Learning to live with novelty: Implications of exotic earthworms and their interactions with seeds, mulch, and wood ash for ecological restoration

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Social and Ecological Sustainability

Waterloo, Ontario, Canada, 2019

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

This thesis consists of five chapters (including an Introduction, three data chapters, and a Conclusion). Michael McTavish is the primary author on all chapters and is exclusively responsible for their preparation. The experiments described in the three data chapters were designed, executed, analyzed, and reported by Michael McTavish under the supervision of Dr. Stephen Murphy. Elements of the earthworm-ash experiments (Chapter 4) were carried out in collaboration with Adam Gorgolewski (PhD student, University of Toronto), who helped source wood ashes for the experiment and was responsible for setting up and collecting earthworm samples from the Haliburton field site. Contents of Chapter 4 will be used alongside soil data analyzed by Adam Gorgolewski not included in this thesis in future manuscripts to be co-authored by Adam Gorgolewski. Donald McTavish helped write the Scilab and MATLAB code to produce the seed and ash burial images in Chapters 2 and 4 respectively. The thesis in its entirety has been reviewed and edited by supervisor Dr. Stephen Murphy and committee members Dr. Brendon Larson, Dr. Heidi Swanson, and Dr. Sandy Smith.

Abstract

A major challenge in restoration ecology is the biological invasion of 'exotic' species, some of which may spread widely and have undesirable impacts as 'invasive' species. Ongoing debates and changing perspectives suggest we may be overlooking opportunities to consider exotic species more broadly, not only as adversaries but also as potential null players or even allies in restoration. This may be exemplified by the invasion of exotic earthworms in North America, a long-term and widespread invasion of ecologically-influential organisms without practical ways to control it. The purpose of this dissertation is to consider the integration of exotic earthworms into restoration by exploring how they interact with three restoration interventions: seeds, mulch, and wood ash. I used laboratory microcosms and field-based experiments with a focus on the ecosystem engineering nightcrawler earthworm (*Lumbricus terrestris* L.). Overall, earthworms had effects that might be contextually beneficial or detrimental to ecological restoration: earthworms selectively consumed and buried seeds which could reduce recruitment from seed mixes or contribute to seed bank formation (Chapter 2); earthworms collected and buried mulch which exposed the soil underneath but could help mix organic matter into degraded soils (Chapter 3); and earthworms responded behaviourally and in population density to different wood ashes and helped mix surface-applied wood ash into the soil (Chapter 4). I propose that by recognizing exotic earthworms as a novel and increasingly common ecosystem feature in North America and by learning how to mitigate their undesirable impacts and take advantage of their benefits, we could more efficiently and effectively restore these changing ecosystems. This dissertation contributes to our expanding knowledge of earthworm ecology, facilitates increased integration of earthworm interactions into restoration, and offers insights into the broader implications of biological invasion for conservation. Studying the case of exotic earthworms in North America raises important questions about why we restore and conserve, the value of case-by-case management of invasions based on impact, the importance of considering the longer-term outcomes of invasion and naturalization, and – in some cases – the merit in learning to live with novelty.

Acknowledgments

This dissertation has been a substantial undertaking and I owe its completion and my personal growth as a researcher to the support I have received from many sources.

I am grateful to partner institutions and their members for the use of their facilities, resources, and expertise, including Haliburton Forest Ltd. and Conservation Halton, with a particular thank you to Nigel Finny of Conservation Halton and to fellow PhD candidate and wood ash colleague Adam Gorgolewski of the University of Toronto.

My path through academia began several years ago and I continue to be grateful for the people who helped me first get started and continue to provide mentorship and support, including Drs. Nathan Basiliko, Tara Sackett, Carolyn Winsborough, Jason Shabaga, Michael Preston, and Varun Gupta.

I was fortunate to conduct my research here as a member of the colourful cast of characters who make up the School of Environment, Resources and Sustainability (SERS) at the University of Waterloo; I have learned a lot here and I know that I will miss it very much. Special thanks to the staff of the Ecology Lab including Bev Raimbault and Anne Grant, administrator Jennifer Nicholson, and Drs. Simon Courtenay, Ian Rowlands, Bob Gibson, Rob de Loë, Andrew Trant, Dan McCarthy, Stephen Quilley, Christine Barbeau, and Maren Oelbermann.

I would like to thank all of my committee members for their support and insightful feedback throughout the program, including Drs. Brendon Larson, Heidi Swanson, and Sandy Smith, and to thank my external examiner, Dr. Peter Kotanen, for his valued contributions. I must of course extend a very special note of appreciation to my supervisor, Dr. Stephen Murphy, who has been there since day one of the project and has made all of this possible.

Thank you to the many friends and colleagues who have greatly enriched my time here with conversations, intramurals, board games, and music. Special thanks to Gwyn Govers, Jay Carter, Jonas Hamberg, Patricia Hyunh, Amanda Shamas, Tomm Mandryk, Vito Lam, Nathan De Carlo, Andrea Bale, Ian Payne, Andrew Wilson, Nilabjo Banerjee, Chelsea Marr, and Devin Shields.

Finally, I could not have done this without the support and encouragement of my family. Thank you to all of the pets avian, feline, equine, and aquatic. Thank you to Dr. Heather Cray for always being there as my partner-in-PhD. Thank you to my mother and father, brother and sister, grandparents and all of the extended family for the ever-present love and support.

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1.1 Challenges of biological invasion for restoration

"[T]he sun never sets on the empire of the dandelion."
- Alfred Crosby, environmental historian (1986)

1.1.1 The conventional intersection of invasion and restoration ecology

Restoration ecology (hereafter, also referred to as 'restoration') is a field that holds much promise to help mitigate and repair stresses we have imposed on global ecosystems (Harris and Van Diggelen, 2006). Ecological restoration is commonly defined as "the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed" (McDonald et al., 2016; SERI, 2004). Driven by diverse ecological, socioeconomic, personal, and cultural values, restoration is an important process furthering the broader goal of conservation: reservation protects what still exists while restoration attempts to return that which was lost (Clewell and Aronson, 2007, 2006; Rosenzweig, 2003).

One of the major challenges to restoration is biological invasion. Increases in human exploration, settlement, and trade have deliberately and accidentally introduced species into new habitats that they would not have been able to reach without human assistance (Meyerson and Mooney, 2007; Ricciardi et al., 2017). Although most of these new 'exotic' species fail to establish, a few generally succeed and an even smaller subset of these may spread widely and have considerable impacts (Williamson and Fitter, 1996). These 'invasive' species are commonly described as the "second greatest threat to biodiversity" (Wilcove et al., 1998; Wilson, 1992), are contributors to a global process of 'biotic homogenization' (Baskin, 1998; McKinney and Lockwood, 1999), and are blamed for billions of dollars in economic damages and harm to human well-being (Mack et al., 2000; Pimentel et al., 2005, 2001, 2000).

The conventional relationship between invasion and restoration has largely been one of problem and solution: depending on the goals of a specific restoration project, exotic species may be undesirable because they are not a part of and may threaten a historical native reference

community (Clewell and Aronson, 2007; McDonald et al., 2016; SERI, 2004; Vitousek et al., 2011), while restoration can be a tool to remove exotic species, remediate the persistent legacy effects of past invasions, or increase the resistance of ecosystems to future invasion (D'Antonio and Meyerson, 2002; D'Antonio and Chambers, 2006; Hobbs and Richardson, 2011; Myers et al., 2000; Simberloff et al., 2013; Zavaleta et al., 2001). However, recent debates in the study of invasions open new possibilities and uncertainties in the relationship between biological invasion and restoration.

1.1.2 Invasion science: a troubled discipline

The academic discipline concerned with biological invasions is often referred to as 'invasion biology', though I will be using the less common but broader label of 'invasion science' (Richardson and Ricciardi, 2013). In its relatively short life since Charles S. Elton's *The Ecology of Invasions by Plants and Animals* (1958) and its rise to prominence in the mid-1980s and 1990s (Sagoff, 2018a; Simberloff, 2003), invasion science has hosted a persistent and extensive disciplinary debate. Critics make repeated "calls for the end" (Valéry et al., 2013) while defenders advocate "an end to calls for the end" (Simberloff and Vitule, 2014), arguing that the debate has descended into a "cavalier bashing of the discipline" (Richardson and Ricciardi, 2013) and science denialism (Ricciardi and Ryan, 2018; Russell and Blackburn, 2017a, 2017b). This debate has solicited researchers to pick a side in high-profile editorials (e.g., Davis et al., 2011; Simberloff, 2011), generated exhaustive exchanges of articles (e.g., on denialism see Briggs, 2017; Crowley et al., 2017; Davis and Chew, 2017; Sagoff, 2018b; Tassin et al., 2017), and appeared in popular science features and books (e.g., *Where Do Camels Belong?* Thompson, 2014). While critical reflection is desirable for academia, this seemingly intractable debate threatens to undermine the progress and value of the discipline and further complicates the relationship between invasion and restoration (Courchamp et al., 2017; Shackelford et al., 2013). Although a more in-depth analysis of this complex debate is beyond the scope of this dissertation, I will provide a summary of four of the major points of contention and briefly explore how they relate to my main invasion of interest and restoration more broadly.

One of the most prominent issues is that core terminology and concepts are often poorly defined and inconsistently used. For example, the distinction between 'native' and 'exotic' species is a guiding principle of the discipline that has also been criticized as fundamentally

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flawed (Chew and Hamilton, 2011). In a world of species constantly on the move, a species is generally identified as exotic to a given habitat if it arrived with human assistance in the 'recent' past (Webb, 1985). Critics argue that this definition is spatially and temporally arbitrary and question the validity of separating human-assisted dispersal as a unique process and assigning species to lasting categories based on an intrinsically ephemeral global biological community (Davis and Thompson, 2000; O'Brien, 2006; Shrader-Frechette, 2001; Warren, 2007). Invasion scientists also disagree as to the definition of 'invasive species', which is at best a misnomer, usually referring to a population of a species that is considered invasive in a given time and place (Colautti and MacIsaac, 2004). Researchers disagree as to whether invasive species should be defined based on demonstrable negative impacts, acknowledging this to be inherently subjective and value-laden (Davis and Thompson, 2001, 2000; Junqueira, 2013; Van der Wal et al., 2015; Warren, 2007), or on ostensibly value-neutral concepts of spread (Colautti and MacIsaac, 2004; Daehler, 2001; Richardson et al., 2000) or 'ecological impacts' (Ricciardi et al., 2013). Confusingly, 'exotic' and 'invasive' are often used somewhat interchangeably in the literature and policy documents, often conflating origin and impact. These issues are well known and while there have been many proposed unifying terminologies (e.g., Blackburn et al., 2011; Colautti and MacIsaac, 2004; Davis and Thompson, 2000), none have been widely adopted.

A second serious point of contention is the actual impact of exotic and invasive species. The primary rationale for acting against exotics is their potential harm, but defining and measuring 'harm' is a daunting ecological and philosophical challenge (Sagoff, 2005; Simberloff, 2005). While some exotic species have clear benefits (e.g., crops) (Pimentel et al., 2005) or undesirable impacts (e.g., disease) (Bonanno, 2016), many effects are more cryptic. Critics suggest that many negative impacts of exotic species are overrepresented or even assumed *a priori* without evidence while many positive benefits are overlooked or under-reported (Goodenough, 2010; Rodriguez, 2006; Sagoff, 2005; Starfinger et al., 2003; Stromberg et al., 2009). Standard 'boilerplate' statements such as the assertion that exotic species pose the "second greatest threat" to global biodiversity (Bellard et al., 2016; Clavero and García-Berthou, 2005; Doherty et al., 2016; Wilcove et al., 1998; Wilson, 1992) have been heavily criticized as inaccurate and misleading (Brown and Sax, 2004; Chew, 2015; Davis, 2003; Dueñas et al., 2018; Gurevitch and Padilla, 2004). The staggering economic costs of exotics (e.g., Pimentel et al.,

2005, 2001, 2000) are also questionable because they often circularly include control costs and rarely factor in benefits (Bonanno, 2016; Pejchar and Mooney, 2009; Thompson, 2014).

These uncertain impacts call into question the ecological uniqueness of exotic vs. native species or of biological invasion vs. colonization (Brown and Sax, 2005; Buckley and Catford, 2016; Cassey et al., 2005; Colautti and MacIsaac, 2004; Davis et al., 2011; Davis and Thompson, 2000; Nackley et al., 2017; Sagoff, 1999). Understanding impacts is further complicated by disentangling multiple factors and distinguishing species as 'drivers' or 'passengers' of change (Bauer, 2012; MacDougall and Turkington, 2005), a lack of pre-impact data (Junqueira, 2013), biased research predisposed to find negative impacts of exotic species (Goodenough, 2010), challenges weighing the relative benefits and costs of any given species (Potgieter et al., 2017), and overgeneralizations based on a few high profile 'worst-case' invaders (Gozlan et al., 2013; Gurevitch and Padilla, 2004; Hulme et al., 2013).

A third common criticism concerns subjectivity and values, and it is reflected in the name of the discipline; invasion science often uses emotive militaristic rhetoric and some researchers have also suggested disturbing parallels between the rhetoric of invasion science and racism, immigration, and xenophobia (Davis et al., 2001; Larson, 2005; Sagoff, 1999; Simberloff, 2003). This language can be accompanied by a nativist bias that equates native with 'good' and exotic with 'bad', echoing ecologically outdated perceptions of a natural balance of native ecosystems disrupted by 'out of place' exotic species (Brown and Sax, 2005, 2004; O'Brien, 2006; Van der Wal et al., 2015). This kind of subjectivity can also be hidden within seemingly more objective assessments; for example, conservation goals are often based on 'biodiversity' or 'ecological integrity' that are defined in a manner that only allows exotic species to have null or negative impacts (e.g., *Canada National Parks Act*, 2018) (Sagoff, 2005, 1999).

This subjectivity also raises concerns about the role of science in informing public opinion and policy, particularly when scientists are not fully aware of their own subjective motivations and biases (Fischer et al., 2014; Schlaepfer et al., 2011a; Starfinger et al., 2003). For example, science communication can conflict with a public that may not know the biogeographic origins of species or value species for other reasons (Bonanno, 2016; García-Llorente et al., 2008; Shackleton et al., 2019; Sharp et al., 2011). Emotive, fear-based rhetoric can create urgency that motivates research and action, but it can also foster inaccurate and misleading perceptions of complex ecological issues (Chew, 2015; Gobster, 2005; Larson, 2005; Larson et

al., 2013). Acknowledging that conservation is inherently value-laden (Larson, 2007), invasion scientists continue to disagree on the appropriate balance of objective fact reporting and subjective advocacy (Brown and Sax, 2004, 2005; Cassey et al., 2005; Colautti and Richardson, 2009; Schlaepfer et al., 2011a).

The fourth major point of debate concerns the capacity of invasion science to predict and control invasions. Predicting which exotic species are likely to be successful or their impacts is considered a priority for the discipline (Mack et al., 2000; Richardson et al., 2000). However, due to site-specific factors, complex species interactions, long-distance dispersal, a changing climate, minimal baseline data, and time lags (Crooks, 2005; Hulme, 2003), predicting invasions based on species traits, taxonomy, or spread has had limited success and general predictive theories of invasibility or impact have proved elusive (Mack et al., 2000; Ricciardi et al., 2013; Ricciardi and Cohen, 2007; Shrader-Frechette, 2001). Given this poor predictive capacity, researchers use risk assessment tools but disagree over the appropriate amount of precaution, with some supporting an uncompromising "guilty until proven innocent" policy (Hulme, 2011; Junqueira, 2013; Leung et al., 2002; Schlaepfer et al., 2011a).

To implement control, the Convention on Biological Diversity recommends a three stage approach of prevention, early eradication, and long-term containment (CBD, 2008). Although control can be successful, particularly when implemented early and when the invasion has not yet spread far (Leung et al., 2002; Simberloff et al., 2013; Zavaleta et al., 2001), control may be an outright expensive failure (Myers et al., 2000) or success could come at the expense of public support or unknown benefits of exotic species, produce unexpected off-target effects, or leave an ecosystem open to future invasion (Bremner and Park, 2007; Buckley et al., 2007; Ewel and Putz, 2004; Hulme, 2006; Zavaleta et al., 2001).

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Before moving on to the following section, given the common inconsistencies in invasion terminology I will explicitly define several key terms that I will use throughout this dissertation. Although I personally question the validity of separating human agency from other 'natural' factors, I will define 'exotic species' as "species currently found outside of the 'normal' distribution they maintain without human assistance" (Convention on Biological Diversity (CBD), 2008; Webb, 1985). While I do believe that the term 'invasive species' has normative

policy value for describing species that have undesirable impacts, I will generally avoid using it. Given the contextuality of defining 'harm' (Sagoff, 1999), I do not think that 'invasive species' is a term that can generally be used meaningfully as a broad species label. I will instead refer to 'invasion' as the overall process that includes the introduction, establishment, and spread of exotic species, which may but need not necessarily result in contextually undesirable impacts. I also acknowledge that both 'exotic' and 'invasive' are labels for populations rather than species (Colautti and MacIsaac, 2004), but for consistency I will perpetuate this particular misnomer. These definitions are a compromise of personal perspectives, clarity, and consistency with the existing discourse; I recognize that they are imprecise and subjective, which are arguably inevitable characteristics of the subject matter (Larson, 2007).

1.1.3 Changing perspectives on invasion and restoration

As the invasion debate continues, it stimulates ongoing critical reflection concerning the relationship between invasion and restoration. While exotic species are conventionally seen primarily as a source or symptom of degradation and restoration as a tool to manage or prevent invasion (Clewell and Aronson, 2006; Hobbs and Richardson, 2011; McDonald et al., 2016; SERI, 2004), changing perspectives on the impacts and implications of invasion open broader possibilities in which exotic species may variably be adversaries, null players, or even allies to restoration (Ewel and Putz, 2004). What role they play depends in large part on the specific impacts of the species in question and how these line up with the conservation goals and restoration targets of a given project (Byers et al., 2006; Shackelford et al., 2013). This may apply to exotic species already present in an ecosystem being restored or to species deliberately introduced (Ewel and Putz, 2004; Schlaepfer et al., 2011b).

Exotic species may potentially fill any role in an ecosystem (Colautti and MacIsaac, 2004; Crooks, 2005). They may affect various ecological processes or ecosystem services, potentially replacing functions previously provided by now extinct or extirpated species or providing novel functions (Ewel and Putz, 2004; Kennedy et al., 2013; Rodriguez, 2006; Schlaepfer et al., 2011b; Shackelford et al., 2013). Some species are likely to have stronger impacts than others (Vitousek et al., 2011), including 'ecosystem engineers' (Byers et al., 2006; Jones et al., 1997, 1994) or 'transformer species' (Richardson et al., 2000; Wells et al., 1986),

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top predators (Rodriguez, 2006), and species that alter disturbance regimes (D'Antonio and Chambers, 2006). In a future of a rapidly changing climate and native species that may be less well adapted to changing conditions, exotic species could also become increasingly important for restoration (Aronson and Vallejo, 2006; Hulvey et al., 2013; Schlaepfer et al., 2011b; Walther et al., 2009).

Embracing new possibilities for exotic species in restoration requires navigating complex trade-offs including the relative costs and benefits of retaining, removing, or even introducing exotic species amidst considerable uncertainty (Ewel and Putz, 2004; Prior et al., 2018; Rodewald et al., 2015; Rodriguez, 2006; Simberloff et al., 2013; Zavaleta et al., 2001). Several new emerging approaches to restoration and conservation have begun to tackle these complex challenges. For example, Rosenzweig's (2003) 'reconciliation ecology' attempts to balance the priorities of biodiversity conservation and human society. Carroll (2011) proposed 'conciliation biology', a branch of invasion science that "focuses not on prevention or eradication of invasive species, but instead predicts and manages outcomes of longer-term native-[exotic] interactions".

Perhaps most influential has been the 'novel ecosystem' framework, which identifies ecosystems defined by species combinations and relative abundances without precedence which are caused but not necessarily maintained by human influence (Hobbs et al., 2006). These novel ecosystems are distinguished from 'hybrid ecosystems' by practical ecological, economic, and social constraints (Hallet et al., 2013; Hobbs et al., 2013) and can help find value in systems that might otherwise be overlooked (Light et al., 2013; Lindenmayer et al., 2008; Seastedt et al., 2008). Additionally, some existing perspectives such as certain traditional ecological knowledge systems may already advocate a similar flexibility in considering the 'purpose' of new species rather than their origins (Reo and Ogden, 2018).

These emerging perspectives have generated new debates of their own. Some researchers argue that the positive impacts and potential conservation value of exotic species have been historically overlooked and they applaud new approaches that allow for them to be considered (Ewel and Putz, 2004; Schlaepfer et al., 2011b). These researchers often emphasize that while invasion can still be a source of problems and should be managed with a precautionary approach, species should be judged and managed based on their impacts, not their origins (Davis et al., 2011; Goodenough, 2010; Shackelford et al., 2013). On the other side of the debate, researchers argue that exotic species do pose unique and substantial threats, are intrinsically incompatible

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with responsible conservation goals, and should be managed accordingly (Crooks, 2005; Preston, 2009; Simberloff and Vitule, 2014; Vitule et al., 2012). These critics suggest that diversifying conservation goals and embracing certain exotic species and novel ecosystems is a dangerous and unacceptable course for conservation (Marris et al., 2013; Murcia et al., 2014; Simberloff et al., 2013; Standish et al., 2013).

In summary, the implications of biological invasion for restoration and conservation is a complex and fiercely debated topic that raises many fundamental questions concerning the role of science in society (Larson et al., 2013), the subjective nature of defining environmental 'harm' (Sagoff, 2005), and the question of why we restore ecosystems in the first place (Clewell and Aronson, 2006). Resolving these larger questions is beyond the scope of this dissertation, but this broader issue frames the biological invasion that will be the focus of this dissertation: the invasion of exotic earthworms into North America. This invasion is an exemplary case of many of the challenges laid out above in the ongoing invasion debate and how this poses additional challenges – and opportunities – for restoration.

1.2. Exotic earthworm invasion in North America

[On earthworms] "It may be doubted whether there are many other animals which played so important a part in the history of the world, as have these lowly organized creatures."
- Charles Darwin (1881)

1.2.1 Patterns of exotic earthworm invasion

Earthworms are hermaphroditic invertebrates of the subclass Oligochaeta in the phylum Annelida. Approximately half of the identified species within Oligochaeta are considered 'terrestrial earthworms', of which approximately 3,700 species have been described (Hendrix et al., 2008; Reynolds, 1994). Beginning in the early 20th century, researchers noticed certain 'peregrine' species found all over the world (Beddard, 1912; Michaelsen, 1900). Today, exotic earthworms are on every continent of the world except Antarctica and in nearly every type of ecosystem (Hendrix et al., 2008). This widespread invasion of exotic earthworms – dubbed "global worming" by Eisenhauer et al. (2012) – is one of the most ubiquitous redistributions of organisms by humans.

With a relatively slow active dispersal rate averaging 1 km every 100 years (Addison, 2009), exotic earthworms are highly dependent on humans for rapid and widespread dispersal. Their typical spread follows a 'jump dispersal' model of long distance passive colonization combined with slower active dispersal (Tiunov et al., 2006). Earthworms and their cocoons are moved over great distances by global trade in ship ballast or imported plants or soils and subsequently distributed locally when people dump fishing bait, dispose of horticultural materials, carry individuals in the treads of shoes or vehicle tires, or regrade roadways (Addison, 2009; Cameron et al., 2007; Hendrix and Bohlen, 2002; Sackett et al., 2012). Most human-facilitated spread of exotic earthworms is unintentional, though some species are globally traded for bait or vermicomposting (Hendrix and Bohlen, 2002) or were historically introduced by settlers to increase agricultural yields (e.g., New Zealand, Baker et al., 2006).

