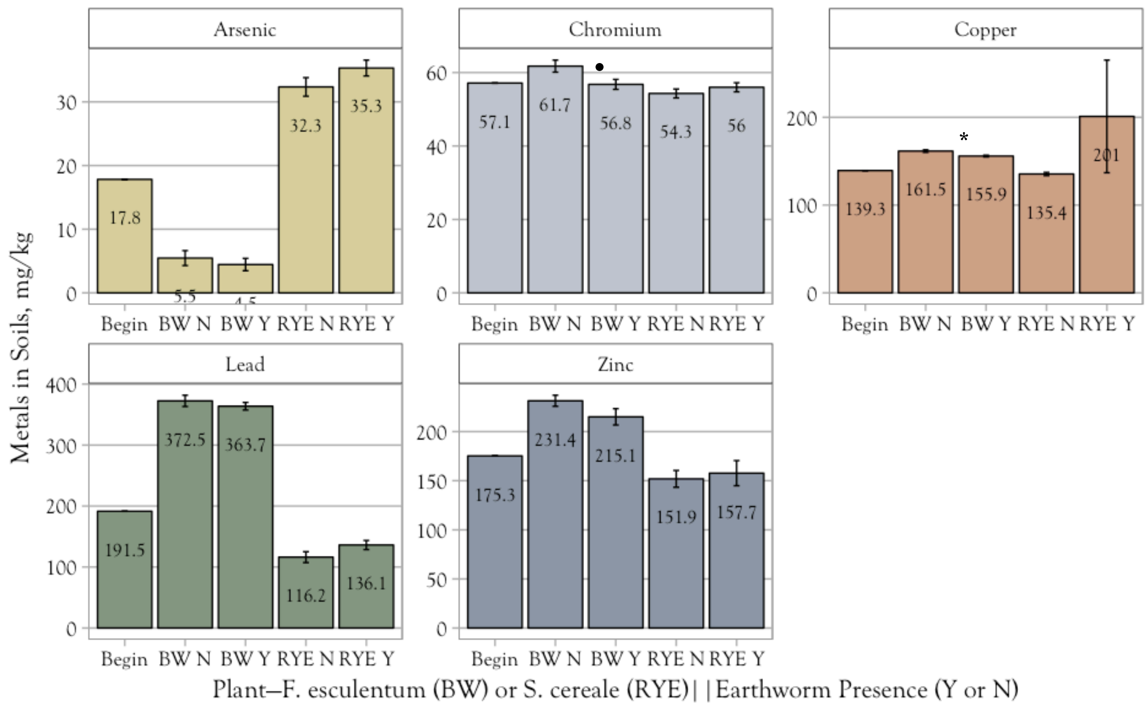
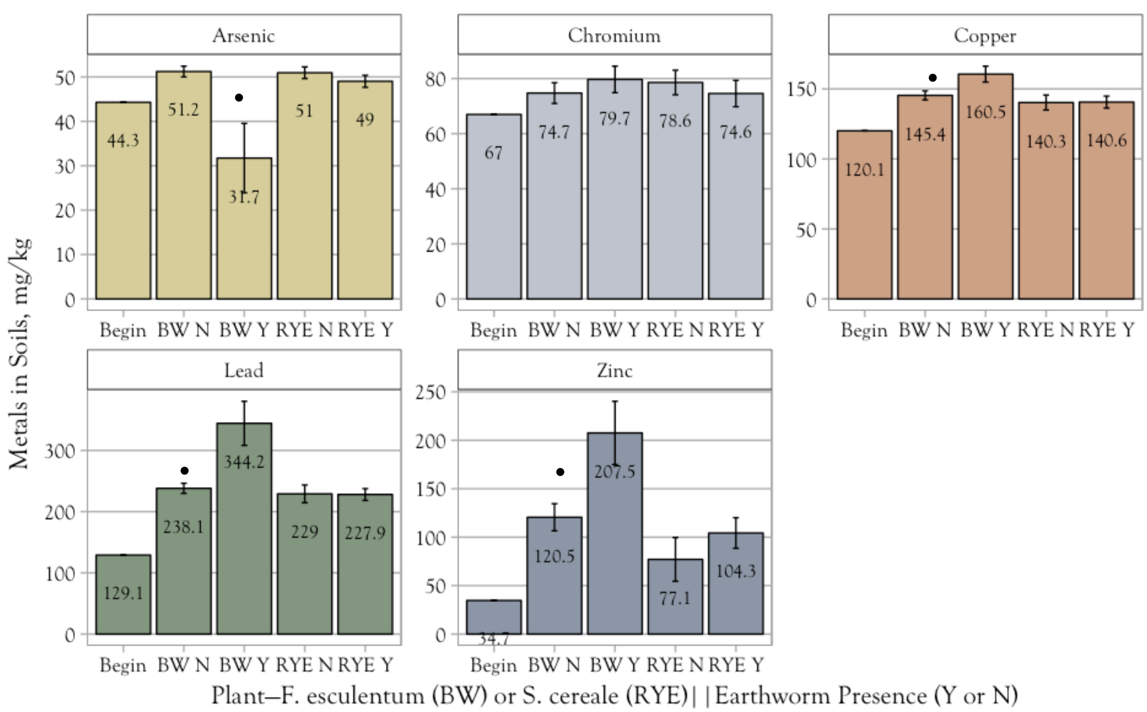


