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Plant-Soil Feedbacks In Heavy Metal Soils

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Abstract

Heavy metal pollution is a major environmental problem. Metal pollutants are long lasting and can be toxic to organisms in all parts of an ecosystem, including humans. Restoring healthy ecosystems in metal contaminated sites is critical for human and environmental health. Plant-soil feedbacks (PSFs), in which plants affect other plants indirectly by modifying the soil they grow in, have been shown to affect many processes critical to successful restoration, but we know little about their generality across ecosystems or how metal contamination will affect them. I conducted a series of experiments to investigate different mechanisms of PSF I predicted to be central to plant succession and metal movement on a mountainside contaminated and devegetated by two nearby zinc smelters. I asked whether soil amendments and seed mixes applied early in restoration affected plant community composition nine years later, how soil metals affect mycorrhizal fungal colonization of plant roots and how both of these factors affect plant metal uptake, and how encroachment into the restored grassland by the pioneer tree gray birch will affect soil chemistry, soil biota, and plant succession.

My results indicate that soil amendments applied at the time seeds are planted can have meaningful and long lasting effects on plant communities. I also found evidence that mycorrhizal fungi are depauperate in my study site and have little to do with plant metal uptake, and that effects of the incoming gray birch trees on nearby plants and soils favor continued succession to forest. I conclude that efforts to restore contaminated areas should focus on remediating soil conditions and fostering desired plant communities, and that soil microbial communities can likely be left alone with little effect on restoration goals. I call for closer integration between restoration research and practice. Restoration treatments constitute experiments performed on ecosystems, and if we study them as such with detailed baseline data, thoughtful controls, and thorough monitoring, we stand to greatly improve our understanding of ecosystem function and the efficacy of future restorations.

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PLANT-SOIL FEEDBACKS IN HEAVY METAL SOILS

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For my family with love

For our children with hope

And for the plants with gratitude

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ABSTRACT

PLANT-SOIL FEEDBACKS IN HEAVY METAL SOILS

Lee H. Dietterich

Brenda B. Casper

Heavy metal pollution is a major environmental problem. Metal pollutants are long lasting and can be toxic to organisms in all parts of an ecosystem, including humans. Restoring healthy ecosystems in metal contaminated sites is critical for human and environmental health. Plant-soil feedbacks (PSFs), in which plants affect other plants indirectly by modifying the soil they grow in, have been shown to affect many processes critical to successful restoration, but we know little about their generality across ecosystems or how metal contamination will affect them. I conducted a series of experiments to investigate different mechanisms of PSF I predicted to be central to plant succession and metal movement on a mountainside contaminated and devegetated by two nearby zinc smelters. I asked whether soil amendments and seed mixes applied early in restoration affected plant community composition nine years later, how soil metals affect mycorrhizal fungal colonization of plant roots and how both of these factors affect plant metal uptake, and how encroachment into the restored grassland by the pioneer tree gray birch will affect soil chemistry, soil biota, and plant succession.

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INTRODUCTION

Many activities essential to modern human existence are highly disruptive to natural ecosystems. We cut down forests to build towns, clear prairies to make farms, and often unintentionally release chemicals and organisms into new places where they may dramatically restructure native ecosystems (Hobbs et al. 2006). The field of ecological restoration has arisen as a way of responding to these perceived damages by taking steps to return ecosystems to their pre-disturbance states, or at least to their pre-disturbance successional trajectories (SER 2004; Clewell & Aronson 2013). In other words, workers seeking to restore a forest may find it more effective to establish a plant community they can expect to develop into a forest over time, rather than trying to plant a fully grown forest immediately.

Restoration offers numerous benefits in terms of our relationship with the biological world around us, not least of which is the mindset of managing ecosystems toward sustained, long-term goals. Such goals might include providing habitat for particular organisms (Chivers et al. 2016), stabilizing a soil against erosion (EPA 2007a; EPA 2007b), or processing water in a particular way (Prosser et al. 2015). Restoration also offers us unparalleled opportunities to learn how ecosystems work. If we control, document, and monitor them well, restoration projects can constitute powerful experiments to test theories about the processes guiding ecosystem structure and function more definitively than observational approaches allow. Restoration experiments are

especially ideal for testing mechanisms of ecological succession (e.g. Connell & Slatyer 1977). Indeed, restoration has been called “an acid test for ecology” (Bradshaw 1987).

I focus here on efforts to recover desired ecosystem functions in habitats decimated by heavy metal pollution. Pollution is one of humanity’s most widespread destructive impacts on the world around us, and heavy metals represent a major component of that (Nriagu & Pacyna 1988; Rauch & Pacyna 2009). Metals are especially pernicious pollutants because they are individual elements, and thus cannot be degraded or broken down. Thus, strategies to remediate metal contaminated landscapes typically must take the form of either removing metals from an ecosystem, or leaving them in place but somehow keeping them out of harm’s way. Historically, we have sought to remediate metal contaminated sites by either manually removing contaminated soils and chemically treating them to remove the metals (Mulligan et al. 2001), or by installing impermeable clay caps to immobilize the metals out of reach of groundwater and aboveground ecosystems (Lee & Jones Lee 1997).

More recently, though, people have begun to harness the metal uptake or exclusion capabilities of plants to remove or immobilize metals in an ecosystem (Pilon-Smits 2005). These technologies are termed phytoextraction for plants removing metals from the soil, or phytostabilization for plants sequestering metals in the soil. Phytoextraction and phytostabilization have the potential to remediate metal pollution for much less expense and labor than traditional methods, but they rely on our ability to understand, predict, and cultivate plant communities with particular metal uptake traits (Pilon-Smits 2005). Often, effective phytoremediation requires not just installing a specific group of plants in a site, but maintaining that plant community over several

generations. For instance, a phytoextraction project may need multiple generations of plants to remove enough metal contamination from a soil to render it safe again (e.g. Wilschut et al. 2013), and phytostabilization requires maintaining plant communities with low metal uptake indefinitely (Mendez & Maier 2007; Walker & del Moral 2009).

When we seek to manage any plant community over time, it is important to take into account the phenomenon of plant-soil feedback (PSF; Bever et al. 1997). PSF is defined as an interaction in which a plant affects the soil in which it grows in such a way as to affect subsequent plants in that soil, whether of the same or different species. In other words, PSF changes the relative performance of co-occurring plant species (van der Putten et al. 2013). PSFs have been shown to have substantial and widespread effects on many of the same ecosystem processes we seek to manipulate in restoration sites and beyond, including plant-plant competition (Casper & Castelli 2007), succession (Kardol et al. 2006; Kulmatiski et al. 2008; Bauer et al. 2015), species invasions (Klironomos 2002; Day et al. 2015), and nutrient cycling (Bragazza 2012). Of particular note here are PSF effects on succession, the semi-orderly turnover of species over time, as in the transition from abandoned agricultural fields to hardwood forests.

Soil metal contamination adds a new dimension to our models of PSF because metals can interact with all of the agents involved – plants, soil chemicals, and soil microbes – as well as the interactions between them (Krumins et al. 2015). These new interactions are important to understand because PSFs in uncontaminated ecosystems often affect the central processes we aim to manipulate in contaminated sites, namely plant community composition and elemental cycling (e.g. Turnau et al. 2010). However, so far little attention has been paid to PSFs in metal contaminated sites despite recent

calls for a better understanding of these processes (Krumins et al. 2015). In my dissertation, I investigate several mechanisms of PSF in metal contaminated soil. I seek to improve our understanding of how metal contamination affects plants, soils, and the intimate interactions between them, and to predict how changes in these players and their interactions will affect ecosystem structure and function in the long term. This knowledge will be deeply useful to the restoration of metal contaminated landscapes.

I focus my studies in the Palmerton Zinc Superfund Site in Palmerton, PA, USA. This site consists of over 2000 acres on the north-facing slope of Blue Mountain in the Kittatinny Ridge. An ecosystem largely representing the northern hardwood forest typical of the region was severely contaminated and devegetated by emissions from two zinc smelters which operated directly upwind of the mountain between 1898-1981. Smelting emissions included most notably the metals Zn, Pb, Cd, and Cu, which contaminated the soil, and SO_x gases, which gave rise to acid rain, further harming plants and causing massive soil erosion (Buchauer 1973; Jordan 1975; EPA 2007a). By the time the smelters shut down, the mountainside was largely barren and was frequently compared to the surface of the moon in appearance.

I conducted most of this research in the portion of the Palmerton site owned and managed by the Lehigh Gap Nature Center (LGNC), which played a leadership role in restoration efforts and now oversees burgeoning educational, recreational, and scientific activities in the site. The most significant restoration activities at LGNC occurred in 2003 and 2006. Fertilizer, lime, compost, and seeds of several prairie grasses were applied to establish a grassland community with low metal uptake, designed to minimize erosion and leaching, and build relatively uncontaminated topsoil over the contaminated

layer (EPA 2007a; EPA 2007b). The planted grasses still thrive in the site, and provide a grassland habitat, rare in PA, for birds, invertebrates, and small mammals, as well as opportunities for public hiking, cycling, recreation, and education. However, without fire or other management to maintain the area as grassland (Van Auken 2009), the site appears to have already begun a trajectory of succession to forest (e.g. Gallagher et al. 2011). Understanding PSF and other processes governing succession is crucial to the long-term success of restoration efforts. Succession determines plant community composition, which is a common goal of restoration efforts and also a key component of other restoration goals such as soil development, erosion control, hydrology, and ecosystem-level cycling of nutrients and metals.

In my first chapter, I ask how initial manipulations of plant and soil communities affected plant community composition in the long term. I take advantage of a revegetation experiment that was set up in the site in 2003. In short, different soil amendments and seed mixes were applied to plots on the contaminated mountainside in 2003, examined a year later to see which treatments gave rise to the fastest revegetation, and then more or less ignored (EPA 2007b). This provided me with an ideal opportunity in 2012 to conduct a census of the plant communities in these plots to ask whether the treatments applied in 2003 had lasting effects on the plant communities in these plots, or whether succession caused all of the plots to converge on similar plant communities regardless of the initial treatments. The answer to this question can shed light on the extent to which the details of early restoration treatments influence long-term trajectories of restoration sites, and generate hypotheses about which characteristics of plants and soils are most important in shaping these trajectories.

In my second chapter, I move to a more detailed investigation of how soil metals affect plant root colonization by arbuscular mycorrhizal fungi (AMF), and how both of these factors together affect plant metal uptake. AMF are some of the most important soil microbes to consider in the context of PSFs. AMF are obligate plant symbionts, which associate with a large majority of land plant species (Wang & Qiu 2006), and provide the plants with phosphorus and other benefits in exchange for carbon compounds (Smith & Read 2008). This mutualism is typically thought to give rise to positive PSFs, in which plants support favorable AMF communities that in turn increase plant performance (e.g. Day et al. 2015). AMF have been shown to affect plant metal uptake (Schützendübel & Polle 2002; Göhre & Paszkowski 2006; Miransari 2011), which may complicate the PSF model but also make them a valuable tool in the remediation of contaminated sites (Pilon-Smits 2005).

Existing literature on AMF and plant metal uptake is highly disjointed and consists almost entirely of greenhouse experiments using just one or two plant species, AMF species, or metals. Thus, it remains an open question whether any general principles govern plant-AMF-soil metal relationships under field conditions. I address this question with a novel approach, measuring soil metal concentrations, AMF colonization of plant roots, and plant aboveground metal concentrations for nine metals in five plant species in the field across the wide range of metal concentrations that exist in the Palmerton site. With this dataset, I can examine the relationships between soil metal concentrations, AMF colonization, and plant metal uptake with greater generality than has yet been achieved. In the process, I also produce valuable information about the

spatial variation in soil metal concentrations in the site, variation in metal uptake among plant species, and AMF abundance following metal pollution.

Finally, in my third chapter I investigate the effects of gray birch, a pioneer tree with much higher leaf metal uptake than the planted grasses, on PSF and succession in the Palmerton site. Encroachment of woody species into grasslands represents an ecological problem worldwide as it contributes to the disappearance of grasslands, one of the most endangered habitats on earth (Browning et al. 2008; Van Auken 2009). Gray birch encroachment into the Palmerton site in particular also has major management implications, as it appears to represent the beginning of succession to forest, a successional trajectory inconsistent with the current restoration goal of maintaining the site as a grassland (EPA 2007a). I examine a series of PSFs and plant-plant interactions to understand how gray birch affects nearby soils and plant communities, with emphasis on the growth and mycorrhizal colonization of later-successional trees expected to follow gray birch in succession. I seek to elucidate the mechanisms by which gray birch affects its environment and predict its consequences for subsequent succession in the site.

As part of this last chapter, I test a particular mechanism of PSF specific to contaminated soils called elemental allelopathy. Elemental allelopathy is a hypothesized phenomenon in which plants with high leaf metal uptake increase surface soil metal concentrations by the decomposition of their leaf litter, thereby making nearby soils toxic to neighboring plants. This phenomenon, first proposed by Baker and Brooks (1989) and named by Wilson and Agnew (1992), has yet to be conclusively tested (Morris et al. 2008). The restoration of the Palmerton site makes it an ideal setting for elemental allelopathy: several inches of uncontaminated compost were applied over the

contaminated soil (EPA 2007a; EPA 2007b), giving gray birch the opportunity to increase surface soil metal concentrations more strongly than would be possible in most other places. I conducted several lines of experiments to test the strength and occurrence of elemental allelopathy in this site, asking whether birch leaves are enriched in metals in the site, whether soil metals are elevated near birches, whether other plants grow differently under birches versus under the surrounding grasses, and whether decomposing birch leaves can poison several target plants. My findings will shed light on the strength and importance of elemental allelopathy in the Palmerton site, suggest possible extents and constraints on this phenomenon in other sites, and inform land managers about the degree of ecological hazard associated with it.

Through these three chapters, I will investigate the long-term effects of soil and seed amendments, the effect of AMF on plant metal uptake in the field, and the consequences of woody colonization of a grassland in which plant and soil metal concentrations play important roles in community and ecosystem dynamics. By doing so, I aim to improve our understanding of these potential mechanisms of PSF in metal contaminated soils, thereby developing insights about how best to manage and remediate polluted sites, and how ecosystems assemble, develop, and function.

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CHAPTER ONE
INITIAL SOIL AMENDMENTS STILL AFFECT PLANT COMMUNITY
COMPOSITION AFTER NINE YEARS OF SUCCESSION ON A HEAVY
METAL CONTAMINATED MOUNTAINSIDE

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1.1 Abstract

Many efforts to restore disturbed landscapes seek to meet ecological goals over time scales from decades to centuries. It is thus crucial to know how different actions available to restoration practitioners may affect ecosystems in the long term, yet few such data exist. Here, we test the effects of seed and compost applications on plant community composition nine years after their application, by taking advantage of a well controlled restoration experiment on a mountainside severely degraded by >80 years of zinc smelting emissions. We asked whether plots have converged on similar plant communities regardless of initial seed and compost treatments, or if these initial treatments have given rise to lasting differences in whole plant communities or in the richness and abundance of native, exotic, and planted species. We found that compost types significantly affected plant communities nine years later, but seed mix species composition did not. Observed differences in species richness and vegetative cover were negatively correlated, and both were related to the differences in plant communities associated with different compost types. These observed differences are due primarily to the number and abundance of species not in original seed mixes, of which notably many are native. Our results underscore the importance of soils in shaping the aboveground composition of ecosystems. Differences in soil characteristics can affect plant diversity and cover, which are both common restoration targets. Even in highly polluted and devegetated sites, compost and seed application can reinstate high vegetative cover and allow continued colonization of native species.

1.2 Implications for Practice

- Application of compost, fertilizer, lime, and C₄ grass seeds effectively restores abundant plant cover to even barren, rocky, metal contaminated soil.
- Initial soil amendments can significantly affect the composition, diversity, and cover of plant communities in the long term (≥ 9 years).
- In temperate climates, C₄ prairie grasses appear better suited for restoration of barren, metal contaminated soils than C₃ grasses.
- Abundant initial seeding of desired species may discourage colonization of unwanted species via priority effects.
- Thorough collection and dissemination of baseline data, including information about the chemical composition and water-holding properties of soil amendments, as well as investment in long-term monitoring, are essential to assessing the efficacy of restoration treatments.

1.3 Introduction

Because we rely on diverse, ecologically healthy landscapes for critical ecosystem services and to support the diversity of organisms with which we share this planet (Janetos et al. 2005), restoring ecological functions to damaged ecosystems is crucial. In severely degraded ecosystems, restoration practitioners often face the task of growing a desired ecosystem from scratch. Revegetation is thus central to many restoration efforts, but plantings alone are rarely sufficient to attain stable, healthy systems (Walker & del Moral 2009). Major changes may occur over time due to ecological succession (Cowles 1911; Clements 1916; Gleason 1926), and these changes may or may not be consistent with restoration goals (Walker & del Moral 2009). In light of this, it is important to understand how initial restoration treatments affect plant communities into the future.

Increasingly, the Anthropocene has brought novel ecosystems (Hobbs et al. 2006) that add a challenging dimension to restoration and the study of how communities may change over time. Novel ecosystems can arise from extreme environmental conditions, such as severe soil contamination by heavy metals or other pollutants, or exotic species invasions (Hobbs et al. 2006; Morse et al. 2014), which may become increasingly difficult to manage over time (e.g. Leung et al. 2002). In addition, some projects actively create novel ecosystems by planting species that would not co-occur without human intervention or are outside the historical trajectory of a given site (e.g. EPA 2007a; Zhang et al. 2012). The creation of novel ecosystems may hinder the original ecosystem's return (Morse et al. 2014). Understanding the trajectories of such ecosystems is crucial to effective long-term restoration planning and management.

The choice to perform active interventions such as applying seed or soil amendments versus leaving an ecosystem to undergo spontaneous succession may affect ecosystem development (Prach & Hobbs 2008). If active interventions are undertaken, the first species planted may enjoy a significant competitive advantage due to priority effects (Grman & Suding 2010; van de Voorde et al. 2011; Grman et al. 2013), although the strength and duration of these effects may vary (Gillhaussen et al. 2014). Differences in seed sources applied to accelerate revegetation may affect the first few years of plant community development at least (Lepš et al. 2007; Baasch et al. 2012). However, little is known about how different treatments applied early in a site's restoration may affect long-term community structure, and there is an urgent need for scientific studies of restoration techniques and outcomes (Clewell & Aronson 2013).

In this study, we utilize an unusually well controlled restoration experiment, in which multiple soil and seed treatments were applied to different parts of a single restoration site, to ask whether subtle differences in initial restoration treatments can have long-lasting effects on plant communities. In particular, we investigate whether initial differences in compost amendments and grass seed mixes affect plant community composition after nine years at a site severely contaminated by >80 years of zinc smelting. We ask whether these differing initial conditions caused lingering differences in plant community composition, and whether herbaceous and woody plants responded similarly. We are particularly interested in whether colonization of native versus exotic species differed as a function of initial treatments.

We use the term restoration here in the broad sense to describe efforts to repair damaged ecosystems. Depending on the management goals and strategies for a site, treatments like the compost and seed applications we investigate may be better described as reclamation, remediation, or rehabilitation. Distinguishing these terms is important in categorizing project goals (Clewell & Aronson 2013) and developing a strong legal definition of restoration (Palmer & Ruhl 2015). However, as this study focuses on biological processes relevant to many different ecosystem improvement projects, we keep the term restoration in the general sense for simplicity.

1.4 Methods

Study site

We conducted this study in the portion of the Palmerton Zinc Superfund Site in Palmerton, PA, USA owned and managed by the Lehigh Gap Nature Center. The site consists of over 2000 acres on the north-facing slope of Blue Mountain spanning Lehigh Gap in the Kittatinny Ridge. This mountainside was heavily contaminated, devegetated, and eroded as a result of airborne emissions (Zn, Pb, Cu, Cd, and SO_x gases) from two upwind zinc smelters operating from 1898-1981 (EPA 2007b). At the height of the pollution, soil metal concentrations within 1 km of the smelter consistently reached for Zn 80,000, Pb 6475, Cu 2000, and Cd 1750 mg kg⁻¹, and the site was largely barren of vegetation (Buchauer 1973; EPA 2007b). The United States Environmental Protection Agency (EPA) formally designated the site a Superfund site in 1983.

Because of the large size and steep, rocky terrain of the contaminated area, land managers used a phytostabilization approach in an attempt to immobilize the metals beneath a developing layer of uncontaminated surface soil. This approach differs from other methods, such as covering the contaminated soil with an impermeable cap (Lee & Jones Lee 1997) or attempting to remove the metals by excavation (EPA 2007a) or phytoextraction (Kumar et al. 1995). In particular, practitioners amended the soil with lime, fertilizer, and compost to increase pH, decrease metal bioavailability, and provide hospitable surface soil. They then sowed mixes of several grass species considered to have low leaf metal uptake and to tolerate shallow, metalliferous soils (EPA 2007a).

In spring 2003, 30 one-acre (0.4 ha) plots were established to test the efficacy of different combinations of soil amendments and seed mixes in restoring vegetative cover (EPA 2007b). These plots constituted a full factorial experiment with two replicates of every combination of three seed mixes and five compost types, assigned to plots in no particular order relative to plot locations. Seed mixes were composed of a core group of seven C₄ grasses either alone or with one of two accessory groups of three C₃ grasses (Table 1.1). Compost types included duck manure, Lehigh County compost, mushroom compost, sewage sludge, and straw mulch. Each plot received approximately 9000 kg (wet weight) of compost, 73 kg N fertilizer (urea), 59 kg P fertilizer (monoammonium phosphate), 132 kg K fertilizer (potash), and 3600 kg lime (wet weight) (Frank & West 2004, 2006). Initial moisture content of the compost types at the time of application was 25-30% for mushroom compost, 18-22% for Lehigh County compost, 16-20% for duck manure, and 0% for straw mulch and sewage sludge (Frank & West 2004). No data was

collected on the chemical composition of compost types. However, mushroom compost typically consists of some combination of straw, hay, poultry manure, cottonseed meal, ground corn cobs, gypsum, and peat moss (Landschoot & McNitt 2016), and Lehigh County compost is primarily composted yard waste. Plots were all 0.4 ha in area, but due to topographical constraints, some were approximately 64x64 m and others were approximately 32x128 m. Another group of grass seeds, similar to the core group of C₄ species applied in 2003, was applied aerially to the entire site, including the plots, in 2006 (Table 1.1).

Vegetation census

Between June and September 2012, nine years after application of seed and compost treatments, we conducted a census of the plant communities in all 30 plots. In each plot, we established either six parallel 60 m transects or three parallel 120 m transects, depending on plot dimensions (Fig. 1.1). Sampling points were established every 10 m along each transect for 36 points per plot. At every third sampling point, we recorded species identity and percent cover of all woody species in a 100 m² (5.6 m radius) circular quadrat centered on the point. At the remaining 24 sampling points, we recorded the same for all herbaceous species in a 1 m² (1x1 m) square quadrat. We determined which sampling points would be used for woody or herbaceous plants arbitrarily in advance (Fig. 1.1). We conducted census of plots in no particular order with

regard to treatments to avoid confounding seasonal and spatial variation in plant community composition.

We identified plants to species whenever possible. For genera in which we could not, we grouped all congeners together to avoid artificially inflating diversity estimates. For instance, because we could not identify non-flowering clovers (genus *Trifolium*) to species, we treated all plants in the genus as “*Trifolium* sp.” even though we positively identified flowering individuals of *T. campestre* (low hop-clover), *T. hybridum* (alsike clover), and *T. reflexum* (buffalo clover). We made an exception for pines (genus *Pinus*), because we were able to differentiate *P. strobus* (white pine) from other *Pinus* spp. Plant species names and native/exotic status follow Rhoads and Block (2007), and are detailed in Table 1.S1.

We defined the herbaceous community as all plants below one meter tall, plus all grasses regardless of height, and we defined the woody community as all woody plants taller than one meter. Our definitions divide the site’s plant community into its most relevant structural fractions better than using a strict height cutoff, which would struggle to differentiate tall (> 2 m) grasses from tree saplings, or a strict criterion based on the presence of woody tissue, which would separate low-lying shrubs (< 1 m) from the grasses and forbs with which they most directly interact. We had no *a priori* reason to expect herbaceous and woody plants to respond to treatments in the same way. Therefore, we performed all analyses on herbaceous and woody communities separately.

Statistical analysis

To ask whether current plant communities differ as a result of initial seed and compost treatments, we performed Canonical Analysis of Principal Coordinates (CAP) (Anderson & Willis 2003), a constrained ordination approach that allows significance testing, using the “vegan” package in R version 3.1.3 (Oksanen et al. 2011). Herbaceous and woody community composition matrices were averaged to the plot level, and their rows and columns were standardized by the Wisconsin method. The permutation-based `anova.cca` test was used to test for effects of compost type (“Compost”), seed mix (“Seed”), and their interaction on plant community composition.