At larger scales, exotic earthworms tend to be closely associated with settlements, farms, and roads (Choi et al., 2017; González et al., 2006; Sackett et al., 2012), while local community composition and structure tend to be more strongly influenced by habitat characteristics (Tiunov et al., 2006). Unlike many invasions, exotic earthworms are often able to spread into relatively undisturbed habitats (Hendrix et al., 2006). Exotic earthworms seem to establish in semi-predictable sequences of species, typically beginning with smaller, fast growing, litter-dwelling species followed by larger, slower-growing species (Hale et al., 2005; Holdsworth et al., 2007a).

The exotic earthworm invasions that have gained the most attention and are the best documented are those in North America. Interest in these invasions is recent and our current understanding of their distributions is largely attributable to decades of surveys by the American zoologist Gordon E. Gates beginning in 1949 and later by his student John Reynolds, who first documented exotic earthworms in Canadian forests in the mid-1970s and created a taxonomic key for the earthworms of Ontario (Hendrix and Bohlen, 2002; Reynolds, 1977).

From these surveys, researchers generally believe that Pleistocene glaciations eliminated most of North America's pre-existing native earthworms, sparing an estimated 100 species of native earthworms that survived along the southern extent of the glaciers and in small refugia along the western and eastern coastlines (Gates, 1970; Hendrix and Bohlen, 2002; Reynolds and Wetzel, 2004). The most recent glacial episode in North America – the Wisconsinan Glaciation – ended approximately 10,000-11,000 y ago, leaving much of the continent earthworm-free. Remaining pockets of native earthworms have been slow to recolonize and have not generally

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spread beyond historical glacial boundaries; most of the earthworms found beyond these boundaries are exotic species thought to have been arriving since widespread European colonization c. 400 y ago (Callaham et al., 2006).

North America (north of Mexico) currently has at least 45 species of exotic earthworms. These are dominated by approximately 25 species from the European family Lumbricidae, likely due to habitat similarity and a long history of European trade and settlement (Hendrix and Bohlen, 2002). Because of more extensive glaciation, Canada has only eight documented native earthworms with very limited distributions and 19 documented exotic earthworms, again dominated primarily by European Lumbricids (Addison, 2009). Exotic earthworms are generally most abundant in areas with the longest histories of European settlement, though certain species have spread into largely undisturbed nearby forests (Addison, 2009) and northward into the boreal forests (Cameron et al., 2007). Further northward expansion is likely to continue, particularly in light of a warming climate and human exploitation (Cameron et al., 2007; Tiunov et al., 2006), though increases in drought conditions accompanying warmer weather could limit expansion (Eisenhauer et al., 2014). While European earthworms have been arriving in North America for the past hundreds of years, the northeastern USA is also more recently experiencing a 'second wave' of invasion by Asian earthworms including *Amynthas* spp. (Szlavecz et al., 2018).

1.2.2 Ecological impacts and current management of exotic earthworms

As a primarily belowground phenomenon, invasions of exotic earthworms have gone largely unnoticed for hundreds of years (Hendrix, 2006). However, earthworms are considered among the most influential soil organisms in global soil systems (Butt, 2008; Jouquet et al., 2006) and scientists are now concerned how exotic earthworms may change invaded ecosystems. Earthworms are 'ecosystem engineers' that physically transform the habitats they live in and modify the resources available to other organisms and can have large and complex effects on ecosystems disproportionate to their biomass (Jones et al., 1997, 1994; Lavelle et al., 2006). The changes brought by exotic earthworms could be particularly dramatic in areas that have been historically earthworm-free for the last several thousand years (Addison, 2009; Callaham et al., 2006; Hendrix and Bohlen, 2002).

The ecological impacts of exotic earthworms are highly variable between earthworm functional groups, species, and ecosystems (Frelich et al., 2006). Earthworms generally affect their environments primarily by burrowing through the soil, consuming a diet dominated by decomposing organic material, and producing nutrient-rich 'casts' as waste (Edwards and Bohlen, 1996). The impacts of specific earthworm species are closely associated with their life histories. Earthworms are often assigned to one or two of three main functional groups described by Bouché (1977): 'epigeic' species live in leaf litter atop the soil, 'endogeic' species form temporary horizontal burrows in the upper mineral horizons, and 'anecic' species form semi-permanent, deep, vertical burrows that open to the soil surface. Earthworm communities with varying functional composition affect ecosystems differently, with the strongest impacts typically resulting from a mix of functional groups (Frelich et al., 2006).

Earthworms can affect soil physical properties including bulk density, porosity, and aggregation (Blanchart et al., 2004; Blouin et al., 2013; Coq et al., 2007; Milleret et al., 2009) and soil chemical properties including nutrient cycling and pH (Chaoui et al., 2003; Materechera, 2002; Whalen et al., 1999). Earthworms can alter the composition and function of microbial communities in their guts, burrows, casts, and surrounding soil (Aira et al., 2009; Binet et al., 1998; Dempsey et al., 2011; Li et al., 2002; McLean et al., 2006). Researchers are also increasingly recognizing the role that earthworms play as granivores and seed dispersers (Forey et al., 2011) and as herbivores of live aboveground leaves, seedlings, and fine roots (Eisenhauer et al., 2010; Griffith et al., 2013; Wolters and Stickan, 1991). The impacts of earthworms on carbon storage are debated (Hendrix et al., 2008).

While earthworms are generally considered beneficial in their native ranges or in agricultural or horticultural systems (Bertrand et al., 2015; Butt, 2008), researchers are concerned about the potential undesirable consequences of exotic earthworms for the structure and function of invaded ecosystems. In North America, most of the concern surrounds exotic earthworms in forests and originates from studies of earthworm invasion fronts in hardwood forests in the northeastern USA that began in the early 2000s (most notably Hale et al., 2006, 2005). These studies have linked exotic earthworm invasion to a 'forest decline syndrome' of simplifications to forest understory plant diversity (Frelich et al., 2006).

The suspected driver of this syndrome is that exotic earthworms decrease the thickness of the forest leaf litter layer by burial, consumption, and accelerated decomposition, exposing

seedlings to desiccation and predation, particularly in habitats with large deer populations (Dobson and Blossey, 2015; Hale et al., 2006). Earthworms are also suspected to alter plant community composition by homogenizing the soil profile (Bohlen et al., 2004; Hale et al., 2008), selectively ingesting the seeds of certain species (Cassin and Kotanen, 2016; Milcu et al., 2006; Nuzzo et al., 2015; Zaller and Saxler, 2007), disrupting mycorrhizal fungi (Dempsey et al., 2011; Lawrence et al., 2003; Milleret et al., 2009), and generally altering soil properties and microbes in ways that disadvantage locally-adapted species (Bohlen et al., 2004; Frelich et al., 2006; McLean et al., 2006). Citing these mechanisms, numerous studies have implicated exotic earthworms in the decline of the abundance and diversity of native forest vegetation and an increase in exotic invasive plants in an 'invasional meltdown' *sensu* Simberloff and Von Holle (1999) (Corio et al., 2009; Hale et al., 2006; Holdsworth et al., 2007b, 2007a; Hopfensperger et al., 2011; Nuzzo et al., 2009; Suárez et al., 2006).

Although much of the existing research has focused on responses of vegetation to exotic earthworms, a relatively small pool of research has also suggested potential associations between earthworms and changes in communities of salamanders (Maerz et al., 2009), millipedes (Snyder et al., 2013), other soil invertebrates (Ferlian et al., 2018; Migge-Kleian et al., 2006), and ground-nesting songbirds (Loss et al., 2012; Loss and Blair, 2014). In some habitats, exotic earthworms may coincide with native earthworms; such interactions are relatively common in the tropics (González et al., 2006) but rare in North America (Addison, 2009). Additionally, exotic earthworms are a high quality, protein-rich food resource that has become a part of the diets of several species including robins (Cameron and Bayne, 2012), salamanders (Maerz et al., 2009), and bears (Mattson et al., 2002).

Given that the prevailing scientific opinion is that exotic earthworms are or could be drivers of substantial and undesirable ecological change in North America, many scientists recommend control (Addison, 2009; Callaham et al., 2006; Hendrix et al., 2008; Hendrix and Bohlen, 2002; Holdsworth et al., 2007a). Researchers recommend interventions to prevent introductions and spread such as trade restrictions, screening and quarantine procedures (Callaham et al., 2006), and education campaigns to raise public awareness and reduce behaviours such as bait dumping (Cameron et al., 2013). Future spread could be predicted based on variables including propagule pressure, habitat matching, distance from human activity, and species identity (Hendrix and Bohlen, 2002; Sackett et al., 2012). Scientists have also proposed

several tools to reduce or control existing populations including chemical poisons (Callaham et al., 2006; Parmelee et al., 1990; Walton, 1928), prescribed burning (Callaham et al., 2003; Ikeda et al., 2015), and biocontrol using the predatory New Zealand flatworm (Great Lakes Worm Watch; Murchie and Gordon, 2013) or parasitoid flies (Choi 2012). However, very few of these recommendations have been enacted. Some exceptions include federal restrictions on intentional earthworm imports into Canada (Hendrix and Bohlen, 2002), a risk assessment of exotic earthworms in Ontario (Evers et al., 2012), and several prominent education programs (e.g., Alberta Worm Invasion Project, Cameron et al., 2013; Great Lakes Worm Watch, Hale, 2013).

1.2.3 Exotic earthworms and the invasion debate

The invasion of exotic earthworms in North America epitomizes many of the critiques and challenges raised in the broader invasion science debate. I postulate that these same issues have resulted in a largely ineffective management stance and has prevented proper consideration of the implications of exotic earthworms for restoration.

Although the historical 'native' or 'exotic' status of different earthworms in North America is generally agreed upon, assigning species to these categories has been a challenge in other parts of the world. For example, in a curious study, Wackett et al. (2018) re-evaluated the status and ecological 'threat' of earthworms on the Fennoscandian Peninsula based on the likelihood that they arrived by themselves or alongside native Sami settlements (making them desirable natives) or more recent settlers (making them exotics and ecologically "potent threats"). As more North American earthworm inventories are completed, similar questions could arise concerning the role of humans in the post-glacial dispersal of 'native' earthworm species. Additionally, many of North America's 'exotic invasive' earthworms that are found in long-settled areas have been living there for hundreds of years (Szlavecz et al., 2018); with no clear consensus on what constitutes a 'naturalized' species (Valéry et al., 2013), it is unclear when the 'exotic' label becomes inappropriate and a poor basis for management decisions.

As seems to be the case for many invasions, the actual impacts of exotic earthworms may be more uncertain than they are often reported. The dominant message from the scientific community has been that exotic earthworms are a "driving force" behind undesirable ecological changes (Craven et al., 2017; Heneghan et al., 2007; Nuzzo et al., 2009). However, many of these claims have arguably been overgeneralized from studies that are geographically biased

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towards hardwood forests in the northeastern USA, focused primarily on the leading edge of active invasion fronts where earthworm densities and impacts may be highest but possibly transient (Addison, 2009; Straube et al., 2009), and use observational data unable to establish causal relationships (Hale et al., 2006; Hopfensperger et al., 2011). The few meta-analyses that have been done document weak and inconsistent effects but still present strong, arguably oversimplified conclusions (Craven et al., 2017; Ferlian et al., 2018). Finally, speculative claims such as how exotic earthworms disrupt mycorrhizae, favour grasses over herbaceous species, interact synergistically with deer browsing (e.g., all mentioned in Hale et al., 2006; Nuzzo et al., 2009), and outcompete native earthworms (Stebbings, 1962) are repeated despite a deficit of supporting evidence and contradictory findings (Dobson and Blossey, 2015; Hendrix et al., 2006; McLean et al., 2006; Paudel et al., 2016). While some exotic earthworm impacts such as the reduction of forest leaf litter thickness have been observed very consistently (e.g., Corio et al., 2009; Maerz et al., 2009; Nuzzo et al., 2009; Suárez et al., 2006), many of their other ecological impacts seem to vary idiosyncratically (Dobson and Blossey, 2015; Hendrix and Bohlen, 2002).

The case of exotic earthworms in North America is also an example of the potential disconnect between the attitudes of the public and the scientific community towards invasions. While many researchers now describe exotic earthworms as a threat, members of the public and certain aboriginal communities primarily see them as normal and desirable soil residents (Callaham et al., 2006; Cameron et al., 2013; Hendrix and Bohlen, 2002; Reo and Ogden, 2018), and education campaigns may struggle to convince people otherwise (Cameron et al., 2013); these generally positive public perceptions can be common for exotic species that have been around for a longer time (Shackleton et al., 2019). Although scientists have been studying earthworms since Darwin (1881), the idea of seeing certain earthworm species as a problem is relatively recent. Besides mentions of controlling earthworms as a pest on golf courses (Walton, 1928), scientists only began to seriously consider exotic earthworms in North America undesirable in the mid-20th century, soon after Elton's seminal 1958 text (e.g., Stebbings, 1962), with most of the research and concern not emerging until the mid-2000s (Callaham et al., 2006). Articles and websites targeted at a more general audience then began to appear, using many of the usual rhetorical devices. These include the provocatively-titled *Attack of the killer worms* news article in The Star (Scrivener, 2007) and an unsettling graphic of the province of Alberta

comprised fully of giant, intermingled earthworms (Alberta Worm Invasion Project, http://worms.biology.ualberta.ca/).

Finally, and perhaps most importantly, the case of exotic earthworms highlights many of the common challenges in predicting or controlling invasions. Overall, our current ability to model and predict the spread and impacts of exotic earthworms is very poor (Hendrix et al., 2008; Holdsworth et al., 2007a), resulting from uncertain and inconsistent ecological impacts (Hendrix and Bohlen, 2002) and minimal large-scale data on the current and historical distributions of earthworms in North America (Addison, 2009). Crucially, there are also no practical management options at this time for eradicating existing populations or controlling the introduction or spread of populations, particularly at large scales; chemical treatments can have severe off-target consequences (Callaham et al., 2006; Parmelee et al., 1990), fire has produced only modest reductions and is difficult to use (Ikeda et al., 2015), there is no clear evidence the New Zealand flatworm has catastrophically impacted European earthworms (Murchie and Gordon, 2013), regulation of global trade and transportation is unlikely to be effective or commercially popular (Callaham et al., 2006; Gates, 1982; Hendrix and Bohlen, 2002), and outreach has been largely ineffective at changing public awareness, attitude, or behaviour (Cameron et al., 2013). Many of the same researchers who advocate for management interventions acknowledge that they are likely unrealistic (Addison, 2009; Callaham et al., 2006; Hendrix et al., 2008; Hendrix and Bohlen, 2002; Holdsworth et al., 2007a). One pragmatic concession has been to prioritize and protect areas that remain mostly earthworm-free or most susceptible to ecological harm (Corio et al., 2009; Hendrix and Bohlen, 2002; Holdsworth et al., 2007a, 2007b; Maerz et al., 2009), but this approach is similarly limited by a lack of data and management options.

1.2.4 Implications of exotic earthworms for restoration

Given the key ecological roles that earthworms play in soils, it is surprising that they have not been more widely considered and used in restoration. The scattered cases of earthworms contributing to restoration are most common in their native habitats, mostly in Europe (Butt, 2008; Curry, 1988). Earthworms may be intentionally inoculated into a site (Forey et al., 2018) or the recolonization of a soil by nearby earthworms may be planned as a part of the restoration (Yvan et al., 2012). Earthworms have helped restore a variety of landscapes including

those affected by including agriculture, mining, and industrial activities (Butt, 1999; Muys et al., 2003). Earthworms can help improve soil macroaggregate structure (Blanchart, 1992), reduce compaction (Ampoorter et al., 2011), increase water infiltration (Yvan et al., 2012), incorporate organic matter (Scullion and Malik, 2000; Sizmur et al., 2011), increase plant productivity (Forey et al., 2018; Muys et al., 2003), and act as bioindicators (Snyder and Hendrix, 2008). They are also useful for restoration outreach because they are relatable and safe to handle (Snyder and Hendrix, 2008). However, the broader restoration potential of earthworms (along with other key soil biota such as millipedes, isopods, and termites) has largely been overlooked in their native ranges because of a traditional disciplinary focus on vegetation (Butt, 2008; Jouquet et al., 2014; Snyder and Hendrix, 2008). Researchers advocate greater consideration of native earthworms in restoration and are trying to trying to determine the best practices of species selection and inoculation (Butt, 2008; Butt et al., 1995).

In contrast, exotic earthworms are primarily considered a source of ecological degradation and restoration as a tool that may help control them (Callaham et al., 2006, 2003; Heneghan et al., 2007; Madritch and Lindroth, 2009). However, some researchers have begun to use exotic earthworms for restoration. To help incorporate organic matter into mine waste soils, Vimmerstedt and Finney (1973) inoculated *L. terrestris* (European) in Ohio, USA and Ganihar (2003) inoculated *P. corethrurus* (South American) in India. Baker et al. (1999) used *Aporrectodea* spp. (European, introduced intentionally into New Zealand) to mix lime into the soil to mitigate acidification. The popular vermicomposting worm *Eisenia fetida* (European) has been used to effectively restore the hydrology of clogged constructed wetlands (Li et al., 2011) and to restore coastal saline soils in China (Zhang et al., 2015). Gut-associated microbes found in *L. terrestris* may also help accelerate the decay of low-density polyethylene and help restore heavily plastic-contaminated soils around the world (Huerta Lwanga et al., 2018).

As uses of exotic earthworms in restoration become more common, Snyder and Hendrix (2008) succinctly posed the question: "Are invasive earthworms always detrimental […] or can they be helpful?" They did not provide an answer, however, suggesting that using invasive species to achieve restoration goals raises "ethical issues" beyond the scope of their paper. The case of exotic earthworms in North America is instructive in this discussion because it restricts our options; we are past the point of prevention and there are no practical options for control, meaning that further spread is effectively inevitable (Hendrix and Bohlen, 2002). Whether the

risk of introducing or not removing an exotic species for restoration is acceptable is irrelevant when the exotic species are already present and cannot be removed. Research that focuses on these species primarily as exotic and undesirable taxa, emphasizes detrimental impacts without proper consideration of possible benefits, and advocates control when it is not realistic is clearly unhelpful. An alternative is considering these species as integrated novel components of ecosystems (Bonanno, 2016) and focusing on the impacts (both positive and negative) that they have in a given context rather than on where they came from (Davis et al., 2011).

This is relevant not only for planning to inoculate earthworms as a part of a restoration project (Ganihar, 2003; Vimmerstedt and Finney, 1973), but also for considering how earthworms already present at a restoration site interact with other management interventions. As ecosystem engineers, earthworms have considerable potential to affect how an ecosystem may respond to management (Bohlen et al., 2004; Byers et al., 2006). Poorly understood earthworm interactions may unexpectedly impair or facilitate different management interventions; modifications designed around these interactions could improve the overall efficiency and efficacy of restoration in the large and growing number of earthworm-invaded ecosystems. However, because of our historical focus on earthworms as 'exotic' species that do not belong in these ecosystems, and as 'invasive' species often expected *a priori* to have predominantly negative impacts, we currently have limited research on how earthworms interact with different restoration interventions and how to integrate their effects.

1.3 Purpose and structure of the dissertation

The purpose of this dissertation is to explore the interactions of exotic earthworms with three restoration interventions: seeds, mulch, and wood ash. I have focused primarily on interactions with the 'nightcrawler' earthworm (*Lumbricus terrestris* L.), a commonly studied, geographically-widespread, and ecologically-influential European Lumbricid (Addison, 2009; Keller et al., 2007). As an anecic earthworm, *L. terrestris* is active both belowground and at the soil surface, collecting surface materials such as leaf litter and woody debris into burrows or raised 'midden' structures that sit atop their burrows (Butt and Grigoropoulou, 2010). I was particularly interested in how these collection, aggregation, and burial behaviours might interact with the application of seeds, mulch, and wood ash at the soil surface.

I designed this research to address current knowledge gaps in basic earthworm ecology and to build the empirical research foundations needed to ultimately integrate earthworm interactions into restoration planning to hopefully improve the efficiency and efficacy of restoration in ecosystems inhabited by earthworms. Although this research is based in North America and addresses exotic earthworms as a form of ecological novelty, many of the findings should be transferable to restoration projects in other locations containing the same or functionally similar species, be they native or exotic.

I have organized the remainder of this dissertation into a preface, three data chapters, and a concluding chapter in accordance with the guidelines for a 'manuscript-style' thesis set out by the University of Waterloo (https://uwaterloo.ca/graduate-studies-postdoctoral-affairs/current-students/thesis-preparation). In the following preface I address earthworm taxonomy and identification and general statistical analyses I have used throughout the dissertation. In each of the following data chapters I then focus on one of the three restoration interventions using a combination of laboratory and field-based experiments. In the first data chapter I examine the effects of earthworm seed preferences, ingestion, egestion, and burial on the seedling recruitment of different types of grass seeds (*Chapter 2*). In the second data chapter I examine the effects of mulch amendment on earthworm communities in the field and describe the short- and long-term impacts of earthworms on the spatial distribution of surface-applied mulch (*Chapter 3*). In the final data chapter, I test the responses of earthworms to different types of wood ash and use a novel method to track the earthworm-facilitated burial of surface-applied wood ash (*Chapter 4*). These are followed by a concluding chapter in which I synthesize the observed interactions with the different interventions and briefly discuss some of the broader implications for the management of exotic earthworms in North America and of invasion for restoration more broadly (*Chapter 5*).

1.4 Preface to the data chapters

1.4.1 Earthworm taxonomy and identification

I identified earthworm specimens using the dichotomous keys and specimen photos and diagrams in Reynolds' (1977) *The earthworms (Lumbricidae and Sparganophilidae) of Ontario* and Hale's (2013) *Earthworms of the Great Lakes* and classified them into epigeic, endogeic, or anecic functional groups *sensu* Bouché (1977). Overall, between my three earthworm-collection

field sites (Glenorchy tallgrass prairie restoration, Waterloo North Campus Environmental Reserve, Haliburton Forest) I documented 10 species of exotic European Lumbricids belonging to six genera. All three functional groups were present including three epigeic species, six endogeic species, and one anecic species (Table 1.1).

Table 1.1: Summary of earthworm species documented across all field sites and the level of taxonomic and functional identification possible for adult and juvenile specimens.