Figure 4-11: Values of Metals in Ending Soils – High Pollution



Significance codes: p ≤ 0.0001 '***' | p ≤ 0.001 '**' | p ≤ 0.01 '*' | p ≤ 0.05 '•' | p ≤ 0.1 '—' | p ≤ 1 'N/S

D. Analysis of Variance:

Two-way ANOVAS were run on each sample type (*Fagopyrum* and *Secale* material). ANOVAS compared relative explanation of variance due to: A.) *pollution*, B.) *earthworm presence*, and C.) *Pollution* • *Earthworm presence*. These full results from the appendix are summarized here in Table 4-1.

Table 4-1: Summary of ANOVA

		Biomass	Arsenic	Chromium	Copper	Lead	Zinc
Fagopyrum (buckwheat) leaves and stems	Pollution level	***	*	***	***	***	***
	Earthworms	n/s	n/s	***	•	•	•
	EW and Pollution	n/s	*	*	n/s	n/s	*
Secale (rye) leaves	Pollution level	***	***	***	***	***	***
	Earthworms	**	*	*	***	*	n/s
	EW and Pollution	n/s	***	***	***	•	n/s

Significance codes: p ≤ 0.0001 '***' | p ≤ 0.001 '**' | p ≤ 0.01 '*' | p ≤ 0.05 '•' | p ≤ 0.1 '–' | p ≤ 1 'N/S'

E. Analysis of Earthworm Hyperaccumulation:

Any earthworms found when the buckets were harvested were immediately euthanized, dried for 48 hours at 60° C before being digested and analyzed in the ICP-OES. There were no earthworms found alive in the most polluted soils, but there were some intact burrows, indicating that perhaps they had lived in the soil for some time. In total, twenty pots had earthworms that were recoverable and had enough dry biomass to analyze.

Table 4-2 shows the results of the analysis on the recovered earthworms. Note that the first number is the concentration (mg/kg) of the metal, and the second column is the standard deviation, indicated by **σ**.

Table 4-2: Summary of Earthworm Metal Accumulation

Soil	Plant	As	σ As	Cr	σ Cr	Cu	σ Cu	Pb	σ Pb	Zn	σ Zn
Low	*F. esculentum*	1.45	0.31	6.32	2.85	44.41	4.50	94.88	1.08	985.50	216.27
Mid	*F. esculentum*	2.93	1.25	4.05	1.87	34.71	3.75	82.62	8.00	1458.94	220.11
Low	*S. cereale*	0.72	0.06	6.47	0.79	42.43	2.02	94.91	0.95	1229.84	119.12
Mid	*S. cereale*	3.17	1.44	6.10	3.07	39.73	5.22	77.37	11.16	1521.43	312.84

V. Discussion:

Plants in this experiment grew very differently than they had in the community-level experiment (Chapter Three). Although the growth period was only seven days shorter than the previous study, biomass was ten times less. In spite of this, there are still some differences between non-polluted soils with and without earthworms in both the *Secale* and *Fagopyrum*.