We used the `by=“terms”` option within `anova.cca` to test the effects of Compost, Seed and their interaction in a single analysis. Because this test is sensitive to the order in which model terms (Compost and Seed) are entered, we repeated the test with model terms entered in the reverse order, to confirm that the order of model terms did not affect our qualitative results. We repeated the analysis on the herbaceous community with short-statured woody plants removed, to confirm that this aspect of our definition of herbaceous and woody plants did not affect qualitative results. Finally, we confirmed that our results were qualitatively similar if we used the `by=“margin”` option within `anova.cca`, which is insensitive to the order of model terms but cannot test the significance of a main effect also present in an interaction. In order to test the main effects of Seed and Compost this way, we created a new CAP model that excluded the Seed \times Compost interaction. The results of all of these analyses were qualitatively

similar. Therefore, we report only results from the first anova.cca analysis, in which Seed, Compost, and their interaction were tested sequentially within a single model.

To better understand variation among plots in plant community composition, we plotted each plant species' contribution to the ordination space. This allowed us to discern which species contributed the most to the observed treatment effects. We also examined correlations with the first two CAP axes, and with initial compost moisture content, to elucidate factors driving differences between plots. As potential correlates, we examined both cover and richness of: native herbaceous species, native woody species, exotic herbaceous species, exotic woody species, planted herbaceous species, all native species, all exotic species, all herbaceous species, and all woody species. Because of the multiple tests performed, we treated only correlations with $|\rho| > 0.5$ and $P < 0.005$ as significant.

We further examined the census data to explore total cover of herbaceous and woody species, and colonization by exotic species and native species not planted in the site. We used analysis of variance (ANOVA) and generalized linear models (GLM) as appropriate to test whether seed and compost treatments affected: plot-level cover of all herbaceous and all woody plants; species richness of herbaceous and woody plants separately and together; and cover and richness of native colonizing species, exotic colonizing species, and planted species.

1.5 Results

We identified 101 plant species, including 10 of the 14 grasses originally planted in 2003, and 91 other species that either colonized the site independently or persisted there throughout the zinc smelting. After grouping together congeners of individuals we could not identify to species, we were left with 90 taxa on which we performed analyses. We will refer to these taxa as species for simplicity, even though some are genera (e.g. *Trifolium* sp.), and one represents the few herbaceous specimens that could only be identified to family. A complete species list is in Table 1.S1.

We recorded 58 of these 90 species in the herbaceous layer only, 17 in the woody layer only, and 15 in both layers. These 15 are woody species with some individuals, usually juveniles, shorter than 1 m, and other individuals taller than 1 m. We recorded 19-38 species per one-acre plot (28.2 ± 0.93 average \pm SE). On average, plots contained 23.3 species in the herbaceous layer, accounting for 83.3% cover, and 7.6 species in the woody layer, accounting for 30.3% cover. Herbaceous and woody species totals do not sum to the average species per plot because of the few species found in both vegetation layers. Similarly, the average percent cover in a plot need not equal 100%.

The 2003 compost treatments significantly affected 2012 plant community composition in both herbaceous ($P < 0.01$, Fig. 1.2a) and woody ($P < 0.05$, Fig. 1.4a) vegetation layers according to CAP analyses. Effects of Seed mixture and the Seed \times Compost interaction were not significant for either the herbaceous or the woody layer ($P > 0.05$). The first two CAP axes accounted for 44.4% of total constrained variation in the

herbaceous community ordination (CAP1, 27.7%; CAP2, 16.7%), and 45.3% of total constrained variation in the woody community ordination (CAP1, 31.4%; CAP2, 14.0%).

Herbaceous community structure

For the herbaceous plant communities, the compost types supporting the highest average vegetative cover showed lower species richness and greater representation of a few species. This statement is supported by the first CAP axis being negatively correlated with native herbaceous cover ($\rho = -.66$, $P < 0.001$) (Fig. 1.2a) and positively correlated with total species richness, total herbaceous species richness, and most specifically native herbaceous species richness ($\rho = .66$, $P < 0.001$). Sewage sludge had the highest herbaceous cover and lowest total richness; Lehigh and mushroom composts had lower cover but the highest richness. Variation among compost treatments in cover and species representation is further illustrated in Fig. 1.3a. Four herbaceous species were overrepresented in plots treated with sewage sludge (*Minuartia patula* [sandwort], *Agrostis perennans* [autumn bent], *Tripsacum dactyloides* [eastern gammagrass], and *Panicum dichotomiflorum* [smooth panic grass]; Fig. 1.2b, 1.3a). Of these, only the C₄ grass *T. dactyloides* was among the species planted in 2003, and at least *M. patula* and *A. perennans* grew at the site even while the smelters were still operating (Pretz 1954; Jordan 1975). These four species occurred at lower abundance in plots amended with the other compost types, which contained more species overall. We found no significant correlations with the second axis of the herbaceous CAP analysis for any of the cover and

richness values we examined, whether for herbaceous or woody species, native, planted, or exotic species, or their combinations. ANOVAs and GLMs examining the effect of Seed, Compost, and their interaction on cover and richness of herbaceous species found significant Compost effects on native and total herbaceous cover ($P < 0.05$, Fig. 1.5a) but no other significant effect. Relatedly, initial compost moisture content was significantly correlated with herbaceous cover ($\rho = -.55, P < 0.005$), and marginally correlated with herbaceous species richness ($\rho = .39, P < 0.05$).

Woody community structure

In the woody community CAP ordination, the first axis is negatively correlated with native herbaceous cover ($\rho = -.51, P < 0.005$) and positively correlated with total species richness ($\rho = .49, P = 0.006$), although we do not consider the latter significant. The second axis of the woody community ordination is correlated with woody species richness ($\rho = .64, P < 0.001$), specifically native woody species richness ($\rho = .63, P < 0.001$). Individual species' contributions to the woody ordination space are not clearly related to compost treatments or even individual CAP axes (Fig. 1.4b). The species that most distinguish plots in ordination space include mature *Quercus montana* (chestnut oak), *Sassafras albidum* (sassafras), and *Nyssa sylvatica* (black gum), from remnant forest patches that predate restoration efforts, as well as newly colonizing *Pinus strobus* (white pine), *Betula populifolia* (gray birch), *Populus tremuloides* (quaking aspen), and *Buddleja davidii* (butterfly bush). These colonizers are native, early successional forest

species except for *B. davidii*, which is an exotic invasive shrub. The importance of *Comptonia peregrina* (sweet fern) in Fig. 1.4b appears exaggerated because it occurred in only one plot. There are no strong patterns in how the most common woody species are distributed across compost types, although *B. populifolia* is most abundant in mushroom compost plots and least abundant in sewage sludge plots. ANOVAs and GLMs showed no effect of Seed, Compost, or their interaction on woody plant cover or richness.

Status of planted species

The seed mixes applied in 2003 included both C₃ and C₄ grasses, but the C₄ grasses dominated in our 2012 census. We recorded each of the seven planted C₄ grasses in 28-30 of the 30 plots, and together they amounted to an average of 43.7% cover per plot, or 52.5% of the total herbaceous cover. The C₃ grasses constituting the differences between seed mixes varied greatly in occurrence. One of these, *Elymus canadensis* (Canada wild rye), was included in the 2006 mountain-wide planting and was recorded in 29 of the 30 plots. Two species, *Festuca rubra* (red fescue) and *F. trachyphylla* (hard fescue), were present but were treated as *Festuca* sp. because of the difficulty of distinguishing nonflowering specimens. The remaining three, *Deschampsia flexuosa* (common hairgrass), *Avena sativa* (oats), and *Lolium perenne* (perennial ryegrass), were not recorded at all, although *D. flexuosa* is abundant at higher elevations on the mountain (Glassman & Casper 2012). On average, *E. canadensis* and *Festuca* sp. together accounted for only 5.8% of herbaceous plant cover, but *Festuca* sp. represented on

average 10.3% of the herbaceous cover in the plots in which it was planted, occurring in eight of those ten plots. Interestingly, *Festuca* sp. was found in only three of the 20 plots in which it was not planted, and each of these is directly across a trail from a plot in which it was planted.

Native and exotic species

Exotic species, or those not native to Pennsylvania according to Rhoads and Block (2007), made up 20 of the 101 species we identified and accounted for only 2.2 of the 28.2 average species per plot. In the herbaceous layer, exotic colonists represented only 3.5% of observed plant cover, compared to 37.0% cover for native colonists and 58.9% cover for planted grasses. In the woody layer, exotics represented 16.1% of total observed plant cover while natives represented 83.7%. No woody species were planted as part of the restoration. Plants of unknown origin constituted <1% of both the herbaceous and woody vegetation layers. Among the exotic species, *B. davidii* was by far the most abundant, accounting for 60.5% of total exotic cover in the herbaceous layer, and 98.3% in the woody layer. The next most abundant exotic herbaceous plants were *Microstegium vimineum* (Japanese stiltgrass) and *Coronilla varia* (crown vetch), which accounted for 15.8% and 12.9% of exotic herbaceous cover, respectively.

Despite clear separation between some plant groups in ordination space, ANOVAs and GLMs revealed almost no significant differences attributable to Seed and Compost treatments for either cover or species richness, when we examined native,

exotic, or planted species separately. This was true whether we analyzed herbaceous and woody vegetation layers separately or together (Fig. 1.5). The one exception is that Compost affected native and total herbaceous cover ($P < 0.05$, Fig. 1.5a).

1.6 Discussion

The types of compost applied to near-barren plots at the Palmerton Zinc Superfund Site gave rise to differences in plant communities nine years later, but initial seed treatments did not. Thus, we demonstrate long-lasting differences in plant communities due to initial soil amendments despite the same applications of fertilizers. While the use of seeds in restoration has received abundant attention due to its major and intuitive role in reestablishing plant communities (e.g. Mijnsbrugge et al. 2010; Merritt & Dixon 2011), soil amendments such as the compost treatments investigated here have been far less well studied. Other researchers working on similar time scales have documented that different seed sources can generate differences in plant communities (Baasch et al. 2012; Auestad et al. 2015), and that soil amendments like those used here can jump-start improvements in soil quality (Tandy et al. 2009; Oldfield et al. 2014; Emam 2015) and affect plant community composition (Paschke et al. 2005). However, few, if any, have connected multiple soil amendment types to plant communities on this time scale.

The particular differences in communities attributable to soil amendments—changes in species richness and cover—relate well to the common restoration goals of

species diversity and productivity (Clewell & Aronson 2013), known to have central roles in ecosystem function (Isbell et al. 2011; Pasari et al. 2013). The negative relationship we found between herbaceous cover and diversity suggests there may be a trade-off between maximizing the diversity and productivity of revegetated landscapes. Such a trade-off is consistent with aspects of both diversity-productivity theory (van Ruijven & Berendse 2005) and productivity-diversity theory (Fraser et al. 2015), though we grant that the relationship between biodiversity and productivity is complex, and that cover is an imperfect proxy for productivity.

Effects of Lehigh Co. and mushroom compost on plant community structure appear more consistent with restoration goals at the Palmerton site than effects of sewage sludge. Although increased vegetative cover was one of the most immediately measurable goals of the revegetation effort, the greater herbaceous plant cover associated with sewage sludge is attributable primarily to small statured *M. patula* and *A. perennans*, which were present even before the smelters shut down (Pretz 1954; Jordan 1975). They are among the few species that occur, often in dense monocultures, on what are thought to be the most contaminated soils on the mountainside. The low biomass and high shoot metal concentrations of *M. patula* (Ch. 2), in particular, make it a poor match for the overarching goal of building an organic rich surface soil with reduced metal concentrations.

The relationships between woody communities and the experimental treatments are less clear than those for herbaceous communities. One caveat is that several species driving the observed differences are mature trees that were clearly present before

revegetation began. However, the other species driving the observed compost effect include those presenting the greatest management concerns in the site, the native pioneer tree *B. populifolia* and the exotic shrub *B. davidii*. Land managers consider both species undesirable and fear they will outcompete the planted grasses. With high leaf metal concentrations, *B. populifolia* has the potential to re-mobilize metals to surface soils, counter to restoration goals (EPA 2007a), and *B. davidii* is known to be invasive. Both of these species colonized the site independently.

We speculate that differences in plant community composition may relate to the biological origin of the soil amendments. Lehigh Co. compost, mushroom compost, and straw mulch, which gave rise to the most diverse plant communities, were derived mainly from plant material, while duck manure and sewage sludge had more immediate animal origins. Organic matter in most well developed soils contains much more decomposed plant material (e.g. leaf litter) than animal material, simply because plants tend to far outweigh animals in terrestrial ecosystems (Elton 1927). Plant-derived composts may thus be closer than animal-derived materials in their nutrient ratios or other abiotic characteristics. This is further supported by pre-application measures of compost moisture content correlating significantly with our measured herbaceous species richness and herbaceous cover. This could indicate that compost water-holding capacity may vary in a meaningful way among treatments, although pre-application moisture content may differ from an amendment's water-holding capacity. Differences in the nutrient, metal, or microbial contents of soil amendments could also clearly affect plant recruitment and succession. Indeed, sewage sludge has drawn concern as a soil amendment because it

commonly contains high concentrations of toxins including heavy metals (Alloway & Jackson 1991).

While the soil amendments used here might have contained viable seeds themselves, we do not think any such additions contributed substantially to the differences in community composition we measured. We found no colonizing species in any of our plots that did not occur elsewhere on the mountain, although some other areas received soil amendments subsequent to the initial applications in these test plots. Moreover, many of the species accounting for differences in cover between plot types were known to grow in the site prior to the application of these seed and compost treatments (Pretz 1954; Jordan 1975).

It is also possible that the different shapes of experimental plots (64x64 m versus 32x128 m) might have affected plant community composition, but we do not believe this to be the case. Plot shape was confounded with compost application such that the oblong plots received mushroom or Lehigh Co. compost and the square plots received straw mulch, duck manure, or sewage sludge. Thus, we cannot fully disentangle the impact of Lehigh Co. and mushroom composts on plant communities from the topographical conditions that led land managers to make these plots oblong rather than square. However, the clear separation in ordination space between herbaceous communities associated with sewage sludge and those associated with any other compost type highlights the ability of compost amendments to induce differences in plant communities even among plots of the same shape.

We also do not expect any direct effects of plot shape on plant community composition in this study. A worry might be that the oblong plots, having greater perimeters, have a greater amount of edge exposed to colonization or different abiotic characteristics. However, such edge effects are not likely relevant here because propagules of novel species would likely be coming from off site, and all experimental plots are located at least 50 m from the nearest edge of the study site and thus the nearest alternative habitat. Furthermore, we accounted for the possibility that plot edges were somehow different from plot interiors by placing all of our sampling quadrats at least 7 m from the nearest edge of the plot. Thus, while we call on restoration practitioners to carefully consider the statistical design of even pilot experiments like this one to ensure that their data are as broadly informative as possible, we maintain that our observed differences in plant community structure are primarily attributable to differences in the applied compost amendments and not plot shape.

We were surprised to find no significant effect of initial seed treatment on any aspect of plant community composition. In this study, the C₄ grasses planted in all plots were abundant, but most of the C₃ grasses that constituted the differences among seed mixes were absent. Several of these C₃ grasses were reportedly present in the first two years after planting, so the taller C₄ species probably outcompeted them. Two of the initial C₃ grasses remain: *E. canadensis*, which is similar in height to the C₄ species and was included in the 2006 site-wide planting, and *Festuca* sp., which demonstrated a small amount of dispersal among plots. In general, the C₄ species appear to be stronger

competitors in this habitat and thus better suited to revegetating similarly contaminated sites.

Neither the compost nor the seed treatment explained colonization by native versus exotic plants when these groups were examined individually, and in general, unwanted exotic species are less common in Palmerton than in other restoration projects (D'Antonio & Meyerson 2002). For example, Simmers and Galatowitsch (2010) found exotic species to comprise 43.6% of plant cover on revegetated oil field access roads compared with 8.8% in nearby reference prairie, and Yurkonis (2013) reported that exotic species comprised 80% of the seed bank in some reconstructed grasslands. At Palmerton, in contrast, exotics make up only 3.5% of observed herbaceous cover and 16.1% of observed woody cover. Most of that cover is comprised of *B. davidii*, which might be much more common without intensive eradication efforts since 2004.

Previous studies have shown that the first group of species to arrive in a site often enjoys a substantial advantage, called a priority effect, likely by establishing and beginning to grow with little competition from other plants (Grman & Suding 2010; van de Voorde et al. 2011; Grman et al. 2013; Gillhaussen et al. 2014). In light of our observations, we hypothesize that a combination of soil contamination, a priority effect favoring the planted C₄ grasses (see Prach et al. 2012), low exotic propagule pressure due to the site's rural location, and concerted efforts to remove *B. davidii* all reduce the abundance of exotic invasives at the site.

We conclude that differences in the composition of soil amendments applied to degraded landscapes can have long-term effects on plant community composition and succession in those landscapes. These effects are highly relevant to restoration and management efforts because most such projects seek to manipulate plant community composition over time in one way or another. Thus, it is important to consider initial restoration treatments, including soil amendments, carefully in light of a project's particular objectives. We also highlight the importance of long-term monitoring in restoration sites to ensure that restoration goals continue to be met over decadal to centurial time scales, and to understand how decisions made early in the process may have lasting consequences.

Finally, we note that thorough collection and dissemination of baseline data provide crucial information for monitoring and assessing the progress of restoration projects. We appreciate the experimental design records and marked plot locations maintained by the site's land managers, which made this study possible. Moving forward, we urge any practitioners performing soil amendments to measure the effects on soil chemistry and water-holding properties in order to help generate a more mechanistic understanding of how soil amendments affect plant communities over short and long time scales.

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Table 1.1: Species planted in different seed treatments in 2003, and in a site-wide planting in 2006. Seed mixes applied in 2003 consisted of the C₄ grasses alone (Seed Mix 1) or with one of two accessory groups of C₃ grasses (Seed Mixes 2, 3). Each plot received one of these three seed mixes and one of five compost types in a full factorial design with two replicates. Species' common names are provided in Table 1.S1.

Notes: Both Seed Mix 2 and the 2006 planting included *E. canadensis*. No establishment was observed for *A. hallii* or *A. virginicus*, and *T. flavus* is present but rare.

		Seed treatments			
		2003 Seed Mix 1	2003 Seed Mix 2	2003 Seed Mix 3	2006 Site-wide Planting
C ₄ grasses	<i>Andropogon gerardii</i>	<i>Andropogon gerardii</i>	<i>Andropogon gerardii</i>	<i>Andropogon gerardii</i>	<i>Andropogon gerardii</i>
	<i>Andropogon hallii</i>	<i>Andropogon hallii</i>	<i>Andropogon hallii</i>	<i>Andropogon hallii</i>	<i>Andropogon virginicus</i>
	<i>Eragrostis trichodes</i>	<i>Eragrostis trichodes</i>	<i>Eragrostis trichodes</i>	<i>Eragrostis trichodes</i>	<i>Eragrostis trichodes</i>
	<i>Panicum amarum</i>	<i>Panicum amarum</i>	<i>Panicum amarum</i>	<i>Panicum amarum</i>	<i>Panicum amarum</i>
	<i>Panicum virgatum</i>	<i>Panicum virgatum</i>	<i>Panicum virgatum</i>	<i>Panicum virgatum</i>	<i>Panicum virgatum</i>
	<i>Schizachyrium scoparium</i>	<i>Schizachyrium scoparium</i>	<i>Schizachyrium scoparium</i>	<i>Schizachyrium scoparium</i>	<i>Schizachyrium scoparium</i>
	<i>Sorghastrum nutans</i>	<i>Sorghastrum nutans</i>	<i>Sorghastrum nutans</i>	<i>Sorghastrum nutans</i>	<i>Sorghastrum nutans</i>
	<i>Tripsacum dactyloides</i>	<i>Tripsacum dactyloides</i>	<i>Tripsacum dactyloides</i>	<i>Tripsacum dactyloides</i>	<i>Tridens flavus</i> <i>Tripsacum dactyloides</i>
C ₃ grasses		<i>Avena sativa</i>	<i>Deschampsia flexuosa</i>	<i>Dichanthelium clandestinum</i>	
		<i>Elymus canadensis</i>	<i>Festuca ovina</i>	<i>Elymus canadensis</i>	
		<i>Lolium perenne</i>	<i>Festuca trachyphylla</i>		

Figure 1.1: Sampling scheme for assessing plant communities in (a) 64x64 m and (b) 32x128 m plots. Dashed lines represent transects, squares represent 1 m² herbaceous quadrats, and circles represent 100 m² woody quadrats. The x- and y-axes run roughly east-west and north-south, respectively.

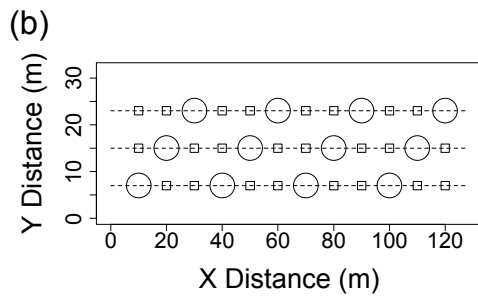
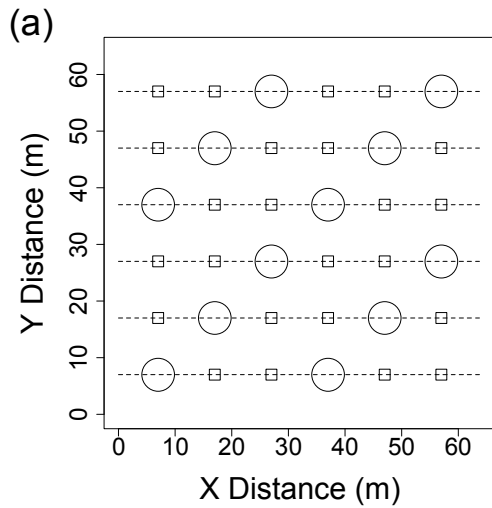


Figure 1.2: (a) Compost treatments applied to plots in 2003 significantly affect herbaceous community composition (CAP, $P < 0.01$). Each red point represents one plot's herbaceous community. Labeled black ellipses represent SE intervals around the average herbaceous community associated with each compost type. Blue contour lines show plot-level total (herbaceous and woody) species richness. Compost types are abbreviated as follows: Duck, duck manure; Lehigh, Lehigh County compost; Mushroom, mushroom compost; Sludge, sewage sludge; Straw, straw mulch. (b) Species' contributions to the herbaceous CAP ordination space. Species on the left, represented by labeled filled circles, are relatively overrepresented in plots amended with sewage sludge. Species near the origin, represented by unlabeled open circles, are distributed relatively uniformly among plot types. Labeled species are abbreviated as follows: MINPAT, *Minuartia patula*; AGRPER, *Agrostis perennans*; TRIDAC, *Tripsacum dactyloides*; PANDIC, *Panicum dichotomiflorum*.

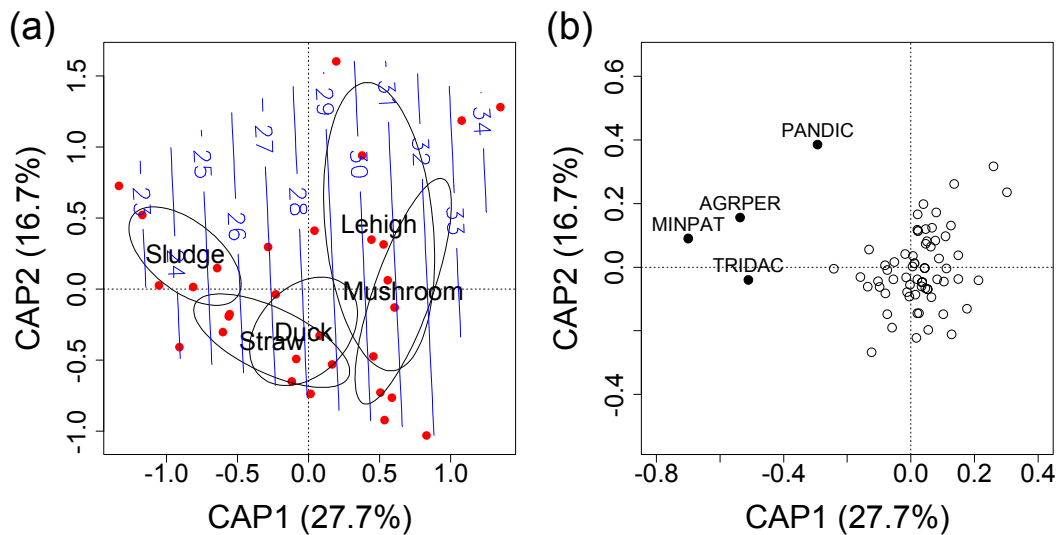


Figure 1.3: Average cover of the six most abundant (a) herbaceous and (b) woody species, and all other herbaceous or woody species observed, by compost treatment. Compost types are arranged from left to right in order of increasing average species richness. Species abbreviations are in Table 1.S1, or as follows: ANDGER, *Andropogon gerardii*; PANVIR, *Panicum virgatum*; MINPAT, *Minuartia patula*; TRIDAC, *Tripsacum dactyloides*; ERATRI, *Eragrostis trichodes*; AGRPER, *Agrostis perennans*; SMIGLA, *Smilax glauca*; BETLEN, *Betula lenta*; NYSSYL, *Nyssa sylvatica*; BUDDAV, *Buddleja davidii*; SASALB, *Sassafras albidum*; BETPOP, *Betula populifolia*.