Earthworm Species	Maturity	Identification	Functional Classification
Allolobophora chlorotica	Adult	*Allolobophora chlorotica*	Endogeic
	Juvenile	*Allolobophora/Aporrectodea* spp.	Endogeic
Aporrectodea rosea	Adult	*Aporrectodea rosea*	Endogeic
	Juvenile	*Allolobophora/Aporrectodea* spp.	Endogeic
Aporrectodea trapezoides	Adult	*Aporrectodea trapezoides*	Endogeic
	Juvenile	*Allolobophora/Aporrectodea* spp.	Endogeic
Aporrectodea tuberculata	Adult	*Aporrectodea tuberculata*	Endogeic
	Juvenile	*Allolobophora/Aporrectodea* spp.	Endogeic
Aporrectodea turgida	Adult	*Aporrectodea turgida*	Endogeic
	Juvenile	*Allolobophora/Aporrectodea* spp.	Endogeic
Dendrobaena octaedra	Adult	*Dendrobaena octaedra*	Epigeic
	Juvenile	*Dendrobaena octaedra*	Epigeic
Dendrodrilus rubidus	Adult	*Dendrodrilus rubidus*	Epigeic
	Juvenile	*Dendrodrilus rubidus*	Epigeic
Lumbricus rubellus	Adult	*Lumbricus rubellus*	Epigeic
	Juvenile	*Lumbricus* spp.	Epigeic/Anecic
Lumbricus terrestris	Adult	*Lumbricus terrestris*	Anecic
	Juvenile	*Lumbricus* spp.	Epigeic/Anecic
Octolasion tyrtaeum	Adult	*Octolasion tyrtaeum*	Endogeic
	Juvenile	*Octolasion* spp.	Endogeic

I made the identifications based on several diagnostic features including the prostomium, setae, male pores, clitellum, genital tumescences, tubercula pubertatis, colour, and size. For many species, full diagnostic features are only visible on reproductive adults, identified by the

presence of the clitellum, a raised, non-segmented structure on the anterior end of the body. The juveniles of some species possess adequate features to make a species-level identification (e.g., *Dendrobaena octaedra*, *Dendrodrilus rubidus*). For other species, juveniles could only be classified to one or more possible genera. In most cases, this still allowed me to confidently assign juveniles to a functional group (Table 1.1). One exception were the *Lumbricus* spp. juveniles, which I could not confidently determine to be either the epigeic *L. rubellus* or anecic *L. terrestris*, unless I found only one of the two species at a given site. If both were present, I counted the juvenile *Lumbricus* spp. towards a separate functional category.

For the most part, different earthworm taxonomic systems agree regarding naming conventions and the functional classification of the species documented in this dissertation. One exception is the naming of the abundant endogeic 'grey worm', which has been classified by Hale (2013) as *Aporrectodea caliginosa* (Savigny, 1826) but by Reynolds (1977) as *Aporrectodea turgida* (Eisen, 1873). Other researchers suggest that it belongs to a species complex (Pérez-Losada et al., 2012, 2009). I have used Reynolds' (1977) convention, which allows a less ambiguous differentiation between three *Aporrectodea* species (*A. turgida, A. tuberculata, A. trapezoides*) and is mostly used in a Canadian context (Reynolds, personal communication). Another exception is the functional classification of *Lumbricus rubellus*, which is sometimes regarded as either an epigeic or epi-endogeic species (Addison, 2009). I consistently observed them almost exclusively in the litter layer as others have as well (e.g., Abail and Whalen, 2018) and have thus categorized them here as epigeic.

1.4.2 Notes on statistical analyses

General statistical approach

In this dissertation I have primarily used frequentist null hypothesis-based inferential statistics. There is ongoing discussion and debate concerning the relative merits and drawbacks of these approaches, with a common point of critique being emphasis placed on finding 'significant' p-value results based on arbitrary criteria such as $\alpha = 0.05$ (Amrhein et al., 2019; Lärää, 2009; Wasserstein et al., 2019; Wilcox and Serang, 2017). To address some of these issues, I have tried to differentiate between the 'statistical significance' of my results (i.e., the probability of observing these findings under a suitable null hypothesis) and their 'ecological significance' (i.e., the ecological importance of an observed effect). When reporting results, I

only discuss an apparent effect if I have found it to be statistically significant. In addition, I have tried to highlight the direction and magnitude of results where appropriate using a combination of different effect size metrics (Kotrlik et al., 2011; Lakens, 2013) and by reporting either absolute or relative differences between treatments.

For tests in which the data fail to meet the required test assumptions, I have generally chosen to use statistical tests robust to those deviations rather than attempt to adjust the data to fit using transformations. While this sometimes requires using alternative tests that are in some ways less desirable than the original tests (e.g., lower statistical power), I decided that this outweighed the limitations of generating clear ecological interpretations for transformed data, limitations that are sometimes ignored by misleadingly presenting and discussing the original untransformed data (Zuur et al., 2010).

When describing the design of an experiment, I use 'n' to report the number of replicate experimental units per treatment level (for single factor designs) or per unique combination of treatment levels (for crossed, multi-factor designs), and 'N' to report the total number of experimental units. I conducted all statistical tests at the conventional critical value of $\alpha = 0.05$.

I report all means in the text and in figures as mean ± standard deviation (SD). In contrast to other measures of spread such as standard error (SE) or confidence intervals (CI) that are primarily useful for describing the accuracy of estimates of a population parameter (or, more cynically, sometimes to produce figures with smaller error bars) (Gotelli and Ellis, 2013), I chose to use standard deviation as my interest is primarily in describing the amount of variability within a given sample and this does not require the data to conform to any assumptions (as is the case for constructing confidence intervals) (Altman and Bland, 2005; Streiner, 1996).

My primary resources for selecting and interpreting appropriate statistical analyses were Quinn and Keough's (2002) *Experimental Design and Data Analysis for Biologists* and Gotelli and Ellis' (2013) *A Primer of Ecological Statistics*. I used the Real Statistics Resource Pack (V5.6) for Microsoft Excel® to calculate Greenhouse-Geisser and Huynh-Feldt corrections (Zaiontz, 2018) and Minitab® 18.1 for all other analyses. I produced graphics in Microsoft Excel®, Microsoft PowerPoint®, Corel PHOTO-PAINT 12©, Scilab 6.0.1, MATLAB®, and Minitab® 18.1.

Testing assumptions

A common assumption of many statistical tests is normality of the original data or test residuals. Although several formal statistical tests can be used to check for data normality (e.g., Anderson-Darling, Shapiro-Wilk, Kolmogorov-Smirnov), their value is debated; at low sample sizes, these tests often have very low power for detecting deviations from normality, while at large sample sizes they can be overly sensitive in detecting minor and practically irrelevant deviations (Lärää, 2009). Additionally, some statistical tests including most ANOVA-based models are relatively robust to all but the most serious deviations from normality, even at smaller sample sizes (Johnson, 1995). For these reasons, some statisticians recommend checking this assumption for severe deviations qualitatively by visually inspecting the data using boxplots, histograms, or residual plots (Henderson, 2006; Lärää, 2009; Zuur et al., 2010). For ANOVA-based analyses, I inspected plots of the residuals generated by the models. For correlation analyses and t-tests, I inspected the original data using histograms.

Another common assumption is that of homoscedasticity, or equal variance between treatment groups (e.g., for ANOVA) or along a best-fit line (e.g., for correlation analyses). I qualitatively assessed this by visually examining residual plots and boxplots of original data or scatterplots to check for any obvious deviations (Lärää, 2009; Zuur et al., 2010). I supplemented this visual inspection with formal hypothesis testing using Levene's Test. I chose this over Bartlett's Test, a common alternative which can be more powerful but is also less robust to non-normal data (Gastwirth et al., 2009).

Testing linear association

For testing linear associations between two continuous variables I first considered Pearson's (Product Moment) Correlation. This test assumes independent data, equal variance across the best-fit line, and a bivariate normal distribution. In most cases in this dissertation, the data failed to meet the bivariate normality assumption. Simulation studies suggest that violations of this assumption can reduce the power and inflate the Type I error rate of the test (Bishara and Hittner, 2012; Puth et al., 2014). As a non-parametric alternative, I instead used Spearman's Rank Correlation, which conducts a standard Pearson Correlation analysis on ranks of the original data to create a new correlation coefficient (r_s) and tests more generally for a monotonic relationship between two variables (Quinn and Keough, 2002).

Testing differences in means

        The most common statistical methods that I used were for comparing the means of response variables across different treatment levels. I used different procedures depending on whether data were naturally paired, how many factors were included in a given model, and whether repeated measures were included.

        For paired data from two different groups (e.g., differences in earthworm densities between paired control-treatment wood ash amendment plots, Chapter 4) I used either a One-Sample t-Test or a Two-Sample Paired t-Test. These are in effect the same test procedure, differing only in whether I was using paired data for which the difference between the two had already been calculated (One-Sample t-Test) or not (Two-Sample Paired t-Test). These tests compare whether the mean of a single sample or the mean difference between two paired samples differs from a specified value (in my case, H_0: $\bar{x} = 0$). I ran both tests as two-tailed tests (i.e., H_A: $\bar{x} \neq 0$). Both tests assume samples are independent and each response variable follows a normal distribution.

        Many of the analyses that I ran used Analysis of Variance (ANOVA) models. As a general family of statistical tools, these models partition the total variance in the data to different sources (e.g., treatments, interactions between treatments, residual error). In a simple analysis, the overall approach is to compare the amount of variation between different levels of a predictor variable or treatment to the amount of variation within treatment level groups; a higher ratio of between-to-within treatment variation is considered indicative of a treatment effect.

        For data with one treatment (e.g., effects of different earthworm densities on total grass seed burial in microcosms, Chapter 2), I used One-Way ANOVA. For data with two treatment variables (e.g., effects of seed type and burial depth on grass seedling emergence, Chapter 2), I used Two-Way ANOVA. When Two-Way ANOVA results indicated no statistically significant interaction between the two treatments, I present the main effects of each predictor averaged across the levels of the other. When a statistically significant interaction was present, I did not discuss main effects and instead conducted additional analyses (where relevant to my research questions) of the simple main effects of each treatment at each level of the other treatment. For data with two treatments in which one was repeated either temporally (e.g., the effects of mulch amendment rate on earthworm density across multiple time periods, Chapter 3) or spatially (e.g.,

the effects of earthworm density on wood ash burial across subsequent depths, Chapter 4), I used a repeated measured approach with Mixed ANOVA, including a replicate identity variable (e.g., plot number) as a random factor nested within the fixed 'between subjects' factor (e.g., mulch amendment rate, earthworm density) which is crossed with the fixed 'within subjects' factor (e.g., year, depth). If I found a statistically significant interaction between the two main fixed factors and I was interested primarily in the between subjects factor, I analyzed the simple main effects at each level of the within subjects factor using One-Way ANOVA. If there was an interaction and I was interested in the within subjects I factor, I analyzed the simple main effects at each level of the between subjects factor using Two-Sample Paired t-Tests if the within subjects factor had two levels (e.g., Year 1, Year 2) and One-Way Repeated Measures ANOVA if it had more than two levels (e.g., eight sequential soil depths).

ANOVA models generally assume independent data, normally distributed test residuals, and equal variance between treatment levels (i.e., homoscedasticity). Most ANOVA procedures are robust to all but the most extreme deviations of residuals from normality and will generally experience only modest reductions in power for non-normal data (Blanca et al., 2017; Liu, 2015). I followed the recommendation to proceed with the standard test unless the departure from normality was particularly severe because the possible loss of power may be preferable to interpreting transformed data (Zuur et al., 2010) or to the intrinsically lower power and susceptibility to other violations of assumptions of alternative procedures (e.g., non-parametric Kruskall-Wallis test) (Blanca et al., 2017; Johnson, 1995; Moder, 2010).

A greater concern, however, is the susceptibility of these models to deviations from the equal variance assumption. When the variance between different treatment levels is larger, the Type I error rate of the test may deviate from nominal levels, especially when treatment group sizes are more unequal (Jan and Shieh, 2014; Moder, 2010). For One-Way ANOVA analyses in which the equal variance assumption was violated, I used Welch's Test (or Welch's ANOVA) as an alternative procedure (Welch, 1951). Welch's Test mitigates unequal variance using a weight term scaled by the sample size and variance of different treatment levels that changes the calculation of the pooled error estimate and reduces the degrees of freedom in the ANOVA F-ratio. Although Welch's Test has lower power and can inflate Type I error rates for very non-normal data, it is still considered one of the best alternatives to One-Way ANOVA for dealing with heteroscedasticity (Jan and Shieh, 2014; Liu, 2015; Moder, 2010).

A related assumption for ANOVA models with repeated measures is sphericity, or equal variance across all pairings of the repeated factor. Although there are statistical tests for sphericity (e.g., Mauchly's Test), these are often considered unreliable (Quinn and Keough, 2002). Given the challenges of testing for sphericity and how commonly it is violated, I used a conservative correction procedure that reduces the degrees of freedom for any model terms that include the repeated factor. I used the Huynh-Feldt correction (ε_{HF}) if estimates of departures from sphericity were small (i.e., $\varepsilon > 0.75$) and the more conservative Greenhouse-Geisser correction (ε_{GG}) if estimates of departure were larger (i.e., $\varepsilon < 0.75$) (Quinn and Keough, 2002).

I was not able to find a suitable procedure for dealing with unequal variance in Two-Way ANOVA models. Fortunately, violations of this assumption were not common in these data sets and their implications are likely minor in the few cases where they did occur because of generally small, non-marginal p-values (i.e., several orders of magnitude distant from $\alpha = 0.05$) and significant interactions that primarily required analyses of simple main effects for which alternative test procedures (e.g., Welch's Test, ε corrections) were available if necessary.

For omnibus tests indicating statistically significant effects of treatments with more than two levels, I used complementary post-hoc tests. Based on the recommendations of Ruxton and Beauchamp (2008), I used Tukey's Honestly Significant Difference (HSD) Test (also known as the Tukey-Kramer Test when modified for unequal sample sizes) for ANOVA models and the less powerful Games-Howell Test for Welch's Test. Both were used to create alphabetic groupings to describe statistically significant differences between treatment levels.

Effect sizes

To help communicate the ecological significance of my results and facilitate comparisons between the relative importance of different experimental factors, I calculated effect size metrics following statistically significant hypothesis tests. I measured effect size using Cohen's D for Paired Samples (d_z) for Paired t-Tests, the correlation coefficient (r_s) for Spearman's Rank Correlation, the coefficient of determination (R^2) for Welch's Test, and the omega-squared (ω^2) family of metrics for ANOVA analyses, which is considered less biased by differences in sample size compared to other metrics (Lakens, 2013; Olejnik and Algina, 2000, 2000; Rosenthal, 1991). Cohen's D is a mean difference scaled by a pooled standard deviation while the other metrics describe a proportion of variance explained by a given factor. For multi-factor ANOVA

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models (e.g., Two-Way ANOVA, Mixed ANOVA) I used partial omega-squared (ω_p^2) to describe the partial variance explained by a single factor in isolation from the variance contributed by other model factors (Keren and Lewis, 1979).

Chapter 2: Anecic earthworms (*Lumbricus terrestris* L.) reduce initial recruitment from grass seed by preferential granivory and burial

Abstract

   Recruitment limitation of surface-sown seeds can be a key limiting factor to restoration success and earthworms may play an underrecognized role in altering seedling recruitment through granivory and seed burial. I used the common and ecologically influential anecic earthworm *Lumbricus terrestris* L. and commercial grass seed mixes as model organisms in a series of experiments including: artificial burial of grass seeds at different depths to determine the sensitivity of recruitment to seed burial (Experiment 1); tracking the depth and position of seed buried by different densities of earthworms (Experiment 2); no-choice feeding trials to determine earthworm granivory preferences based on seed size category and the presence/absence of a water-absorbent seed coating (Experiment 3); germination experiments to determine the effects of earthworm ingestion-egestion on seed germination (Experiment 4); monitoring initial recruitment from seed mixes with different seed coatings (uncoated or coated) in the presence or absence of earthworms (Experiment 5); and field exclosures that controlled the access of earthworms and other granivores (e.g., birds, rodents) to determine the interactive effects of different taxa on seedling recruitment (Experiment 6). Earthworms reduced initial seedling recruitment by burying seed too deeply to germinate and reach the surface. Shallowly buried seed that could still germinate was spatially aggregated. Earthworms reduced recruitment from both seed mixes but caused stronger reductions for the mix with the preferred water-absorbent seed coating. Earthworms also reduced seedling recruitment under field conditions, but their effects were only detectable when other granivores were excluded. Modifying seed mix composition on a case-by-case basis to take advantage of or compensate for earthworm seed preferences could help improve the efficiency of seed use in soils with abundant earthworm communities.

2.1 Introduction

Constraints on seedling recruitment from surface-sown seed can be a key limitation to restoration success (James et al., 2011; Standish et al., 2007). Recruitment can be influenced by biotic interactions spanning a complex range of antagonistic (e.g., granivory) and beneficial effects (e.g., dispersal) (Chambers and MacMahon, 1994). Although mammals and birds have historically been considered the dominant sources of seed predation and dispersal in many systems (Grant, 1983), researchers have been interested in the importance of earthworm-seed interactions dating back to Darwin (1881), with a renewed interest in recent years (Forey et al., 2011).

Deep-burrowing, surface-foraging anecic earthworms (*sensu* Bouché, 1977) are considered the most ecologically important earthworm granivores and seed dispersers (Asshoff et al., 2010; Grant, 1983). Anecic earthworms can ingest seeds selectively or coincidentally while burrowing through the soil (McCormick et al., 2013), while seeds too large to be ingested may be cached belowground or in 'middens' formed atop burrow openings at the soil surface (Eisenhauer and Scheu, 2008; Regnier et al., 2008). Ingested seeds are subsequently exposed to physical and chemical digestive processes and may be destroyed or egested back into the soil (Curry and Schmidt, 2007). These egested seeds may germinate at higher or lower rates (Decaëns et al., 2003; Eisenhauer et al., 2009a; Grant, 1983). Earthworms also transport seeds vertically through the soil (McCormick et al., 2013; Regnier et al., 2008; Willems and Huijsmans, 1994) and can consume and destroy recently germinated seedlings (Eisenhauer et al., 2010; Griffith et al., 2013). Overall, earthworm-seed interactions can have variable positive or negative outcomes for plants that vary between different combinations of earthworm and plant species (Eisenhauer et al., 2009a; Grant, 1983).

While there is a growing body of literature concerning how earthworm-seed interactions influence plant community composition over longer time scales (Forey et al., 2011; Frelich et al., 2006; Hale et al., 2006, 2005; Nuzzo et al., 2015, 2009), few studies have focused on how earthworms affect initial seedling recruitment from seed mixes, which may be important for restoration. Seeding introduces high densities of seed that may be subject to immediate density-dependent granivory and dispersal (James et al., 2011; Mitchell and Brown, 1990). Earthworms may influence the fates of these seeds as they can detect and respond to high densities of aboveground food (Butt et al., 2003), have foraging strategies that are highly density dependent

(McTavish and Murphy, 2019), and can ingest and bury large quantities of seed very quickly (Cassin and Kotanen, 2016; Eisenhauer and Scheu, 2008; Milcu et al., 2006; Quackenbush et al., 2012; Regnier et al., 2008).

Seed burial is suspected to be a leading cause of seedling recruitment limitation by earthworms (Cassin and Kotanen, 2016; McCormick et al., 2013; Milcu et al., 2006; Regnier et al., 2008) but is poorly understood because of the challenges of studying a primarily nocturnal and belowground behaviour (Butt and Grigoropoulou, 2010). Burial can occur by ingestion and egestion, collection and caching, and coincidental adhesion to the earthworm mucus coating and burrowing (Milcu et al., 2006; Regnier et al., 2008; Shumway and Koide, 1994). Burial can reduce recruitment when seeds are buried too deeply in the soil to germinate or reach the surface (Regnier et al., 2008) or beyond shallow-living fungi needed by some plant species to help acquire water and nutrients (McCormick et al., 2013). Notably, earthworms can rapidly bury large quantities of seed from the soil surface within the first several days following seed addition (Cassin and Kotanen, 2016; Eisenhauer and Scheu, 2008; Milcu et al., 2006; Quackenbush et al., 2012; Regnier et al., 2008). Despite the importance of seed burial to seed bank formation, seedling recruitment, and seedling aggregation (Grant, 1983), few studies have documented the actual spatial patterns of seed burial by earthworms in great detail (Milcu et al., 2006; Regnier et al., 2008; Zaller and Saxler, 2007).

Initial recruitment may also be affected by earthworms preferentially ingesting certain seeds over others. Earthworms use physical and chemical traits such as seed size, shape, oil content, coat texture, and plant functional identity to identify and selectively consume different seeds (Clause et al., 2017, 2011; Curry and Schmidt, 2007; Eisenhauer et al., 2010; Shumway and Koide, 1994; Willems and Huijsmans, 1994). Seeds preferred by earthworms will generally be ingested in greater numbers, which can result in more seeds being destroyed by digestion, experiencing boosted or impaired germination (Decaëns et al., 2003), or being buried. More strongly earthworm-preferred seeds may benefit or be negatively impacted by these interactions depending on environmental conditions and the species of earthworm and plant (Eisenhauer et al., 2009a; Grant, 1983). While other researchers have considered the implications of earthworm seed preferences for seedbank dynamics and plant community structure and function (Aira and Piearce, 2009; Donath and Eckstein, 2012; Drouin et al., 2014), the consequences of earthworm

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preferences for recruitment limitation have received minimal study (Eisenhauer and Scheu, 2008; Milcu et al., 2006; Regnier et al., 2008).

Finally, while earthworm granivory and seed dispersal have mostly been studied in isolation from other taxa, earthworm impacts in a natural field setting will often occur alongside the effects of other granivores and seed dispersers. In North America, small mammals including grey squirrels (*Sciurus carolinesis*), eastern chipmunk (*Tamias stratus*), and deer mice (*Peromyscus* spp.) and various bird species are generally thought to be the primary contributors to seed removal (Cassin and Kotanen, 2016; Grant, 1983). More recently, soil invertebrates including earthworms, isopods, millipedes, and beetles have also been implicated as major seed predators and dispersers (Cromar et al., 1999; Pufal and Klein, 2013; Westerman et al., 2003), but their interactions with other granivores in the field have seldom been examined experimentally, with few exceptions (e.g., Cassin and Kotanen, 2016).

A better understanding of earthworm-seed interactions could help optimize restoration project decisions such as seed mix composition, quantity, and timing of application and ultimately improve the efficiency of seed mix use in soils with abundant earthworm communities. In this study I explore how earthworms affect the fate of seed added to the soil surface. It was guided by three primary research questions: (1) How does earthworm seed burial affect recruitment from seed? (2) How do earthworm seed preferences affect the initial performance of different seed mixes? and (3) How do earthworm impacts on seedling recruitment compare to and interact with impacts from other taxa? I addressed these research questions with laboratory and field experiments using the geographically-widespread and ecologically-influential anecic earthworm *Lumbricus terrestris* L. (Addison, 2009; Keller et al., 2007) and commercial grass seed mixes.

2.2 Methods

2.2.1 Earthworms, seeds, soil, and litter

Several experiments used common materials or methods described here. I purchased adult *L. terrestris* earthworms from a commercial bait vendor (Waterloo, Ontario, Canada) prior to each experiment and stored them at c. 6 °C for a maximum of two weeks prior to use. I purchased commercial grass seed including a standard 'uncoated' grass seed mix (Scotts Turf Builder® Grass Seed, Sun & Shade Mix) and a 'coated' version of the same seed mix treated

with WaterSmart® PLUS Coated Seed Technology (Scotts). The coating is 96 % (by weight) limestone and 4 % ZEBA®, a patented, starch-based product designed to increase water uptake and nutrient retention (Zeba). Both products contain a mix of three common lawn grass species: 42 % (by abundance) Creeping Red Fescue (*Festuca rubra* L.), 34 % Kentucky Bluegrass (*Poa pratensis* L.), and 24 % Turf-Type Perennial Ryegrass (*Lolium perenne* L.). By weight, the coated seed mix is 50 % seeds and 50 % coating. The three grasses are Eurasian species widely used for turf and considered naturalized or exotic in different parts of North America (USDA PLANTS Database). I used grass seed because of high and fast germinability and the availability of otherwise similar seed mixes that differed only in seed coating.