Total metal concentrations in *Fagopyrum esculentum* and *Secale cereale* did vary slightly with the presence of earthworms, especially as pollution levels increased. Although overall the soils from Liberty State Park are very polluted with metal toxicants, the particular small patch from which these field soils were retrieved were not significantly more metalliferous compared to the bulk non-polluted topsoil from the home improvement center.

Analysis of variance measures demonstrate the variability that can be explained by pollution level, earthworm presence, or both. When interpreting the p-values (Table 4–1), it is expected that the pollution levels account for the majority of the variation in metalliferous content. Earthworm presence or absence can account for little to no variation in *Fagopyrum* in arsenic, but does have significant effects on all the other metals. As in

Chapter Three, the overall differences in *Fagopyrum* with and without earthworms are less significant than effects on *Secale*. While earthworms do have an effect on phytoremediative capabilities of *Fagopyrum*, they play a much bigger role in the same abilities of *Secale*.

A. Arsenic:

Concentrations of arsenic in plants are relatively similar regardless of earthworm presence, with two notable exceptions (Figure 4–4): In mid-level contamination (starting soil 17.8mg/kg As), both plants sequestered more As in the presence of earthworms ($p = \leq 0.001$). In the most polluted soils (starting value 44 As mg/kg), both plants absorbed slightly less than the mid-level pollution, which is a curious result given higher pollution levels. Mains et al. (2007) report results on hyperaccumulation of arsenic with *Secale*, but these results are not echoed in this study. Unexpectedly, Mains' result of high As accumulation is more pronounced in the least polluted soils, which had a starting concentration of 1.3 mg As/kg, while the plants accumulated 4.5 mg As/kg. *Fagopyrum* absorbed the same amount (4.5 mg As/kg) in the most polluted soils, despite containing a starting concentration over ten times more than what was absorbed.

B. Chromium:

Cr concentrations in plants with and without earthworms overall achieved varying levels of significant differences (Figure 4-5). Pots containing low and mid chromium polluted soils and *Fagopyrum* showed significantly increased uptake in the presence of earthworms as well. The ANOVA scores are significant ($p \leq 0.01$) across all treatments with earthworms in phytoextraction of chromium, and it is believed that with better plant growth, the concentration in plants would increase. This is in agreement with Arillo & Melodia (1991), in which they report not only increased phytoextraction with earthworms, but also that certain processes in earthworms (*E. foetida,* in their experiment) could reduce the toxicity of hexavalent chromium by modifying oxidation levels.

C. Copper:

Phytoextraction of copper, shown in Figure 4-6 shows minimal variation across all treatments and replicates. In light of similar concentrations of Cu in non–polluted soils and polluted soils, little variation in accumulations should be expected, and both species of plants sequestered similar amounts with and without earthworms.

D. Lead:

Pb accumulations as shown in Figure 4-7 are remarkably similar across all soil and earthworm treatments. Honda et al. (2007) report that *Fagopyrum* is an excellent hyperaccumulator of Pb, which explains at least some of why it absorbed more lead overall. Interestingly, earthworm treatments were highly significant in the mid-level polluted pots ($p \leq 0.001$) of *Secale*. This is a similar result to Chapter Three, where *Fagopyrum* sequestered more lead overall, but the earthworms still made a significant difference in concentrations in *Secale*.

E. Zinc:

Figure 4-8 shows that the quantities of Zn that have accumulated in the plants are largely indifferent between earthworm treatments. This is further supported by ANOVA scores, in which earthworms only had a slight difference ($p \leq 0.05$) in Zn uptake. Further confounding these results is that zinc concentrations were higher in the non-polluted soil. Normal ranges for zinc in soils are 10–300 (Deuel & Holliday 1994), so although the low pollution topsoil was higher in zinc, it was not, by definition, to a toxicant level. In spite of an indeterminate result with plant growth, the earthworms, which accumulated little more metal than the plants, truly hyperaccumulated Zn. Earthworm tissue was on average 1,299 mg/kg — over six times higher than the soils (which were average 212 mg/kg) in which they were living. This finding exceeds the ratios of concentration in many manuscripts, including Panda et al. (1999), Morgan and Morgan (1998), Spurgeon and Hopkin (1999), and Andre et al. (2010). It is slightly suspicious that with several dozen papers on the topic, similar results were not found that showed accumulation of 6X over bulk soil.