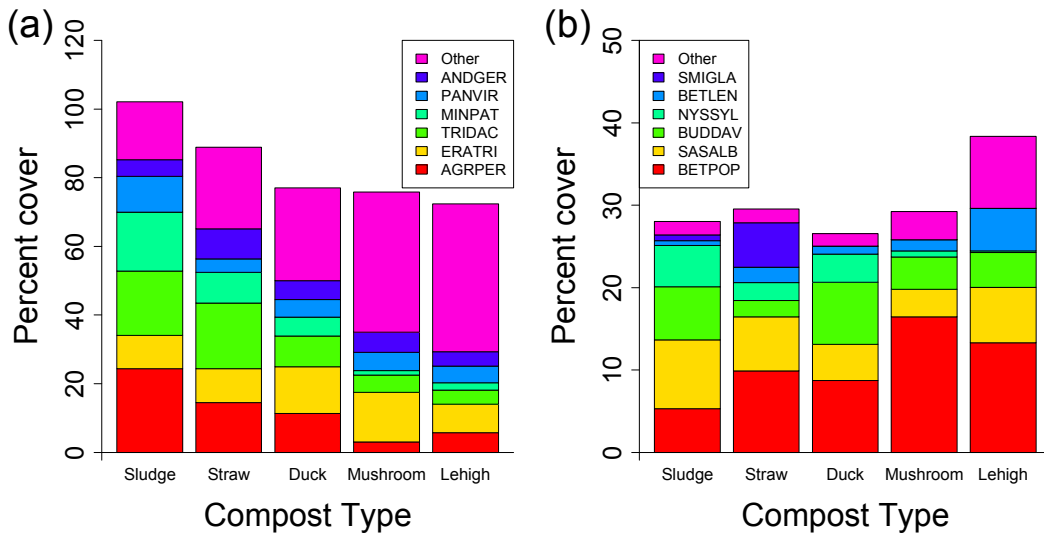


Figure 1.4: (a) Compost treatments applied to plots in 2003 significantly affect woody community composition (CAP, $P < 0.05$). Each red point represents one plot's woody community. Labeled black ellipses represent SE intervals around the average woody community associated with each compost type. Blue contour lines show plot-level total (herbaceous and woody) species richness. Compost types are abbreviated as in Fig. 1.2. (b) Species' contributions to the woody CAP ordination space. Species near the origin, represented by unlabeled open circles, are distributed relatively uniformly among plot types. Species further from the origin, represented by labeled filled circles, are distributed relatively unevenly among plot types. Labeled species are abbreviated as follows: QUEMON, *Quercus montana*; SASALB, *Sassafras albidum*; NYSSYL, *Nyssa sylvatica*; BUDDAV, *Buddleja davidii*; BETPOP, *Betula populifolia*; PINSTR, *Pinus strobus*; COMPER, *Comptonia peregrina*; POPTRE, *Populus tremuloides*.

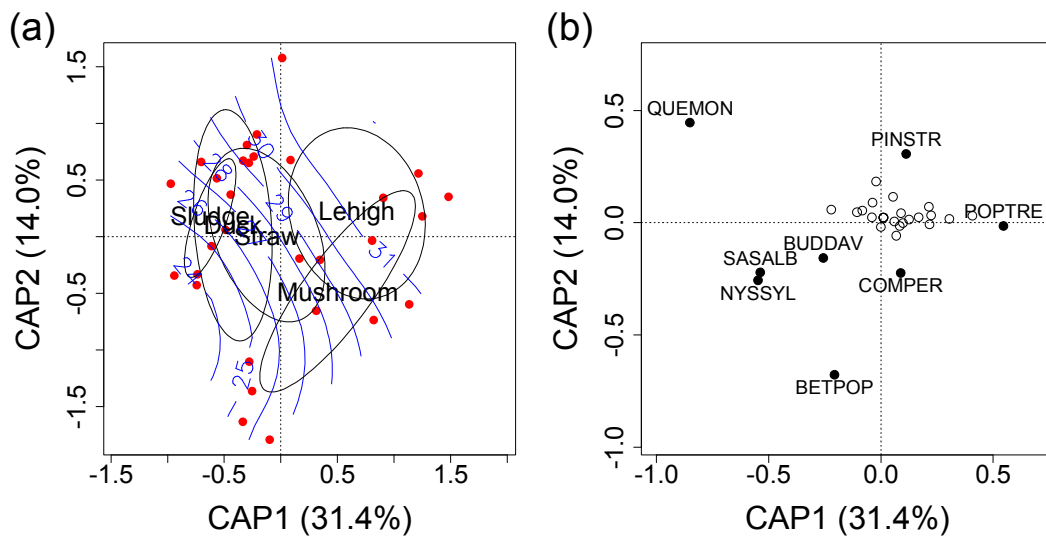
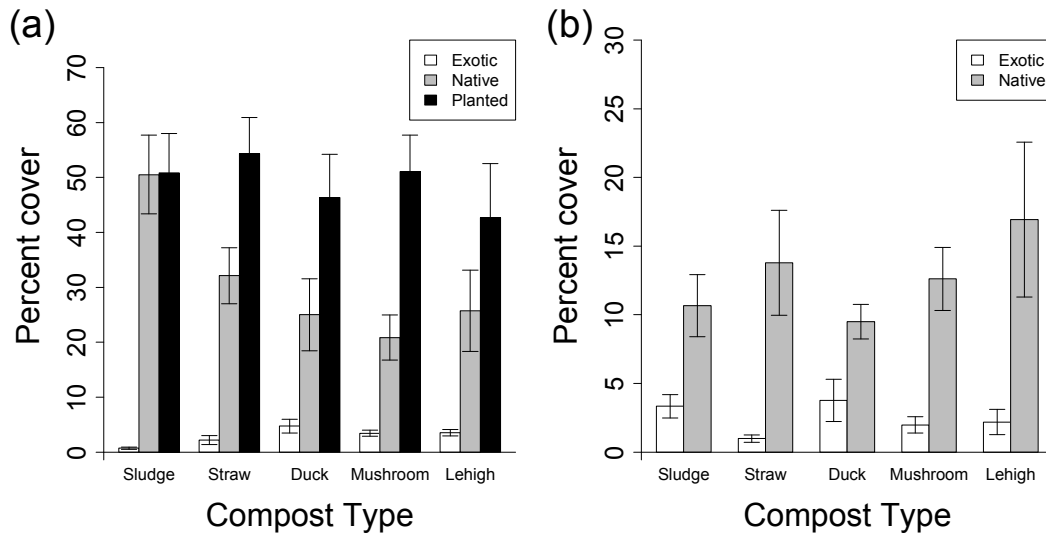


Figure 1.5: Total cover (average \pm SE) of native, exotic, and planted herbaceous (a) and woody (b) species in plots of each compost type. Compost types are arranged from left to right in order of increasing average species richness. No woody species were planted in the course of this experiment.



Supporting Information

Table 1.S1. Complete species list we recorded at Palmerton in summer 2012.

“Code.recorded” is a unique six-letter code for each species as it was identified in the field or in the lab. “Code.analyzed” gives the species codes used in our data analysis, adjusted as described in Materials and Methods. A “Code.analyzed” value of “NA” denotes species we observed in plots but not in any of our quadrats; we did not include these species in our analysis but document their occurrence in the site here. Columns “Genus,” “Species,” and “Family” and “Growth.form” are self-explanatory and follow Rhoads and Block (2007). In “Growth.form,” values “VineH,” “VineW,” and “UnknownH” denote herbaceous vines, woody vines, and unknown herbaceous species, respectively. Column “Native.Exotic.Planted” denotes whether each species is native or exotic to Pennsylvania following Rhoads and Block (2007), or whether it was planted in the site (Table 1). Taxa are marked “Unknown” if native/exotic status could not be determined; this was the case for specimens that we could only identify to a genus or family that contains both native and exotic species. Column “Common.Name” reports the common name of each species following Rhoads and Block (2007).

Code.recorded	Code.analyzed	Genus	Species	Family	Growth.form	Native.Exotic.Planted	Common.Name
ACEPL A	ACEPLA	<i>Acer</i>	<i>platanoides</i>	Sapindaceae	Tree	Exotic	Norway maple
ACERU B	ACERUB	<i>Acer</i>	<i>rubrum</i>	Sapindaceae	Tree	Native	Red maple
ACHMI L	ACHMIL	<i>Achillea</i>	<i>millefolium</i>	Asteraceae	Forb	Exotic	Common yarrow
AGEAL T	AGEALT	<i>Ageratina</i>	<i>altissima</i>	Asteraceae	Forb	Native	White snakeroot

AGRPER	AGRPER	<i>Agrostis</i>	<i>perennans</i>	Poaceae	Grass	Native	Autumn bent
AILALT	AILALT	<i>Ailanthus</i>	<i>altissima</i>	Simaroubaceae	Tree	Exotic	Tree-of-heaven
AMBAR T	AMBAR T	<i>Ambrosia</i>	<i>artemisiifolia</i>	Asteraceae	Forb	Native	Ragweed
ANAMAR	ANAMAR	<i>Anaphalis</i>	<i>margaritacea</i>	Asteraceae	Forb	Native	Pearly everlasting
ANDGER	ANDGER	<i>Andropogon</i>	<i>gerardii</i>	Poaceae	Grass	Planted	Big bluestem
ARATHA	ARATHA	<i>Arabidopsis</i>	<i>thaliana</i>	Brassicaceae	Forb	Exotic	Mouse-ear cress
ARCMIN	ARCMIN	<i>Arctium</i>	<i>minus</i>	Asteraceae	Grass	Exotic	Common burdock
ARTANN	ARTANN	<i>Artemisia</i>	<i>annua</i>	Asteraceae	Forb	Exotic	Sweet wormwood
ASCCAN	ASCCAN	<i>Asclepias</i>	<i>cannabinum</i>	Apocynaceae	Forb	Native	Milkweed
ASCSYR	NA	<i>Asclepias</i>	<i>syriaca</i>	Apocynaceae	Forb	Native	Dogbane
BERTHU	BERTHU	<i>Berberis</i>	<i>thunbergii</i>	Berberidaceae	Shrub	Exotic	Japanese barberry
BETLEN	BETLEN	<i>Betula</i>	<i>lenta</i>	Betulaceae	Tree	Native	Black birch
BETPOP	BETPOP	<i>Betula</i>	<i>populifolia</i>	Betulaceae	Tree	Native	Gray birch
BUDDAV	BUDDAV	<i>Buddleja</i>	<i>dauidii</i>	Scrophulariaceae	Shrub	Exotic	Butterfly bush
CARGLA	CARGLA	<i>Carya</i>	<i>glabra</i>	Juglandaceae	Tree	Native	Pignut hickory
CARHIR	CARHIR	<i>Carya</i>	<i>hirsuta</i>	Juglandaceae	Tree	Native	Mockernut hickory
CASMOL	CASMOL	<i>Castanea</i>	<i>mollissima</i>	Fagaceae	Tree	Exotic	Chinese chestnut
CATBIG	CATBIG	<i>Catalpa</i>	<i>bignonioides</i>	Bignoniaceae	Tree	Native	Catalpa
CELSPP	CELSPP	<i>Celastrus</i>	<i>sp</i>	Celastraceae	Vine	Unknown	Bittersweet
CENSTO	CENSTO	<i>Centaurea</i>	<i>stoebe</i>	Asteraceae	Forb	Exotic	Spotted knapweed
CHEALB	CHEALB	<i>Chenopodium</i>	<i>album</i>	Amaranthaceae	Forb	Native	Lamb's-quarters
CLEVIR	CLEVIR	<i>Clematis</i>	<i>virginiana</i>	Ranunculaceae	VineH	Native	Virgin's-bower

COMPER	COMPER	<i>Comptonia</i>	<i>peregrina</i>	Myricaceae	Shrub	Native	Sweet-fern
CONCAN	CONCAN	<i>Conyza</i>	<i>canadensis</i>	Asteraceae	Forb	Native	Horseweed
CORVAR	CORVAR	<i>Coronilla</i>	<i>varia</i>	Fabaceae	Forb	Exotic	Crown-vetch
DAUCAR	DAUCAR	<i>Daucus</i>	<i>carota</i>	Apiaceae	Forb	Exotic	Queen Anne's lace
DENPUN	DENPUN	<i>Dennstaedtia</i>	<i>punctilobula</i>	Polypodiaceae	Fern	Native	Hay-scented fern
DESSPP	DESSPP	<i>Desmodium</i>	<i>sp</i>	Fabaceae	Forb	Native	Tick-trefoil
DICCLA	DICCLA	<i>Dichanthelium</i>	<i>clandestinum</i>	Poaceae	Grass	Planted	Deer-tongue grass
DICEXI	DICEXI	<i>Dicentra</i>	<i>eximia</i>	Papaveraceae	Forb	Native	Wild bleeding-heart
DIGCIL	DIGCIL	<i>Digitaria</i>	<i>ciliaris</i>	Poaceae	Grass	Native	Southern crabgrass
DRYMAR	DRYMAR	<i>Dryopteris</i>	<i>marginalis</i>	Polypodiaceae	Fern	Native	Marginal wood fern
ELYCAN	ELYCAN	<i>Elymus</i>	<i>canadensis</i>	Poaceae	Grass	Planted	Canada wild-rye
EPICOL	EPISPP	<i>Epilobium</i>	<i>coloratum</i>	Onagraceae	Forb	Native	Purple-leaved willow-herb
EPISPP	EPISPP	<i>Epilobium</i>	<i>sp</i>	Onagraceae	Forb	Unknown	Willow-herb
ERATRI	ERATRI	<i>Eragrostis</i>	<i>trichodes</i>	Poaceae	Grass	Planted	Sand love-grass
EREHIE	EREHIE	<i>Erechtites</i>	<i>hieracifolia</i>	Asteraceae	Forb	Native	Fireweed
EUPPER	EUPPER	<i>Eupatorium</i>	<i>perfoliatum</i>	Asteraceae	Forb	Native	Boneset
EUPSER	EUPSER	<i>Eupatorium</i>	<i>serotinum</i>	Asteraceae	Forb	Native	Late thoroughwort
EUTGRA	EUTGRA	<i>Euthamia</i>	<i>graminifolia</i>	Asteraceae	Forb	Native	Grass-leaved goldenrod
FESRUB	FESSPP	<i>Festuca</i>	<i>rubra</i>	Poaceae	Grass	Planted	Red fescue
FESSPP	FESSPP	<i>Festuca</i>	<i>sp</i>	Poaceae	Grass	Planted	Fescue
FESTRA	FESSPP	<i>Festuca</i>	<i>trachyphylla</i>	Poaceae	Grass	Planted	Hard fescue
GLETRI	GLETRI	<i>Gleditsia</i>	<i>triacanthos</i>	Fabaceae	Tree	Native	Honey-locust
HAMVIR	HAMVIR	<i>Hamamelis</i>	<i>virginiana</i>	Hamamelidaceae	Shrub	Native	Witch-hazel
IMPCAP	IMPCAP	<i>Impatiens</i>	<i>capensis</i>	Balsaminaceae	Forb	Native	Jewelweed

LACCAN	LACCAN	<i>Lactuca</i>	<i>canadensis</i>	Asteraceae	Forb	Native	Wild lettuce
LACSER	LACSER	<i>Lactuca</i>	<i>serriola</i>	Asteraceae	Forb	Native	Prickly lettuce
LIRTUL	LIRTUL	<i>Liriodendron</i>	<i>tulipifera</i>	Magnoliaceae	Tree	Native	Tulip tree
LONSPP	LONSPP	<i>Lonicera</i>	<i>japonica</i>	Caprifoliaceae	VineH	Exotic	Japanese honeysuckle
LONSPP	LONSPP	<i>Lonicera</i>	<i>sp</i>	Caprifoliaceae	VineH	Unknown	Honeysuckle
LYSQUA	LYSQUA	<i>Lysimachia</i>	<i>quadrifolia</i>	Myrsinaceae	Forb	Native	Whorled loosestrife
MACCORA	NA	<i>Macleaya</i>	<i>cordata</i>	Papaveraceae	Shrub	Exotic	Plume poppy
MICVIM	MICVIM	<i>Microstegium</i>	<i>vimineum</i>	Poaceae	Grass	Exotic	Japanese stiltgrass
MINPAT	MINPAT	<i>Minuartia</i>	<i>patula</i>	Caryophyllaceae	Forb	Native	Sandwort
NYSSYL	NYSSYL	<i>Nyssa</i>	<i>sylvatica</i>	Nyssaceae	Tree	Native	Black gum
ONOSEN	ONOSEN	<i>Onoclea</i>	<i>sensibilis</i>	Polypodiaceae	Fern	Native	Sensitive fern
PANAMA	PANAMA	<i>Panicum</i>	<i>amarum</i>	Poaceae	Grass	Planted	Atlantic coastal panicgrass
PANDIC	PANDIC	<i>Panicum</i>	<i>dichotomiflorum</i>	Poaceae	Grass	Native	Smooth panicgrass
PANVIR	PANVIR	<i>Panicum</i>	<i>virgatum</i>	Poaceae	Grass	Planted	Switchgrass
PARPEN	PARPEN	<i>Parietaria</i>	<i>pensylvanica</i>	Urticaceae	Forb	Native	Pellitory
PINSPP	PINSPP	<i>Pinus</i>	<i>sp</i>	Pinaceae	Tree	Unknown	Pine
PINSTR	PINSTR	<i>Pinus</i>	<i>strobus</i>	Pinaceae	Tree	Native	White pine
PINVIR	PINSPP	<i>Pinus</i>	<i>virginiana</i>	Pinaceae	Tree	Native	Virginia pine
POACOM	POACOM	<i>Poa</i>	<i>compressa</i>	Poaceae	Grass	Exotic	Canada bluegrass
POLACR	POLACR	<i>Polystichum</i>	<i>acrostichoides</i>	Polypodiaceae	Fern	Native	Christmas fern
POPGRA	POPGRA	<i>Populus</i>	<i>grandidentata</i>	Salicaceae	Tree	Native	Bigtooth aspen
POPTRE	POPTRE	<i>Populus</i>	<i>tremuloides</i>	Salicaceae	Tree	Native	Quaking aspen
PYCVIR	PYCVIR	<i>Pycnanthemum</i>	<i>virginianum</i>	Lamiaceae	Forb	Native	Mountain-mint

QUEALB	QUEALB	<i>Quercus</i>	<i>alba</i>	Fagaceae	Tree	Native	White oak
QUECO C	QUECOC	<i>Quercus</i>	<i>coccinea</i>	Fagaceae	Tree	Native	Scarlet oak
QUEMON	QUEMON	<i>Quercus</i>	<i>montana</i>	Fagaceae	Tree	Native	Chestnut oak
QUERUB	QUERUB	<i>Quercus</i>	<i>rubra</i>	Fagaceae	Tree	Native	Red oak
RHAFA	RHAFA	<i>Rhamnus</i>	<i>frangula</i>	Rhamnaceae	Shrub	Exotic	Alder buckthorn
RHUGLA	RHUGLA	<i>Rhus</i>	<i>glabra</i>	Anacardiaceae	Shrub	Native	Smooth sumac
RHUTYP	RHUTYP	<i>Rhus</i>	<i>typhina</i>	Anacardiaceae	Shrub	Native	Staghorn sumac
RUBOC C	RUBSPP	<i>Rubus</i>	<i>occidentalis</i>	Rosaceae	Shrub	Native	Black raspberry
RUBSPP	RUBSPP	<i>Rubus</i>	<i>sp</i>	Rosaceae	Shrub	Unknown	Raspberry/blackberry
SALDIS	SALDIS	<i>Salix</i>	<i>discolor</i>	Salicaceae	Shrub	Native	Pussy willow
SASALB	SASALB	<i>Sassafras</i>	<i>albidum</i>	Lauraceae	Tree	Native	Sassafras
SCHSCO	SCHSCO	<i>Schizachyrium</i>	<i>scoparium</i>	Poaceae	Grass	Planted	Little bluestem
SMIGLA	SMIGLA	<i>Smilax</i>	<i>glauca</i>	Smilacaceae	VineW	Native	Catbrier
SOLAME	SOLAME	<i>Solanum</i>	<i>americanum</i>	Solanaceae	Forb	Native	Black nightshade
SOLCAN	SOLSPP	<i>Solidago</i>	<i>canadensis</i>	Asteraceae	Forb	Native	Canada goldenrod
SOLGIG	SOLSPP	<i>Solidago</i>	<i>gigantea</i>	Asteraceae	Forb	Native	Smooth goldenrod
SOLJUN	SOLSPP	<i>Solidago</i>	<i>juncea</i>	Asteraceae	Forb	Native	Early goldenrod
SOLNEM	SOLSPP	<i>Solidago</i>	<i>nemoralis</i>	Asteraceae	Forb	Native	Gray goldenrod
SOLRUG	SOLSPP	<i>Solidago</i>	<i>rugosa</i>	Asteraceae	Forb	Native	Wrinkle-leaf goldenrod
SOLSPP	SOLSPP	<i>Solidago</i>	<i>sp</i>	Asteraceae	Forb	Native	Goldenrod
SORNUT	SORNUT	<i>Sorghastrum</i>	<i>nutans</i>	Poaceae	Grass	Planted	Indian-grass
SPIALB	SPIALB	<i>Spiraea</i>	<i>alba</i>	Rosaceae	Shrub	Native	Meadow-sweet
SYMSP	SYMSP	<i>Symphotri</i>	<i>sp</i>	Asteraceae	Forb	Native	Aster

P		<i>chum</i>					
TILAME	TILAME	<i>Tilia</i>	<i>americana</i>	Malvaceae	Tree	Native	Basswood
TOXRAD	TOXRAD	<i>Toxicodendron</i>	<i>radicans</i>	Anacardiaceae	VineH	Native	Poison ivy
TRICAM	TRISPP	<i>Trifolium</i>	<i>campestre</i>	Fabaceae	Forb	Exotic	Low hop-clover
TRIDAC	TRIDAC	<i>Tripsacum</i>	<i>dactyloides</i>	Poaceae	Grass	Planted	Gammagrass
TRIHYP	TRISPP	<i>Trifolium</i>	<i>hybridum</i>	Fabaceae	Forb	Exotic	Alsike clover
TRIREF	TRISPP	<i>Trifolium</i>	<i>reflexum</i>	Fabaceae	Forb	Native	Buffalo clover
TRISPP	TRISPP	<i>Trifolium</i>	<i>sp</i>	Fabaceae	Forb	Unknown	Clover
TSUCAN	TSUCAN	<i>Tsuga</i>	<i>canadensis</i>	Pinaceae	Tree	Native	Canada hemlock
UNKAST	UNKSPP	<i>Unknown</i>	<i>sp</i>	Asteraceae	Forb	Unknown	Unknown
UNKFAB	UNKSPP	<i>Unknown</i>	<i>sp</i>	Fabaceae	Forb	Unknown	Unknown
UNKGRA	UNKSPP	<i>Unknown</i>	<i>sp</i>	Poaceae	Grass	Unknown	Unknown
UNKSPP	UNKSPP	<i>Unknown</i>	<i>sp</i>	Unknown	UnknownH	Unknown	Unknown
VERALT	NA	<i>Verbesina</i>	<i>alternifolia</i>	Asteraceae	Forb	Native	Wingstem
VICTET	VICTET	<i>Vicia</i>	<i>tetrasperma</i>	Fabaceae	VineH	Exotic	Slender vetch

CHAPTER TWO

ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT METAL UPTAKE: A FIELD STUDY

Lee H. Dietterich, Cédric Gonneau, and Brenda B. Casper

2.1 Abstract

The factors affecting plant uptake of heavy metals from contaminated soils are of deep importance to efforts to remediate these lands. Arbuscular mycorrhizal fungi (AMF), soil-dwelling fungi that engage in an intimate exchange of nutrients with plant roots, have been suggested to be involved in plant metal uptake as well. Here, we investigated the effects of AMF on plant metal uptake from contaminated soils using a novel field-based approach. We examined direct and AMF-mediated effects of soil metal concentrations on plant metal concentrations, improving on the generality of previous work by measuring AMF colonization and plant and soil concentrations of 8-13 metals simultaneously in five plant species sampled in the field across a gradient of Zn, Pb, Cd, and Cu contamination. Plant and soil metal concentration profiles were closely matched despite high variability in soil metal concentrations even at small spatial scales. However, we observed few effects of soil metals on AMF colonization, and no effects of AMF colonization on plant metal uptake. Manipulating soil chemistry or plant community composition directly may control landscape-level plant metal uptake more effectively than altering AMF communities. Plant species may serve as highly local indicators of soil chemical characteristics.

2.2 Introduction

Heavy metal pollution is a global phenomenon having widespread effects on diverse ecosystems and people who depend on them. Plant uptake of metals from contaminated soils is commonly the first step in a metal's pathway from the soil to the aboveground ecosystem, from where it can be mobilized to organisms at upper trophic levels including humans. Understanding the factors affecting plant metal uptake is crucial to effectively remediating contaminated sites, whether plants are used to remove pollutants (phytoextraction) or to sequester them in place (phytostabilization) (Pilon-Smits 2005). Plant metal uptake also has important implications for agriculture, especially the rising urban agriculture movement, in which many social, ecological, and public health benefits rest on our ability to safely grow crops in often-polluted urban soils (Romic & Romic 2003; EPA 2011). Arbuscular mycorrhizal fungi (AMF), obligate symbionts associated with a large majority of plant species, may have important effects on plant metal uptake (Alford et al. 2010; Miransari 2010), but the literature does not yet support a general understanding of what these effects might be.