Different experiments used 'seed category' (which sorted different seed types by size) and 'seed coating' as treatment variables. For these purposes, I hand sorted mixes into 'large' seeds (*F. rubra* and *L. perenne*) and 'small' seeds (*P. pratensis*). Coating had a negligible impact on seed size but increased individual seed weight by approximately 50 %, 23 %, and 80 % for *F. rubra, L. perenne,* and *P. pratensis* respectively. The 'large' seeds had lower oil content and marginally higher protein content compared to the 'small' *P. pratensis* seed (Table 2.1) (Seed Information Database: Royal Botanic Gardens, Kew).

Table 2.1: Summary of grass seed properties. Length and width estimated from averages of 10 random seeds and individual seed mass from the average mass of 50 seeds. Whole seed oil and protein content for *L. perenne* and *P. pratensis* from Jones and Earle (1966) and Earle and Jones (1962) respectively. Oil and protein content for *F. rubra* based on data from Barclay and Earle (1974) for the phylogenetically similar *F. ovina* (Cheng et al., 2016). All results given as mean ± standard deviation (SD).

Seed Species	Size Class	Coating	Length (mm)	Width (mm)	Mass (mg)	Oil (%)	Protein (%)
Creeping Red Fescue (*Festuca rubra* L.)	'Large'	Uncoated	5.9 ± 0.7	1.1 ± 0.2	1.4	2.2	26.1
		Coated	5.6 ± 0.7	1.2 ± 0.2	2.1		
Perennial Ryegrass (*Lolium perenne* L.)	'Large'	Uncoated	5.3 ± 0.6	1.4 ± 0.2	2.6	1.8	18.8
		Coated	5.2 ± 0.6	1.3 ± 0.1	3.2		
Kentucky Bluegrass (*Poa pratensis* L.)	'Small'	Uncoated	2.8 ± 0.2	0.8 ± 0.1	0.5	8.9	14.4
		Coated	2.6 ± 0.4	0.8 ± 0.1	0.9		

For the earthworm seed burial (Experiment 2) and grass growth microcosm experiments (Experiment 5) I used an artificial soil with a neutral pH of 7.3 made by mixing three parts (by volume) Circle H Farms Black Earth potting soil with two parts screened topsoil known from past experiments to be suitable for earthworm survivourship, growth, and burrowing behaviour. I provided leaf litter as an alternate food source for earthworms in the earthworm seed burial (Experiment 2), grass growth (Experiment 5), and granivore exclosure experiments (Experiment 6). The leaf litter was a mix of sugar maple (*Acer saccharum* Marshall) and Norway maple (*Acer platanoides* L.) I collected from a woodlot on the University of Waterloo campus and air-dried for five days.

2.2.2 Earthworm seed burial

I examined how earthworms bury seed and how this affects recruitment using: a microcosm experiment to assess the sensitivity of recruitment to artificial burial in the absence of earthworms (Experiment 1); and a microcosm experiment to track the burial of surface-sown seeds belowground in the presence of different earthworm densities (Experiment 2).

Experiment 1: Artificial burial experiment

I assessed the effects of seed type (uncoated, coated) and sowing depth (0 cm, 1 cm, 2 cm, 6 cm) on grass recruitment in a two-way factorial microcosm experiment (n = 4 microcosms per unique treatment combination, total N = 32). I chose depths to compare non-buried seed (0 cm depth) to near surface burial (1 cm, 2 cm) and deeper burial (6 cm). I filled opaque plastic cylinders (7 cm diameter, 13 cm height) with a commercial potting soil (Circle H Farms, Black Earth) to a depth of 10 cm, placing 25 seeds at one of the four depths, and positioned the cylinders randomly on an open-air plant stand (22.5 °C, RH = 60%, 14 h light (5,000 lux):10 h dark) (Figure 1.1). I watered the cylinders every two days with 50 mL deionized (DI) water. After 21 days, I measured emergence (%) and harvested, dried (72 h at 60°C), and weighed all aboveground grass biomass. Response variables were total seedling emergence (%) and aboveground grass dry biomass. I assessed the effects of seed type and sowing depth on total seedling emergence and aboveground grass dry biomass using Two-Way ANOVAs (for all statistical tests in this dissertation, see *1.4.2 Notes on statistical analyses* for general details

regarding testing of assumptions, alternative tests used when assumptions were not met, and post-hoc testing).

Figure 2.1: Artificial burial cylinders on a plant stand with grass seed artificially sown at different soil depths (0 cm, 1 cm, 2 cm, 6 cm).

Experiment 2: Earthworm seed burial experiment

I used a complementary microcosm experiment to test the effects of different earthworm densities (none, 'low', 'high') on the burial and emergence of surface-sown grass seed (n = 6 microcosms per earthworm density, total N = 18). I only used coated grass seed because I found it was ingested in greater quantities than uncoated seed and would result in more seed burial to measure. The 'low' and 'high' earthworm density treatments consisted of one or three earthworms per microcosm respectively, corresponding to earthworm densities of 57 m^{-2} and 170 m^{-2} and simulating the range of *L. terrestris* densities I observed in local forests.

I cut opaque cylindrical microcosms (0.16 m diameter, 0.30 m height) vertically in half to allow removal of soil by depth, taped together, filled them to a depth of 14 cm with the artificial soil, and watered them with 0.5 L tap water. I added adult *L. terrestris* at rates of zero earthworms per microcosm (none), one earthworm per microcosm ('low' density), and three earthworms per microcosm ('high' density). I applied 1 g of crushed maple litter to the surface to provide an initial food source. I then covered microcosms with window screen mesh and transferred them to a growth chamber to approximate local late spring conditions (21°C, 50% RH, 14 h light:10 h dark). After five days of acclimation, I removed any remaining surface leaf

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litter and randomly spread 230 coated grass over the microcosm surface according to the supplier's recommended seeding rate (16 g·m^{-2}).

   After seven days, I opened each soil column and separated them into horizontal cross-sections including the surface soil (0-1 cm) and six 2 cm-thick subsurface cross sections (1-3 cm, 3-5 cm, 5-7 cm, 7-9 cm, 9-11 cm, 11-13 cm) (Figure 2.2a). I harvested, dried (72 h at 60°C), and weighed aboveground grass biomass. I transferred the soil cross sections to plastic plates, placed them on an open-air plant stand (22.5 °C, RH = 60%, 14 h light (5,000 lux):10 h dark), and watered them with 30 mL tap water every 2-3 days (Figure 2.2b). After two weeks, I marked the position of each emerging grass shoot with a plastic straw and took a photograph (Figure 2.2c). I measured the position for each emerging seed and the centroid of all seeds buried in each cross section using ImageJ (V1.52a) and averaged the distance of each seed from the centroid for its cross section across all depths for each microcosm.

Figure 2.2: Deconstruction of an earthworm seed burial microcosm including (a) removal of a 2 cm-thick soil cross section; (b) grass seed growing out of soil slices transferred to a plant stand; and (c) plastic straws marking grass seedling positions.

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I analyzed the spatial dispersion of seeds buried in the top 1-5 cm of the soil using a sector-based method adapted from Milcu et al. (2006). I plotted the two-dimensional positions of each seed in the top 1-5 cm of the soil on a circle divided into 14 approximately equal-sized sectors. I analyzed the spatial dispersion of seeds using the Variance to Mean Ratio (VMR) and Morisita's Index (I_M), which describes how more or less likely two points chosen randomly will be in the same sector relative to randomly distributed points (Morisita, 1959). For both indices, values of < 1 are indicative of over-dispersion, close to 1 of a random distribution, and > 1 of clustering. I have included VMR despite criticisms that different spatial patterns can produce the same ratios and its notable scale-dependence (Horne and Schneider, 1995; Hurlbert, 1990) because it is still widely used and has been supplemented by Morisita's Index.

Response variables were aboveground grass dry biomass, total seed burial (%), seed burial to a given soil depth, mean seed distance from depth centroid, and indices of dispersion of seeds in the top 1-5 cm (VMR, I_M). I assessed the effects of earthworm density on aboveground grass dry biomass, total seed burial, seed distance from centroid, and indices of dispersion with One-Way ANOVAs. I assessed the effects of earthworm density on seed burial by depth using a Mixed Effects ANOVA with microcosm as a random factor nested within earthworm density and earthworm density and depth as fixed factors (see *1.4.2 Notes on statistical analyses*).

2.2.3 Impacts of earthworm seed preferences on recruitment

I assessed the impacts of earthworm seed preferences on initial recruitment from seed mixes using: an initial feeding experiment to determine the effects of seed category and seed coating on seed ingestion and egestion (Experiment 3); a follow-up germination experiment to determine the effects of earthworm egestion on seed germination (Experiment 4); and a microcosm growth experiment to determine the performance of grass seed mixes with different seed coatings in the presence and absence of earthworms (Experiment 5). Based on the findings of the feeding experiment, I selected coating as the seed characteristic to be assessed further in the growth experiment.

Experiment 3: Feeding experiment

I assessed the effects of seed category ('large' seeds, 'small' seeds) and seed coating (uncoated, coated) on earthworm granivory (seed ingestion, seed egestion) in a two-way factorial

feeding experiment adapted from McRill and Sagar (1973) (n = 10 feeding dishes per combination of size and coating, total N = 40). After a 24 h fast, I weighed the earthworms and transferred them to individual Petri dishes (15 cm diameter) lined with filter paper (cellulose, Grade 1, 11 µm pore size). Each dish received 20 seeds of a given category and coating type at a seed density of 1132 m^{-2} chosen to be consistent with similar studies (e.g., Flinn, 2017; Grant, 1983) (Figure 2.3). Following an 18 h feeding period, I rinsed the earthworms and transferred them to identically prepared, seed-free dishes for a 48 h egestion period. I determined seed ingestion from how many seeds were removed during the feeding period. After the egestion period, I removed the earthworms and hand searched the remaining casts for egested seeds. I retained the egested seed for the germination experiment (*Experiment 4*). I conducted the feeding trials with the Petri dishes placed randomly in a growth chamber (24 h dark, 80 % RH, 15 °C).

Figure 2.3: Earthworms in no-choice feeding trial dishes with seeds added.

Response variables were seed ingestion (total number of ingested seeds) and seed egestion (% of ingested seed subsequently egested). I assessed the effects of seed category and seed coating on each of ingestion and egestion using Two-Way ANOVAs and tested associations between earthworm fresh mass and seed ingestion or egestion for each unique treatment combination using Spearman's Rho Correlation (r_s) (see *1.4.2 Notes on statistical analyses*).

Experiment 4: Germination experiment

I assessed the impacts of seed type (uncoated 'large', uncoated 'small', coated 'large', coated 'small') and seed egestion (control, earthworm egested) on seed germination (cumulative total germination, days to peak germination) in a two-way factorial experiment (n = 3 replicates of 25 seeds per unique treatment combination, total N = 24). Because the relative germinability of different types of seeds was not my primary interest, I treated combinations of seed category and coating as a single 'seed type' variable to avoid a three-way analysis (i.e., coating × category × egestion). I subjected control seeds to the same conditions as in the seed ingestion and preference trials (*Experiment 3*) but without earthworms.

For each treatment combination, I transferred groups of 25 seeds to Petri dishes (9 cm diameter) lined with filter paper (cellulose, Grade 1, 11 µm pore size), moistened with 20 mL DI water, and covered with a perforated lid. I placed the labeled dishes randomly on an open plant stand (23 °C, RH = 30%, 12 h light (5,000 lux):12 h dark) (Figure 2.4). I measured germination and re-moistened the filter paper every two to four days. I counted a seed as germinated once the seedling had grown to a height of ≥ 1 cm. Response variables were total seed germination (cumulative percentage of seeds germinated) and the number of days to peak germination (i.e., the number of days after which the highest cumulative germination was reached). I assessed the effects of seed type and previous seed egestion on total seed germination and days to peak germination using Two-Way ANOVAs (see *1.4.2 Notes on statistical analyses*).

Figure 2.4: Grass seeds (egested by earthworms or control seeds) germinating on plant stand.

Experiment 5: Grass growth experiment

I used a two-way factorial microcosm experiment to assess the impacts of seed coating (uncoated, coated) and earthworm presence (earthworms absent, earthworms present) on the growth of the different grass seed mixes (n = 3 microcosms per unique treatment combination, total N = 12). I filled plastic nursery pots (28 cm diameter, 28 cm height) to a depth of 18 cm with the artificial soil and placed them in 10 cm of standing tap water to maintain soil moisture. I fasted healthy adult *L. terrestris* for 24 hours, and then weighed and randomly added them in groups of four to half of the microcosms at an earthworm density of 65 m^{-2} to simulate population densities I observed in local forests. As in the seed burial experiment, I gave each microcosm 4 g of maple litter and then covered and transferred them to a growth chamber set to approximate local spring conditions (21 °C, 50% RH, 14 h light:10 h dark).

I watered the microcosms with c. 0.5 L of tap water every 2-3 days. After an 11-day acclimation period, I removed any remaining surface litter to simulate seeding onto bare soil and hand applied seed according to the supplier's recommended seeding rate (16 g·m^{-2}). I monitored microcosms and gave them c. 0.5 L tap water every 2-3 days (Figure 2.5). After 16 days, I harvested, dried (72 h at 60°C), and weighed aboveground grass biomass. The response variable was aboveground grass dry biomass. I assessed the effects of seed coating and earthworm presence on aboveground grass biomass using a Two-Way ANOVA (see *1.4.2 Notes on statistical analyses*).
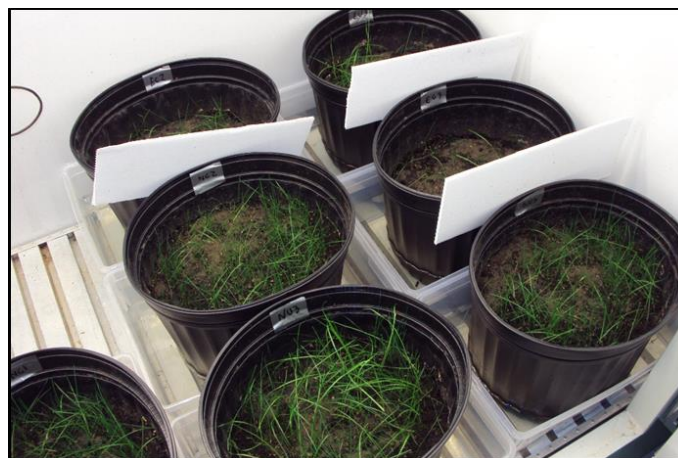
Figure 2.5: Grass growth microcosms (with earthworms absent or present) in growth chamber before harvesting of aboveground grass biomass.

2.2.4 Interactive effects of earthworms and other granivores on recruitment

Experiment 6: Granivore exclosure field experiment

I assessed the interactive effects of earthworms and other granivores on initial recruitment from seed in the field using a field microcosm experiment in which grass seed was sown in an open field with exclosures to control earthworm access to the plots (earthworms excluded, earthworms allowed) and the access of other granivores such as mammals and birds (others excluded, others allowed) in a two-way factorial experiment (n = 6 microcosms per unique treatment combination, total N = 24).

I conducted the field trial in early October 2016 in a bare, recently tilled soil surrounded by lawn (Columbia Lake, Waterloo, Ontario) (Figure 2.6a). I chose this site because of its proximity to a nearby waterbody and woodlot as potential sources of wildlife and a low density of earthworms in the soil due to recent tilling. I placed nursery pots (28 cm diameter, 28 cm height) 20 cm apart in a trench, using soil from the trench to backfill the spaces between the pots and fill the pots level with the surrounding soil. I gave each pot 5 g of maple litter and 0.5 L of tap water and added groups of five earthworms to half of the microcosms to create a realistic population density (81 m^{-2}) while also attempting to offset potential losses due to mortality or escape.

After a 14-day acclimation period, I removed any remaining surface litter and hand seeded the pots with 1 g of uncoated commercial grass seed to match the supplier's recommended seeding rate ($16 \text{ g}\cdot\text{m}^{-2}$). I only used uncoated grass seed due to a limited availability of the coated grass seed. I fit pots assigned to the 'other granivore absent' treatments with cylindrical exclusion covers made of ¼" hardware cloth (35 cm diameter, 6.5 cm height) (Figure 2.6b). I watered pots with c. 1 L tap water and monitored them every 2-3 days. To avoid a confounding effect of the exclusion collars on grass growth, once I observed growth (which occurred after 14 days), I removed the top 2 cm of soil from each pot and transferred it to plastic plates on an open, indoor plant stand to allow seeds to grow under equal conditions (23 °C, 30 % RH, and 12 h light (5,000 lux): 12 h dark). After 16 days, I harvested, dried (72 h at 60°C), and weighed aboveground grass biomass. I assessed the effects of earthworm and other granivore exclusion on grass dry biomass using a Two-Way ANOVA (see *1.4.2 Notes on statistical analyses*).
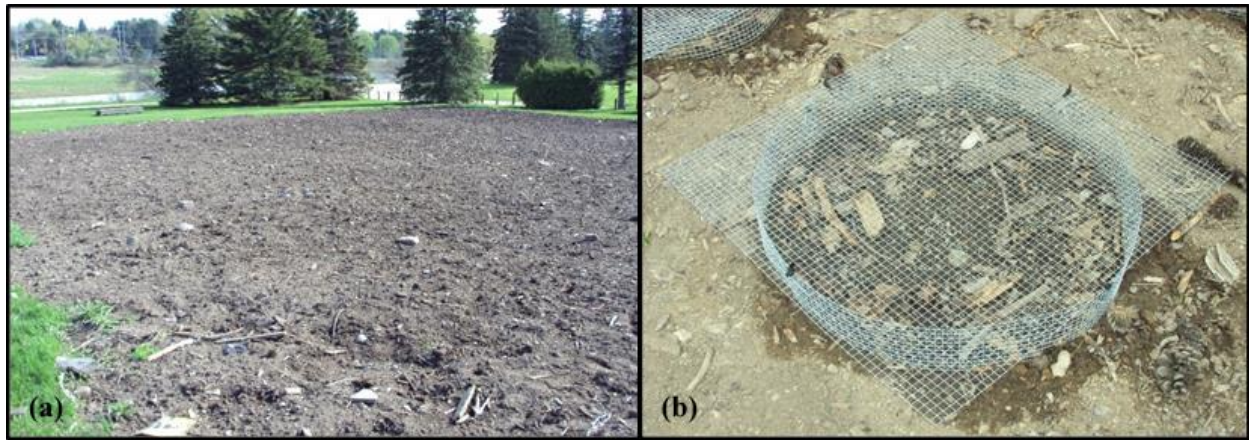
Figure 2.6: (a) Tilled field beside Columbia Lake where I established the granivore exclosure field experiment. (b) An exclusion collar on a buried pot to exclude non-earthworm granivores.

2.3 Results

2.3.1 Earthworm seed burial

In the absence of earthworms, overall seedling emergence was not statistically significantly different at the surface and 1 cm and 2 cm depths but was 68 % lower when seed was sown 6 cm deep (Two-Way ANOVA, $F_{3,24} = 72.33$, $p < 0.001$, $\omega_p^2 = 0.87$) (Table 2.2). Additionally, the overall emergence of the coated seed was 25 % lower than that of the uncoated seed (Two-Way ANOVA, $F_{1,24} = 37.45$, $p < 0.001$, $\omega_p^2 = 0.53$) (Table 2.2).

Table 2.2: Summary of main effects of seed type (n = 16 per seed type) and sowing depth (n = 8 per depth) on seedling emergence (% of total seed). Superscript letters indicate groupings from Tukey's HSD Test (capital letters: seed type, lower case letters: sowing depth). All results given as mean ± standard deviation (SD).

Factor	Factor Level	Seedling Emergence (%)
Seed Type	Uncoated	$76.2^A \pm 27.5$
	Coated	$57.0^B \pm 23.2$
Sowing Depth	0 cm (Surface)	$85.5^a \pm 13.7$
	1 cm	$82.0^a \pm 9.8$
	2 cm	$71.5^a \pm 17.2$
	6 cm	$27.5^b \pm 12.6$

In the seed burial experiment with earthworms added, aboveground grass biomass was highest in the absence of earthworms and was reduced by 59-65 % in the presence of 'low' or 'high' earthworm density (One-Way ANOVA, $F_{2,15}$ = 17.79, p < 0.001, ω^2 = 0.65) (Table 2.3). In the absence of earthworms, no seed was buried over the seven days of the experiment. Higher densities of earthworms buried more seed, burying 6.5 % of total seeds in the 'low' density treatment and 17.4 % at the 'high' density treatment (One-Way ANOVA, $F_{1,10}$ = 10.78, p = 0.008, ω^2 = 0.45) (Table 2.3). The number of seeds buried to a given depth was affected only by earthworm density (Mixed Effects ANOVA, $F_{1,10}$ = 10.78, p = 0.008, ω_p^2 = 0.45); earthworms buried an average of 2.5 ± 2.8 seeds at 'low' density to each of the six measured depths and 6.7 ± 6.6 seeds at 'high' density.

Table 2.3: Summary of effects of earthworm density (none, 'low', 'high') on grass seeds in burial microcosms (n = 6 per earthworm density). Superscript letters indicate groupings from Tukey's HSD Test (capital letters: grass biomass, lower case letters: seed burial, lower case italic letters: seed distance). All results given as mean ± standard deviation (SD).