VI. Conclusion:

Plants in this experiment did show a small amount of phytoextraction ability, and in many cases, that ability was influenced either positively or negatively by the presence of earthworms. Plants did not grow with the vigor in the greenhouse that they did for Chapter Three (outside). Growth was reduced across all soil treatments, meaning it was unlikely the polluted soil had a toxicant that was slowing plant productivity. Light, water, or some other simple factor is the most likely result of the stunting. Compared to Chapter Three's plant growth, the plants were on average 10% as large. The greenhouse location was chosen to minimize variables like overwatering, leaching, or storm and herbivore damage; all of which may have been factors in Chapter Three. Additionally, the tall sapling pots used for this current experiment were made of black plastic, and had a much smaller surface to volume ratio. It was likely that the pots would get too hot in the summer sun outside and the earthworms could not burrow far enough from the sides to be insulated from the heat.

Despite less–than–ideal growth, and less–than–polluted soils, some findings did come from this experiment. Chromium, for example, showed significant differences in uptake in all plants when earthworms were present. This could be very helpful because although chromium spills are rare, their toxicity (depending on oxidation level) can be extremely dangerous (Arillo & Melodia 1991).

Copper concentrations represented a paradox in this study. Although copper is an essential micronutrient, it is highly toxic to earthworms even at relatively low concentrations (Neuhauser et al. 1995). Ireland (1979) reports that the copper accumulation for earthworms living in polluted soils was at least an order of magnitude lower than bulk soil concentrations.

Lead also had improved uptake in the presence of earthworms. The analysis of variance shows differences at least $p \leq 0.05$ for all soil treatment levels in their presence. This p-value is in agreement with the findings in Chapter Three, albeit on a much smaller scale of contamination. It is believed that given more biomass, *Fagopyrum* would have continued to uptake Pb, and given more complete root development in *Secale*, root hairs and mycorrhizae would have also shown a significant impact.

Finally, it is appropriate to attempt to answer the major question of this dissertation. During the last glacial event, earthworms were extirpated far south in North

America, and the species living in the Northeast now are largely non-native or invasive. Earthworms can improve plant growth, and some plants absorb large quantities of toxic metals. If the contamination levels do not kill the earthworms, it has been shown in Asia, Europe, and the Middle East that hyperaccumulating plants increase productivity in their presence. Is the same result to be expected in areas that earthworms have not adapted to over millennia? If so, what species of earthworms are found in metalliferous sites that could tolerate the harsh soils and improve growth of phytoremediative plants?

As with many questions in the natural sciences, the answer is complicated. Earthworms can increase plant productivity most if they are anecic or endogeic burrowing species. Epi–endogeic earthworms like *Amynthas spp.* can actually reduce plant productivity by removing the leaf litter and allowing erosion and desiccation of exposed soils. *Amynthas spp.* were the most common specimens collected in both brownfield and non-polluted sites, followed by *Lumbricus rubellus* and *Lumbricus terrestris*, both of which burrow in and around plant roots and increase growth.

Plants that do not have extensive root systems or rely on symbiotic mycorrhizal fungi for nutrient transfer are not as likely to be impacted by the presence of earthworms as phytoremediative plants that do. Plants that rely on arbuscular mycorrhizal fungi for some of their nutrient transfer needs also rely on them for protection from metal toxicants in the soil. Earthworms living in and around the rhizosphere consume the fungal hyphae, effectively removing the filter that protects the plant from contamination. There is also a threshold of improvement in relation to earthworm presence. If soils are relatively nutrient rich with low levels of pollution, they have less impact on plant growth than in soils that have more toxicants.