Heavy metals can have toxic effects on plants, adversely affecting their fitness (Lin & Aarts 2012). This can occur by several mechanisms, including metal ions substituting for chemically similar metals as cofactors in enzymes, generating reactive oxygen species, or interfering with the uptake of chemically similar micronutrients (Brady et al. 2005; Bothe et al. 2010). Even metals known to have essential biochemical functions can be toxic in sufficiently large concentrations (Broadley et al. 2007). However, some plants, considered metallophytes, appear adapted to elevated soil metal

concentrations. Endemic serpentine plants, for instance, seem to grow only where the stress associated with naturally metalliferous serpentine soils reduces competition with other species that would outcompete them on uncontaminated soils (Brady et al. 2005). Plants may use any of several metal tolerance strategies, including hyperaccumulating metals or excluding them (Baker 1981), and they have diverse cellular and molecular strategies for alleviating the toxicity of metals they take up or even their bioavailability in the soil (Bothe et al. 2010).

Soil metals may have similar direct effects on arbuscular mycorrhizal fungi (AMF). Fungal nutrition is generally less well studied than plant nutrition, but many of the same principles apply. Some metals such as Zn are essential nutrients for fungi as well as plants, and become toxic only at sufficiently elevated concentrations, and others such as Pb or Cd have no known biological function and can be toxic at any concentration (Bothe et al. 2010). The biochemical mechanisms of metal toxicity to fungi are likely similar to those in plants in general, although of course the details of the metals, enzymes, and other cellular components involved may vary. Elevated soil metal concentrations have been shown to decrease AMF colonization in some field systems (Khan 2001), and increase it in others (Vogel-Mikuš et al. 2006). Like plants, fungi may tolerate metal stress by limiting uptake of nonessential metals, or by absorbing and sequestering metals in tissues like cell walls or intracellular compartments where they are less likely to induce toxicity (Weiersbye et al. 1999). Fungi can also produce exudates that chelate or bind metals to affect their mobility and availability in the soil (Bothe et al. 2010).

To better understand the effects of metals on plant-AMF systems, we must consider the plant-fungal responses together, as soil metals may affect plants either directly or via their mycorrhizae (Fig. 2.1). The relationship between AMF colonization and plant metal uptake can determine whether AMF may exacerbate or alleviate metal toxicity to plants in metalliferous soils. For instance, if colonization increases plant metal uptake, then AMF would be expected to exacerbate plant metal toxicity. Alternatively, if colonization decreases plant metal uptake, then AMF could alleviate plant metal toxicity. Differing relationships between soil metal concentrations and AMF colonization could further complicate plant-soil metal dynamics. Numerous mechanisms have been proposed for how AMF may affect plant metal uptake, including AMF reducing plant uptake by altering soil metal bioavailability, sequestering metals in their own tissues, or AMF increasing plant uptake by actively translocating metals into plants through pathways presumably evolved for nutrient transfer (reviewed by Schützendübel & Polle 2002; Göhre & Paszkowski 2006; Miransari 2011). However, little is known about which mechanisms might be dominant in any given situation.

Curiously, at a larger taxonomic scale, many metal hyperaccumulating plant species occur in predominantly nonmycorrhizal plant families and are themselves nonmycorrhizal. It remains to be seen how closely (non)-association with mycorrhizal fungi is related to metal hyperaccumulation across the plant kingdom. It has been suggested that hyperaccumulation and mycorrhization both require substantial carbon investment on the part of the plant so there may be a trade-off discouraging plants from both hyperaccumulating trace elements and forming mycorrhizae (Audet 2013).

However, since then, many mycorrhizal hyperaccumulators have been documented (e.g. Turnau & Mešjasz-Przybyłowicz 2003; Vogel-Mikuš et al. 2005), leading others to wonder where there is a meaningful relationship between hyperaccumulation and mycorrhization at all (Alford et al. 2010).

Numerous studies have investigated the effects of AMF on plant metal uptake, however, these studies are almost invariably greenhouse experiments in which one or two plant species were grown with one or two kinds of AMF inocula, in soil spiked with one or two metals. This approach is difficult to generalize to field conditions, in which many different plant species, AMF species, and metals interact simultaneously. Indeed, in different systems, these studies have found that AMF increase (Chen et al. 2005; Orłowska et al. 2013), decrease (Abdel Aziz et al. 1997; Jiang et al. 2016), have mixed effects (Weissenhorn et al. 1995; Wang et al. 2007), or no effects (Tonin et al. 2001; Lagrange et al. 2013) on plant metal uptake. However, it is difficult to synthesize these disparate results, and many of them do not take into account the possibility that soil metals affect plants directly and indirectly via AMF. Thus, we still lack an understanding of any general principles that might govern plant-AMF-metal interactions in the field.

Here, we take the approach that soil metal concentrations could affect plant metal concentrations either directly by root uptake, or indirectly via effects on AMF colonization (Fig. 2.1). Thus, at a site polluted by Zn, Cd, Pb, and Cu, and for five different plant species, we examine the following specific relationships: soil metal concentrations and plant metal concentrations, soil metal concentrations and AMF colonization, and AMF colonization and plant metal concentrations (Fig. 2.1). We

improve on the generality of other studies by (1) sampling plants that spent their entire lives in the field, (2) examining five plant species across three families under similar environmental conditions, (3) using continuous variation in soil metal concentrations and AMF colonization rather than single doses of either, and (4) analyzing many metals simultaneously. We aim to better understand how plants, AMF, and soil metals interact in field conditions where they are most relevant.

2.3 Methods

Study site

We collected samples on the north facing slope of Blue Mountain just west of Lehigh Gap in Carbon County, PA, USA, on lands owned and managed by the Lehigh Gap Nature Center (LGNC) and the National Park Service (NPS). This area constitutes the western portion of the Palmerton Zinc Superfund Site, a >2000 acre area of mountainside severely contaminated and devegetated due to airborne Zn, Cd, Pb, Cu, and SO_x emissions from two zinc smelters operating upwind between 1898-1980 (EPA 2007). Deposition of smelting emissions is thought to have produced a gradient of soil heavy metal contamination on the property, with concentrations in our sampling area predicted to increase northward and eastward toward the smelters (Buchauer 1973; Johnson & Richter 2010; Glassman & Casper 2012). However, this contamination gradient has not yet been verified to exist at the spatial scale of this study.

Study species

Remediation efforts began in 2003 with the seeding of a suite of C₄ grasses, which have since become dominant in much of the site. However, to extend the generality of our study to contaminated sites regardless of restoration strategy, we chose to focus on species that had not been planted. We sampled 15-30 individuals each of three forbs: *Minuartia patula* (Caryophyllaceae), *Ageratina altissima* (Asteraceae), *Eupatorium serotinum* (Asteraceae), and two C₃ grasses, *Agrostis perennans* (Poaceae) and *Deschampsia flexuosa* (Poaceae) (Rhoads & Block 2007). All are abundant and distributed widely across the expected contamination gradient. Three of these species, *M. patula*, *A. perennans*, and *D. flexuosa*, were documented in the site well before restoration began (Pretz 1954; Jordan 1975). We have not been able to find records of *A. altissima* or *E. serotinum* in the site before remediation, so we do not know when they arrived on the mountain.

We collected samples along two hiking trails, the North Trail and the LNE Trail, which served as approximately east-west transects across the upper and lower slopes of the mountain, respectively. Along both trails, we established sampling locations at least 50 m apart and at least 5 m from the trail where we could find at least two target species growing within 10 m, and recorded the GPS coordinates of each location. When possible, we sampled multiple species, and up to two individuals per species, at each sampling location, in order to document both fine-scale and large-scale variation in soil characteristics. In one sampling location, we made an exception and collected five individuals of *D. flexuosa*. For each individual plant sampled, we collected aboveground

tissue and rooting soil for elemental analysis, and roots for AMF colonization. Trowels used to collect soil and roots were washed and sterilized with 70% ethanol between samples to prevent cross-contamination.

AMF colonization

In the lab, roots were separated from soil, cleaned in tap water, placed in plastic cassettes (Fisher 15-182-701E) and stored in water at 4 °C until staining. Roots were then cleared in hot 10% KOH (~6 min), bleached in room temperature 1:10 household ammonia : household H₂O₂ (~2 min), acidified in room temperature 5% HCl, (10-20 min), and stained in hot 0.1% trypan blue in 1:1:1 water : lactic acid : glycerol (~5 min). At least 10 1-cm long root segments were mounted on a microscope slide, fixed with polyvinyl lactic acid glycerol, and cured at 60 °C for at least 48 h (INVAM 2014). Percent colonization was measured by recording the presence or absence of AMF structures at intersections spaced 1 mm apart on each root segment (McGonigle et al. 1990). We considered blue-staining hyphae without septa, as well as any associated vesicles and arbuscules, to be AMF structures. While the goal was to record presence or absence of AMF at 100 intersections per slide, we were frequently unable to do so, most commonly because of missing root cortex, dark background staining, or abundant non-mycorrhizal structures obscuring our view. We included data only from samples with more than 30 intersections in our analysis. We also searched the literature for studies

presenting colonization data for our study species and congeners to determine how AMF colonization in our system compares to colonization elsewhere.

Plant and soil metal concentrations and integrative soil variables

We measured metal concentrations of plant aboveground tissue, and both total extractible and exchangeable metal concentrations of soils. Soil total extractible metal concentrations are close to the samples' total metal concentrations (Brümmer 1986), and we interpret these concentrations as reflecting the amount of a metal that a long-lived perennial plant may have access to in its lifetime. Exchangeable soil metal concentrations are less than total extractible concentrations and represent the amount of metal a plant may encounter on shorter time scales from the soil solution or via cation exchange (Brümmer 1986). As total extractible and exchangeable soil metal concentrations may behave very differently (Remon et al. 2013), we analyzed both to better understand the soil chemical factors affecting AMF colonization and plant metal uptake.

Plant aboveground tissue used for elemental analysis was washed thoroughly with tap water, oven-dried at 60 °C for at least 48 h, and ground using mortar and pestle with liquid nitrogen as necessary. Ground plant samples were stored sealed at room temperature until they could be digested. Soils were sieved to 2 mm, air-dried for at least one week, and stored in sealed plastic bags before digestion.

Plant metal concentrations and total extractible soil metal concentrations were measured as follows. Samples were weighed into ceramic crucibles, covered, ashed at 475 °C for at least 4 h, allowed to cool, and weighed again to estimate organic matter content by loss on ignition (LOI). Ashed samples were digested in 2 mL concentrated HCl at 90-100 °C for 10 min, diluted to 25 mL with deionized water, and stored at 4 °C until their metal concentrations could be measured. In each batch of samples we digested, we included a reagent blank as well as two standard reference materials, peach leaves (NIST 1547) and either olive leaves (BCR 062) or citrus leaves (NIST 1572), to check the quality of the digest.

From these digest solutions, we measured plant and total extractible soil metal concentrations of the contaminants Zn, Pb, Cd, and Cu, the macronutrients Ca, Mg, and K, and the micronutrients Ni and Mn with a Spectro Genesis inductively coupled plasma optical emissions spectrometer (ICP-OES). In each ICP-OES run, digested experimental samples and standard reference materials were interspersed with standard solutions containing known concentrations of each element measured, in order to ensure the quality of the run.

For the subset of soils for which we had sufficient soil remaining after measuring total extractible metal concentrations, we also measured exchangeable soil metal concentrations and the integrative soil variables pH, cation exchange capacity (CEC), and base saturation. We measured soil pH in a 1:5 soil : water ratio according to the ISO 10390 standard method. We used cobaltihexammine extraction (ISO 23470) to

determine the CEC at soil pH, and to determine the exchangeable concentrations of soil Ca, K, Mg, Na, Al, Fe, Mn, Cd, Cr, Cu, Ni, Pb, and Zn by ICP-OES.

Statistical analysis

Plant and soil element concentrations were \log_{10} -transformed before analysis to improve normality. We removed total extractible soil Mn from relevant analyses including *D. flexuosa* because of insufficient usable measurements, and we removed exchangeable soil Cu, Ni, Fe, and Cr from the entire dataset because they were consistently below the detection limit of the ICP-OES. We also removed *D. flexuosa* from analyses including exchangeable metals and integrative soil variables because of insufficient sample size.

We used multivariate analysis of variance (MANOVA) to investigate the effects of soil metal concentrations on AMF colonization, and of soil metal concentrations and AMF colonization on plant aboveground metal concentrations. We used MANOVA rather than univariate ANOVAs to take advantage of the correlation structure of both the plant and soil metal concentration datasets. Because total extractible and exchangeable metal concentrations may behave differently (Remon et al. 2013), and we were not able to measure both sets of metal concentrations from all soil samples, we used separate MANOVA models to examine these sets of variables separately and with maximal sample size.

To investigate the effects of soil chemistry on AMF colonization, we ran two different MANOVA models (Table 2.1). In the first model, we included exchangeable metal concentrations, and the integrative soil variables pH, CEC, and base saturation, and plant species identity. In the second model, we included only total extractable metal concentrations and plant species identity. This second model allowed us to increase our sample size by including samples for which we did not have sufficient material to measure exchangeable metal concentrations or integrative variables, and this model was the only one in which we could include *D. flexuosa*. To investigate the effects of soil metals and AMF colonization on plant aboveground metal concentrations, we ran a similar pair of MANOVA models except that these models also included AMF colonization as a predictor variable (Table 2.1). Because of the large number of predictor variables included in these models, we tested all main effects but no interactions.

Because plant species identity was a significant predictor of plant aboveground metal concentrations in both of our models, we then examined interspecific differences in plant and soil metal concentrations. We observed that *M. patula* was strongly distinct from the other species in many of its plant and soil metal concentrations, so we repeated the MANOVA models above with that species removed. To better understand these interspecific differences, we also tested for associations between plant species identity and each total extractable and exchangeable metal concentration, integrative soil variable, and AMF colonization using one-way ANOVAs, using the Dunn–Šidák correction to account for the large number of multiple comparisons.

We used constrained analysis of proximities (CAP), a constrained ordination technique (Anderson & Willis 2003), to visualize the relationships between plant species identity and plant and soil chemical characteristics. We constructed CAP models using plant species identity as a predictor of plant metal uptake profiles, soil total extractable chemical profiles, and soil exchangeable chemical profiles, respectively. We used the permutation-based “anova.cca” method to test the significance of species as a predictor of plant and soil chemistry, and the “plot.cca” method to visualize the results (Oksanen et al. 2013). CAP significance testing yielded results very similar to those from our MANOVAs, so we report only the latter because MANOVA more explicitly takes into account the correlation structure of the data.

Spatial analysis

We used a multipronged statistical approach to investigate the spatial distributions of soil metal concentrations in the Palmerton site. In particular, we sought to determine the spatial scale of variation in soil metal concentrations, and to compare the strength of plant species identity and proximity of soil samples as predictors of soil metal concentrations.

We first performed a series of Mantel tests to test whether soil samples collected near each other had more similar metal concentration profiles than samples collected farther apart. We constructed a geographic distance matrix of sampling points using the geosphere package in R (Hijmans et al. 2016), and constructed dissimilarity matrices of

soil metal concentrations using total extractible or exchangeable concentrations of all metals measured or only the known contaminants Zn, Cd, Pb, and Cu using the *vegan* package in R (Oksanen et al. 2013). We used Euclidean distance between \log_{10} -transformed soil metal concentrations to construct soil metal dissimilarity matrices.

To examine how soil metal concentrations varied with position on the mountain and distance from the smelters, we performed principal components analysis (PCA) on \log_{10} -transformed, scaled, and centered soil total extractible or exchangeable concentrations of all metals or just the contaminants, for a total of four PCAs. We then extracted each soil sample's coordinates on the first two PCA axes and regressed those coordinates against each sample's distance from the two smelters.

We further visualized the relationships between soil metal concentrations and position on the mountain by repeating the above PCAs but with distance from the smelters included. Plotting biplot arrows of these variables helps illustrate the correlation structure of the data; longer arrows pointed in more similar directions represent stronger positive correlations.

2.4 Results

Site conditions

Soil total extractible metal concentrations varied over 1-3 orders of magnitude per element. Compared to the first quartile to third quartile range of topsoil metal concentrations in the United States (Smith et al. 2013), soils in the Palmerton site were

low in Ca and K, similar in Mg and Ni, and high in Zn, Cd, Pb, and Cu. Soil exchangeable metal concentrations were consistently 5-10 times lower than total extractible concentrations but also varied over 1-3 orders of magnitude per element. Plant metal concentrations varied over 1-2 orders of magnitude per element, and were comparable to metal concentrations of our standard reference materials and the standard reference plant described by van der Ent et al. (2013), except for Zn and Cd for which our samples averaged 1-3 orders of magnitude higher (Figs. 2.2, 2.S1, 2.S2). Root colonization by AMF reached 40% but was below 10% for most samples. The integrative soil variables fell between 4.0-7.2 for pH, 1.8-41.6 for CEC, and 25-104% for base saturation (Fig. 2.3).

Soil metal concentrations and AMF colonization

Depending on the statistical model, soil concentrations of Zn, Cu, and LOI were found associated with AMF colonization. Of these, the relationship with Cu was the strongest, followed by LOI and then Zn. In the model including exchangeable soil metals, integrative soil variables, and plant species, exchangeable Zn was the only significant predictor of AMF colonization. Removing the potentially anomalous species *M. patula* from the analysis resulted in the addition of LOI as a significant predictor of AMF colonization. In the model including only total extractible metals and plant species, only total extractible Cu significantly predicted AMF colonization, whether or not *M. patula* was included (Table 2.1). AMF colonization rates were nonzero but notably

reduced from previously recorded values in all of our study species except *M. patula*, for which the near-zero colonization rates we observed were expected (Table 2.2, Fig. 2.3).

Following up on these results by separate univariate regressions revealed that exchangeable Zn, LOI, and total extractible Cu were all negatively related to AMF colonization. These effects appear to be driven in part by interspecific differences in soil metal concentrations: the aster species we examined tended to have greater AMF colonization, and grew in soil with lower LOI, exchangeable Zn, and total extractible Cu, than the other species (Figs. 2.2, 2.3, 2.S1, 2.S2). However, plant species identity was never a significant predictor of AMF colonization (Table 2.1).

Indeed, when we examined the univariate relationships between soil element concentrations and AMF colonization for each element and plant species separately, significant relationships were few and weak. Only *E. serotinum* samples had any significant relationships between total extractible soil metals and AMF colonization (negative relationships with Cu, Mn, Ni, and Pb, and positive relationship with Mg). For exchangeable metals and integrative variables, AMF colonization in *A. ageratina* was positively related to base saturation, in *E. serotinum* negatively related to exchangeable Zn, and in *M. patula* negatively related to exchangeable Mn. However, among all of these relationships, only two had $R^2 > 0.4$ (*E. serotinum* colonization and total extractible soil Ni, $R^2 = 0.43$, and *A. altissima* colonization and soil base saturation, $R^2 = 0.55$). None of them remained significant following the Dunn–Šidák correction for multiple comparisons.

Effects of soil metal concentrations and AMF colonization on plant metal concentrations

Our MANOVA models consistently showed significant effects of plant species identity, total extractible or exchangeable soil Zn, and total extractible soil Pb concentrations on plant metal profiles (Table 2.1). Univariate regressions showed soil total extractible or exchangeable Zn to have positive relationships with plant Zn. Soil total extractible Pb, though, had a negative relationship with plant Pb, which appears to be largely driven by interspecific variation (Fig. 2.2). Exchangeable Mg and total extractible K were also significant predictors of plant metal profiles in their respective models, but only when *M. patula* samples were included. AMF colonization never affected plant metal profiles in any model (Table 2.1).

Species effects

Plant species identity was a highly significant predictor of plant metal concentration profiles, soil total extractible metal profiles, and soil exchangeable metal profiles (CAP; $P < 0.001$ for each; Fig. 2.4). Furthermore, when examined by individual ANOVAs, all plant metal concentrations, soil metal concentrations, integrative soil variables, and AMF colonization differed significantly with plant species except for exchangeable soil Na and plant Ni, even after the Dunn–Šidák correction for multiple comparisons ($P < 0.00165$). As a general rule, soils under *M. patula* had the highest total extractible and exchangeable concentrations of heavy metals, except that they had

relatively low exchangeable Pb. Compared with the other plant species analyzed, *M. patula* had higher aboveground tissue concentrations of Zn and Cd, but lower concentrations of Pb, Cu, and Ni. The two aster species, *A. altissima* and *E. serotinum*, had the greatest leaf Ca, Mg, Cu, and Pb concentrations. For Ca and Mg, these higher leaf concentrations appeared to follow higher soil concentrations, but for Cu, differences in concentration between plant species did not appear to relate to differences in soil concentrations. Plant Pb concentrations followed a similar pattern to exchangeable soil Pb but opposite to total extractible soil Pb. The two grasses, *A. perennans* and *D. flexuosa*, consistently had among the lowest leaf metal concentrations of the species examined, and their soils had the lowest Ca and Mg concentrations and the greatest LOI (Figs. 2.2, 2.S1, 2.S2).

AMF colonization, soil pH, CEC, and base saturation were highest in samples from the asters *A. altissima* and *E. serotinum*. AMF colonization was lowest in *M. patula* and *A. perennans*. Soil pH and base saturation were lowest under *D. flexuosa*, with intermediate values associated with *A. perennans* and *M. patula*. Soil CEC was lowest under the two grasses (Fig. 2.3).

Spatial analysis

Mantel tests showed significant associations between soil chemical dissimilarity and spatial proximity of samples only when soil chemical dissimilarity was calculated from all soil total extractible metal concentrations measured ($P < 0.01$). However, there

was no significant relationship between spatial distance and chemical dissimilarity of metal concentrations when soil chemical dissimilarity was calculated from total extractible contaminant concentrations, exchangeable contaminant concentrations, or exchangeable concentrations of all metals measured.

Principal components analysis (PCA) of soil metal concentrations again showed significant spatial patterns in total extractible but not exchangeable metal concentrations. When all total extractible metal concentrations were considered, the first PCA axis (PC1) was negatively related to the concentrations of all contaminants as well as Ni and K, and PC2 was positively related to Ca and Mg (Fig. 2.5a). In regressions against distance from the smelters, PC1 was positively related to distance from the east and west smelters ($P < 0.01$ and $P < 0.05$, respectively), and PC2 was negatively related to distance from either smelter ($P < 0.001$ for both). A similar analysis including just the contaminants also indicated the contaminants increasing in concentration toward the smelters as expected.

Similar PCA-based analysis of exchangeable metal concentrations revealed few significant results. Analysis of all exchangeable metals together indicated that all exchangeable metal concentrations measured increase weakly toward the west smelter, and analysis of only exchangeable contaminant concentrations found no significant relationships with distance to either smelter.

We then added distance from the smelters back into the above PCAs to more directly examine the importance of spatial patterns in soil metal concentrations. Distances from the two smelters were consistently positively correlated with each other,

but most metal concentrations, including those of the contaminants, were not strongly correlated with distance from the smelters. The primary contaminants Zn and Cd tended to be closely correlated with each other but not necessarily with the other contaminants Cu or Pb. Otherwise, the most consistent pattern observed in this analysis is that concentrations of the base cations Ca and Mg tend to increase toward the smelters (Fig. 2.5b). For simplicity, we present this data only for total extractible metal concentrations, but the pattern is similar for total extractible contaminants, all exchangeable metals, and exchangeable contaminants by themselves.

2.5 Discussion

Contrary to our expectations, AMF do not appear to play a major role in the relationships between plant and soil metal concentrations at this site polluted by Zn, Cd, Pb, and Cu. While a few soil metals appear to affect AMF colonization rate, the relationship may be driven in part by differences among plant species in both AMF colonization and the metal content of their rhizosphere soils. We find no evidence for an effect of AMF colonization rate on plant metal concentrations. Instead, we find that plant metal concentrations are strongly related to plant species identity and soil metal concentrations, which are highly variable even at small spatial scales.

Our results from the field contrast with many results from greenhouse experiments investigating these same relationships between soil metals, AMF, and plant metals. In other studies, metals have typically been found to decrease AMF colonization

and/or diversity (Del Val et al. 1999; Khan 2001; Chen et al. 2005), although Tan et al. (2015) found no effect of applied Cd on AMF colonization of *Solanum photeinocarpum*, Al Agely et al. (2005) found no effect of high As concentrations on AMF colonization of *Pteris vittata*, and Vogel-Mikus et al. (2006) found that soil Cd and Pb increased AMF colonization of the hyperaccumulator *Thlaspi praecox*. Similarly, numerous greenhouse studies have shown increased AMF colonization to have positive, negative, or mixed effects on plant metal uptake (e.g. Diaz et al. 1996; Turnau & Mesjasz-Przybylowicz 2003; Chen et al. 2005; Jiang et al. 2016).