Earthworm Density	Grass Dry Aboveground Biomass (mg)	Total Seed Burial (no.)	Seed Distance from Centroid (cm)
None	142.9^A ± 35.5	-	-
'Low'	58.6^B ± 27.9	15.0^a ± 8.6	1.9^a ± 0.6
'High'	49.4^B ± 25.4	40.0^b ± 16.6	3.4^b ± 0.8

Due to a low number of replicates and the absence of seed location data for certain depths, I could not analyze patterns of mean seed distance from the centroid of seed locations by depth. However, when comparing the overall seed distance from centroids averaged across all depths for each microcosm, this distance was approximately 79 % greater in the 'high' earthworm density treatment compared to the 'low' density treatment (One-Way ANOVA, $F_{1,10}$ = 13.76, p = 0.004, ω_p^2 = 0.52), suggesting that buried seeds were more spread out horizontally at any given depth at higher earthworm densities (Table 2.3). I often found buried seeds in small clusters and in several microcosms they seemed to fall along a single line that crossed obliquely between depths (Figure 2.7).
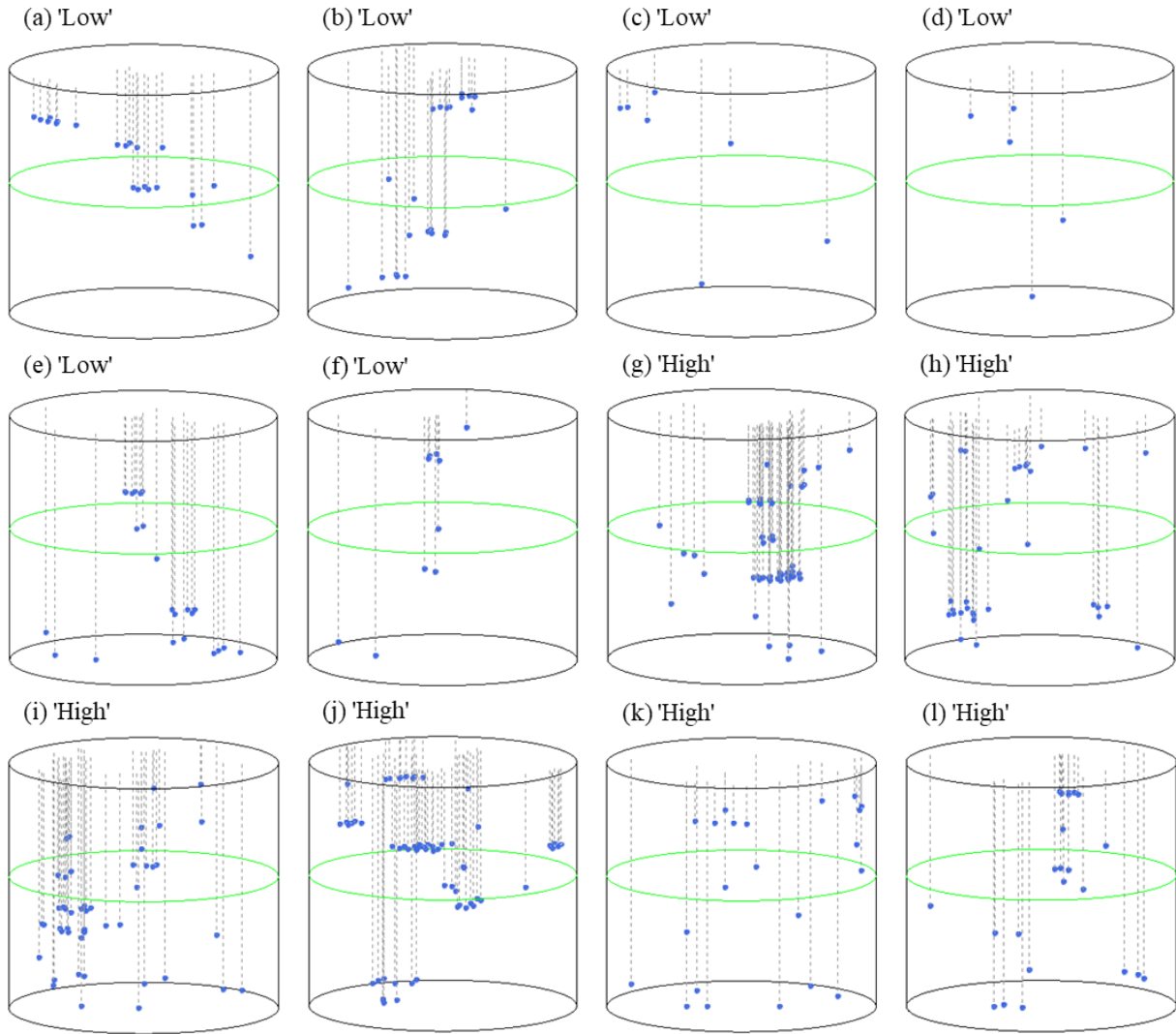
Figure 2.7: Plots of coated grass seed buried by *L. terrestris* at 'low' earthworm density (panels a-f) and 'high' density (panels g-l) in cylindrical microcosms (8 cm radius, 13 cm height). Each dot represents one seed found within 2 cm-deep horizontal cross-sections (1-3 cm, 3-5 cm…11-13 cm) depicted in the center of each cross-section (i.e., at 2 cm, 4 cm…12 cm). The green ring marks the 6 cm depth at which seedling emergence was reduced by c. 68 %.

Overall, 44 ± 13 % of buried seeds were found in the top 1-7 cm and 28 ± 7 % in the top 1-5 cm. Shallowly earthworm buried seeds in the top 1-5 cm were spatially aggregated, with both VMR and Morisita's Index values greater than one at 2.7 ± 1.3 and 3.5 ± 2.6 respectively (Figure 2.8). The spatial distribution of shallow seeds was not affected by differences between 'low' and 'high' earthworm density (One-Way ANOVA, $F_{1,10} = 0.0008$, $p = 0.98$).
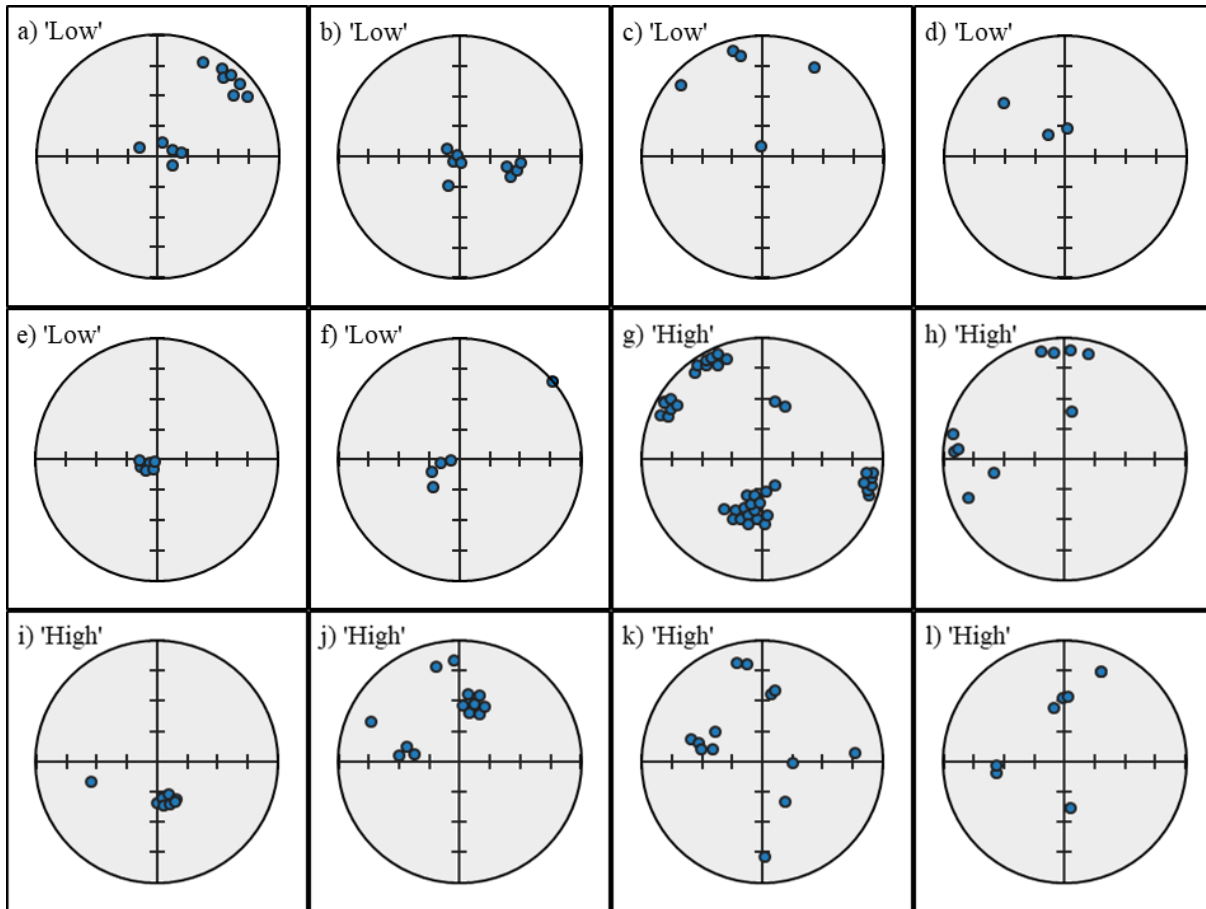
Figure 2.8: Scatterplots of grass seeds shallowly buried in the top 1-5 cm of soil in microcosms (16 cm radius) by *L. terrestris* at 'low' earthworm densities (Panels a-f) or 'high' earthworm densities (Panels g-l). Each dot represents one grass seed. Axis tick marks every 2 cm.

2.3.2 Impacts of earthworm seed preferences on recruitment

In the feeding experiment, ingestion of the smaller seed category was 116 % greater than ingestion of the larger seed category (main effect, Two-Way ANOVA, $F_{1,36} = 11.40$, $p = 0.002$, $\omega_p^2 = 0.21$) and ingestion of coated seed was 100 % greater than ingestion of uncoated seed (main effect, Two-Way ANOVA, $F_{1,36} = 9.42$, $p = 0.004$, $\omega_p^2 = 0.17$) (Table 2.4). Seed egestion was only affected by seed category (main effect, Two-Way ANOVA, $F_{1,30} = 8.20$, $p = 0.008$, $\omega_p^2 = 0.18$), with 76 % higher egestion of 'larger' seeds compared to 'smaller' seeds (Table 2.4). I

did not find any significant correlations between earthworm fresh mass and either seed ingestion or egestion for any of the combinations of seed coating and seed category (data not shown).

Table 2.4: Summary of main effects of seed category and seed coating on the ingestion of grass seed by *L. terrestris* (n = 20 per seed category and seed coating level) and the egestion of grass seed (n = 19 for 'small' seed, n = 15 for 'large' seed, n = 14 for uncoated seed, n = 20 for coated seed; replicates omitted for trials with no seeds ingested). Superscript letters indicate groupings from Tukey's HSD Test for each seed treatment (capital letters: ingestion, lower case letters: egestion). All results given as mean ± standard deviation (SD).

Seed Treatment	Treatment Level	Ingestion (no. of seeds)	Egestion (% of ingested seed)
Category	'Large'	$3.8^A \pm 4.0$	$77.8^a \pm 30.1$
	'Small'	$8.2^B \pm 5.0$	$44.3^b \pm 29.4$
Coating	Uncoated	$4.0^A \pm 4.6$	$47.0^{n.s.} \pm 36.2$
	Coated	$8.0^B \pm 4.6$	$67.5^{n.s.} \pm 30.0$

Total seed germination was significantly affected by seed type (main effect, Two-Way ANOVA, $F_{3,16} = 6.96$, p = 0.003, $\omega_p^2 = 0.43$), but not by seed egestion (main effect, $F_{1,16} = 0.70$, p = 0.42) or an interaction ($F_{3,16} = 1.86$, p = 0.180) (Table 2.5). Similarly, the number of days to peak germination was significantly affected only by seed type (main effect, Two-Way ANOVA, $F_{3,16} = 41.91$, p < 0.001, $\omega_p^2 = 0.84$), with no statistically significant effects of seed egestion (main effect, $F_{1,16} = 4.28$, p = 0.055) or an interaction ($F_{3,16} = 0.46$, p = 0.720) (Table 2.5).

In the growth microcosms, seed coating and earthworm presence had an interactive effect on aboveground grass biomass (Two-Way ANOVA, $F_{1,8} = 17.88$, p = 0.003, $\omega_p^2 = 0.58$). The growth of uncoated and coated seed was the same in the absence of earthworms (simple main effect, One-Way ANOVA, $F_{1,4} = 0.58$, p = 0.49) but different when earthworms were present (simple main effect, One-Way ANOVA, $F_{1,4} = 63.22$, p = 0.001, $\omega^2 = 0.91$). Earthworms reduced aboveground grass biomass for both seed mixes, but the reduction was greater for the coated seed (- 78 %) (simple main effect, One-Way ANOVA, $F_{1,4} = 79.79$, p = 0.001, $\omega^2 = 0.93$) than for the uncoated seed (- 36 %) (simple main effect, One-Way ANOVA, $F_{1,4} = 28.86$, p = 0.006, $\omega^2 = 0.82$) (Figure 2.9). Earthworms qualitatively increased the apparent patchiness of grass

growth, with growth aggregated around earthworm casts and burrows separated by relatively bare ground (Figure 2.10a). I frequently observed tufts of grass growing out of earthworm burrows (Figure 2.10b).

Table 2.5: Summary of main effects of seed type (n = 3 per seed type, 25 seeds per replicate) on total germination and time to peak germination. Superscript letters indicate groupings from Tukey's HSD Test (capital letters: total germination, lower case letters: time to peak germination). All results given as mean ± standard deviation (SD).

Seed Type	Total Germination (%)	Time to Peak Germination (days)
Uncoated 'Large'	$96.7^A \pm 4.7$	$10.8^a \pm 2.5$
Uncoated 'Small'	$95.3^A \pm 1.6$	$28.8^{bc} \pm 3.1$
Coated 'Large'	$91.3^{AB} \pm 3.0$	$25.2^b \pm 4.2$
Coated 'Small'	$87.3^B \pm 5.9$	$32.2^c \pm 4.6$
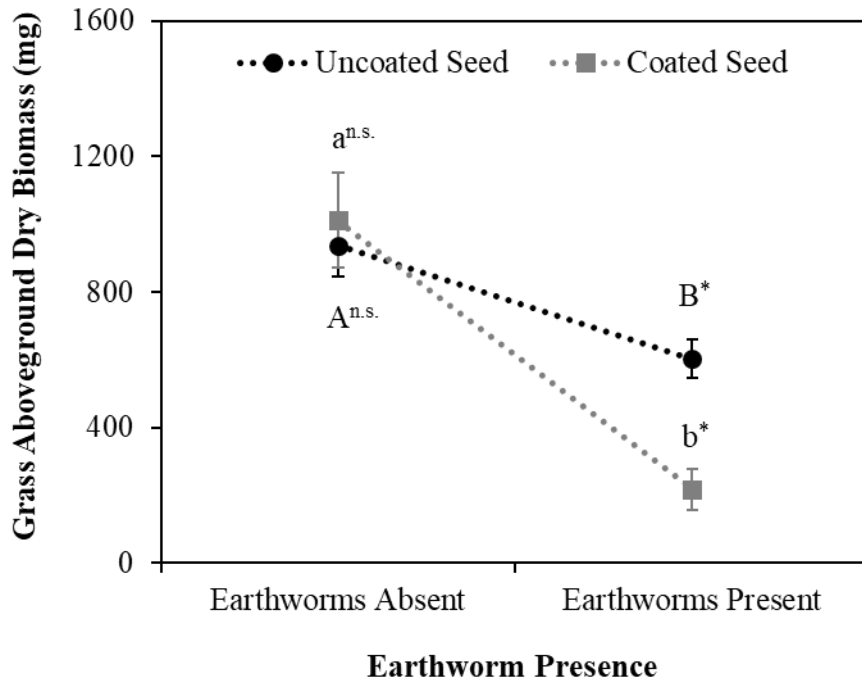
Figure 2.9: Line plot of aboveground grass dry biomass (mg) grown in the absence or presence of *L. terrestris* and from uncoated (black) or coated (grey) grass seed (n = 3 per unique treatment combination). Letters denote groupings from Tukey's HSD Test for significant simple main effects of earthworm presence (capital letters: uncoated seed, lower case letters: coated seed).

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Superscripts indicate statistically significant (*) or non-significant (n.s.) simple main effects of seed coating. Error bars depict standard deviation (SD).
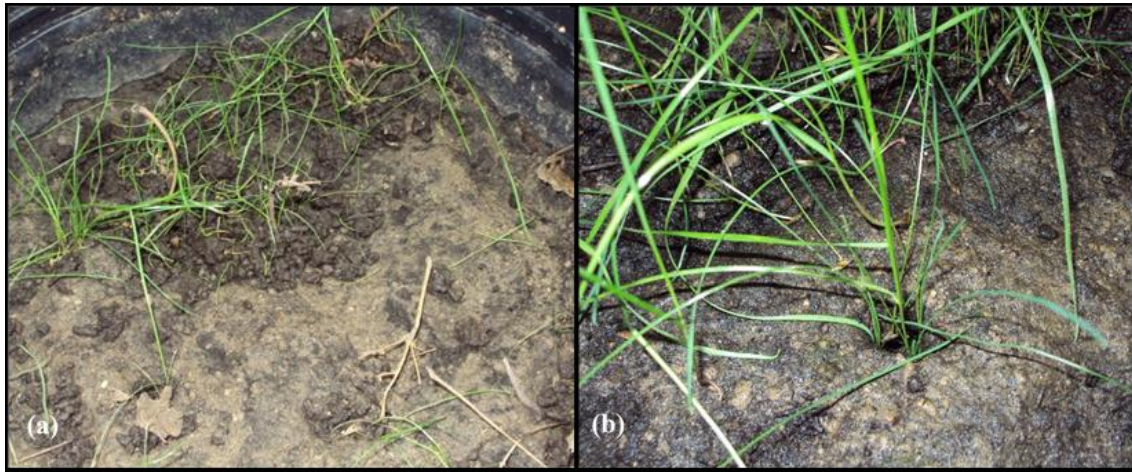
Figure 2.10: Grass growth microcosm surfaces after 16 days showing (a) patchy growth aggregated around earthworm casts and burrows at 'high' earthworm density, and (b) grass growing out of an earthworm burrow.

2.3.3 Interactive effects of earthworms and other granivores on recruitment

In the field exclusion experiment, aboveground grass biomass was affected by an interaction between the access of earthworms and other granivores (Two-Way ANOVA, $F_{1,20} = 8.93$, p = 0.007, $\omega_p^2 = 0.24$). Earthworms reduced grass biomass by 22 % when other granivores were excluded (simple main effect, One-Way ANOVA, $F_{1,10} = 14.02$, p = 0.004, $\omega^2 = 0.52$) but had no observable effect when other granivores were present (simple main effect, One-Way ANOVA, $F_{1,10} = 0.25$, p = 0.629) (Figure 2.11). In contrast, other granivores caused greater reductions of 80-83 % that were the same whether earthworms were excluded (simple main effect, One-Way ANOVA, $F_{1,10} = 266.23$, p < 0.001, $\omega^2 = 0.95$) or present (simple main effect, One-Way ANOVA, $F_{1,10} = 69.81$, p < 0.001, $\omega^2 = 0.85$) (Figure 2.11).
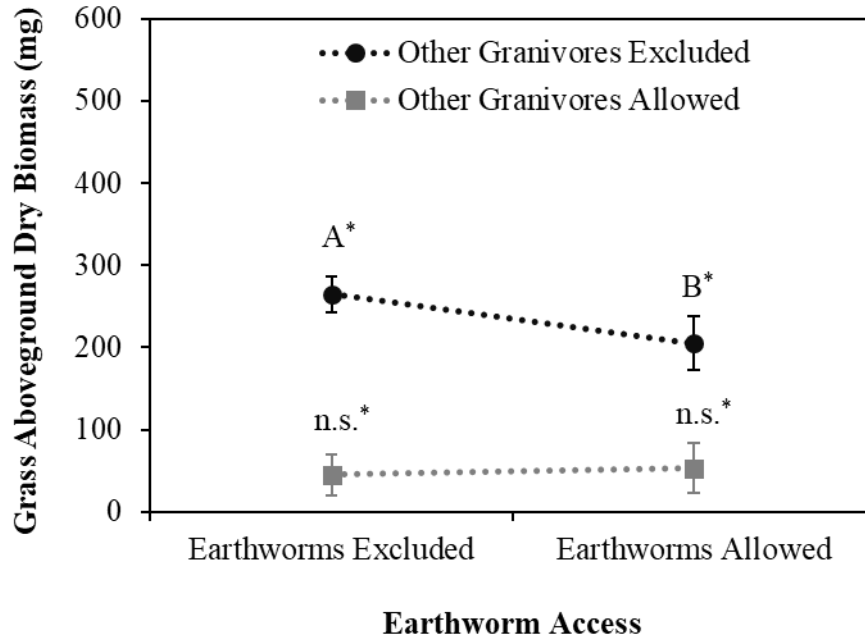
Figure 2.11: Line plot of aboveground grass dry biomass (mg) grown in the exclusion (dark grey) or allowance (light grey) of other granivores and *L. terrestris* (n = 6 per unique treatment combination). Letters denote groupings from Tukey's HSD Test for significant simple main effects of earthworm access (capital letters: other granivores excluded, lower case letters: other granivores allowed). Superscripts indicate statistically significant (*) or non-significant (n.s.) simple main effects of other granivore access. Error bars depict standard deviation (SD).

2.4 Discussion

2.4.1 Earthworm seed burial

In the seed burial experiment, *L. terrestris* reduced grass seed recruitment, in part by rapidly and deeply burying surface-sown seeds. Compared to abiotic seed burial, which will generally bury seeds shallowly in the soil over longer periods of time (Chambers and MacMahon, 1994), earthworm burial was considerably faster and deeper (Table 2.2). Seed burial was relatively even across the 13 cm deep microcosms, with approximately half of the earthworm buried seed transported to depths below the 6 cm depth demonstrated to limit seedling emergence and reduce grass biomass (Table 2.2). This seed burial was proportional to earthworm density, suggesting that the magnitude of earthworm seed burial in the field may vary with earthworm population density (Table 2.3). Curiously, however, 'low' and 'high' earthworm

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densities comparably reduced grass biomass (Table 2.3), which could suggest a positive effect of higher earthworm densities on seedling growth (Forey et al., 2011).

The emergence assay technique used in the seed burial experiment allowed me to create one of the most detailed spatial mappings of earthworm-buried seed currently available and illustrates the ability of earthworms to affect not only the vertical dispersal of seeds, but the horizontal dispersal as well (Smith et al., 2005). At a given depth, I generally found seeds in clusters or along single oblique lines traced through the centroid of seeds at each depth (Figure 2.7). These patterns are suggestive of seeds deposited in middens or casts near the surface and in burrow walls and would be consistent with the typically singular, unbranching vertical burrows characteristic of *L. terrestris* (Pitkänen and Nuutinen, 1997)*.* The apparent absence of similarly clear lines in the 'high' density microcosms and the greater mean distance of seeds from depth centroids (Table 2.3) is presumably due to more burrows occurring in the same soil volume.

This spatial aggregation of buried seed has notable implications for the spatial patterning of the plant community. Seed buried shallowly enough (e.g., 1-5 cm for these grass seed mixes) can still reach the surface and contribute substantively to aboveground vegetation. While other studies noting the spatial aggregation of plants by earthworms have attributed this primarily to plant growth occurring out of earthworm middens or burrows on the soil surface (Grant, 1983; Milcu et al., 2006), this study suggests that aggregation of shallowly-buried seed may also contribute to this effect. This horizontal aggregation of seed does not necessarily alter initial recruitment, but instead has longer-term implications for plant community structure and function (Regnier et al., 2008).

This study focused on and demonstrated the generally negative effects of earthworms burying seed on recruitment, but seed burial also involves complex longer-term trade-offs for plant communities. For example, buried seed is generally protected from other granivores and can contribute to a seed bank (Regnier et al., 2008). Earthworms can also transport buried seed back up to the surface (Zaller and Saxler, 2007), variably helping seeds from the seed bank germinate (Blanchart et al., 2004; Grant, 1983; Nuzzo et al., 2015; Willems and Huijsmans, 1994) or increasing seed mortality through exposure and desiccation (Drouin et al., 2014). For restoration, these longer-term trade-off effects of earthworm seed burial should be considered alongside the initial impacts on early seedling recruitment.

2.4.2 Impacts of earthworm seed preferences on recruitment

In the feeding experiment, *L. terrestris* preferentially ingested grass seed based on seed category and coating, ingesting larger quantities of the smaller category of seeds and coated seeds (Table 2.4). Although it is important to note that the seed categories differed in more ways than simply seed size, size is commonly reported as a relatively strong predictor of earthworm ingestion, with typically higher ingestion of smaller seeds (Cassin and Kotanen, 2016; Clause et al., 2015; Eisenhauer et al., 2009a, 2010; Quackenbush et al., 2012). Smaller seeds can be easier to ingest and often have higher oil content (which was true of seeds used in this study, Table 2.1) that may increase their nutritive value (Clause et al., 2011; Shumway and Koide, 1994; Zaller and Saxler, 2007). It is less clear why earthworms ingested more of the coated seed. They may be attracted to the limestone component of the coating as earthworms require grit for their gizzards (Marhan and Scheu, 2005) and have previously been observed to prefer mixes of mineral and organic materials when feeding on litter (Doube et al., 1997). Alternatively, the starch component of the coating may increase the nutritive value of coated seed (Clause et al., 2017; Eisenhauer et al., 2010). Either the limestone or starch may also make it easier for earthworms to chemically detect the seeds (Clause et al., 2017; Willems and Huijsmans, 1994).