Earthworms make plants deal with metal contamination differentially. For example, arsenic uptake in plants improves significantly when earthworms are present. Cadmium uptake is relatively unaffected by earthworm presence, but chromium absorption is significantly improved. Copper, lead and zinc are all taken up into some plants more in the presence of earthworms, especially if those plants rely on mycorrhizae for protection. In the case of non–mycorrhizal dependent plants, earthworm presence actually reduces uptake of metals in this study.

The presence or absence of earthworms does have an impact on metal accumulations and growth in plants. For most effective toxicant removal from soil, this study shows that high biomass, large root systems, and mycorrhizal dependence are characteristics desired in hyperaccumulating plants. In order for earthworms to give plants an improvement in phytoextraction, they need to be anecic or endogeic species with tunnel networks through the rhizosphere that will improve plant growth, and an appetite for mycorrhizal hyphae to remove the protection the plant roots have from the pollutants.

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VIII. Appendix:

A. Metals in Plants:

The following charts are the results of testing the digested biomass in the ICP—OES. Biomass is given in g, and all metals are shown as mg/kg^{-1} (ppm). Lowercase sigma (σ) is the notation used for standard deviation, and all p–values are the results of a Student's two–tailed t-test of equal variance.

Fagopyrum esculentum (Buckwheat)		Biomass	σ Biomass	p–value Biomass	As (mg/kg)	σ As	p–value As	Cr (mg/kg)	σ Cr	p–value Cr	Cu (mg/kg)	σ Cu	p–value Cu	Pb (mg/kg)	σ Pb	p–value Pb	Zn (mg/kg)	σ Zn	p–value Zn
Low	EW	1.699	0.367	0.007	4.541	0.696	0.784	3.205	0.070	0.002	92.980	3.020	0.035	176.473	0.457	0.007	148.127	11.308	0.495
Low	No EW	2.175	0.298		4.469	0.347		3.099	0.052		90.434	1.453		175.956	0.230		152.142	13.061	
Mid	Ew	2.439	0.659	0.957	4.417	0.348	0.009	3.033	0.089	0.705	86.669	1.637	0.655	176.232	0.389	0.576	122.838	6.985	0.280
Mid	No EW	2.421	0.714		3.869	0.442		3.047	0.069		87.158	2.785		176.321	0.254		143.596	55.445	
Most	EW	1.436	0.277	0.195	4.405	0.520	0.153	3.245	0.138	0.001	93.397	2.966	0.081	178.717	0.764	0.528	352.668	47.649	0.049
Most	No EW	1.249	0.310		4.768	0.515		3.053	0.057		91.176	2.053		178.491	0.722		312.903	30.201	

Significance codes: $p \leq 0.0001$ '***' | $p \leq 0.001$ '**' | $p \leq 0.01$ '*' | $p \leq 0.05$ '•' | $p \leq 0.1$ '–' | $p \leq 1$ 'N/S'

Secale cereale (Rye)		Biomass	σ Biomass	p–value Biomass	As (mg/kg)	σ As	p–value As	Cr (mg/kg)	σ Cr	p–value Cr	Cu (mg/kg)	σ Cu	p–value Cu	Pb (mg/kg)	σ Pb	p–value Pb	Zn (mg/kg)	σ Zn	p–value Zn
Low	EW	1.210	0.303	0.017	4.519	0.691	0.946	3.355	0.098	0.021	90.207	1.261	0.470	176.422	0.266	0.346	136.979	14.813	0.965
Low	No EW	0.829	0.315		4.537	0.360		3.261	0.052		89.840	0.792		176.306	0.241		137.345	19.612	
Medium	Ew	1.496	0.365	0.188	3.626	1.035	0.000	2.323	1.140	0.002	77.108	14.790	0.002	163.797	15.782	0.001	101.752	26.624	0.307
Medium	No EW	1.185	0.575		2.035	0.270		0.955	0.085		58.889	1.207		144.091	0.254		90.068	20.045	
Most	EW	0.749	0.154	0.274	2.023	0.398	0.105	1.006	0.242	0.088	59.584	1.114	0.527	146.215	1.788	0.390	184.831	75.411	0.397
Most	No EW	0.659	0.183		2.279	0.209		1.314	0.451		60.005	1.610		147.386	3.566		211.854	55.063	

Significance codes: $p \leq 0.0001$ '***' | $p \leq 0.001$ '**' | $p \leq 0.01$ '*' | $p \leq 0.05$ '•' | $p \leq 0.1$ '–' | $p \leq 1$ 'N/S'

B. ANOVA Output for Variables in Pots:

Scores below are based on a two-way analysis of variance, assessing the relative importance or explanatory power of three different factors:

1. The impact that earthworms had on overall plant growth
2. The impact that soil quality or pollution level had on overall plant growth
3. The impact that BOTH earthworms AND soil pollution had on overall plant growth, versus just normal variability in consistent treatments (H_0).