There may be several explanations for why our results contrast with other studies showing relationships between AMF and plant and soil metals. Most of these studies took place in greenhouses, where many conditions are different from the field conditions we investigated. Jankong et al. (2007) did include a field component in a study of the effects of different inocula on growth and As uptake in *Pityrogramma calomelanos* ferns, in which they found that bacterial inocula, but not fungal inocula, increased root and front As concentrations relative to un-inoculated controls. However, because AMF cannot grow without a host plant (Smith & Read 2008), their culture-based preparation of fungal inocula likely resulted in their inocula containing few, if any, AMF propagules. Furthermore, in most studies of AMF and plant metal uptake to date, plants are transplanted into contaminated soil as seedlings, thereby bypassing germination, exposing them to concentrations or forms of trace elements that may be unrepresentative of field conditions, and possibly changing their growth and metal uptake dynamics substantially.

In contrast to greenhouse experiments, our study plants were exposed to soil metals and AMF for their entire life cycles, including germination, which has been shown to be especially sensitive to soil metal concentrations (Bae et al. 2016 and sources therein). Our plants also likely experienced a wider range of environmental conditions, such as temperature and water availability, than exist in most greenhouses. We suggest that one or more components of these more variable, natural conditions may overwhelm the effects of AMF on leaf metal concentrations seen in some greenhouse studies.

It is also possible that conditions at our research site serve as an ecological filter favoring species with low reliance on AMF. Smelting pollution in the site is thought to have reduced the diversity, abundance, and activity of soil dwelling microbes, potentially including AMF (Jordan and Lechevalier 1975; Strojan 1978; Latham et al. 2007), so that plants colonizing the site soon after the disturbance may have benefited from low AMF reliance. The relatively low AMF colonization rates we observed in our study species further support this idea. We also consider the possibility that AMF do indeed play important roles in plant-soil metal dynamics in the field, but that root colonization rates do not consistently reflect the strength or function of mycorrhizal symbioses (Smith et al. 2004; Smith & Read 2008, pp.84-85). Supporting this idea with regard to trace element uptake, Liu et al. (2009) found similar effects of AMF on As uptake by *Pteris vittata* whether all or just half of the plant's root system was exposed to AMF.

AMF colonization of our study plants responded to only two of the four major contaminants in the site. While soil concentrations of Zn and Cu had the expected negative effect on AMF colonization in one model each, Pb and Cd never significantly

affected AMF colonization. We expected Pb and Cd to be more toxic to AMF than Cu because they occur at similar or greater concentrations and Cu is an essential micronutrient for many organisms, including fungi (Ding et al. 2014), but Pb and Cd have no known biological function in most organisms. However, longtime use of Cu as an agricultural fungicide (Winston et al. 1923) and recent studies showing high Cu sensitivity of soil fungi (Klimek & Niklińska 2007) support our finding that Cu is one of the dominant toxins to fungi in the Palmerton site.

Our most striking result is a strong relationship between plant species identity and leaf and soil metal concentrations. While our total extractible metal concentration data does support the existence of a site-wide gradient of contaminant concentrations, most of the variation in soil metal concentrations is not associated with distance from the smelters, and much of that residual variation is associated with plant species identity. Plant species may preferentially establish in soil with fairly specific trace element concentration profiles, or there could be species-specific effects on soil trace element chemistry, or both. These possibilities deserve further investigation to disentangle the effects of varying soil chemistry on plant establishment and competition (e.g. McCormick & Gibble 2014), and the effects of plants on the trace element chemistry of the soils in which they grow (e.g. Waring et al. 2015). Either could be a mechanism for locally positive intraspecific plant-soil feedback that could help maintain high environmental heterogeneity, with potential but complex implications for biodiversity (van der Putten et al. 2013; Yang et al. 2015). This idea is further supported by the high

variability of soil physical and chemical characteristics we observed even among soils collected less than 10 m apart.

Our findings also provide empirical support for *M. patula* being a nonmycorrhizal or nearly so Zn hyperaccumulator (van der Ent et al. 2013) and tolerating some of the highest levels of soil contamination in the site. Land managers have long noticed that despite the nearby presence of taller plants that would be expected to outcompete it, *M. patula* forms near-monocultures on characteristic black, powdery soils in the site. We hypothesize that these soils, which are higher in total extractible concentrations of all major contaminants, and exchangeable concentrations of Zn and Cd which are thought to be most enriched due to the smelting pollution, are too toxic to support most of the other plant species in the site. The small-statured *M. patula*, then, may remain dominant in these areas by tolerating soil metal concentrations toxic to its larger neighbors. This could also explain *M. patula*'s failure to disperse out of the contaminated region despite living there for over 60 years (Pretz 1954); this species is still found nowhere else in Pennsylvania (Rhoads & Klein 1993; Latham et al. 2007; Rhoads & Block 2007).

The other associations we observed between plant species and soil chemistry appear to separate plant taxa at the family level. The two Asteraceae we studied, *A. altissima* and *E. serotinum*, seem to favor soils with higher pH and base cation concentrations, and lower contaminant concentrations than the other species. In contrast, our study grasses, *A. perennans* and *D. flexuosa*, grew in soils with lowest base cation concentrations, intermediate contaminant concentrations, and highest organic matter. Soil pH was also lowest under *D. flexuosa* and then *A. perennans*, but that seems to be

confounded with their location on the mountain. We found *D. flexuosa* exclusively on the upper slope, *A. perennans* on both slopes, and the other three species primarily on the lower slope of the mountain. Soils on the upper slope are substantially more acidic than on the lower slope, likely due to remediation activities. The smelting pollution acidified soils all over the mountain, but the lower slope received more soil amendments in the course of restoration because of technical and logistical constraints associated with accessing the upper slope (EPA 2007).

Our spatial analyses suggest that the distribution of soil metals is substantially more complex than would be predicted based on the point source nature of the smelting pollution. We observed some evidence of total extractable contaminant concentrations decreasing with distance from the smelters, as expected (Fig. 2.5a; Buchauer 1973; Johnson & Richter 2010; Glassman & Casper 2012). However, the relative independence of contaminant concentrations and distance to the smelters we observed in Fig. 2.5b, and the consistent non-significance of our Mantel tests for association between spatial distance and chemical dissimilarity suggests that most of the variation in the site's soil metal concentrations needs to be explained by factors other than space. Such factors might include microsite-specific differences in hydrology, erosion, or metal binding sites in soils. The complex set of associations between soil metals we observed in our ordination analyses speaks further to the multidimensionality of soil chemistry and plants' interactions with it.

We suggest that AMF colonization has little if any effect on plant metal uptake in the field. Therefore, while we acknowledge that AMF are still important in improving

plant growth under a variety of stresses, manipulating AMF colonization is not likely to affect plant metal uptake under field conditions. Land managers seeking to modulate a plant community's metal uptake may be better served by seeding desired species or using soil amendments such as compost, fertilizer, or lime, in the hopes of altering soil chemistry and/or plant community composition (Dietterich & Casper 2016).

We also highlight that, in light of the high local variability of soil chemistry and its close association with plant species observed here, the particular plant species growing in a patch of soil could provide significant information about the chemical composition of that soil. Thus, plant community composition may be able to help us understand soil chemical characteristics to a first approximation. This insight could be useful to restoration, agriculture, mining, or other settings where it is important to understand fine-scale variation in soil chemistry.

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Table 2.1: Significant drivers of mycorrhizal colonization and plant metal uptake. Models are specified as “response ~ predictor 1 + predictor 2 + ...” Model terms are abbreviated as follows: Plant, plant metal concentrations; AMF, percent root colonization by AMF; tot, total extractable soil metal concentrations; cohex, exchangeable soil metal concentrations; int, integrative soil variables (pH, CEC, base saturation); Species, plant species identity; MP, *Minuartia patula*. Significant metal concentrations are notated either XX.tot or XX.cohex, where XX is the chemical symbol of the element in question. Significance codes: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Model	Significant terms (all species in model)	Significant terms (species less MP)
AMF ~ cohex + int + species	Zn.cohex *	Zn.cohex *, LOI.s *
AMF ~ tot + species	Cu.tot *	Cu.tot *
Plant ~ cohex + int + AMF + Species	Mg.cohex **, Zn.cohex ***, Species ***	Zn.cohex **, Species ***
Plant ~ tot + AMF + Species	K.tot **, Pb.tot **, Zn.tot ***, Species ***	Pb.tot ***, Zn.tot ***, Species **

Table 2.2: Measured AMF colonization of our target species at Palmerton is low compared to previously reported colonization data for these species and their close relatives, except for *M. patula*, which was expected to have near-zero colonization. For method, “magnified intersections” follows McGonigle et al. (1990) and “gridline intersect” follows Giovannetti and Mosse (1980).

Species	Colonization (%)	Method	Study
<i>Ageratina altissima</i>	9.1±1.5	Magnified intersections	Present study
<i>Ageratina espinosarum</i>	0-10	Unclear	Camargo-Ricalde et al. (2003)
<i>Agrostis perennans</i>	2.4±0.71	Magnified intersections	Present study
<i>Agrostis scabra</i>	25.7	Gridline intersect	Titus and Tsuyuzaki (2002)
<i>Agrostis scabra</i>	2.2-55	Magnified intersections	Bunn et al. (2009)
<i>Agrostis stolonifera</i>	50.0	Gridline intersect	Pawlowska et al. (1996)
<i>Agrostis stolonifera</i>	32.8	Gridline intersect	Wilson and Hartnett (1998)
<i>Deschampsia flexuosa</i>	6.0±2.1	Magnified intersections	Present study
<i>Deschampsia flexuosa</i>	13-30	Magnified intersections	Alaoja (2013)
<i>Deschampsia flexuosa</i>	23-93	Gridline intersect	Vosatka and Dodd (1998)
<i>Deschampsia flexuosa</i>	60.9-80.4	Magnified intersections	Ruotsalainen et al. (2007)
<i>Deschampsia flexuosa</i>	27-58	Gridline intersect	Malcová et al. (1999)
<i>Deschampsia flexuosa</i>	38	Unclear	Read and Haselwandter (1981)
<i>Eupatorium serotinum</i>	13.4±2.5	Magnified intersections	Present study
<i>Eupatorium serotinum</i>	24	Magnified intersections	Turner et al. (2000)
<i>Eupatorium serotinum</i>	“quite abundant”	Unclear	McDougall and Glasgow (1929)
<i>Eupatorium coelestinum</i>	0	Unclear	McDougall and Glasgow (1929)
<i>Eupatorium purpureum</i>	“present but scarce”	Unclear	McDougall and Glasgow (1929)
<i>Eupatorium urticaefolium</i>	“present. Arbuscules observed”	Unclear	McDougall and Glasgow (1929)
<i>Minuartia patula</i>	1.1±0.53	Magnified intersections	Present study
<i>Minuartia sp.</i>	0-5	Unclear	Harley and Harley (1987)

Figure 2.1: Conceptual diagram of the relationships examined in this study. Soil metal concentrations may relate to plant metal concentrations directly, or indirectly via mycorrhizal fungal colonization.

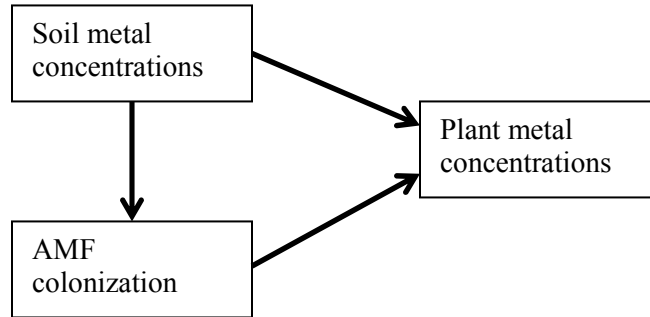


Figure 2.2: Interspecific differences in plant (A-C), soil total extractable (D-F), and soil exchangeable (G-I) concentrations of the primary pollutants Zn (A,D,G), Cd (B,E,H), and Pb (C,F,I). Note that the vertical axes are different for each pool of each element. Species are abbreviated as follows: AA, *Ageratina altissima* (Asteraceae); AP, *Agrostis perennans* (Poaceae); DF, *Deschampsia flexuosa* (Poaceae); ES, *Eupatorium serotinum* (Asteraceae); MP, *Minuartia patula* (Caryophyllaceae). Families are color-coded as follows: Asteraceae, blue; Poaceae, red; Caryophyllaceae, gray.

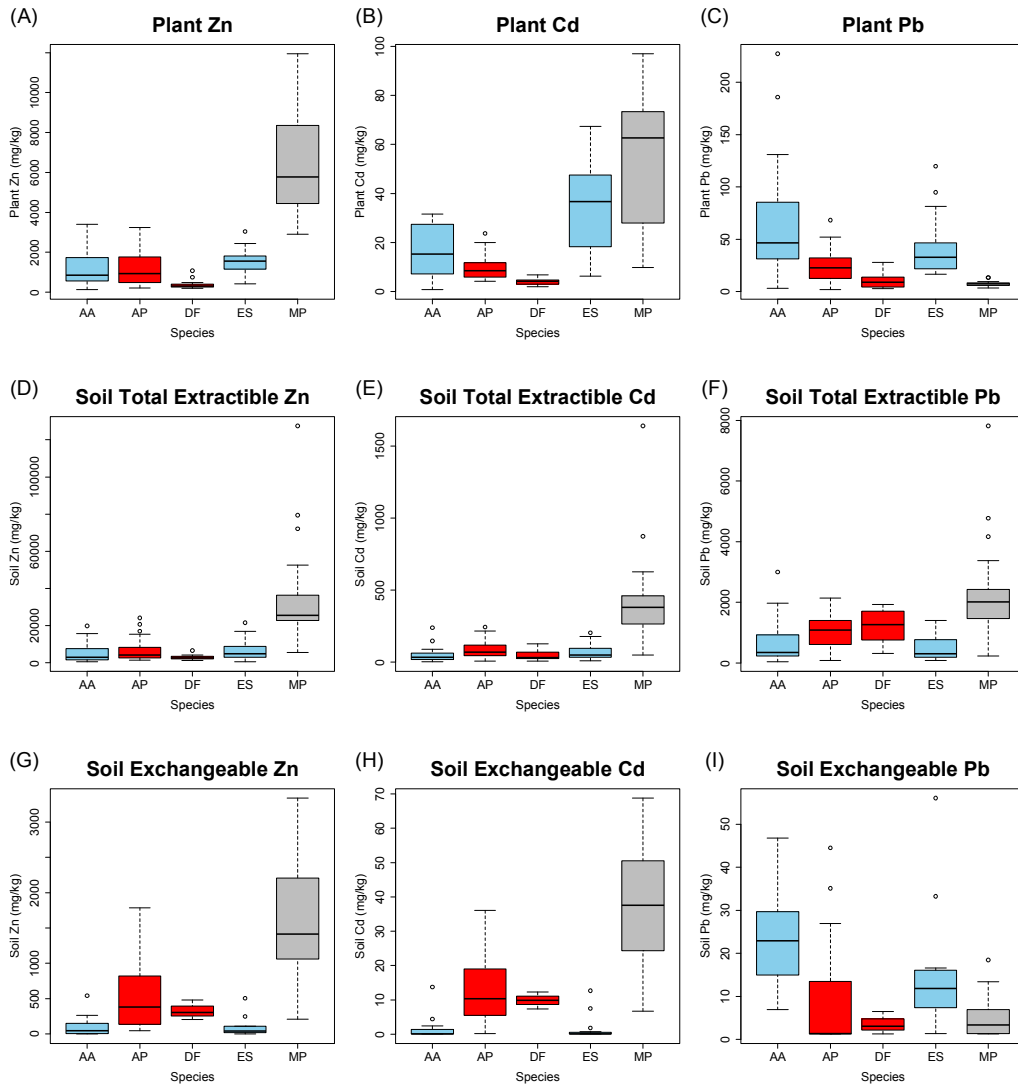


Figure 2.3: Interspecific differences in plant root colonization by AMF (A) and in the integrative soil variables pH (B), CEC (C), and base saturation (D). Species and families are abbreviated and color-coded as in Fig. 2.2.

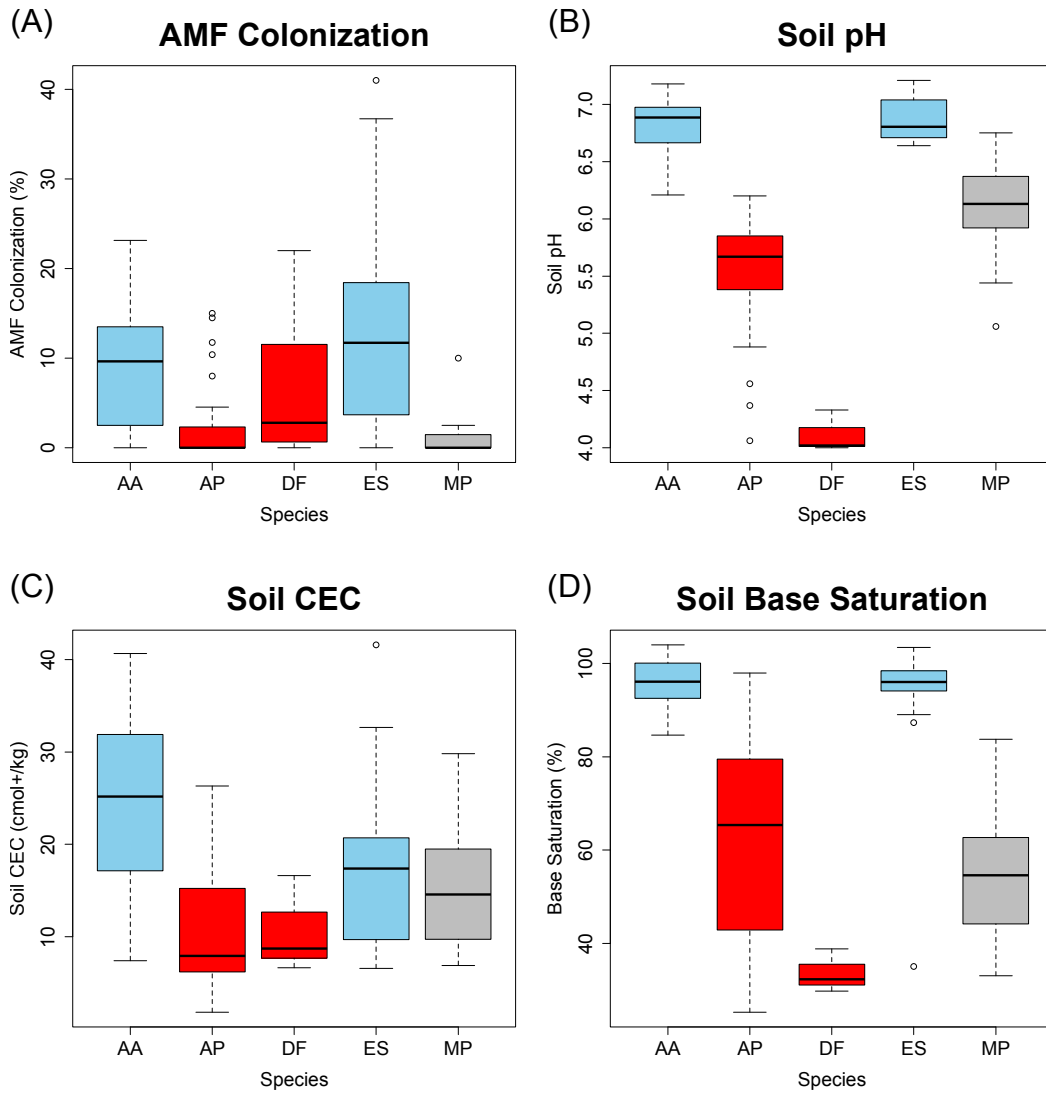


Figure 2.4: (A) Plant metal concentration profiles, (B) soil total extractable metal concentration profiles, and (C) soil exchangeable metal concentration profiles each clearly segregate plant species in CAP ordination space. Species and families are abbreviated and color-coded as in Fig. 2.2. (D-F) Contributions of individual metal concentrations, LOI, and AMF colonization (D only) to the ordination spaces in (A-C), respectively. Percentages on axis labels show the amount of constrained variation accounted for by individual CAP axes. Species DF does not appear in (C) because of insufficient sample size.

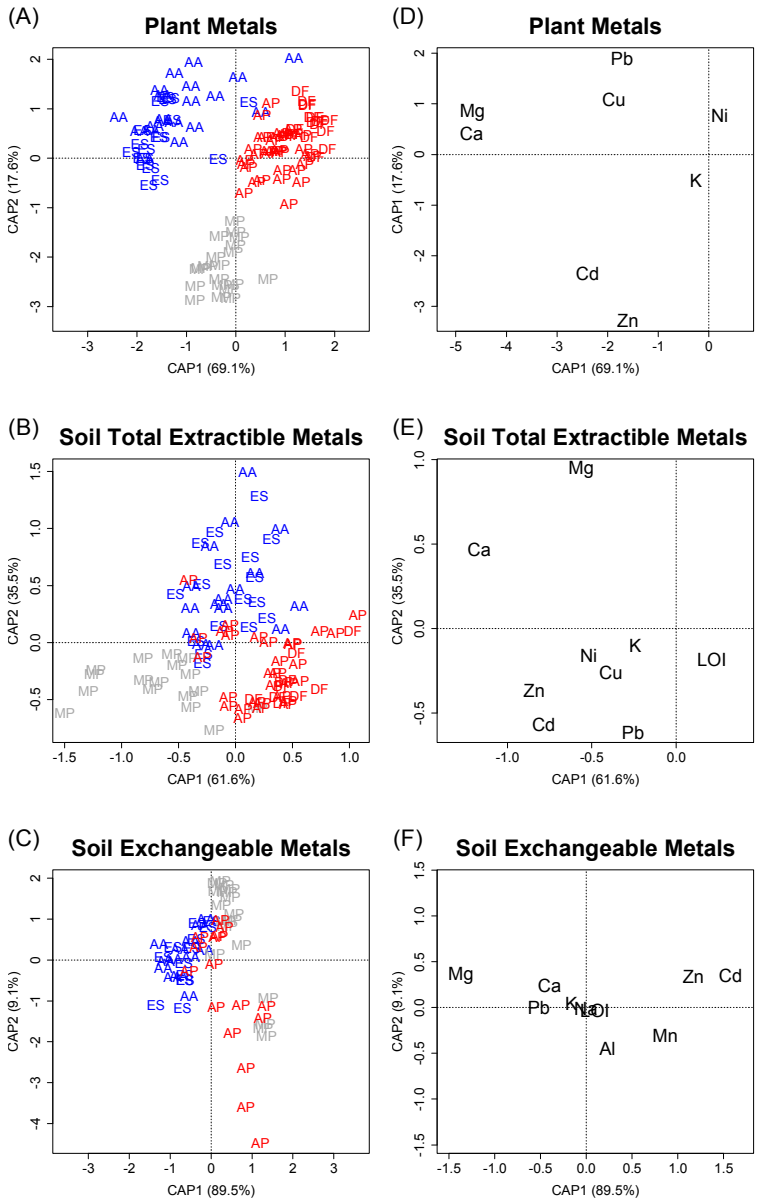
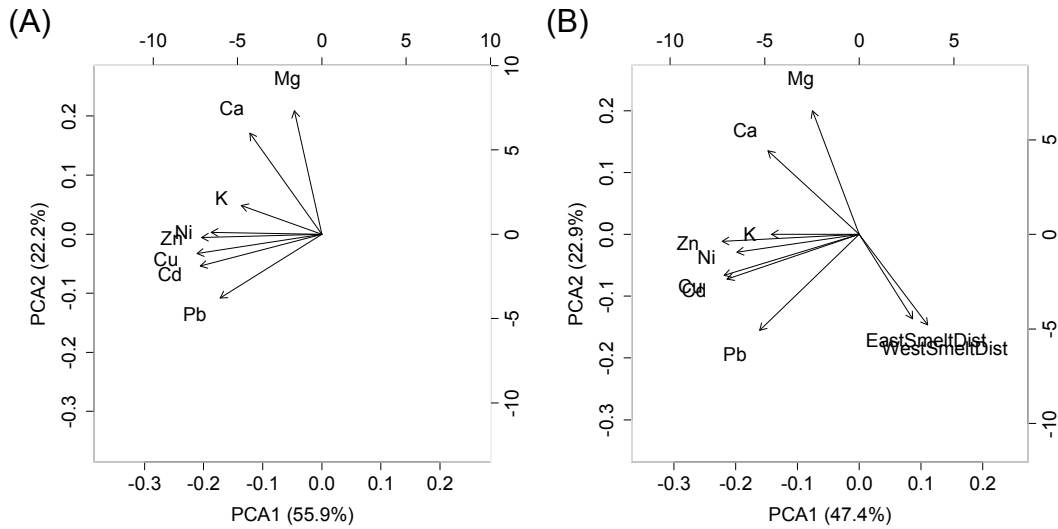


Figure 2.5: PCA ordination plots showing the correlation structure of (A) all total extractable metal concentrations measured and (B) all total extractable metal concentrations plus distance from the smelters. Arrows are biplots, which indicate the strength and direction of correlations between variables: longer arrows pointing in more similar directions are more highly correlated. In (A), PC1 is significantly positively related to distance from both smelters, and PC2 is significantly negatively related to distance from both smelters. Percentages on axis labels show the amount of variation accounted for by individual PCA axes.