Earthworm seed preferences I observed in the feeding experiment were consistent with the comparative performances of the uncoated and coated grass seed mixes in the subsequent growth experiment: earthworms reduced seedling recruitment from both seed mixes, but the reduction was approximately twice as high for the earthworm-preferred coated seed mix (Figure 2.9). Earthworms presumably ingested greater quantities of the preferred coated seed, reducing recruitment by digesting more seeds and burying more egested seeds below emergence depth. Additionally, although earthworm egestion did not affect the germination of these particular seeds, the coated seeds tended to be slower to germinate than uncoated seed (Table 2.5), which may have left the coated seed exposed to predation for a longer period of time (Eisenhauer et al., 2010). Anecdotally, grass growth in the earthworm-inoculated microcosms was spatially patchy, with tufts of growth occurring around burrows or casts separated by large areas of bare soil (Figure 2.10). This pattern of growth was even more pronounced for the coated seed mix when earthworms were present and would be undesirable in most restoration applications attempting to establish more contiguous ground cover.

In this study, increased earthworm ingestion had an overall negative impact on seedling recruitment of these seed mixes. Therefore, all else being equal, using the less earthworm-preferred seed mix might produce more satisfactory results (e.g., in terms of recruitment per unit seed applied) when earthworm communities are abundant. Although increased preferability and higher ingestion will generally increase recruitment losses due to seed digestion, other seed species could alternatively benefit from being preferred by earthworms. For example, earthworm-preferred seeds could experience increased germination or growth (Decaëns et al., 2003; Drouin et al., 2014; Eisenhauer et al., 2009a; Grant, 1983) or seedbank formation (Regnier et al., 2008). Seeds with tough seed coats (Clause et al., 2017), dormancy tolerance (Donath and Eckstein, 2012), or able to emerge from deep in the soil (Regnier et al., 2008) might be particularly immune to the negative effects of earthworm ingestion and able to benefit from the positive effects. Using seed that ultimately benefits from these interactions and is preferred by earthworms or minimizing preferred seed that is negatively impacted could improve seeding outcomes in soils with abundant earthworm communities.

2.4.3 Interactive effects of earthworms and other granivores on recruitment

In the field exclusion experiment, earthworms reduced the biomass of grass grown in the field, but their effect was less than that of other granivores at the site (presumably birds, rodents, etc.) and only observed when other granivores were excluded (Figure 2.11). This result is consistent with Cassin and Kotanen (2016), who found moderate earthworm impacts on seed removal that were subsequently masked by seed predation from other taxa (primarily rodents). I am unsure why the earthworm effect disappeared when other granivores were not excluded, although it is possible that an earthworm effect was present but obscured by variability in the larger recruitment reductions caused by other taxa. Alternatively, near simultaneous seed removal from different sources may slow the rate of subsequent foraging by each source over time due to diminishing food density (Mitchell and Brown, 1990), or the presence of rodents or birds as potential predators of the earthworms themselves may reduce earthworm surface foraging.

There is ongoing discussion over the broader ecological significance of earthworms as granivores (Cassin and Kotanen, 2016; Grant, 1983). While this study suggests that earthworms may remove less seed than other species, earthworm granivory can affect seedling recruitment in

other ecologically significant ways. For example, while larger vertebrate taxa often prefer larger seeds, earthworms generally prefer smaller seeds (Cassin and Kotanen, 2016; Thompson, 1987). Earthworms can also ingest seed buried beneath the surface that is inaccessible to surface foraging species such as birds (Bakker et al., 1996). Finally, as earthworms did reduce grass biomass detectably in this study when other granivores were excluded, earthworms may be dominant agents of seed removal in scenarios with lower abundances of other taxa (e.g., times when rodent or avian activity may be lower, open construction sites with a scarcity of aboveground wildlife).

2.4.4 Implications of earthworm-seed interactions for restoration and future research

This study raises three primary considerations that could improve the efficiency of seeding-based restoration in soils with abundant earthworm communities. First, earthworm preferences for different seeds can alter the recruitment from and overall performance of different seed mixes. Second, the magnitude of some earthworm impacts on recruitment (e.g., seed burial) may vary with the density of the earthworm population. Third, although the influence of earthworms on recruitment may be generally weaker than that of other taxa, it may be stronger for certain types of seed and when other granivores are less abundant. Based on whether earthworm interactions tend to benefit or negatively impact specific seed used in a given application, these considerations could help inform the design and use of seed mixes that specifically take advantage of these benefits or help offset negative impacts.

Future investigation of several key topics will help facilitate practical implementation of these results. Although this study examined only the effects of the anecic *L. terrestris*, other species and functional groups may also affect seedling recruitment individually and through interactions (Asshoff et al., 2010; Eisenhauer et al., 2009b, 2009a, 2008; McCormick et al., 2013). Earthworms may also limit initial seedling recruitment through herbivory of recently germinated seedlings, though earthworm herbivory has only been documented in a few cases (Eisenhauer et al., 2010; Griffith et al., 2013). Finally, since L. terrestris burrows may be > 80 cm deep depending on soil conditions (Pitkänen and Nuutinen, 1997), the emergence assay for tracking seed burial positions could be used to track the burial of seeds across a wider range of soil depths.

Practical integration of earthworm-seed interactions into restoration planning would need to be highly contextual and case-by-case. The seed preferences of earthworms and the responses of seeds to the complex trade-offs of granivory and dispersal are difficult to generalize between different earthworm and plant species (Clause et al., 2017, 2011; Eisenhauer et al., 2009a; McRill and Sagar, 1973) and would have to be assessed for relevant specific combinations as needed. Determining the best means of efficiently testing preferences that will be consistent between feeding experiments and field conditions could also be useful (Burilo 2019, Honours thesis project in progress co-supervised by McTavish). Additionally, site-specific differences such as earthworm density or the relative abundances of other granivores can change how earthworms affect seedling recruitment.

The diverse goals of different restoration projects may also influence preferred management options concerning earthworm granivory. For example, if the target vegetation community is relatively flexible, it may be more economical to design a seed mix that either reduces negatively-impacted, earthworm-preferred seed or uses more positively-affected, earthworm-preferred seed. Alternatively, if a highly desired target species was particularly disadvantaged by earthworm predation, granivory pressure could be reduced by including supplemental or 'sacrificial' seed (Riebkes et al., 2018) or increasing the amount of target seed to offset or oversaturate earthworm interactions, similar to the strategy of mast seeding (Kelly, 1994). Individual projects will also have to weigh the short-term impacts of earthworms on initial seedling recruitment against longer-term impacts on seedbank dynamics and plant growth (Grant, 1983; Willems and Huijsmans, 1994; Zaller and Saxler, 2007).

Chapter 3: Mulch amendments increase earthworm density and are spatially aggregated by anecic earthworms (*Lumbricus terrestris* L.)

Abstract

Mulch amendment is used in restoration to improve soil conditions, limit plant invasion, and protect seedlings. The effectiveness of mulch may be altered, however, when amendments are physically collected and buried by ecosystem engineering anecic earthworms. In this study, I investigated how mulch amendments affect earthworm communities and how anecic earthworms change the distribution of mulch on the soil surface using: a field experiment applying straw mulch in a tallgrass prairie restoration to monitor earthworm community change (Experiment 1); a laboratory microcosm experiment assessing how the anecic earthworm *Lumbricus terrestris* L. collects mulch in the first six weeks following amendment (Experiment 2); and an observational field study of how *L. terrestris* influences the longer-term spatial distribution of natural corn stubble mulch in an abandoned agricultural field (Experiment 3). Mulch amendment in the field generally increased earthworm densities, although specific impacts on earthworm functional groups varied over time and with the amount of mulch applied. In the microcosm experiment, *L. terrestris* reduced initial mulch coverage as early as the first week post amendment but higher mulch rates helped mitigate this loss. In the observational field study, earthworms aggregated mulch around their burrows, leaving bare, exposed soil in between. Although earthworms can improve some benefits of mulch, increased earthworm densities under mulch and anecic earthworms aggregating mulch can reduce coverage and evenness in the short and long-term; this may require larger or more frequent mulch applications to compensate. Learning to better understand and plan for both beneficial and detrimental earthworm-mulch interactions could help improve the effectiveness of mulch amendment in earthworm-inhabited soils.

3.1 Introduction

Various terminology has been used to describe the dead and decaying organic material found on the soil surface; 'litter' has been typically favoured in forests while 'mulch' has been used for grasslands and in horticulture (Dyksterhuis and Schmutz, 1947). Mulch may refer to naturally occurring organic residues on the soil surface or materials intentionally added by humans. Anthropogenic mulch amendments may be different plant-based materials including bark, wood chips, leaf litter, or stems, or manufactured materials such as cardboard or plastic (Andersen et al., 2013; Zhang et al., 2018). Mulch is widely used in agriculture, horticulture, and ecological restoration and can have various benefits including soil temperature stabilization, retention of soil moisture, reduced soil erosion, organic matter input, seedling protection, and weed suppression (Bakker et al., 2003; Dyksterhuis and Schmutz, 1947; Thomson and Hoffmann, 2007; Wong, 2003). In restoration, mulch amendment is recommended in the management of a diversity of ecosystems, including grasslands (Bakker et al., 2003), woodlands (Vallejo et al., 2009), coastal scrublands (Zink and Allen, 1998), and peatlands (Rochefort, 2000).

In addition to the direct effects of mulch amendment on soil properties, mulch also interacts with various soil biotic communities including microbes and invertebrates, including earthworms (Andriuzzi et al., 2016; Cromar et al., 1999; Thomson and Hoffmann, 2007). As soil-dwelling organisms, earthworms are potentially susceptible to physical or chemical soil changes caused by mulch. Furthermore, as ecosystem engineers that physically modify, maintain, and create habitats and alter the distribution of resources in an ecosystem (Jones et al., 1994; Lavelle et al., 2006), earthworms may alter the effects of mulch added to earthworm-inhabited soils.

Much of the existing research of earthworm-mulch interactions has focused on how earthworm communities respond to mulch. Most of these studies concern agricultural systems, in which earthworms are thought to have predominantly positive effects and land managers are interested in residue management that increases earthworm populations (Bertrand et al., 2015; Lal, 1978; Mackay and Kladivko, 1985; Mele and Carter, 1999; Ortiz-Ceballos et al., 2007; Sizmur et al., 2017). Mulch amendment generally benefits earthworm survivourship, growth, and reproduction by reducing variations in soil temperature (Acharya et al., 1998; Tian et al., 1997), increasing moisture retention (Blanco-Canqui and Lal, 2007a; Lal, 1978), and providing food

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(Ortiz-Ceballos et al., 2007) and material for burrow construction (Stroud et al., 2016). In some systems, earthworm communities are strongly dependent on mulch for survival and are strictly spatially limited to mulched areas (Lal, 1978). Removal of surface mulch can even reduce or completely eliminate earthworms (Blanco-Canqui and Lal, 2007b).

In contrast, negative effects of mulch on earthworms are relatively rare and seem to occur primarily when naturally cool, moist soil conditions render potential mulch benefits redundant (Eriksen-Hamel et al., 2009), or when mulch physically impedes the input of other organic inputs or raises soil temperatures too high (Andersen et al., 2013). Mulch can also have varying effects on different earthworm species and functional groups, including litter-dwelling epigeics, horizontal burrowing geophagous endogeics, and deep vertical burrowing anecics (*sensu* Bouché, 1977). While some past studies have found minimal changes in earthworm community composition (Abail and Whalen, 2018), others have observed functional group shifts that variably favoured epigeics (Fusilero et al., 2013), endogeics (Frøseth et al., 2014), or anecics (Pelosi et al., 2015). The effects of mulch on earthworms can also depend on amendment rate (Blanco-Canqui and Lal, 2007a) and may change over time (Pelosi et al., 2015).

Research into how earthworms affect mulch amendments is considerably rarer. Existing studies have focused primarily on how earthworms affect mulch decomposition, with implications for agricultural soil fertility (Teotia et al., 1950). Earthworms generally accelerate mulch decomposition by increasing residue surface area (Tian et al., 1997) and enhancing microbial activity (Subler and Kirsch, 1998; Wolfarth et al., 2011). These impacts may vary between different types of mulch based on their physical and chemical properties (Mackay and Kladivko, 1985; Tian et al., 1997, 1995). Earthworms may be particularly influential in accelerating the normally slower breakdown of 'tougher' woody mulches with higher C:N ratios or lignin concentrations (Fraser et al., 2003). This accelerated decomposition by earthworms may be desirable or detrimental for the management of mulched systems. While decomposition incorporates mulch into the soil, prevents excessive residue build-up, and improves soil fertility (Mackay and Kladivko, 1985; Tian et al., 1997), it may also accelerate loss of cover and physical protection (Shuster et al., 2000) or rapidly release nutrients asynchronously with plant uptake (Tian et al., 1997).

Even fewer studies have investigated the potential of ecosystem engineering earthworms (Jones et al., 1994; Lavelle et al., 2006) – particularly anecic species such as *Lumbricus terrestris*

L. – to change the spatial distribution of surface applied mulch. Anecic earthworms forage aboveground out of deep semi-permanent burrows and collect organic materials into burrows or into raised middens, mixtures of casts and organic matter thought to store food and physically protect burrow openings (Butt and Grigoropoulou, 2010). With an estimated foraging radius of 30 cm (Nuutinen and Butt, 2005), anecic earthworms such as *L. terrestris* can collect residues from the surrounding soil surface (Stephens et al., 1994, 1993; Zhang et al., 2018) or 'steal' them from the middens of conspecifics, allowing them to 'relay' material horizontally across the surface (Butt et al., 2003; Nuutinen and Butt, 2019). Although these collection behaviours are known, their consequences for the spatial distribution and effectiveness of amendments such as mulch remain poorly understood. Earthworm middens tend to be over-dispersed at small scales (Grigoropoulou and Butt, 2010) and aggregated at larger scales (Jiménez et al., 2001; Nuutinen et al., 1998; Rossi and Nuutinen, 2004). Aggregation of mulch into middens could substantially alter the spatial distribution of the amendment itself, with potential implications for coverage, evenness, and effectiveness (Shuster et al., 2000; Subler and Kirsch, 1998).

The purpose of this study was to investigate how mulch amendments affect earthworm communities and how anecic earthworms change the spatial distribution of mulch on the soil surface. This study was guided by three primary research questions: (1) How do applications of mulch at different amendment rates affect earthworm communities? (2) How do earthworms affect the initial spatial distribution of surface-applied mulches? And (3) How do earthworms affect the longer-term spatial distribution of mulch on the soil surface? I addressed these research questions with field studies conducted at a tallgrass prairie restoration site in Ontario, Canada and laboratory microcosm experiments using the geographically-widespread and ecologically-influential anecic earthworm *L. terrestris* (Addison, 2009; Keller et al., 2007).

3.2 Methods

3.2.1 Experiment 1: Tallgrass prairie mulch amendment field experiment

I conducted a field plot experiment in a tallgrass prairie restoration site to determine the effects of different mulch amendment rates (none, 'low', 'high') on earthworm communities (n = 10 plots per mulch rate, total N = 30). The site was a 6.3 ha field in the Glenorchy Conservation Area (Conservation Halton, Ontario, Canada) used to grow soy in 2012 and corn in 2013. In 2014, the field was sprayed, lightly disked, and hand-broadcast seeded with a tallgrass prairie

mix consisting of warm season grasses and wildflowers. When the mulch application experiment began in summer 2016, overall vegetation cover consisted of a mix of target grass and herbaceous species (e.g., *Andropogon gerardi*) and agricultural weeds (e.g., *Trifolium repens* L., *Trifolium pratense* L.) and was relatively sparse (visual estimate of 30-50 % bare ground across the field). The western half of the field lay on a slight downslope and was characterized by mesic conditions with higher vegetation cover and was used for the mulch application experiment (Figure 3.1). The eastern half of the field was more upland with drier conditions and sparser vegetation and was used for the field midden survey (*Experiment 3*).

Figure 3.1: Western end of Glenorchy tallgrass prairie restoration field (looking north). Flag markers for mulch amendment plots are visible in the foreground.

In preliminary earthworm samples across the field I found a community of at least four endogeic earthworm species dominated by *Allolobophora chlorotica* (Savigny 1826) but also including *Aporrectodea turgida* (Savigny 1826), *Aporrectodea tuberculata* (Eisen 1874), and *Aporrectodea rosea* (Savigny 1826), and one anecic earthworm (*L. terrestris*). I did not find any litter-dwelling epigeic taxa. From mulch-free control plots sampled in November 2016, the background earthworm community was functionally dominated by endogeic taxa (Figure 3.2). I could see abundant surface middens created by *L. terrestris* throughout the field.
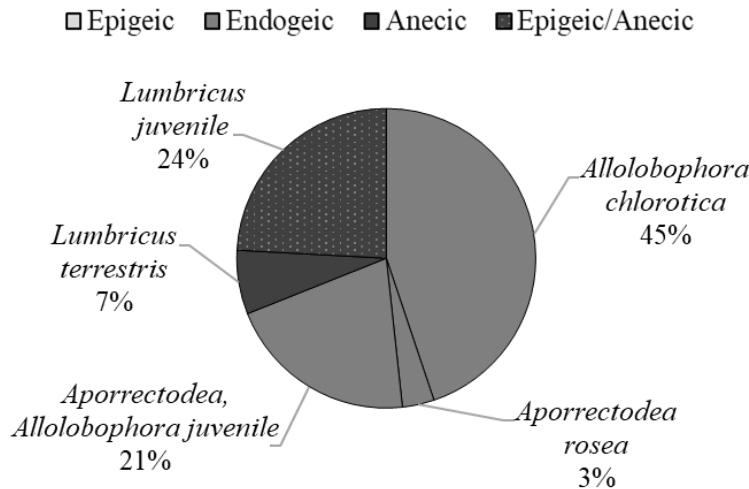
Figure 3.2: Summary of background earthworm community at Glenorchy field site based on proportional population density (% of total earthworms, total earthworm density 40 ± 39 m^{-2}) of species collected from mulch-free control plots sampled in November 2016 (n = 8 plots). Wedge colour denotes functional group: epigeic (light grey), endogeic (medium grey), anecic (dark grey), epigeic/anecic (dotted dark grey).

I established thirty 1 m × 1 m plots approximately 10 m out from the western hedgerow of the field, leaving 1 m spacing between adjacent plots. I randomly assigned plots to one of three mulch amendment rates: no mulch, 'low' mulch (2.5 USG·m^{-2}), or 'high' mulch (10 USG·m^{-2}) (n = 10 plots per mulch rate). The 'low' rate was the minimum amount of mulch able to fully cover the plots and the 'high' rate was four times higher (Figure 3.3). I purchased a straw mulch from a local farmer and applied it evenly over plots in June 2016 by hand.

I sampled the amendment plots c. 5 months post mulch application on November 3, 2016 and resampled them c. 16 months post mulch application on October 19, 2017. I measured soil temperature using a probe inserted into the center of each plot and air temperature from an average of five measurements taken with the same probe. After clearing surface mulch, I took pairs of soil cores from each plot using a 5 cm diameter corer and aggregated the top 0-10 cm of soil from each core per plot. I collected earthworms from a random subset of eight plots of each mulch amendment rate (of a possible ten plots) by clearing all mulch within a 30 cm × 30 cm quadrat and driving earthworms to the surface by applying 2.5 L of mustard solution (10 g dry mustard powder to 1 L tap water) over 15-minutes (Lawrence and Bowers, 2002; Singh et al.,

2016). I euthanized earthworms in isopropyl, transferred them to a 10% formalin solution for 24-48 h for fixation, and transferred them back to isopropyl for storage (Hale, 2013).
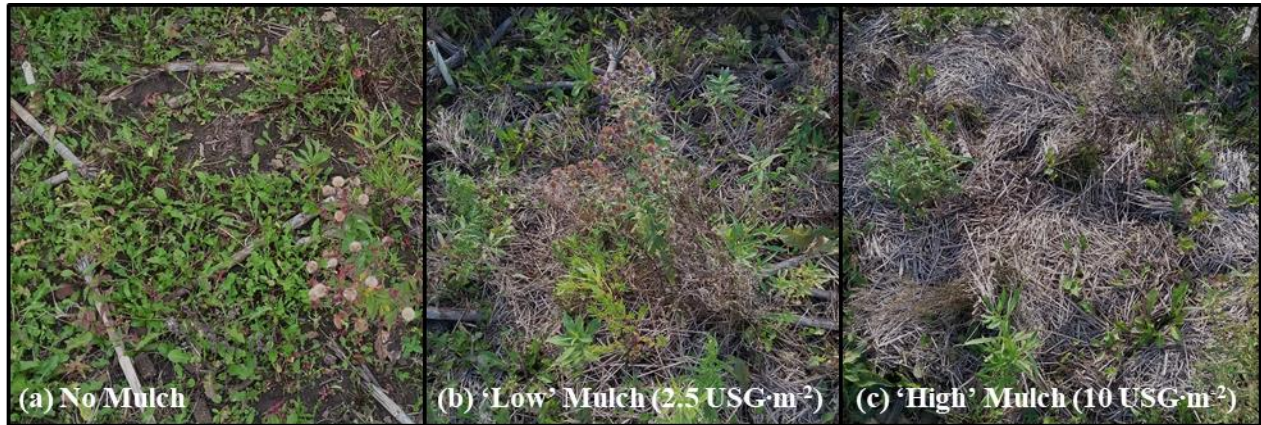
Figure 3.3: Mulch application rates on 1 m × 1 m plots immediately after amendment: (a) No Mulch, (b) 'Low' Mulch, and (c) 'High' mulch.

In the lab, I thawed and homogenized soil samples and took 5 g wet samples from each plot to measure moisture content by drying for 18 h at 110° C. I transferred the dry soil samples to a muffle furnace for 4 h at 550° C to estimate organic matter content based on mass loss-on-ignition (% LOI). I identified earthworms identified to genus, species, and functional group where possible (see *1.4.1 Earthworm taxonomy and identification*). I assessed the effects of mulch amendment rate and time since amendment on soil properties (temperature, moisture content, LOI), earthworm community composition (% endogeic), and earthworm densities for each functional group present using Mixed ANOVAs, with mulch amendment and time as fixed factors and plot identity as a random factor nested within mulch amendment (for all statistical tests in this dissertation, see *1.4.2 Notes on statistical analyses* for general details regarding testing of assumptions, alternative tests used when assumptions were not met, and post-hoc testing).

3.2.2 Experiment 2: Mulch collection microcosm experiment

I used a laboratory microcosm experiment to examine how *L. terrestris* affects the spatial distribution of mulch added at two amendment rates ('low', 'high') in the first zero to six weeks

following amendment (n = 6 microcosms per mulch amendment rate, total N = 12 microcosms). I filled large nursery pots (30 cm diameter, 28 cm height) 15 cm deep with field soil I collected from a nearby mixed upland forest known to support earthworms (North Campus Environmental Reserve, University of Waterloo, Ontario, Canada) and sieved (5 mm) to remove larger particulates and soil fauna. I placed the pots in 10 cm of standing tap water to help maintain soil moisture. I crushed 1.5 g of maple litter (mixed *Acer saccharum* Marshall and *Acer platanoides* L., collected from a woodlot on the University of Waterloo campus, air dried for 5 days) and applied it to the surface of each microcosm to provide an initial food source. I added four healthy adult *L. terrestris* to each microcosm for an earthworm density of approximately 65 m^{-2}, simulating densities I have observed in local forests. I prepared an additional set of microcosms identically but with the omission of earthworms to determine the effects of earthworm presence on surface mulch loss (n = 6 microcosms per mulch amendment rate, total N = 12 earthworm-free microcosms). I covered the microcosms with window screen mesh, transferred them to a growth chamber to approximate early fall conditions (18°C, 80% RH, 12 h light:12 h dark), and left them for four days to allow earthworms to acclimate.