Thus far, results have only been to compare one variable (earthworm presence) against the same soil treatment without earthworms, ten pots against ten pots. The ANOVA takes into account all 120 pots at the same time, comparing both earthworm and pollution levels.

Arsenic in *Fagopyrum esculentum*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	2.391	1.1955	4.368	0.0174 *
Earthworms	1	0.130	0.1300	0.475	0.4937
EW and Poll	2	2.217	1.1084	4.050	0.0230 *
Residuals	54	14.780	0.2737		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Arsenic in *Secale cereale*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	27.823	13.912	52.189	2.41e-13 ***
Earthworms	1	1.232	1.232	4.623	0.03603 *
EW and Poll	2	5.551	2.775	10.412	0.00015 ***
Residuals	54	14.394	0.267		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Chromium in *Fagopyrum esculentum*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	0.2098	0.10488	10.915	0.000104 ***
Earthworms	1	0.1646	0.16459	17.129	0.000123 ***
EW and Poll	2	0.0803	0.04014	4.178	0.020563 *
Residuals	54	0.5189	0.00961		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Chromium in *Secale cereale*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	23.313	11.656	69.176	1.27e-15 ***
Earthworms	1	0.877	0.877	5.204	0.0265 *
EW and Poll	2	4.063	2.031	12.055	4.69e-05 ***
Residuals	54	9.099	0.169		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Copper in *Fagopyrum esculentum*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	436.6	218.30	30.338	1.47e-09 ***
Earthworms	1	49.4	49.44	6.871	0.0114 *
EW and Poll	2	10.9	5.43	0.755	0.4751
Residuals	54	388.5	7.20		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Copper in *Secale cereale*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	5341	2670.5	113.70	< 2e-16 ***
Earthworms	1	299	298.9	12.73	0.000765 ***
EW and Poll	2	607	303.3	12.91	2.61e-05 ***
Residuals	54	1268	23.5		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Lead in *Fagopyrum esculentum*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	86.35	43.17	127.184	<2e-16 ***
Earthworms	1	1.08	1.08	3.175	0.0804 .
EW and Poll	2	0.74	0.37	1.085	0.3451
Residuals	54	18.33	0.34		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Lead in *Secale cereale*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	676.1	338.1	73.397	3.98e-16 ***
Earthworms	1	26.3	26.3	5.712	0.0204 *
EW and Poll	2	22.6	11.3	2.451	0.0957 .
Residuals	54	248.7	4.6		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Zinc in *Fagopyrum esculentum*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	543376	271688	369.400	<2e-16 ***
Earthworms	1	2526	2526	3.435	0.0693 .
EW and Poll	2	5766	2883	3.920	0.0257 *
Residuals	54	39716	735		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Zinc in *Secale cereale*

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)
Pollution Level	2	132738	66369	35.317	1.56e-10 ***
Earthworms	1	1535	1535	0.817	0.370
EW and Poll	2	2161	1080	0.575	0.566
Residuals	54	101479	1879		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Fagopyrum esculentum Biomass Growth

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Earthworms	1	11.856	5.928	23.879	3.72e-08 ***
Pollution Level	2	0.123	0.123	0.495	0.485
EW and Poll	2	1.184	0.592	2.385	0.102
Residuals	54	13.406	0.248		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Secale cereale Biomass Growth

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Earthworms	1	4.057	2.0283	15.363	5.23e-06 ***
Pollution Level	2	1.020	1.0202	7.728	0.00747 **
EW and Poll	2	0.231	0.1156	0.875	0.42258
Residuals	54	7.129	0.1320		

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1