Supporting Information

Figure 2.S1: Interspecific differences in plant (A-C), soil total extractable (D-F), and soil exchangeable (G-I) concentrations of the major elements Ca (A,D,G), K (B,E,H), and Mg (C,F,I). Note that the vertical axes are different for each pool of each element. Species are abbreviated as follows: AA, *Ageratina altissima* (Asteraceae); AP, *Agrostis perennans* (Poaceae); DF, *Deschampsia flexuosa* (Poaceae); ES, *Eupatorium serotinum* (Asteraceae); MP, *Minuartia patula* (Caryophyllaceae). Families are color-coded as follows: Asteraceae, blue; Poaceae, red; Caryophyllaceae, gray.

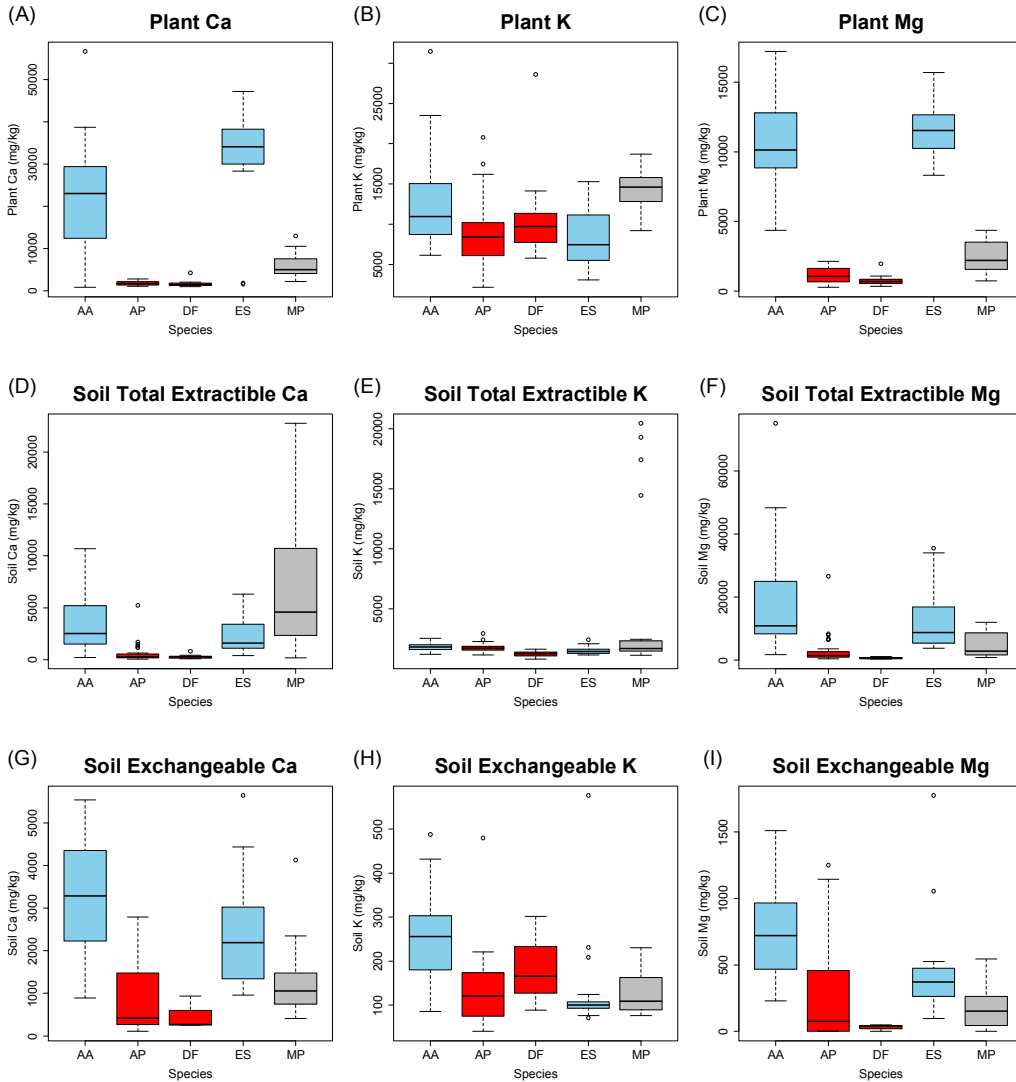
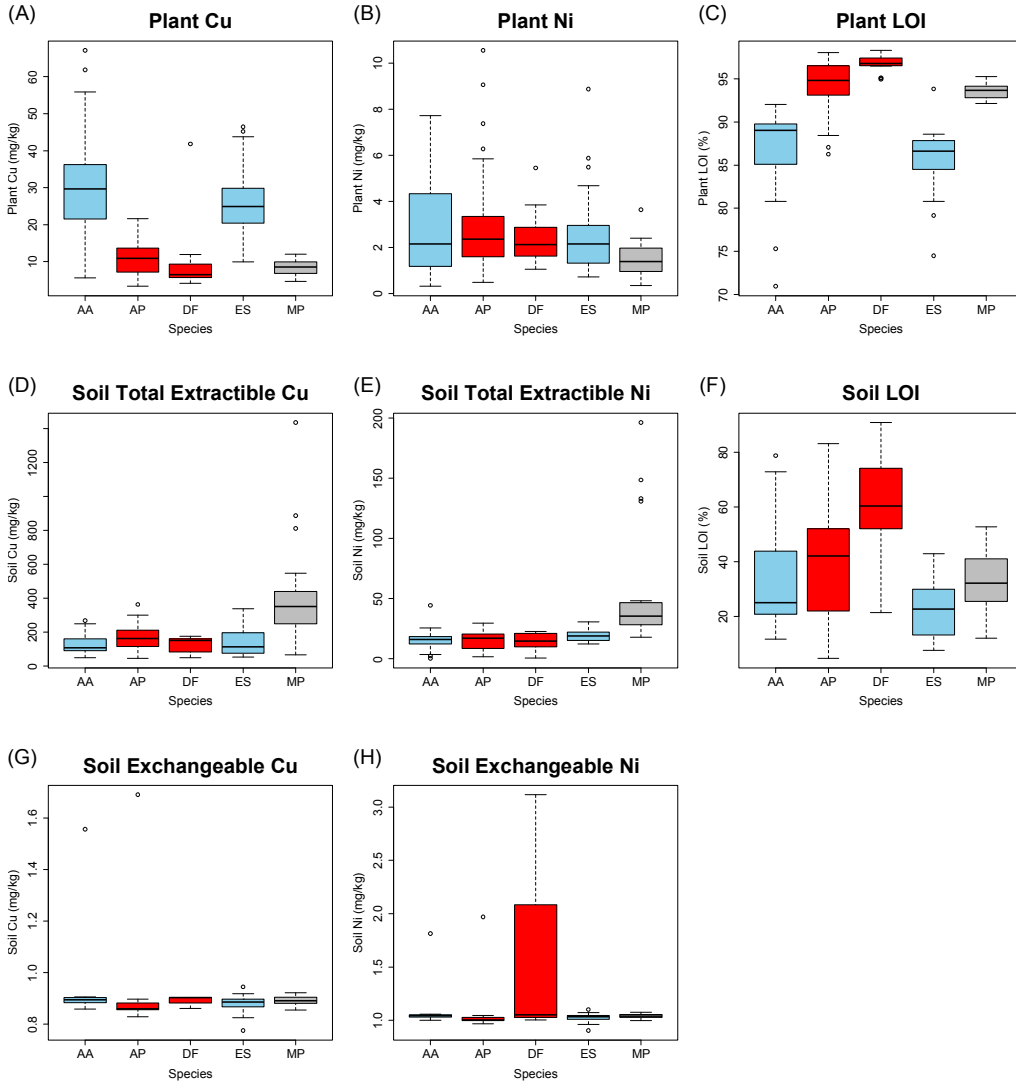


Figure 2.S2: Interspecific differences in plant (A-C), soil total extractable (D-E) and soil exchangeable (G-H) concentrations of the heavy metals Cu (A,D,G) and Ni (B,E,H), as well as organic matter measured by loss on ignition (C,F). Species and families are abbreviated and color-coded as in Fig. 2.S1.



CHAPTER THREE
CONSEQUENCES OF WOODY ENCROACHMENT INTO A RESTORED
GRASSLAND

Lee H. Dietterich, Amy Li, Sarah Garvey, and Brenda B. Casper

3.1 Abstract

Encroachment into grasslands by woody plants threatens grassland habitats and the organisms that inhabit them. Metal contamination in soils may complicate mechanisms and consequences of encroachment, as well as land managers' potential responses. We performed a series of field and greenhouse experiments to investigate the mechanisms and consequences of gray birch (*Betula populifolia*) encroachment into a grassland recently restored on a mountainside contaminated with zinc, lead, and cadmium. Over two growing seasons, we assessed the effects of gray birch on soil chemistry, soil microbes, neighboring plant community composition, and the germination and seedling performance of black oak (*Quercus velutina*) and sugar maple (*Acer saccharum*), two tree species expected to follow gray birch in succession. Gray birch was associated with increased diversity of neighboring plant communities relative to the surrounding grassland, as well as changes in mycorrhizal colonization of black oak and sugar maple seedlings, despite little effect on soil chemistry or seedling performance. We also tested whether gray birch is engaging in elemental allelopathy, a hypothesized interaction in which species with high leaf metal uptake poison their neighbors via the decomposition of contaminated leaf litter, but found no evidence of this phenomenon in our study system. We conclude that gray birch alters its environment in such a way as to

promote continued encroachment and succession of woody species. These mechanisms were independent of soil metal concentrations, suggesting that woody encroachment may behave similarly in contaminated and uncontaminated areas.

3.2 Introduction

Grasslands are endangered worldwide. They used to cover a huge area and are now reduced to just a few percent of their original extent (Browning et al. 2008; Van Auken 2009). They are important, though, because they harbor rich plant and microbial diversity (Adler et al. 2009; Frank et al. 2010; Wilson et al. 2012). They also provide valuable ecosystem services, including the formation and development of some of the most fertile soils in the world, as well as a great capacity to store carbon (Brady & Weil 2008, pp.104-106).

Encroachment by woody species can start native or restored grasslands on successional trajectories toward forest or savanna ecosystems (Browning et al. 2008; Van Auken 2009). Historically, factors such as extensive fire and grazing have maintained grasslands by preferentially killing woody colonizing species before they can become established (Van Auken 2009). However, humans have dramatically reduced fire and grazing regimes and increased habitat fragmentation, such that even pristine grasslands are increasingly vulnerable to takeover by shrubs and trees. As woody species begin to establish in grasslands, they may shade out grassland species, reduce plant diversity (Ratajczak et al. 2012), and potentially begin a series of plant-soil feedbacks that encourage succession to more woody-dominated communities. Such feedbacks could include altering soil chemistry or the composition of soil-dwelling communities of mycorrhizal fungi and other microbes in ways that favor forest species over grassland species. Plant-soil feedbacks have been shown broadly to be involved in succession (Kardol et al. 2006; Kulmatiski et al. 2008; Bauer et al. 2015), but connecting specific

feedbacks to restoration and succession remains rare in the literature, and there have been calls for more studies linking plant-soil feedbacks and succession (van der Putten et al. 2013), in particular in heavy metal contaminated soils (Krumins et al. 2015).

Here, we study how the invasion of a recently restored grassland by the pioneer tree gray birch (*Betula populifolia*) will influence succession to native deciduous forest. This rapidly colonizing species is native to the region but not to our study site (Latham et al. 2007). We compare existing plant community composition, soil chemistry, and the germination, growth, and metal uptake of representative later-successional tree seedlings in areas dominated by birch versus areas dominated by planted C₄ grasses. In our study site, the effects of gray birch and the grasses on the ecosystem are of particular interest because they are highly relevant to continued restoration and management, as well as our understanding of plant-soil feedbacks and succession.

We conducted our study at the Lehigh Gap Nature Center (Slatington, PA, USA). This land is part of the Palmerton Zinc Superfund Site (Palmerton, PA, USA), a >2000 acre mountainside severely contaminated and devegetated by Zn, Pb, Cd, Cu, and SO_x emissions from two zinc smelters that operated for approximately 80 years (Buchauer 1973; Jordan 1975; EPA 2007a; EPA 2007b). Because of the steep, rocky topography of the site, the land managers chose a phytostabilization approach to sequester the metals in place rather than attempting to remove them. They applied compost, fertilizer, lime, and seeds of primarily C₄ grasses known to have low metal uptake rates on top of the contaminated soil. The goals are for the grasses to reduce soil erosion and metal leaching

while developing healthy, uncontaminated surface soil, thereby sequestering the metals underground away from humans and the rest of the food web (EPA 2007a; EPA 2007b).

Because our grassland study system is a product of restoration on heavy metal contaminated soils, we were interested in how high metal uptake by the incoming birches might affect plant-soil feedbacks and succession. We were particularly interested in the possibility that high birch metal uptake could result in differences in soil metal concentrations between birch- and grass-dominated areas, which could then affect the growth of other plants and soil microbes including mycorrhizae. This idea that metal hyperaccumulating plants may locally enrich soil metals via contaminated leaf litter, with negative impacts on neighboring plants, is called elemental allelopathy (Wilson and Agnew 1992). Elemental allelopathy has been hypothesized to exist in metalliferous soils (Baker & Brooks 1989), where it could have major effects on metal biogeochemistry and plant community composition. However, despite a few tentative cases in the literature (Boyd & Jaffré 2001; Morris et al. 2006; Mehdawi et al. 2011), it has not yet been conclusively demonstrated (Morris et al. 2008). Due to its restoration strategy, the Palmerton site appears an ideal setting for elemental allelopathy. Birches here have the opportunity to pump metals from belowground into the uncontaminated surface layer, thereby increasing topsoil metal concentrations more strongly than might be possible elsewhere.

Thus, our study had two main goals: (1) To discern the impact of gray birch on the trajectory of grassland succession to deciduous forest, and (2) To explore the role of plant-soil feedbacks including elemental allelopathy in the succession process. To

address (1) above, we measured soil metal and nutrient concentrations, plant community composition, and the germination, growth, metal uptake, and mycorrhizal colonization of seedlings of later-successional trees in grass- and birch-dominated areas in the field. To address (2), we conducted a field experiment to ask whether soil metal and nutrient concentrations varied with distance from focal birch trees, and conducted a greenhouse experiment to ask whether contaminated gray birch leaves in particular could affect other plants' growth and metal and nutrient uptake.

We hypothesized that gray birch would increase soil metal concentrations compared to the planted grasses, and that increased soil metal concentrations combined with shading would reduce plant diversity under birch stands. Because the grasses and sugar maple host AMF exclusively while gray birch and black oak also support ECM (Harley & Harley 1987; Dickie et al. 2001; Wang & Qiu 2006), we hypothesized that colonization would be greater when target trees shared the initial vegetation's preference for mycorrhizal association, and in cleared plots where the injured birch or grasses may represent less desirable hosts to the fungi. Thus, we expected that sugar maple would grow the most in cleared grass plots, and black oak would grow the most in cleared birch plots despite increased soil metal concentrations. In the greenhouse experiment, we hypothesized that soils amended with contaminated gray birch leaves would decrease plant growth and increase plant metal concentrations compared to soils amended with uncontaminated leaves, but that the addition of any leaves may increase soil nutrients, thus increasing plant growth compared to soils amended with no leaves at all.

3.3 Methods

Gray birch effects on soil characteristics and community composition

The heart of this study is a two-year field experiment to determine to determine how seedling performance of black oak (*Quercus velutina*; Fagaceae) and sugar maple (*Acer saccharum*; Sapindaceae), both later-successional tree species, respond to grass vs birch cover. Before planting this experiment, we compared soil metal concentrations and plant community composition in experimental plots in grass vs birch dominated areas. We identified 10 locations in the Palmerton site where grassland communities grew in close proximity to dense gray birch stands. In each location, we established four 1.5 x 3.0 m experimental pots; two initially dominated by gray birch, and two initially dominated by the grasses.

We collected surface soil (primarily O_A horizon material from the top ~10 cm of soil) from near the center of each plot at the beginning of the experiment to measure its elemental concentrations. The trowel used to collect soil samples was thoroughly cleaned and sterilized with 70% ethanol between samples to avoid cross-contamination. Soils were air-dried in the lab for at least one week, and sieved to 2 mm. We then used inductively coupled plasma optical emissions spectroscopy (ICP-OES; details below) to measure their concentrations of the major nutrients Ca, Mg, and K, the known contaminants Zn, Cu, Pb, and Cd, and the heavy metals Mn and Ni that were not expected to be enriched in the site. Our procedure yields total extractible concentrations of these metals, which we interpret as the amount of metal a long-lived plant may have

access to in its lifetime, but likely more metal than is available to a plant at any given time. We also measured soil organic matter content as percent loss on ignition (LOI).

To further characterize growing conditions in our experimental plots, we measured soil moisture, temperature, and shading during the second growing season of the field experiment. At multiple time points during the season, we recorded soil moisture and temperature at three points in each experimental plot with a WET-2 sensor connected to an HH2 moisture meter (Delta-T Devices Ltd), and measured leaf area index as a proxy for shading intensity with a CI-110 Plant Canopy Imager (CID Bio-Science, Inc.).

Just before setting up the experiment in May 2014, we conducted a census of the plant community in each plot. We identified all plants in each plot to species or as precisely as possible, and estimated their percent cover within the plot. Because the planted C₄ grasses could not consistently be identified to species at this time of year, we grouped them all together into a single taxon for analysis. In September 2015, shortly before harvesting our experimental oak and maple seedlings at the end of the second growing season, we conducted another census of the plant communities in each plot, excluding our experimental tree seedlings. In this census, we could consistently identify almost all plants to species, including all of the planted grasses. In both cases, we examined species richness, total vegetative cover, cover of the planted grasses, and Shannon diversity, all with and without birch cover included.

Gray birch and aboveground competition impacts on woody seedling performance, mycorrhizal colonization, and leaf metal content

We investigated the potentially interacting effects of vegetation type and aboveground competition on the germination and seedling performance of black oak and sugar maple. In each of the ten locations above where grass and birch dominated areas grew near each other, we cleared the aboveground vegetation in one plot of each initial vegetation type. This left one plot of each initial vegetation type intact in each location. We chose not to remove belowground vegetation in order to avoid disrupting soil structure and possible variation in soil chemistry with depth.

We then planted six seedlings each of black oak and sugar maple (Musser Forests) in each plot in May 2014 and left them for two growing seasons before harvesting them in September 2015. We chose these species in particular because they are common later-successional trees in this region with congeners growing in remnant forest patches in the study site, so it is likely that they could naturally establish there. Furthermore, sugar maple, like the planted grasses, associates with arbuscular mycorrhizal fungi (AMF), whereas black oak, like gray birch, also associates with ectomycorrhizal fungi (ECM). We recognized that vegetation type (birch vs grass) could impact soils' mycorrhizal inoculum potential. Each seedling received 10-20 mL of water upon planting and again one or two days after planting, but otherwise seedlings were irrigated entirely by rainfall. We used plastic mesh tubes (Forestry Suppliers, Stock No. 17048), supported by wooden garden stakes and cable ties, to protect seedlings from deer while minimizing changes to the microclimate surrounding the trees. Every 2-3 weeks

during both growing seasons, we re-cleared all aboveground vegetation from cleared plots, and examined all planted tree seedlings, adjusting displaced protective tubes as necessary to avoid injuring the seedlings.

We measured the initial height of each tree seedling upon planting. We assessed oak and maple seedling performance nondestructively at the end of the first growing season, and harvested them for more thorough measurement at the end of the second growing season. We also assessed germination of these species by planting 20 cold-stratified seeds of each of them (Sheffield's Seed Co., Inc.) near the center of each plot and counting how many had germinated after nine weeks. At the end of the first growing season, in September 2014, we collected one leaf from each seedling with three leaves, and we collected two leaves from each seedling with four leaves or more, combined them by species and plot, and measured their metal concentrations by ICP-OES as below.

We harvested seedlings in September 2015 at the end of the second growing season, and separated their aboveground and belowground parts by clipping at the top of the root collar. We recorded aboveground plant height, fresh and dry leaf biomass, dry stem biomass, leaf area, number of leaves, and the length of apical and lateral branches grown during the current and previous growing seasons. We estimated whether leaf herbivory was absent, mild (5-20% of leaf area), moderate (20-50% of leaf area), or severe (>50% of leaf area), and noted whether leaf herbivory appeared to be due to insects, deer, or both. We also recorded whether each sample had clear signs of stem herbivory, which we expect to be primarily due to deer.

We also measured biomass and fungal colonization of seedlings' roots. We washed roots thoroughly with tap water and recorded the fresh weight of the whole root system. For black oak seedlings, we recorded percent colonization by ECM as the proportion of colonized root tips out of total root tips in a representative sample of the root system. For sugar maples, we then took and weighed sub-samples of fine roots to clear and stain for colonization by AMF and dark septate endophytes (DSE), non-mycorrhizal fungi that have been suggested to have diverse and important effects on plant growth especially in stressful habitats (Jumpponen & Trappe 1998; Lukešová et al. 2015; Wang et al. 2016). Because recording AMF colonization is incompatible with measuring the dry biomass of root samples, we took additional subsamples of sugar maple fine root systems to weigh both before and after drying in order to generate a conversion factor we could use to estimate the dry biomass of roots sampled for AMF colonization. We then dried root systems at 60°C for at least 48 h, and recorded the dry biomass of fine (≤ 1 mm diam.) and coarse (> 1 mm diam.) roots.

To quantify AMF colonization of sugar maple roots, subsamples of the fine root system were cleared in 10% KOH for 12-24 hours or as needed to remove abundant dark pigments, bleached in 9:1 household H_2O_2 : household NH_3 , acidified in 5% HCl for 15-30 minutes, and stained in hot 0.01% Trypan blue in 1:1:1 lactic acid : glycerol : water. For each sample, at least 10 root segments at least 1 cm long were mounted in parallel on a microscope slide and fixed with polyvinyl lactic acid glycerol (PVLG; INVAM 2014). We recorded percent colonization of AMF on a subset of sugar maple samples by

assessing the presence or absence of AMF structures on each 1-mm section of each root segment (modified from McGonigle et al. 1990).

To measure tree seedlings' leaf metal content, we washed leaves thoroughly with tap water, dried them at 60°C for at least 48 h, and ground them with mortar and pestle with liquid nitrogen as necessary. We then weighed the leaves into ceramic crucibles, ashed them in a muffle furnace at 475°C for at least 4 hours, let them cool, weighed them again to estimate organic matter content by loss on ignition (LOI), and digested them in 2 mL concentrated HCl for 10 minutes at 90-100°C. Digest solutions were diluted to 25 mL with ultrapure (18 Ω) water and stored at 4°C. Solution concentrations of Ca, Mg, K, Zn, Cu, Pb, Cd, Ni, and Mn were measured by inductively coupled plasma optical emissions spectroscopy (ICP-OES) using standard methods modified from Zarcinas et al. (1987). We included the standard reference materials peach leaves (NIST 1547) and either olive leaves (BCR 062) or citrus leaves (NIST 1572), as well as a reagent blank, in each digest to confirm its quality, and we further verified ICP-OES measurements by including standard solutions of known concentrations in each run. We used a similar method to measure soil metal concentrations except that soils were air-dried and sieved to 2 mm, rather than ground, prior to ashing and digestion.

Testing elemental allelopathy

To determine the extent that gray birch trees accumulate heavy metals in their leaves relative to their neighbors and explore the possibility that they engage in elemental

allelopathy, we compared leaf metal concentrations in birch leaves and the nearby planted grasses, measured topsoil metal concentrations as a function of distance from gray birch trees in the field, and conducted a greenhouse experiment to determine the phytotoxicity of gray birch leaf litter.

In July 2014, we collected leaf samples from 12 gray birch trees and 4-8 individuals each of nine planted grass species and measured their metal and nutrient concentrations by ICP-OES as described above.

We next assessed the impact of gray birch on soil chemistry, including known contaminants and major nutrients, in the field. To determine potential effects of birch on soil chemistry, in August 2013, we sampled the top ~10 cm of soil at points located 0, 50, 100, and 200 cm from the trunks of 18 gray birch trees located throughout the Palmerton site. We chose trees that were relatively large for the site (~5-10 years old, 2-4 m tall, canopy radius <2 m), and established our soil collection points along a cardinal direction chosen randomly for each tree such that the focal tree was the closest gray birch tree to each collection point. We then measured soil metal concentrations and organic matter content as described above.

We finally performed a greenhouse experiment to determine the phytotoxicity of contaminated gray birch leaves. We grew seedlings of autumn bent (*Agrostis perennans*; Poaceae), white snakeroot (*Ageratina altissima*; Asteraceae), black oak, and sugar maple individually in pots of contaminated soil to which we mixed in manually crushed gray birch leaves collected from inside or outside the contaminated site, or no leaves at all.