After four days, I removed any remaining surface litter. To determine whether mulch affects earthworm leaf litter foraging, I cut sets of 12 squares of air-dried *A. saccharum* leaves (1.5 cm × 1.5 cm) and placed them regularly on each of two halves of each microcosm surface. I added straw mulch to one randomly selected half of each microcosm surface at a 'low' amendment rate (2.5 USG·m^{-2}) or a 'high' amendment rate (10 USG·m^{-2}) (matching mulch amendments used in Experiment 1) (Figure 3.4). I watered microcosms with c. 0.5 L tap water every two to three days. Immediately following mulch application (week 0) and at one-week intervals post-application for a total of six weeks (week 1 to week 6), I took photographs of the surface of each microcosm and converted them into black and white images to determine mulch coverage (% of soil surface area) from pixel counts on the application area, initially bare area, and total area of each microcosm using ImageJ (V1.52a). After six weeks, I removed any remaining surface mulch, air dried it for 72 h, and weighed it to determine relative surface mulch loss (% of initial amendments loss by mass). I counted any leaf squares remaining on the application and initially bare sides of each microcosm to determine leaf burial (% of initial leaves buried).

Figure 3.4: Mulch collection microcosms immediately after setup. 12 additional leaf squares beneath mulch amendments not visible. Examples of 'Low' (2.5 USG·m^{-2}) and 'High' mulch application rates (10 USG·m^{-2}) with different container areas labeled.

I assessed the effects of mulch amendment rate and time since amendment on mulch coverage in the application side, initially bare side, and total area of the earthworm-inoculated microcosms and the effects of mulch amendment rate and container side on leaf burial using Mixed ANOVAs, with microcosm identity as a random factor nested within mulch amendment rate. I assessed the effects of mulch amendment rate and earthworm presence on surface mulch loss using a Two-Way ANOVA (see *1.4.2 Notes on statistical analyses*).

3.2.3 Experiment 3: Earthworm midden field survey

In early November 2015 I conducted a survey of a former agricultural field being restored to tallgrass prairie to measure the distribution and composition of earthworm middens containing natural woody mulches. I conducted the survey in the eastern half of the same field I used for the mulch field application experiment (Figure 3.5a). The field was taken out of agriculture with a final harvest in 2013, leaving standing corn stubble with sparse vegetation cover. During early site visits, I observed dried woody fragments of corn 'stubble' sticking out of earthworm middens. Since other organic residues (e.g., leaf litter) were very rare, I use 'mulch' hereafter in this survey to refer exclusively to these woody fragments.

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I randomly established nine circular plots in the northeast corner of the field by walking 4 m in a random direction away from a previous plot, throwing a hoop (0.35 m radius) a random distance in a random direction, and repeating the process. Using a method adapted from Grigoropoulou and Butt (2010), within each plot, I visually identified earthworm middens, outlined their perimeters with string, and took a photograph from a height of 1.5 m (Figure 3.5b). I removed middens to ground level using a metal spatula. To measure the density of mulch on bare ground, I used the same random placement procedure described above with a smaller hoop (0.078 m radius) to collect 40 bare ground samples. If a sample contained any earthworm middens, I did not count it and instead repeated the sample. Within each sample, I collected all mulch on the soil surface, and air dried it and the midden samples for 2 weeks before weighing them. After drying, I further analyzed the composition of middens from five randomly selected plots (of nine total), hand sorting the middens into four components and weighing each of soil, rocks (diameter > 5 mm), leaves, and woody mulch.
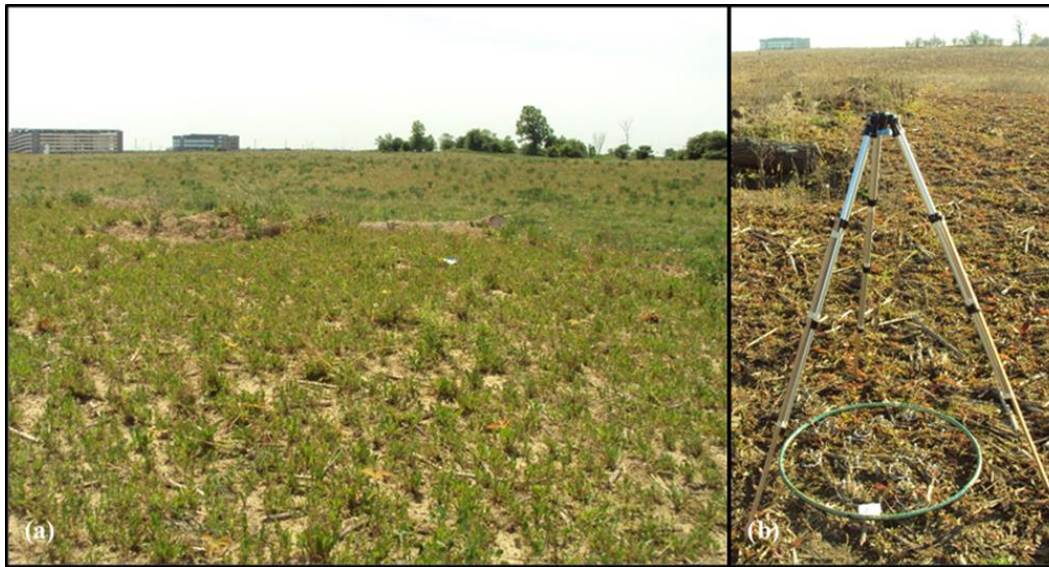
Figure 3.5: (a) Eastern end of Glenorchy tallgrass prairie restoration field (looking south). (b) Sampling apparatus in place with earthworm middens marked off in the circular plot.

I converted the plot photos into black and white images in Corel PHOTO-PAINT 12© and used ImageJ (V1.52a) to determine the area, centroid, and circularity of each midden. Circularity uses a ratio of area to perimeter to measure the similarity of a shape to a circle, with

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values ranging from 0 (irregular shape with increased perimeter : area) to 1 (for a perfect circle) (Cervantes et al., 2016). I analyzed the spatial dispersion of midden centroids using the same sector-based method adapted from Milcu et al. (2006) and dispersion metrics (VMR, I_M) that I described for the seed burial experiment in Chapter 2 (see *2.2.2 Earthworm seed burial*).

I described the overall plot level arrangement of middens based on averages from the nine plots of midden density, midden cover (% of total plot area), total midden dry weight density, and the two indices of dispersion (VMR, I_M). I described individual midden dry weight, midden area, and circularity based on averages from individual middens from all plots (total n = 108 middens). I described midden composition of soil, rocks, leaves, and wood mulch (% by weight) based on averages from individual middens from five random plots (total n = 45 middens). I assessed correlations between two measures of midden size (individual midden area, individual midden weight) and each other, mulch content, and proportional mulch content (% of midden weight) (n = 45 middens) using Spearman's Rank Correlation (r_S) (see *1.4.2 Notes on statistical analyses*).

3.3 Results

3.3.1 Experiment 1: Tallgrass prairie mulch amendment field experiment

Mulch amendment rate and time since amendment had a statistically significant interactive effect on soil temperature (Mixed ANOVA, $F_{2,27} = 12.46$, $p < 0.001$, $\omega_p^2 = 0.26$) and LOI (Mixed ANOVA, $F_{2,27} = 4.28$, $p = 0.024$, $\omega_p^2 = 0.28$), with weaker but statistically significant interactive effects on soil moisture (Mixed ANOVA, $F_{2,27} = 5.57$, $p = 0.048$, $\omega_p^2 = 0.10$) (simple main effects analyses summarized in Table 3.1).

For soil temperature, compared to mulch free plots, when the ambient air temperature was cooler after 5 months, mulch amendment increased soil temperature at both 'low' (+ 7 %) and 'high' rates (+ 13 %). When the air temperature was warmer after 16 months, mulch amendment decreased soil temperature with an intermediate reduction at the 'low' rate (- 4 %) and a larger reduction at the 'high' rate (- 9 %) (Figure 3.6a). For soil moisture, mulch amended plots had higher soil moisture after 5 months, with an intermediate increase at the 'low' rate (+ 7 %) and a larger increase at the 'high' rate (+ 13 %). After 16 months, soil moisture was lower overall, with the lowest moisture in the 'low' mulch plots, intermediate levels in the mulch-free plots (+ 4 %), and the highest moisture in the 'high' plots (+ 7 %) (Figure 3.6b). For soil organic

matter, LOI was not statistically significantly different between amendment treatments after 5 months but was higher in mulch plots after 16 months, with intermediate increases at the 'low' rate (+ 5 %) and higher increases at the 'high' rate (+ 9 %) (Figure 3.6c).

Table 3.1: Summary of omnibus tests of simple main effects of (a) mulch amendment at each time post-amendment (One-Way ANOVA) and (b) time since amendment for each mulch amendment level (Paired t-Test) on soil temperature, moisture content (%), and loss-on-ignition (LOI) (%) (n = 10 plots per mulch rate per year). P-values for significant tests are in bold.

(a) Mulch Amendment by Time				
Time Since Amendment	**Soil Property**	**F**	**p**	**ω^2**
	Temperature	30.20	**< 0.001**	0.661
5 months	Moisture	8.25	**0.002**	0.326
	LOI	1.79	0.186	-
	Temperature	3.97	**0.031**	0.165
16 months	Moisture	4.27	**0.024**	0.179
	LOI	7.42	**0.003**	0.300
(b) Time by Mulch Amendment				
Mulch Amendment	**Earthworm Category**	**t**	**p**	**d_z**
	Temperature	-20.51	**< 0.001**	6.486
No Mulch	Moisture	1.17	0.274	-
	LOI	4.17	**0.002**	1.319
	Temperature	-15.29	**< 0.001**	4.835
'Low'	Moisture	5.19	**0.001**	1.641
	LOI	1.36	0.206	-
	Temperature	-20.70	**< 0.001**	6.546
'High'	Moisture	5.27	**0.001**	1.667
	LOI	-0.86	0.412	-
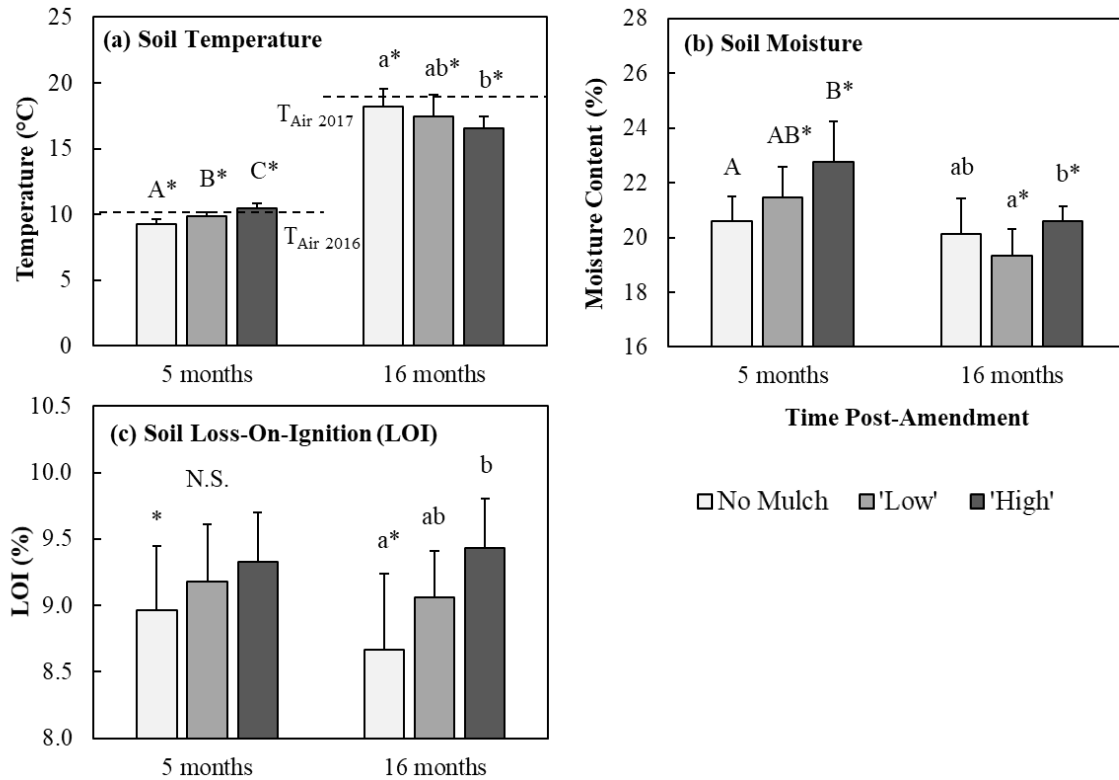
Figure 3.6: Bar charts of simple main effects of mulch amendment (no mulch, 'low' mulch, 'high' mulch) by time since amendment (5 months, 16 months) on (a) soil temperature (°C), (b) soil moisture (%, w/w), and (c) loss on-ignition (LOI) (%, w/w) (n = 10 per amendment rate and sampling year). Horizontal dotted lines in panel (a) show ambient air temperature at times of sampling. Letters denote groupings from Tukey's HSD Test for each sampling period (Capital letters: 5 months, lower case letters: 16 months). Asterisks denote significant pairwise differences between sampling times within a given mulch amendment rate for each soil property. Error bars depict standard deviation (SD).

Overall, mulch amendment generally increased earthworm densities. Endogeic earthworm density was affected by an interaction between mulch and time since amendment (Mixed ANOVA, $F_{2,21} = 3.62$, $p = 0.045$, $\omega_p^2 = 0.14$). After 5 months, 'high' mulch increased endogeic density relative to mulch-free controls (+ 245 %) while 'low' mulch had an intermediate effect (+ 140 %) (simple main effect, One-Way ANOVA, $F_{2,21} = 8.92$, $p = 0.002$, $\omega^2 = 0.40$). After 16 months, 'low' mulch increased endogeic density relative to mulch-free controls (+ 170 %) while 'high' mulch had an intermediate effect (+ 55 %) (simple main effect, One-Way

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ANOVA, $F_{2,21} = 6.56$, $p = 0.006$, $\omega^2 = 0.32$). Endogeic density changed over time only in the 'high' mulch plots, decreasing from 5 months to 16 months post-amendment (- 55 %) (simple main effect, Paired t-Test, $t = -2.65$, $p = 0.033$, $d_z = 0.94$) (Figure 3.7a).

Anecic earthworm density was affected by both mulch (Mixed ANOVA, $F_{2,21} = 4.98$, $p = 0.017$, $\omega_p^2 = 0.25$) and time since amendment ($F_{1,21} = 26.29$, $p < 0.001$, $\omega_p^2 = 0.37$). Overall, anecic density increased relative to mulch-free controls under 'low' mulch (+ 91 %) with an intermediate effect of 'high' mulch (+ 72 %) (Figure 3.7b) and increased from 5 months to 16 months post-amendment (+ 146 %).
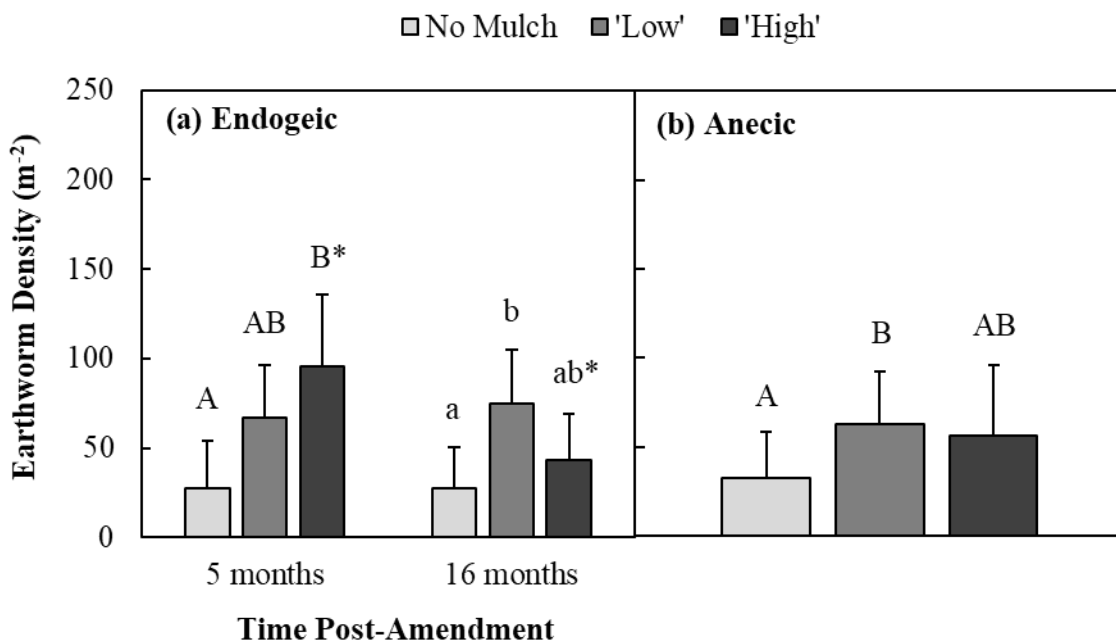
Figure 3.7: Bar chart of effects of mulch amendment (white: no mulch, light grey: 'low' mulch, dark grey: 'high' mulch) (n = 8 plots per mulch amendment rate) on the density of (a) endogeic earthworms (simple main effects of mulch 5 months and 16 months post-amendment) and (b) anecic earthworms (main effects of mulch averaged over time). Letters denote groupings from Tukey's HSD Test groupings comparing mulch amendments for each earthworm category and time. Asterisks denote significant pairwise differences between sampling times within a given mulch amendment rate for endogeic earthworms. Error bars depict standard deviation (SD).

Overall earthworm community composition (measured as % endogeic earthworms by density) was statistically significantly affected by time since amendment (Mixed ANOVA, $F_{1,18}$ = 16.20, p = 0.001, ω_p^2 = 0.13), but not by mulch amendment ($F_{2,18}$ = 0.51, p = 0.608) or an interaction ($F_{2,18}$ = 0.08, p = 0.924). Overall endogeic representation in the earthworm community was higher after 5 months (69 ± 19%) than after 16 months (50 ± 27%).

3.3.2 Mulch collection microcosm experiment

Earthworms changed mulch coverage in all areas of the collection microcosms (Figure 3.8). In the application side, the change in coverage was affected by an interaction between mulch amendment rate and time (Mixed ANOVA, $F_{3.6,35.7}$ = 11.99, p < 0.001, ε_{GG} = 0.60, ω_p^2 = 0.42). The simple main effects of mulch amendment rate and time are summarized in Table 3.2. Overall, application side mulch coverage decreased in the first week post-amendment and continued to decrease in the 'low' application treatment but remained relatively stable in the 'high' application treatment (Figure 3.8a).

Earthworms spread mulch into the initially bare side of the microcosms in the first week post-application, increasing coverage from zero to 53-55 % with no detectable change over the following six weeks (main effect, Mixed ANOVA, $F_{2.7,27.1}$ = 0.59, p = 0.612, ε_{GG} = 0.54). Overall mulch coverage in the initially bare side of the container was 17 % higher over weeks one to six in the 'high' mulch treatment compared to the 'low' mulch treatment (main effect, Mixed ANOVA, $F_{1,10}$ = 8.31, p = 0.016, ω_p^2 = 0.38) (Figure 3.8b).

Earthworms changed total mulch coverage across the whole surface with an interaction between mulch amendment rate and time (Mixed ANOVA, $F_{3.1,30.9}$ = 9.72, p < 0.001, ε_{GG} = 0.52, ω_p^2 = 0.34). The simple main effects of mulch amendment rate and time are summarized in Table 3.2. Earthworms increased total mulch coverage (which began at 50 %) in the first week post-amendment, with a larger increase in the 'high' mulch treatment (+ 41 %) than in the 'low' mulch treatment (+ 26 %). Total coverage decreased over the following several weeks in the 'low' mulch treatment close to initial total coverage (+ 6 %), while total coverage remained higher and more consistent in the 'high' mulch treatment (Figure 3.8c).

Table 3.2: Summary of simple main effects of (a) week at each mulch amendment rate (One-Way Repeated Measures ANOVA), and (b) mulch amendment rate at each week (One-Way ANOVA) on mulch coverage (% area) in the application side and total area of containers (n = 6 per container area, mulch amendment rate, and week). P-values for significant tests are in bold.