We used ICP-OES to measure the metal and nutrient concentrations of separate samples of contaminated and uncontaminated birch leaves. For autumn bent and white snakeroot, we collected seeds from the contaminated site in fall 2013, germinated them in autoclave-sterilized sand in spring 2014, and transplanted seedlings into experimental pots.

Seedlings of black oak and sugar maple were purchased in the same batch as those used for the field experiment above, and transplanted into experimental pots upon arrival. We chose to add the two herbaceous species to this experiment because they are understory species that colonized the Palmerton site naturally and are currently abundant there. To avoid biasing results due to transplant shock, we replaced seedlings that died within the first week of the experiment.

We performed 10 replicates of this experiment, each replicate using soil collected under a different grassland-dominated area in the Palmerton site. All soils were mixed with sand in a 6:1 soil : sand ratio to improve drainage. For each species, we planted each replicate with similarly sized seedlings to avoid confounding experimental treatments with initial seedling size. Pots used for autumn bent and white snakeroot were filled with 150 ml of soil mixed with 3 g of crushed leaves, and pots used for black oak and sugar maple were filled with 550 ml of soil mixed with 4 g of crushed leaves. The spatial arrangement of pots was randomized with respect to replicates and treatments upon planting and then repeatedly throughout the experiment. Because of differences in germination and life history, we grew autumn bent for 11 weeks, white snakeroot for 21 weeks, and black oak and sugar maple for 23 weeks.

Because our target species had different growth forms, we assessed performance differently for each. For the grass autumn bent, we measured plant height, number of tillers, and aboveground and belowground biomass. For the forb white snakeroot, we measured plant height, number of leaves, and aboveground and belowground biomass. For the trees black oak and sugar maple, we measured plant height, the length of apical and lateral branches from current and past growing seasons, the number of leaves, and the biomass of leaves, woody aboveground tissue, fine roots, and coarse roots. For a subset of five replicates of each species, we also measured leaf metal and nutrient concentrations by ICP-OES.

Statistical analysis

We used analysis of variance (ANOVA) to test for effects of vegetation type (birch vs grass) and aboveground competition (cleared vs intact) on soil metal concentrations, moisture, temperature, and light availability, as well as the growth, metal uptake, and root colonization parameters of black oak and sugar maple seedlings separately. For all parameters except soil metal concentrations, we had multiple measurements per plot and thus included plot as a random effect in those models. Models were either ANOVAs for continuous variables such as branch lengths and biomass, which were \log_{10} -transformed as necessary to improve normality, or generalized linear models (GLMs) for leaf number (poisson) or root mycorrhizal colonization (binomial).

We analyzed plant community composition data by testing for effects of vegetation type, aboveground competition, and their interaction on total cover, planted C₄ grass cover, Shannon diversity (ANOVA), and species richness (GLM) of plant communities with gray birch excluded. When relevant, we repeated analyses with gray birch included to disentangle this species' own cover from its effects on the cover of other plants.

To investigate herbivory on our field oak and maple seedlings, we used chi-squared tests to test for associations between experimental treatments and insect, deer, and combined leaf herbivory. We constructed three contingency tables per herbivory type per plant species to test for associations between leaf herbivory and our experimental variables, vegetation type and aboveground competition, separately and together. Because of the low incidence of moderate and severe leaf herbivory on sugar maples, we re-coded sugar maple leaf herbivory as presence/absence of herbivory to avoid violating the assumptions of the chi-squared test. We used a similar chi-squared approach to ask whether the presence or absence of stem herbivory was associated with our experimental treatments.

We used two-tailed t-tests to test for differences in birch versus grass leaf concentrations of each metal individually. We used two-tailed tests rather than one-tailed tests to account for the possibility that certain elements, especially those not emitted by the smelters, might have greater concentrations in soils outside of the contaminated site. Because of the multiple tests performed, we adjusted our significance thresholds using the Dunn–Šidák correction for multiple comparisons.

We used linear regressions to test whether soil concentrations of each element varied significantly with distance from the nearest gray birch tree, applying the Dunn–Šidák correction to account for multiple tests. Element concentration data was \log_{10} -transformed before analysis to improve normality. To account for potential differences in background metal concentrations underneath the trees we sampled, we repeated this analysis with each metal concentration expressed as the change in concentration from the base of the trunk.

In our greenhouse experiment, we used analysis of covariance (ANCOVA) to test for an effect of litter treatment (contaminated, uncontaminated, or none) on each plant performance and metal concentration measure. We included depth to soil surface as a covariate in all models to account for potential differences in the amount of soil, and therefore nutrients, available to individual plants. We repeated these analyses excluding samples with no litter added to be sure to detect effects predicted by elemental allelopathy, namely decreased performance in soils amended with contaminated leaves than with uncontaminated leaves, if they were present.

3.4 Results

Gray birch effects on soil characteristics and community composition

Soil organic matter content was marginally higher in grass plots than birch plots (34.6% vs 25.9%; $P < 0.1$), but soil heavy metal and base cation concentrations did not differ among plot types at the beginning of the experiment. Soil moisture was marginally

greater in grass plots and cleared plots (cleared grass 16.5%, intact grass 14.4%, cleared birch 13.4%, intact birch 9.42%; $P < 0.1$ for both main effects). Soil temperature was significantly greater in cleared plots than intact plots (30.7°C vs 29.0°C; $P < 0.05$), but did not differ between grass and birch plots. Leaf area index, a proxy for aboveground competition, was significantly greater in intact plots than cleared plots (1.36 vs 0.37; $P < 0.001$), but did not differ between birch and grass plots (Table 3.1).

Our spring 2014 census of the field plots before clearing and planting showed that birch plots had lower cover of species excluding gray birch (68.8% vs 97.1%; $P < 0.001$), similar species richness, but greater Shannon diversity (0.23 vs 0.043; $P < 0.05$) than grass plots. Cover of the planted C₄ grasses did not differ significantly among plot types. When cover by gray birch trees was included in the models, birch plots had significantly greater vegetative cover (144% vs 97.2%; $P < 0.001$), greater species richness (5.5 vs 3.5; $P < 0.05$), and greater Shannon diversity (0.97 vs 0.43; $P < 0.001$) than grass plots. Richness and diversity here are underestimates of their true values because we grouped all planted C₄ grasses into a single taxon for this analysis. Plots designated to be cleared did not differ significantly from plots designated to be left intact.

Our fall 2015 census just before harvesting the experiment, in which we could distinguish the planted grass species, showed that vegetative cover excluding gray birch trees was about twice as high in intact plots (96.4% vs 48.3%; $P < 0.001$), but did not differ between birch and grass plots. Cover of the planted grass species was greater in intact plots than cleared plots (60.6% vs 26.3%; $P < 0.001$), but did not differ between birch and grass plots. Species richness did not significantly differ among plot types, but

birch plots again had greater Shannon diversity than grass plots (0.55 vs 0.27; $P < 0.001$). Adding birch cover into the analysis resulted in a significant interaction ($P < 0.01$) in which intact birch plots had the greatest vegetative cover (182%), followed by intact grass plots (99.0%), followed by cleared grass and birch plots (54.8% and 42.6%, respectively) (Fig. 3.1).

Gray birch and aboveground competition impacts on woody seedling performance, mycorrhizal colonization, and leaf metal content

Black oak seed germination was significantly greater in cleared grass plots and intact birch plots, but sugar maple seed germination was not affected by experimental treatments (Fig. 3.2a,b). After two growing seasons, black oak seedlings had significantly more leaves (Fig. 3.2c) and lateral branches, and greater lateral branch length, in cleared plots than in intact plots. Black oak seedlings also had marginally greater stem and total biomass in cleared plots (Fig. 3.2e). Sugar maple seedlings showed no significant effects of experimental treatments on branch length, branch number, leaf number (Fig. 3.2d), leaf biomass, or stem biomass. Neither species' fine, coarse, or total root fresh or dry weight, or whole plant biomass (Fig. 3.2e,f) responded significantly to experimental treatments. Leaf area and the fresh weight of leaves and stems did not respond to experimental treatments for either species. Seedling height increments from the beginning to the end of the experiment were frequently negative, and did not respond significantly to experimental treatments (Fig. 3.2g,h).

Colonization of black oak roots by ECM was marginally greater in birch plots than grass plots (25.7% vs 14.7%; $P < 0.1$ by GLM; Fig. 3.3a). Colonization of sugar maple roots by AMF experienced a significant interaction in which cleared grass plots had greater colonization than the other plot types ($P < 0.001$; Fig. 3.3b). Sugar maple root colonization by DSE was greater in birch plots than in grass plots (29.3% vs 11.4%; $P < 0.001$; Fig. 3.3c), and greater in cleared plots than intact plots (21.4% vs 19.3%; $P < 0.05$; Fig. 3.3c).

After the first growing season, black oak leaves had marginally higher Al, K, and Cu concentrations in cleared plots than intact plots ($P < 0.1$). Black oak leaf Cu concentrations were also marginally higher in birch plots than grass plots ($P < 0.1$). Sugar maple leaves had marginally greater Zn and Pb ($P < 0.1$), and significantly greater Cd ($P < 0.05$) in cleared plots than intact plots.

Herbivory on leaves of both black oak and sugar maple was primarily attributed to insects. We observed a significant increase in oak leaf herbivory in birch plots relative to grass plots, whether we examined all herbivory or insect herbivory, but not deer herbivory (Table 3.2a). There was no association between leaf herbivory and any of our experimental treatments for sugar maple. Herbivory on stems of black oak and sugar maple also did not significantly relate to our experimental treatments (Table 3.2b).

Testing elemental allelopathy

Gray birch leaf concentrations had approximately tenfold greater concentrations than grass leaves of the pollutants Zn and Cd, but not Pb (Fig. 3.4). Despite this difference in leaf metal concentrations, there were no significant trends in soil organic matter, base cation, or heavy metal concentration concentrations with distance from gray birch trees. Analyzing data with the concentration at the base of each tree subtracted to account for differences in background soil metal contamination among different trees showed soil Ni and Pb concentrations increasing with distance from gray birch tree ($P < 0.05$), but these effects were weak ($R^2 < 0.06$), and were not significant after correcting for multiple comparisons.

For our greenhouse experiment, we validated the use of uncontaminated gray birch leaves as a control by confirming that gray birch leaves collected inside the Palmerton site had significantly higher concentrations than those collected in uncontaminated sites for the contaminants Zn (2846 ppm vs. 1410 ppm), Pb (31.5 ppm vs. 10.2 ppm), and Cd (17.8 ppm vs 8.4 ppm) ($P < 0.006$, the threshold determined by the Dunn-Šidák correction). Contaminated and uncontaminated gray birch leaves did not differ significantly in the concentration of any of the other elements we measured, including the base cations Ca, K, and Mg, the suspected contaminant Cu, and the heavy metal Ni that was not emitted by the smelters.

Growth and element uptake responses of our four target species exposed to these litter treatments were species-specific and often minimal. Moreover, none of these species showed predicted signatures of elemental allelopathy, which would be decreased

growth and/or increased Zn, Pb, or Cd concentrations in the contaminated litter treatment relative to the uncontaminated litter treatment.

One species showed reduced growth in the presence of birch leaves regardless of contamination level. Aboveground biomass, belowground biomass, and tiller number of the grass autumn bent were about twice as large in pots without litter added (Fig. 3.5a). However, while this species' growth responded negatively to the addition of gray birch leaf litter, it did not respond differently to contaminated versus uncontaminated litter. The later-successional tree black oak had increased more in height and had greater fine root biomass in pots amended with contaminated gray birch leaf litter than either uncontaminated or no litter (Fig. 3.5b,c). Our other two target species, the forb white snakeroot and the tree sugar maple, did not respond to the litter treatments in either their growth or their leaf contaminant concentrations.

3.5 Discussion

We found that gray birch, the primary woody colonizing species in our study grassland, is associated with changes in the ecosystem that may feed back to increase succession to forest. In particular, birch-dominated areas had lower cover but higher diversity of other plants than grass-dominated areas. We suggest that birches foster greater diversity but lower productivity of herbaceous species by creating partially shaded areas where the grasses are less dominant, thereby facilitating colonization by other species. Birch trees may further facilitate colonization by providing perches for

seed-dispersing birds (e.g. Wenny & Levey 1998). Our mycorrhizal colonization results also suggest that gray birch fosters a shift in mycorrhizal communities, from AMF-dominated under grasses to a more mixed community of AMF, ECM, and non-mycorrhizal endophytes under birches. Shifts between AMF and ECM have been associated with succession and other plant community shifts before (Treseder et al. 2004 AMF to ECM; Williams et al. 2013 ECM to AMF), and in our site this could facilitate continued succession via increased recruitment or growth of ECM-reliant woody species (Pringle et al. 2009).

Patterns of black oak germination and leaf herbivory support the idea that grass and birch dominated areas differ in a way that is meaningful to later-successional trees. We speculate that the ECM favored by the oaks are more abundant under birch than grasses, and that these fungi may improve seedling survival even in the first few weeks of life. As for herbivory, it is possible that birch trees create microhabitats more favorable to the particular insects eating our seedlings' leaves, but it is also possible that in another growing season with different conditions, this trend might disappear or be reversed.

However, for the majority of parameters we measured, we found no differences in the growth or metal uptake of our target black oak and sugar maple seedlings in birch versus grass dominated areas. We hypothesize multiple possible explanations for this. First, it is possible that the differences we observed in mycorrhizal colonization could represent a mechanism of plant-soil feedback giving rise to important differences in plant performance over time. However, the effects on the performance of our experimental tree seedlings may take longer than our two growing season experiment to appear, or

some other variable may be counteracting them. Despite our efforts to protect seedlings from deer, many seedlings showed clear signs of insect and deer herbivory. We find it unlikely that insect herbivory would distort our data dramatically, but if deer preferentially ate larger, healthier seedlings (Price 1991; Blaisdell et al. 2015), that could cause significant discrepancies between our seedlings' actual growth and the growth we observed at the end of the season.

Alternatively, it is possible that while the differences in mycorrhizal colonization we observed were statistically significant, they are not biologically meaningful or may not persist over subsequent growing seasons. While percent colonization is the most commonly used predictor of the strength of a mycorrhizal symbiosis, this is difficult to verify and the method has potentially serious limitations (Smith & Read 2008, pp.81-86). Furthermore, the similarity of soil chemistry, moisture, temperature, and light availability among plot types, combined with the non-differences we observed in most plant performance measures, suggest that our experimental plots may look more similar from the perspective of a tree seedling than they do to us.

We conclude that woody colonizers of grasslands can affect nearby plant and soil microbial communities in ways that facilitate continued succession to forest communities, even in the absence of clear effects on the growth of later-successional tree seedlings. This is consistent with literature on PSFs and succession (e.g. Kardol et al. 2006; Kulmatiski et al. 2008; Bauer et al. 2015) and observed declines in grasslands that lack the fire or grazing disturbances that maintain them.

Despite our study system appearing to be an ideal setting for elemental allelopathy, we consistently saw no evidence of this interaction. Birch leaves are substantially enriched in the pollutants Zn and Cd compared to the surrounding grasses and to most other plants in the site. Furthermore, relatively uncontaminated compost was applied throughout our study area during restoration (EPA 2007a; EPA 2007b), thereby giving birch trees a seemingly ideal opportunity to dramatically enrich topsoil metal concentrations around them. However, our soil metal concentration data indicates that topsoil metal concentrations remain high and vary widely, even among samples collected within less than a meter of each other. Such variation in soil metal concentrations is not unprecedented (Ch. 2; Yang et al. 2013), and appears to mask any effects that birch versus grass litter may have on soil chemistry.

Our greenhouse experiment, designed to isolate contaminated birch leaves as phytotoxic agents, showed no difference in the growth of plants given contaminated versus uncontaminated birch litter, except that black oak seedlings counterintuitively grew slightly greater height and fine root biomass when given contaminated litter. Further examination would be needed to determine whether the reduction in biomass of the grass autumn bent when amended with contaminated or uncontaminated birch litter is due to traditional allelopathy or to some other effect of gray birch leaf litter on the chemistry or biology of the soil. Finally, our field experiment also did not show the reduction of oak and maple performance in birch plots relative to grass plots that would be predicted under elemental allelopathy.

We hypothesize that more stringent conditions must be necessary for elemental allelopathy to play an important role in the field. Effective elemental allelopaths may need some combination of greater leaf metal concentrations, greater leaf biomass, accumulation of more toxic elements, or more time to condition soils, than are found at the Palmerton site. Furthermore, while we do not dispute previously reported cases of elemental allelopathy (e.g. Mehdawi et al. 2011; see also Morris et al. 2008), we emphasize that researchers seeking to demonstrate this phenomenon must show that the focal plant species actually causes local increases in soil metal concentrations, and does not simply establish preferentially in pockets of high soil metal concentration (Morris et al. 2008). Because instances of high plant heavy metal uptake are likely to far outnumber instances of elemental allelopathy, we must consider alternate explanations for the evolution of high plant metal uptake rates, including metal tolerance mechanisms and defense against herbivory (Baker & Brooks 1989; Boyd & Martens 1998).

Our finding that gray birch is not engaging in elemental allelopathy in the Palmerton site alleviates some, but not all, of the management concerns associated with this species there. We find it unlikely that the site will turn into a gray birch monoculture over time as some managers fear. We are further encouraged by recent findings of red oak recruitment in gray birch dominated areas of Liberty State Park in New Jersey (Cullen et al. 2016) and personal observations of other coniferous and broad-leaved species coexisting with gray birch in all but the steepest, rockiest slopes of a series of hills near coal mines between Lansford, Nesquehoning, and Coaldale, PA. However, lingering high topsoil metal concentrations and mobilization of metals from gray birch

leaves into the local food chain remain major concerns. We urge other researchers to investigate the extent of these environmental and human health threats.

The implications of our findings on succession for site management are less straightforward. The current grassland in the site represents a habitat rare in Pennsylvania, which hosts many rare plant and insect species (Latham et al. 2007), so many stakeholders strongly desire to maintain the site as a grassland. Continuation of controlled burns is likely to be effective in suppressing birch and other woody species in favor of the grasses (Van Auken 2009). However, repeatedly burning such a large area at sufficient frequency to maintain the grassland would be extremely expensive, and risks releasing metals into the air with smoke. On the other hand, allowing continued succession to forest would likely result in a plant community with soil stabilization and low plant metal uptake properties similar to the grasses, though abundant deer herbivory may compromise the return of many desirable native forest trees (Horsley et al. 2003; White 2012), and erosion may be increased depending on the condition of the understory (e.g. Puttock et al. 2014). In light of the mixed benefits and drawbacks of these different habitat types, we support a recent suggestion (Lehigh Gap Nature Center “Desired Future Conditions,” July 8, 2016) that parts of the site be managed to maintain grassland ecosystems, and others be allowed to return to forest. This approach, combined with careful and proactive monitoring, will also provide valuable opportunities to study the ecology of grasslands under these two very different management strategies.

3.6 Acknowledgments

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Table 3.1: Average soil moisture, temperature, organic matter concentration, and leaf area index (a proxy for aboveground competition) in experimental field plots. Plot treatments are abbreviated as follows: Birch, B; Grass, G; Cleared, C; Intact, I.

Variable	Plot type				Difference
	BC	BI	GC	GI	
Soil moisture (%)	13.5±1.7	9.42±1.4	16.5±1.8	14.4±2.0	G>B (.); C>I (.)
Soil temperature (°C)	31.2±0.50	29.0±0.44	30.2±0.46	29.0±0.44	C>I *
Soil organic matter (% LOI)	22.5±3.9	29.2±5.2	35.3±4.8	33.9±5.1	G>B (.)
Leaf area index	0.313±.045	1.55±0.23	0.383±0.24	0.954±0.11	I>C ***

Table 3.2: Number of seedlings experiencing different classes of leaf herbivory in (A) black oak and (B) sugar maple seedlings grown in birch (B) or grass (G) dominated areas in the field with aboveground competition cleared (C) or left intact (I). Chi-squared tests of these and related tables showed that black oak leaf herbivory was significantly associated with vegetation type, but not with aboveground competition or their combination. Sugar maple leaf herbivory was not significantly associated with any experimental treatment. Classes of leaf herbivory are defined as follows by the amount of leaf area apparently missing: None, <5%; Minimal, 5-20%, Moderate, 20-50%; Severe, >50%.

(A) Black oak leaf herbivory				
Treatment	None	Minimal	Moderate	Severe
BC	28	18	11	1
BI	23	18	11	2
GC	30	14	8	1
GI	41	10	4	2

(B) Sugar maple leaf herbivory				
Treatment	None	Minimal	Moderate	Severe
BC	42	7	1	0
BI	43	9	3	0
GC	36	13	4	0
GI	40	8	2	0

Figure 3.1: Average \pm SE cover of gray birch (orange bars), the planted grasses (green bars), and all other species (blue bars) in experimental field plots at the end of the experiment in September 2015. Plot treatments are abbreviated as follows: Birch, B; Grass, G; Cleared, C; Intact, I.

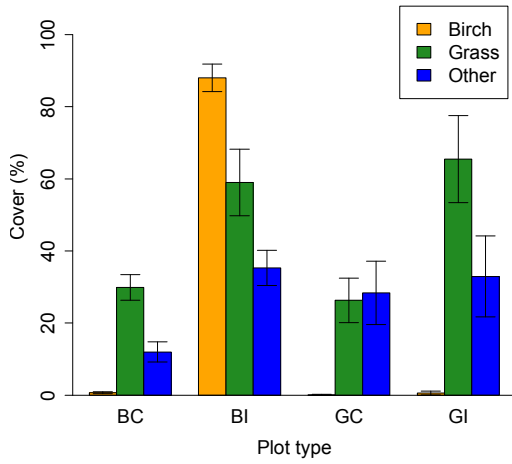


Figure 3.2: Germination (A,B), leaf number (C,D), biomass (E,F), and height increment distribution (G,H) of black oak (A,C,E,G) and sugar maple (B,D,F,H) seedlings grown in the field experiment. Black oak leaf number and biomass were significantly greater in cleared plots than intact plots ($P < 0.05$), and oak germination experienced a significant interaction between the vegetation and competition treatments ($P < 0.05$). Maple leaf number and germination, and both species' biomass and height increments did not significantly differ with experimental treatments. Significance codes: *, $P < 0.05$; NS, not significant.

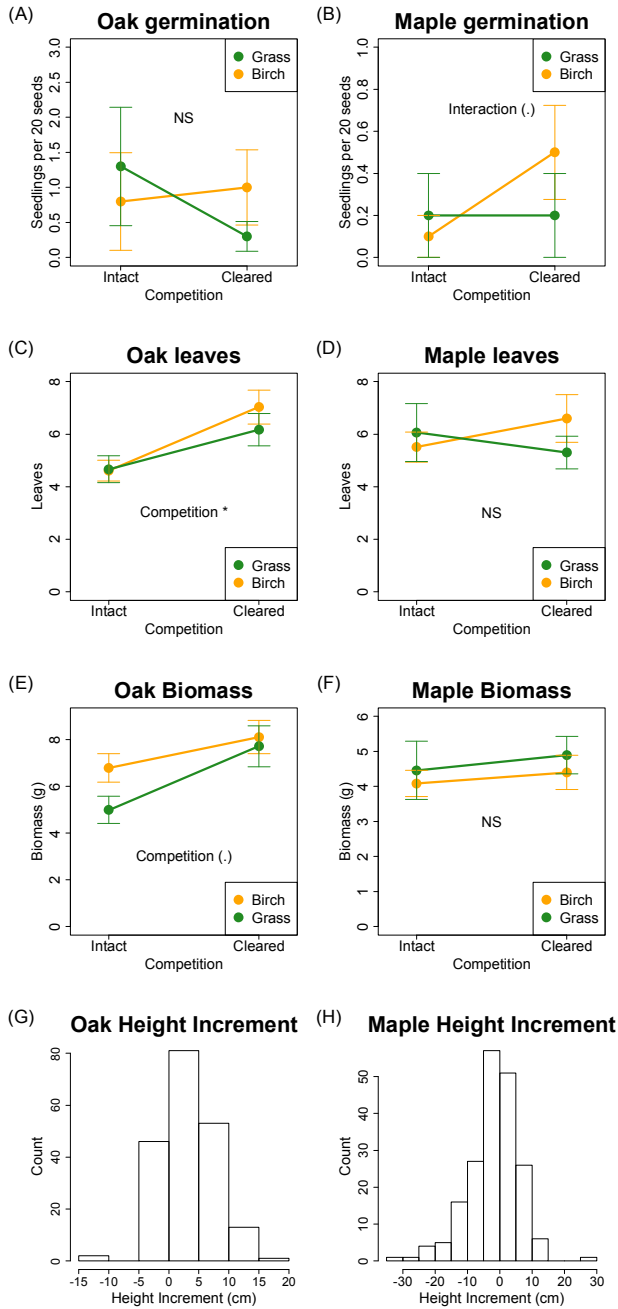


Figure 3.3: Average \pm SE percent colonization of (A) black oak roots by ECM, (B) sugar maple roots by AMF, and (C) sugar maple roots by dark staining fungal endophytes (DSE) after two growing seasons in the field.

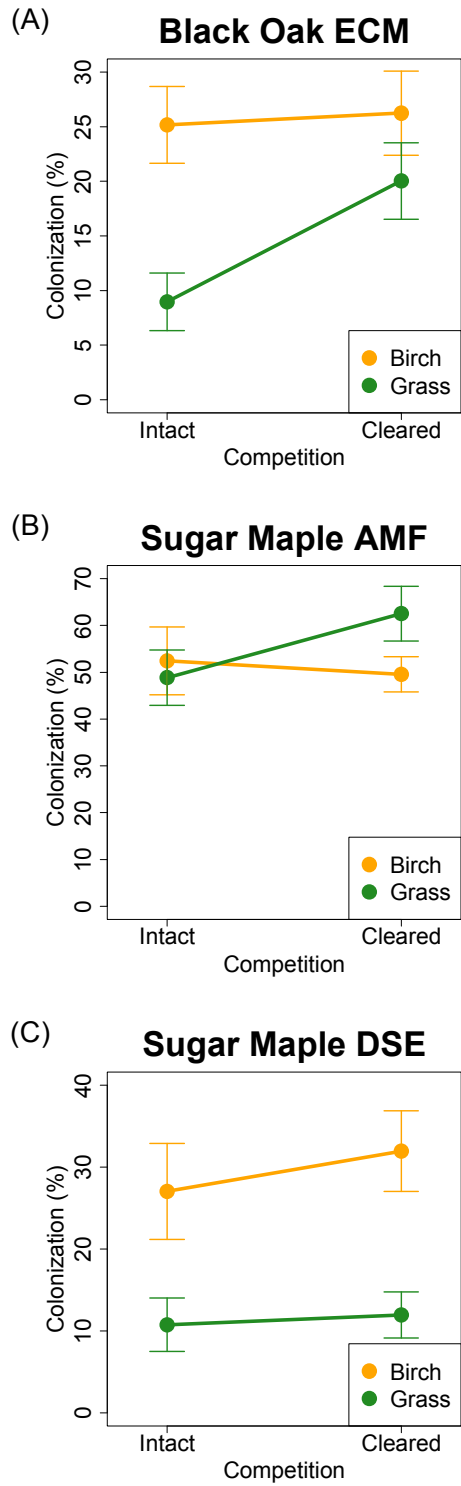


Figure 3.4: Average \pm SE concentrations of the contaminants Zn, Cd, and Pb in leaves of gray birch (orange bars) and the planted grasses (green bars) in the Palmerton site.

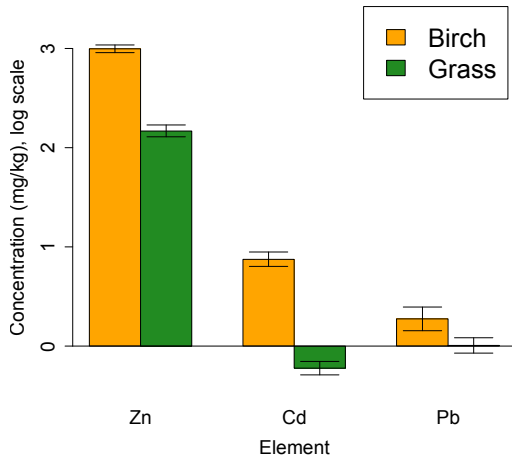
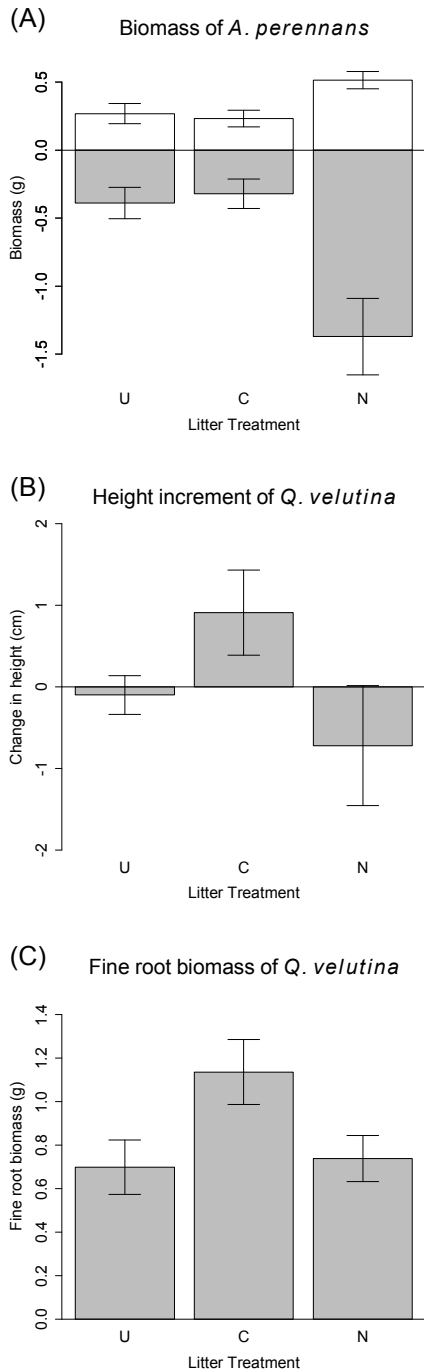


Figure 3.5: (A) Average \pm SE aboveground and belowground biomass of *A. perennans* exposed in the greenhouse to contaminated (C), uncontaminated (U), and no (N) gray birch leaf litter. Bars above the x-axis represent aboveground biomass, and bars below the x-axis represent belowground biomass. (B) Average \pm SE change in height and (C) average \pm SE fine root biomass in *Q. velutina* seedlings exposed to the same gray birch leaf litter treatments.



CONCLUSION

Implications for plant-soil feedbacks and restoration

In my first chapter, I showed that even subtle differences in the characteristics of compost amendments applied early in restoration can have long-lasting effects on plant community composition rivaling effects of different species composition of seed mixes. This shows that even seemingly minor differences in initial restoration conditions can impact succession, and that practitioners must carefully consider the many details of restoration strategies in order to come up with the most effective option. Surprisingly, the group of grass species that differed among the different seed mixes did not give rise to plant-soil feedbacks (PSFs) with detectable signatures on plant community composition nine years after planting. Rather, most of these species appeared to die out due to competition with taller grasses found in all seed mixes. However, if seed mixes were to have completely non-overlapping species composition and be planted over sufficiently large areas, the resulting communities would surely differ. There is much well placed interest in connecting the details of seed applications to subsequent community structure (Mijnsbrugge et al. 2010; Merritt & Dixon 2011). However, the separation we observed between plant- and animal-derived composts speaks in a broader sense to the importance of plant-induced soil development to the long-term succession of plant communities.

In my second chapter, I found a close relationship between plant and soil chemical characteristics largely independent of root colonization by arbuscular mycorrhizal fungi (AMF). The existing literature on the relationships between soil contamination, AMF colonization, and plant metal uptake is messy and consists mainly of disjointed case studies conducted under more or less unrealistic greenhouse conditions. I used a more thorough field-based approach, in which I simultaneously analyzed plant and soil metals and AMF colonization for nine metals and five plant species growing in similar conditions. This is more plant species and metals than any such study has investigated at once so far, and apparently the first study to meaningfully examine mycorrhizal colonization and plant metal uptake in the field. Thus, with these data I can make more general conclusions about the relationships between soil metals, AMF, and plant metals than have yet been possible.

As for the role of AMF in this system, I found that AMF colonization of my study plants' roots was consistently lower than has been previously reported for these species elsewhere. AMF colonization rates responded little to variation in soil contamination, though, and had no effect on plant metal uptake. I hypothesize that the metal contaminants reduced AMF abundance at Palmerton, resulting in the site favoring colonizing plants with little to no reliance on AMF. On the other hand, many of the planted grasses that continue to flourish on the site's lower slope are known to be heavily reliant on AMF (Wilson & Hartnett 1998; Glassman & Casper 2012). It is likely that the compost amendments applied there contained AMF propagules and thus bolstered the remnants of the native AMF community. The absence of soil amendments as a possible

source of AMF propagules at higher elevations may also help explain why the planted grasses are being so slow to colonize the upper slope (Latham et al. 2007), even though AMF spores were applied there with fertilizer (J. Lansing, pers. comm.).

This project also demonstrated that soil metal contamination in the site is highly variable locally, with a distribution far more complex than the simple gradient away from the smelters that has been assumed (Buchauer 1973; Johnson & Richter 2010; Glassman & Casper 2012). Based on this information, I also propose a solution to the longstanding mystery of why the small-statured *Minuartia patula*, a known zinc hyperaccumulator, persists in near-monocultures in patches. This species typically occurs on patches of dark, powdery soil, rarely co-occurs with other, taller species, and has not been documented outside the contaminated area despite being there for over 60 years (Pretz 1954). Based on the exceptionally high contamination I measured in these soils, I suggest that *M. patula* maintains its dominance in these patches by tolerating metal concentrations toxic to most other plant species.

In my third chapter, I have shown that gray birch, a pioneer tree encroaching on the restored grassland, affects its environment in such a way as to facilitate continued succession to forest. This result helps explain the difficulty of reversing woody encroachment once it has begun. Importantly, these effects were independent of soil metal contamination, suggesting that the mechanisms and consequences of woody encroachment into grasslands may be similar in metalliferous and nonmetalliferous sites. In terms of plant-soil feedback, this study also provided evidence that the abundance and types of mycorrhizal fungi colonizing roots of young later-successional trees depends on

local conditions including species composition and aboveground competition. Although the variation in colonization did not yet affect the growth of my experimental tree seedlings, these results suggest a clear mechanism of plant-soil feedback that could have major and long-lasting effects on plant community composition and succession. In systems threatened by unwanted woody encroachment, I advocate early, proactive management to remove woody species before they are able to displace much of the herbaceous community.

Implications for the Palmerton site

The Palmerton restoration has been successful in many ways. Applying compost where possible, and then fertilizer, lime, and grass seeds throughout the mountain has resulted in abundant growth of the desired grasses throughout the restoration area. The grasses appear to provide many of their predicted benefits: they provide lush vegetative cover on top of the mountain's rocky, contaminated substrate (Ch. 1), and clearly slow erosion and transpire abundantly, presumably reducing mobility of the metal contaminants toward groundwater. They have lower foliar metal uptake rates than most of the other species on the mountain (Ch. 3 and unpublished data), which also likely reduces metal movement into the food chain.

However, numerous challenges remain for restoring the mountain. High surface soil metal concentrations clearly present continued threats to human health and the health of plants and animals living there, and will likely impact the ecosystem for centuries. I

have shown evidence for continued effects of the Palmerton metals on the ecosystem in the form of elevated metal concentrations in gray birch leaves, the existence of *M. patula* inside but not outside the site, and low root colonization by arbuscular mycorrhizal fungi in the site compared to colonization data from the literature (Ch. 2 and 3). I offer further anecdotal supporting evidence in my observations of numerous ants and occasional grubs, but a complete lack of earthworms in the hundreds of holes I have dug in the site in the course of this work.

Gray birch encroachment into the site represents a major juncture in terms of management. I have shown in Chapter 3 that gray birch is altering its environment in a way that appears to facilitate continued succession to forest. I predict that the resulting forest will be similar in appearance to nearby forests, though with a somewhat different competition of dominant trees because deer herbivory on many native tree species is severe in the region, can affect forest regeneration (Horsley et al. 2003; White 2012), and was substantial in my experiment despite my precautions. This succession could ostensibly be reversed by widespread and aggressive intervention, likely in the form of frequent controlled burns, to return birch-dominated areas to grasslands. However, such burns would require greater logistical and financial resources than appear to be available, and also risk creating a public health hazard by releasing significant quantities of metals in smoke.

If some or all of the Palmerton site were to return to forest, it would remain largely consistent with the site's restoration goals, which are to cost-effectively increase vegetative cover, transpiration, and soil development, and reduce erosion and metal

leaching. Indeed, before planting the current grassland, restoration efforts actively sought to re-establish forest on the site (EPA 2007). Like grasslands, forests are effective at stabilizing soil and reducing erosion and leaching. With the exception of gray birch, most of the woody species currently growing in the site that would be most likely to dominate a forest have relatively low leaf metal uptake rates (Li, Dietterich, and Casper unpublished data). The land managers are concerned that such a forest would become a gray birch monoculture, citing observations of birch-dominated communities on coal mining waste piles in the nearby towns of Nesquehoning, Coaldale, and Lansford, PA. My own observations of these sites, though, suggest that while it is possible for a slope to be sufficiently steep and rocky that only gray birch can live there, even minor ameliorations in slope or soil quality can allow colonization by pines and other deciduous trees. Thus, while gray birch may initially dominate Palmerton forests simply because of its current initial abundance (a priority effect, e.g. Grman & Suding 2010; van de Voorde et al. 2011; Grman et al. 2013), I am optimistic that other forest species will replace it in due time. Dense birch stands may even provide environments where other plant species can grow with some physical protection from deer herbivory.

The greatest concerns associated with the Palmerton site's returning to forest are the loss of the current grasslands and metal uptake by the gray birch trees that are likely to remain dominant for decades before they are replaced. Above and beyond the benefits the grasses provide in terms of phytostabilizing the metal contaminants, grasslands are rare in Pennsylvania. Therefore, this grassland provides valuable habitat for plants and animals that are similarly rare in the state (Latham et al. 2007). High metal uptake into

the birch leaves presents a problem in that it counteracts the restoration goal of keeping metals sequestered underground. Instead, the birches are moving metals aboveground where they could enter the food chain and potentially cause widespread toxicity via biomagnification (e.g. LeBlanc 1995; Croteau et al. 2005). The question of whether problematic amounts of metals are being introduced into upper trophic levels, primarily via birch, is my most urgent concern with respect to the management of the site.

In my opinion, allowing continued succession to forest is preferable to trying to maintain the site indefinitely as grassland. While the loss of restored grassland habitat would be unfortunate, perhaps it could be used to gather support for efforts to construct or restore other grasslands on nearby soils that are less steep, rocky, and contaminated and therefore more feasible to maintain. As for the environmental impacts of birch leaf metal uptake, I have shown that birch leaf litter has no measurable effects on soil metal concentrations or the growth of representative later-successional trees, though it may adversely affect some herbaceous species (Ch. 3). Furthermore, I have observed that herbivory on birch leaves is notably scarce in this site, so I believe there is little risk that substantial amounts of metals will move into the food chain via birch leaves. However, Beyer (2011) reports that metal incorporation into higher trophic levels was a problem before restoration began, and this issue requires further study in the current ecosystem. Another intuitive option could be to use birch to remove soil metals, but this is countered by our observation that birches did not affect local soil metal concentrations, as well as technical challenges imposed by the steep, rocky topography of the site.

In light of these findings, I support the idea, suggested recently at an LGNC-run meeting (Lehigh Gap Nature Center “Desired Future Conditions,” July 8, 2016), of maintaining a portion of the Palmerton site as grassland while allowing the rest to continue succession to forest. This approach will allow the preservation of some of the desired grassland habitat without requiring too many resources to be spent on controlled burns. Simultaneously, such an approach will be a great opportunity for further experimentation and monitoring to learn more about succession in contaminated sites. For instance, managers could plant seeds or seedlings of more desirable tree species in some areas to attempt to speed succession, or apply further soil amendments to improve soil chemistry, leaving other areas untouched as controls. Managers could also investigate the effects of herbivory on succession in this area by constructing different kinds of animal enclosures and monitoring the course of succession inside and outside of them.

Future directions

There is a significant need, especially at the community scale, to investigate mechanisms by which different soil amendments give rise to different plant communities as we saw in Chapter 1. Repeating experiments like this one, in which multiple restoration treatments were applied to plots within the same study site in a well-controlled and fully factorial design, can be very valuable to this end. More thorough recording of baseline conditions and repeated monitoring of plant communities and soil

conditions would be especially helpful toward elucidating the time course and duration of the effects. We should understand, for instance, how long initial differences in soil amendments or in the plant communities associated with them may persist, or to have a record of the order in which different species colonized a site.

In Palmerton in particular, it would be interesting to continue monitoring the plots in which I conducted my census to see how long the compost-induced differences in plant community composition continue to last. This would also be an effective way to gain an understanding of the extent of year-to-year variation in these communities. The site's restoration history makes it possible to add to the analysis plots receiving no compost, as well as plots receiving similar amendments but three years later. Incorporating these plots into subsequent analyses would be a valuable way of separately assessing the effects of these different treatments. Such knowledge will be invaluable toward improving restoration efforts in other contaminated sites.

In both of my first two chapters, I found variation in soil biology and chemistry associated with variation in plant species identity or community composition. This suggests an intriguing potential mechanism of plant-soil feedback. Plants may assort to soils with chemical characteristics favorable to them (Baker & Brooks 1989), or they may condition soils over time to have chemistry similar to their leaf and root litter (Waring et al. 2015). These hypotheses are not mutually exclusive. Disentangling the extent to which they contribute to associations between plant species and soil chemistry is important in understanding the extent of possible PSF mechanisms. Thus, I call for manipulative studies with careful monitoring to tease these hypotheses apart. For

instance, repeating germination and competition experiments in soils with different and well-characterized chemical and biological characteristics could shed valuable light on the dominant mechanisms by which soil affects plant community composition.

It would also be well worth developing studies and techniques to improve our understanding of how plants influence the heavy metal chemistry of the soil in which they grow. Such studies would ideally carefully control or standardize initial soil properties and growing conditions, with thorough monitoring over multiple growing seasons to include and capture effects of root exudates, root turnover, leaf litter decomposition, and conditioning of soil microbial communities on the spatial distribution and chemical associations of soil metals. To differentiate plant effects from other changes that may occur in a soil over time, it may be important to include non-vegetated control soils.

For researchers studying succession, as I did in Chapters 1 and 3, the most robust approach is clearly to conduct experiments on ecosystems, with careful monitoring over decades to centuries as succession occurs. Because plant community succession happens at the level of habitats to ecosystems, ecological restoration provides many valuable opportunities to observe succession from a relatively well-known baseline. If the Palmerton land managers indeed separate their ecosystem into areas to be maintained as grassland and others to continue succession to forest, that would be an ideal setting for such a study. However, even for the majority of us who do not have whole ecosystems or decades at our disposal to conduct succession experiments in real time, I emphasize the value of finding sites with known ages since planting or abandonment, and collecting

data over more than one growing season. Stochastic factors such as differences in weather or pathogen abundance may change results from year to year, and by integrating results across multiple years we can better determine which factors are consistently important, which are sporadic, and which are consistently unimportant.

In light of the interest and limitations of our mycorrhizal colonization results (Ch. 2-3), we recognize a need for improved techniques for studying mycorrhizal fungal ecology. The percentage of root length colonized is currently our standard measure of the strength of the mycorrhizal relationship (Smith & Read 2008 p. 81). However, measuring colonization is time and labor intensive, does not allow for fungal identification, and can easily be biased by seemingly common problems including differences among samples in rates of root growth or in the functional intensity of plant-fungal interactions (Smith & Read 2008 pp. 81-86).

Metagenomic sequencing of root-associated fungi, on the other hand, promises to be a highly effective way of identifying all of the fungi in a given system once its own technical challenges are better resolved (Hart et al. 2015). However, the best information it can give us about the relative abundance of different species is by differences in the number of sequence reads, which are often unreliable due to the complexity of metagenomic data and its analysis (De Filippo et al. 2012; Sharpton 2014). Furthermore, metagenomics tells us even less about fungal function and activity than percent colonization does. It would be ideal to have techniques that allowed simultaneous identification of mycorrhizal fungi and quantification of their metabolic activity, in particular as it relates to nutrient exchange with host plants.

Research into mycorrhizal fungi and other soil microbes also suffers from difficulties manipulating soil microbial communities in meaningful ways. The most common method of sterilizing soil is to autoclave it, but there remains an unsatisfying trade-off between ensuring sterility and avoiding significant alterations to soil chemistry (e.g. Glassman & Casper 2012). Irradiation avoids these problems but at greater costs (e.g. Yang et al. 2015). Furthermore, regardless of the system, even the spike of dead microbial biomass resulting from sterilization may have significant effects on soil chemistry by mechanisms such as priming (Luo et al. 2015). Existing techniques for inoculating soil with specific organisms or communities are also clumsy. We can inoculate soil with single microbial taxa (Castelli & Casper 2003), groups of microbes separated by size (Glassman & Casper 2012), or whole-soil microbial communities (Emam 2015). However, given that any given plant likely interacts with thousands of microbial taxa and that the most relevant microbial functional groups may be poorly distinguished by size, I conclude that the existing techniques will be largely unsatisfactory for meaningful investigations of the functional ecology of soil microbes. Techniques that allow for easier manipulation of soil microbial communities are urgently needed, especially if they can be applied in the field as well as the lab and greenhouse. For instance, it would be fascinating and valuable to determine whether manipulating the abundance of AMF versus ECM in the field could alter the course of succession by facilitating the establishment of plants with different mycorrhizal requirements.

Indeed, integrating the structure and function of soil microbial communities into our understanding of aboveground ecosystem processes remains one of the most

interesting and challenging questions in terrestrial ecology. In this work, we have investigated soil microbes only very primitively, measuring the extent of root colonization by phylogenetically broad groups of fungi (AMF, ECM, and DSE) as a proxy for the intensity of their symbiosis with their host plants. Investigating fungi at this level is still valuable, as many studies have found considerable functional redundancy within these different groups of organisms; in other words, replacing one species of AMF with another often does not strongly affect host plant growth (Doherty 2010; Gosling et al. 2015). However, even among mycorrhizal fungi, different combinations of plant and/or fungal species can result in very different fungal effects on plant performance even under similar environmental conditions (Diaz et al. 1996; Wang et al. 2007; Ji et al. 2010). We must also better integrate bacteria, archaea, and other eukaryotic soil microorganisms, about which we tend to know even less than we do about mycorrhizal fungi, into our functional understanding of soil and rhizosphere ecology.

Concluding thoughts

Pollution with heavy metals or other toxins can decimate ecosystems and complicate their ecology. Heavy metal pollution combined with acid rain devegetated the Palmerton site, where I conducted this research, so severely that for years local residents described it as looking like the surface of the moon. Even after the remarkable success of restoration efforts to establish a grassland there, metal contaminants still alter soil biology and chemistry, affect plant distributions and community composition, and

complicate the myriad interactions between plants and soils. Plants in the site also influence soil biology, soil chemistry, and the movement of metals through the ecosystem. These dynamics, in which metal pollutants shape a multitude of major ecosystem processes, will continue as long as the metals are present in the ecosystem – likely centuries at least. Despite advances in the reclamation of severely disturbed “lunar landscapes” like the Palmerton site (Tischew & Kirmer 2007; Kirmer et al. 2008; Prach et al. 2012), we have not yet found cheap, easy, or reliable ways to return these landscapes to their pre-disturbance conditions. In terms of ecosystem conservation, there remains no substitute for not releasing pollutants in the first place.

However, many workers involved with such sites tout their ecological benefits (Morse et al. 2014). The chief ecological benefit of restored “lunar landscapes” seem to be that they constitute unusual habitats and thus can provide homes for many species not commonly found in a given area. While there are well-documented cases of this happening (Latham et al. 2007; Hofer et al. 2010), for almost all such species, it is intuitive that landscapes with legacies of severe pollution should be second-rate habitat compared to otherwise similar sites that are less contaminated. Clearly, conserving first-rate habitats would be preferable.

However, this brings us to the second main benefit of polluted sites, which is that people tend to avoid them. Thus, they are conserved not because we value them highly but because we consider them to be wastelands. This is perhaps most evident in the area surrounding the Chernobyl nuclear disaster, where in our absence plant communities have recovered enough to support populations of large animals such as deer, elk, boar,

and wolves on par with those in nearby nature reserves (Deryabina et al. 2015). We also see this in Liberty State Park, a heavy metal polluted rail yard fenced off and abandoned for ~60 years that now serves as a valuable green oasis for migrating birds in an otherwise heavily urbanized area near New York City (Gallagher et al. 2008; Hofer et al. 2010).

Even in Palmerton, land managers extol the benefits of the planted grassland in supporting many plant and animal species – again, migrating birds in particular – seldom found in the forests dominating Pennsylvania and much of the northeastern United States (Latham et al. 2007; Husic et al. 2010). I emphasize again that actively preserving relatively uncontaminated ecosystems would be preferable to using polluted sites as inadvertent conservation areas, and advocate strongly for the reduction of future pollution. This is especially important given the expense and difficulty of remediating polluted sites. However, given that intentional conservation can also be expensive and difficult, perhaps we should not discount the possibility that polluted landscapes may supplement traditional conservation strategies in providing usable homes for imperiled organisms.

Ultimately, we as humans must decide how we want to interact with ecosystems around us, and we must decide this in a way that is both more internationally unified and more locally supported than we have yet been able to achieve. We are one species living on one planet that represents one source of air, water, food, and shelter for us all. Even the most difficult of the divisions we encounter – country, culture, race, religion – are products of our own making. We must not let them hinder us from making sure that the

Earth remains a good home for our children, our grandchildren, and all of the other living things with which we share it.

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