(a) Week by Mulch Amendment Rate					
Container Area	**Mulch Rate**	**F**	**p**	**ε_{GG}**	**ω^2**
Application Side	'Low'	64.34	**< 0.001**	0.35	0.88
	'High'	15.59	**0.0002**	0.45	0.65
Total Area	'Low'	8.78	**0.006**	0.34	0.44
	'High'	24.95	**< 0.001**	0.38	0.73

(b) Mulch Amendment Rate by Week				
Container Area	**Week**	**F**	**p**	**ω^2**
Application Side	0	13.33	**0.004**	0.51
	1	54.97	**< 0.001**	0.82
	2	46.71	**< 0.001**	0.79
	3	109.84	**< 0.001**	0.90
	4	51.07	**< 0.001**	0.81
	5	87.67	**< 0.001**	0.88
	6	77.39	**< 0.001**	0.86
Total Area	0	13.33	**0.004**	0.51
	1	19.73	**0.001**	0.61
	2	18.39	**0.002**	0.59
	3	69.82	**< 0.001**	0.85
	4	28.09	**< 0.001**	0.69
	5	66.48	**< 0.001**	0.84
	6	76.06	**< 0.001**	0.86
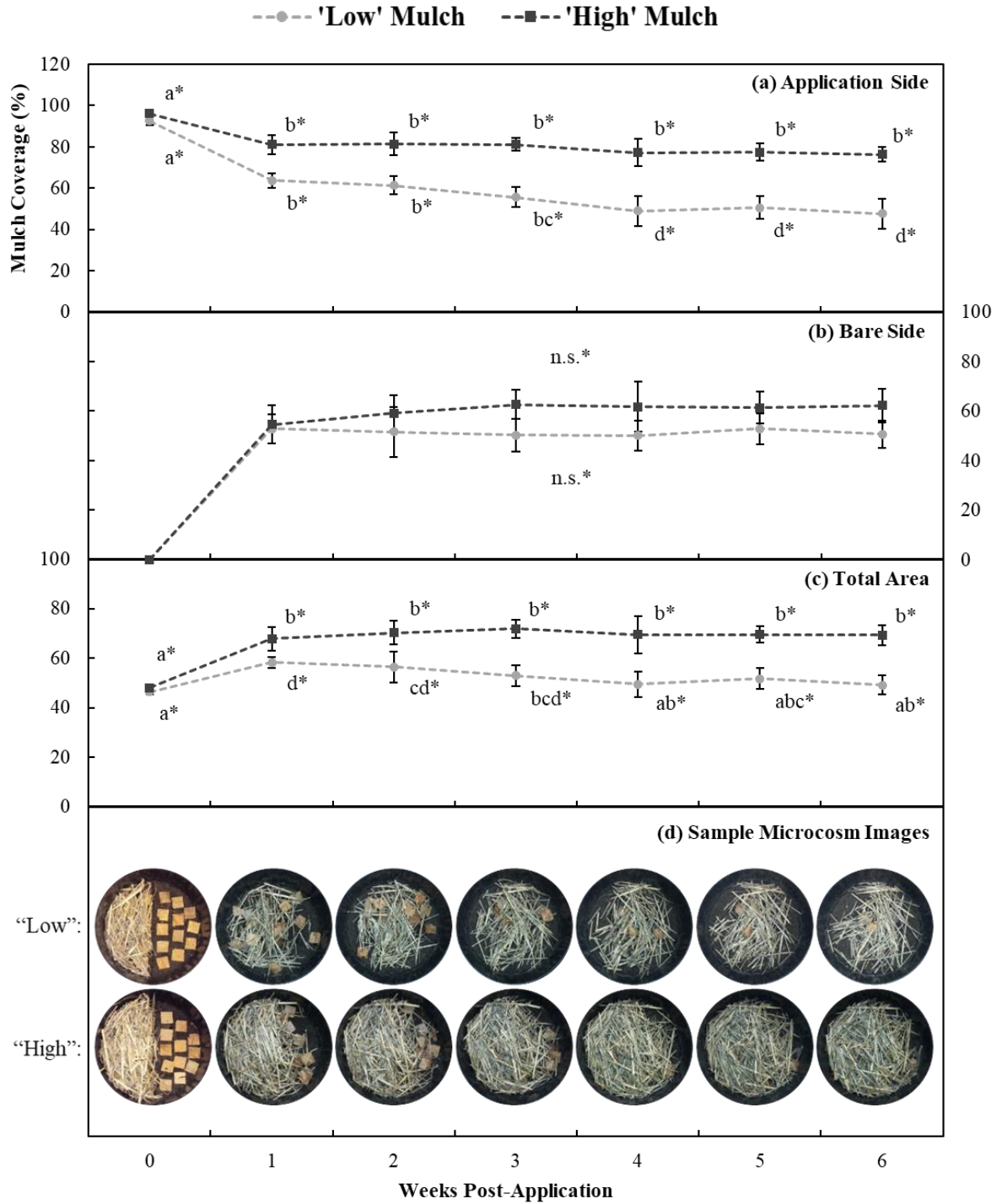
Figure 3.8: Line plots of mulch coverage (% area) in the (a) application side, (b) initially bare side, and (c) total area of earthworm-inoculated microcosms over six weeks post mulch application at two mulch amendment rates (light grey: 'low' mulch, dark grey: 'high' mulch), and (d) sample images of mulch coverage in the microcosms over time (n = 6 microcosms per

mulch amendment rate). Letters denote groupings from Tukey's HSD Test comparing mulch coverage over time within a mulch amendment rate. Asterisks denote significant differences between mulch coverage between different mulch amendment rates by week. Simple main effects across weeks 0 to 6 shown for mulch coverage in the application side (a) and total area (c), main effects averaged across weeks 1 to 6 shown for the initially bare side (b). Error bars depict standard deviation (SD).

Relative mass loss of surface mulch was affected by an interaction between mulch amendment rate and earthworm presence (Two-Way ANOVA, $F_{1,20}= 25.80$, $p < 0.001$, $\omega_p^2 = 0.51$). Earthworms did not affect mulch loss at the 'low' amendment rate (simple main effect, Welch's Test, $F_{1,6.6} = 4.29$, $p = 0.079$) but significantly increased mulch loss at the 'high' amendment rate by approximately 21-fold (simple main effect, Welch's Test, $F_{1,5.0} = 32.12$, $p = 0.002$, $R^2 = 0.76$). Comparing 'low' and 'high' mulch rates, relative mulch loss was comparable when earthworms were absent (simple main effect, Welch's Test, $F_{1,5.1} = 0.70$, $p = 0.441$) but higher in 'high' mulch microcosms when earthworms were present (simple main effect, Welch's Test, $F_{1,5.1} = 25.15$, $p = 0.004$, $R^2 = 0.72$) (Figure 3.9). Upon clearing surface mulch at the end of the experiment, I often observed mulch fragments sticking out of earthworm middens and burrows at the soil surface (Figure 3.10).

Total leaf burial after six weeks was not statistically significantly affected by mulch amendment rates (Mixed ANOVA, $F_{1,10} = 0.45$, $p = 0.515$), microcosm position ($F_{1,11} = 0.48$, $p = 0.504$), or their interaction ($F_{1,11} = 1.05$, $p = 0.464$). Mean leaf burial across all microcosms and positions was 93.1 ± 9.7 %.
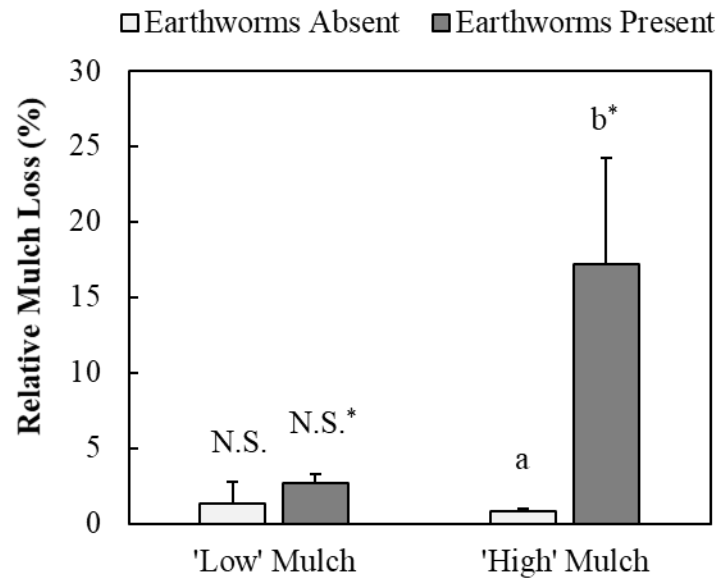
Figure 3.9: Bar chart of simple main effects of earthworm presence (light grey: earthworms absent, dark grey: earthworms present) on relative surface mulch loss (% weight) for two mulch amendment rates ('low', 'high') (n = 6 microcosms per earthworm presence and mulch amendment rate combination). Letters denote groupings based on the Games-Howell test within mulch rates (Capital letters: 'low' mulch, Lower case letters: 'High' mulch). Asterisks denote significant differences between mulch rates. Error bars depict standard deviation (SD).
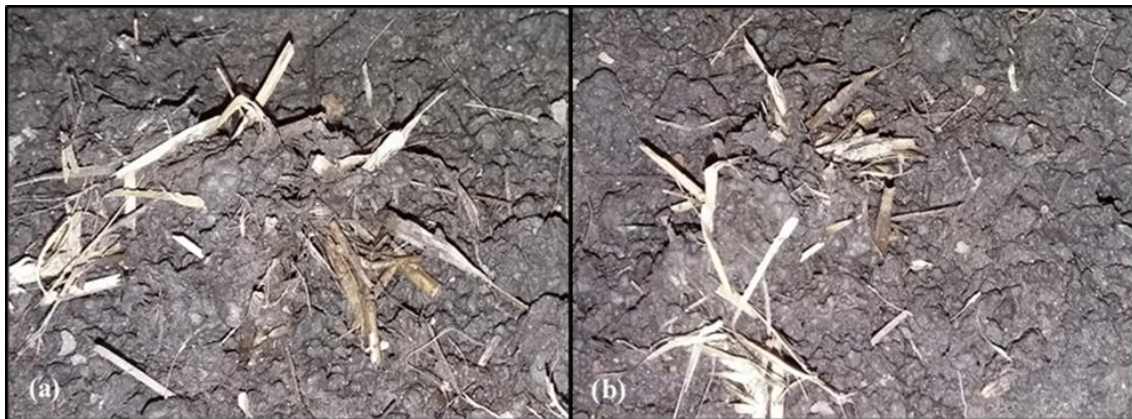
Figure 3.10: Mulch burial and incorporation into earthworm middens and burrows visible after clearing surface mulch six weeks post mulch amendment.

3.3.3 Earthworm midden field survey

I observed that middens occurred at an average density of 30 ± 4 m^{-2}, weighed 1.3 ± 0.2 kg·m^{-2}, and covered 17 ± 3 % of the soil surface by area. Mean values of both the Index of Dispersion (0.55 ± 0.12) and Morisita's Index (0.47 ± 0.15) were below unity, suggesting that when analyzed using sectors of an average area of 12.6 cm^2 covering a total area of 0.38 m^2, middens were spatially over-dispersed (i.e., regularly distributed) (Figure 3.11).
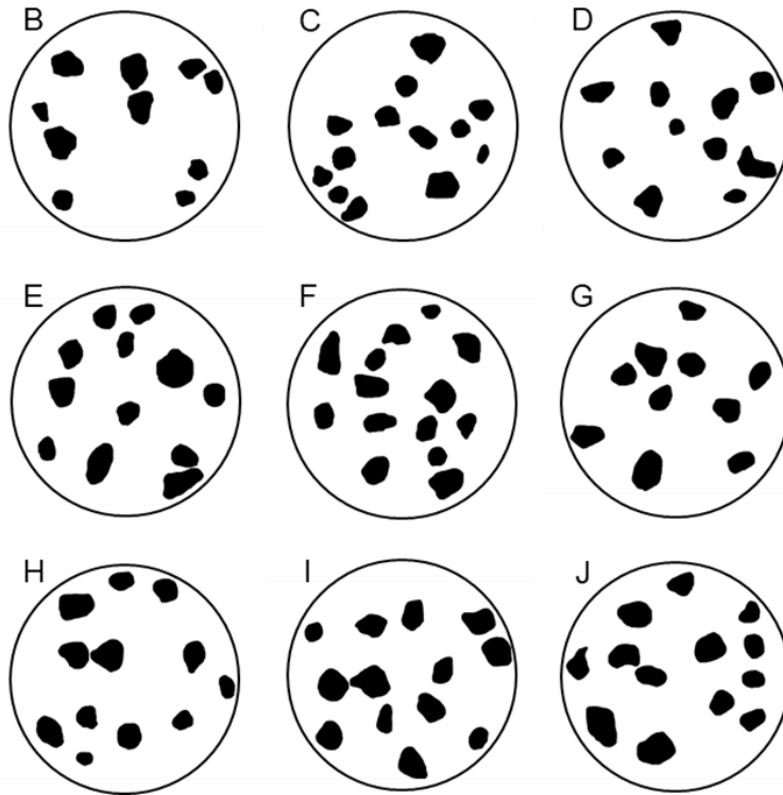
Figure 3.11: Earthworm middens (in black) in random circular plots (Plot ID B-J, 0.35 m radius).

Analyzing a total of 108 middens, individual middens were relatively circular (circularity index = 0.80 ± 0.05) and had a mean dry weight of 42 ± 17 g and a mean area of 55 ± 20 cm^2. By weight, middens were mostly soil, with the remaining material comprised of a mix of woody mulch, rocks, and dead leaf matter (Figure 3.12). A single midden contained an average of 1.3 ± 1.1 g of mulch (Figure 3.13a) and the largest midden collected contained 7.2 g of mulch in an area of 57 cm^2 (Figure 3.13b).
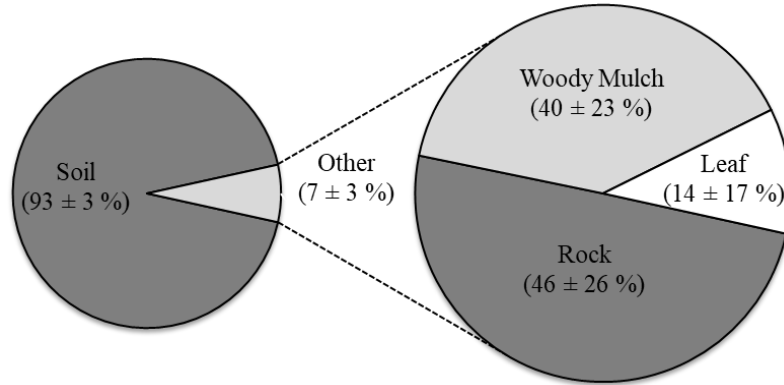
Figure 3.12: Breakdown of contents (% w/w) of *L. terrestris* middens (n = 45 middens).
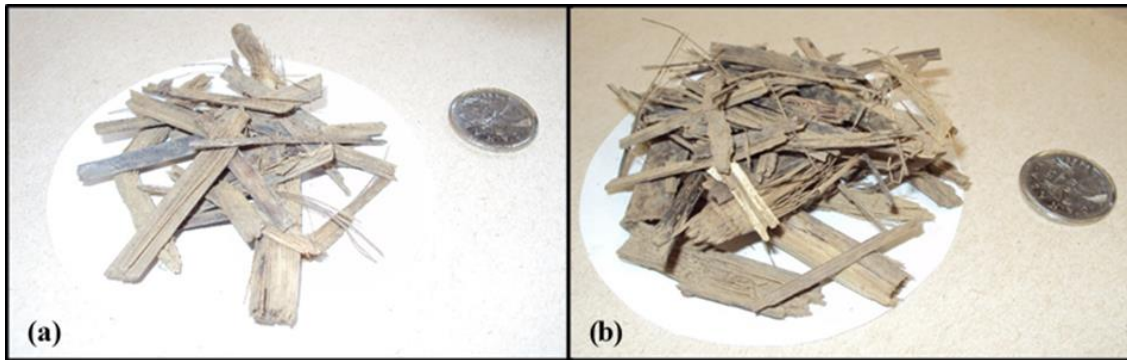
Figure 3.13: Quantities of mulch found in (a) the average earthworm midden (1.3 g in 55 cm^2), and (b) the midden containing the highest quantity collected (7.2 g in 57 cm^2). The white circles depict midden area. A coin is shown in both panels for scale (2.4 cm diameter).

Midden area and weight were positively correlated with one another, i.e., middens covering a larger surface area tended to be heavier (Table 3.3). Both measures of size were positively correlated with mulch content, indicating that larger middens tended to contain higher quantities of mulch. Additionally, midden weight only (not midden area) was positively correlated with the proportional mulch content of middens (% w/w), with mulch comprising a greater proportion by weight of heavier middens.

Table 3.3: Correlation coefficients (Spearman's Rho, r_S) and corresponding p-values for tests of associations between measures of midden size and contents (D.F. = 43 for all tests). Statistically significant p-values are in bold text.

	Midden Area (cm^2)	Midden Weight (g)	Mulch Content (g)	Mulch Content (% w/w)
Midden Area (cm^2)	-	0.645 (**p < 0.001**)	0.409 (**p = 0.005**)	0.212 (p = 0.163)
Midden Weight (g)	-	-	0.654 (**p < 0.001**)	0.377 (**p = 0.011**)
Mulch Content (g)	-	-	-	0.928 (**p < 0.001**)
Mulch Content (% w/w)	-	-	-	-

Figure 3.14: Earthworm middens containing natural corn stubble mulch (a) distributed across the soil surface and (b), (c) in close-up.

The mean density of mulch on non-midden bare ground was 63 ± 81 g·m^{-2}. The mean density of mulch in earthworm middens was 206 ± 178 g·m^{-2} (approximately 3.3 times higher than on bare ground). Using these mulch densities, I estimated that earthworm middens cover

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approximately 17 % of the field surface area but contain 40 % of total surface mulch. Anecdotally, I observed that middens were generally surrounded by stretches of relatively bare soil (Figure 3.14a) and individual middens were often very prominent and well-defined mounds containing reasonably large (> 10 cm long) corn stubble fragments (Figure 3.14b,c).

3.4. Discussion

3.4.1 Earthworm community responses to tallgrass prairie mulch amendment

The results of the tallgrass prairie mulch field experiment indicated a generally positive effect of mulch amendment on earthworm densities. For both endogeic and anecic earthworms, 'low' or 'high' mulch amendment resulted in either substantial or intermediate increases in earthworm density compared to mulch-free controls (+ 55-245 %) (Figure 3.7). These results are consistent with other studies that have observed positive effects of mulch on earthworms (Abail and Whalen, 2018; Frøseth et al., 2014; Pelosi et al., 2015). Mulch generally kept soils warmer when the air temperature was cooler and cooler when the air was warmer, raised soil moisture, and increased soil organic matter (Figure 3.6), suggesting that these positive effects of mulch on earthworms were likely attributable to the benefits of temperature moderation (Acharya et al., 1998; Tian et al., 1997), moisture retention (Blanco-Canqui and Lal, 2007a; Lal, 1978), and provisioning of organic residues for food and midden construction (Stroud et al., 2016). These measured changes in earthworm densities may indicate changes in the actual population size resulting from some combination of changes in dispersal, survivourship, reproduction, or activity and ease of sampling. Dispersal and activity in these small (1 m × 1 m) plots may be most important shortly after amendment, while changes in survivourship or reproduction may become more important over time.

Mulch effects on earthworms depended on mulch amendment rate and differed between endogeic and anecic functional groups, varying for endogeic earthworms over time post amendment. Notably, after 16 months post-amendment, the largest increases in earthworm density occurred under 'low' mulch amendments, with intermediate effects of 'high' mulch amendment. For endogeic earthworms, earthworm density was originally highest under 'high' mulch amendment after 5 months but decreased over the subsequent year (Figure 3.7). While some past studies have found 'more is better' in terms of mulch addition benefiting earthworm communities (Abail and Whalen, 2018; Blanco-Canqui and Lal, 2007a; Teotia et al., 1950),

others have observed no increases in benefits beyond lower mulch rates (Mele and Carter, 1999). While the benefits of mulch can increase as additional amendment is added, particularly high quantities can have negative impacts by impairing mobility (Cromar et al., 1999), blocking inputs of other organic matter (e.g., litter) (Andersen et al., 2013) or water (Blanco-Canqui and Lal, 2007a) into the soil. In general, applying any amount of mulch may be sufficient to improve soil conditions such as temperature and moisture in the short-term, particularly if applied to otherwise bare soil, improving survivourship and encouraging earthworms from adjacent non-mulched soil to migrate in. Positive effects of mulch in the subsequent year may include improvements to earthworm growth and reproduction (Chen et al., 2017; Sizmur et al., 2017), resulting in further density increases in the 'low' plots while possibly being offset by mobility or organic matter limitations in the 'high' mulch plots.

In other studies, the time required for mulch impacts on earthworms to be detectable varies from as short as a single month (Tian et al., 1997), to several months or a year (Abail and Whalen, 2018; Frøseth et al., 2014; Mele and Carter, 1999; Sizmur et al., 2017), or to as long as a decade (Pelosi et al., 2015). In this study, the impacts on earthworm density were observable five months post-application. Since anecic earthworms can increase foraging in response to mulch within days of application (Butt et al., 2003; Zhang et al., 2018) and positive effects of mulch on soil temperature and moisture content occurred five months post amendment, early earthworm responses to mulch are unsurprising.

Further changes in the effect of mulch on earthworms over subsequent years are also likely, particularly in the absence of additional mulch inputs. Decomposition and burial of mulch may increase food palatability and availability (Abail and Whalen, 2018), especially for endogeic species that are not able to immediately access surface organic matter as easily as anecic species (Chen et al., 2017; Snyder and Hendrix, 2008; Wolfarth et al., 2011). In contrast, loss of total mulch biomass and more labile nutrient components may eventually reduce food resources (Sizmur et al., 2017; Tian et al., 1997) and leave soils exposed to greater temperature fluctuations, moisture loss, etc. (Shuster et al., 2000). Long-term effects of mulch on earthworm communities may be difficult to predict without continued monitoring (Pelosi et al., 2015).

3.4.2 Initial short-term mulch collection and burial by anecic earthworms

In the mulch collection microcosms free of earthworms, mulch cover did not change and losses in mulch mass were minimal over the six week experiment (Figure 3.9). This is consistent with the typically slow processes of abiotic organic residue burial and decomposition (Stephens et al., 1994, 1993). In contrast, earthworms reduced mulch coverage in the application areas and generally increased total mulch loss from the surface (Figure 3.8). These losses in mulch coverage are primarily attributable to the spatial aggregation of mulch into middens and burial (Butt et al., 2003), though over a longer time period I would also expect accelerated decomposition of organic material by earthworm-enhanced microbial activity (Mackay and Kladivko, 1985; Teotia et al., 1950; Tian et al., 1997; Wolfarth et al., 2011).

The collection microcosm experiment exhibited the ability of earthworms to redistribute mulch not only when it is placed directly on top of them, but also when it is located more distantly across the soil surface (Butt et al., 2003; Nuutinen and Butt, 2019). Although earthworms likely buried some of the mulch, total surface coverage remained high (Figure 3.8), suggesting that most of the loss of mulch from the initial application area was the result of horizontal redistribution by direct collection or 'stealing' from middens of conspecifics (Butt et al., 2003; Nuutinen and Butt, 2019). This redistribution of mulch may have a limited impact on large mulch applications (e.g., mulching whole agricultural fields) beyond a minor 'bleed' at the field perimeter but may be important at smaller scale uses such as gardens or mulching individual plants, where small patches of mulch could be quickly removed and redistributed.

The mulch collection experiment demonstrated that the effects of earthworms on initial mulch distribution also depend on the amount of mulch used. Overall, applying more mulch helped mitigate losses in coverage. The 'high' mulch amendment rate required more mulch to be removed to expose the underlying soil and increased total cover slightly after six weeks through more mulch spread onto the adjacent initially bare soil (Figure 3.8). Importantly, however, the distribution of a fixed quantity of mulch over a larger area must necessarily require a thinning of depth which may reduce mulch benefits. In addition to the thinning, earthworms only increased the relative mass loss of surface mulch under the 'high' amendment treatment (Figure 3.9). This may be a result of higher intensity foraging and burial in response to the higher resource density (Butt et al., 2003) or of the burial of mulch beneath a moist horizontal layer of non-consolidated earthworm casts that I found at the soil surface in the 'high' amendment treatment only. Similar

cast layers have been observed underlying mulch in the field and may indicate increased activity and casting under particularly thick layers of mulch (Dyksterhuis and Schmutz, 1947).

I detected changes in mulch coverage within just one week following mulch amendment. Notably, the relatively large 51-62 % increase in coverage of the initially bare areas occurred during the first week of the experiment and did not change over the subsequent five weeks (Figure 3.8). These results are consistent with the ability of *L. terrestris* to respond rapidly to changes in resource availability, beginning to collect amendments within days of application (Butt et al., 2003; Zhang et al., 2018). While other studies have emphasized the importance of longer-term interactions of earthworms and mulch (Pelosi et al., 2015), these results underscore the importance of also considering initial, fast-acting interactions. For example, if mulch is applied with a relatively short term objective such as the protection of vulnerable developing seedlings (Bakker et al., 2003), rapid initial redistribution by earthworms may severely compromise amendment effectiveness by rapidly exposing soils or plants (Shuster et al., 2000).

3.4.3 Longer-term effects of anecic earthworms on mulch distribution

The midden survey of the abandoned agricultural field documented longer-term mulch aggregation by anecic earthworms into middens. A large proportion of the non-soil contents of middens consisted of mulch (Figure 3.12), presumably collected by *L. terrestris*. Earthworm midden density and cover in this field were consistent with observations made in other agricultural fields and woodlots in North America and Europe (Grigoropoulou and Butt, 2010; Hamilton and Sillman, 1989; Simonsen et al., 2010; Subler and Kirsch, 1998), accounting for 17 % of the total area of the field but containing an estimated 40 % of the total surface mulch. As a result of earthworm foraging, the overall distribution of mulch on the soil surface in the field was strongly tied to the distribution of the middens themselves.

At a relatively small spatial scale, earthworm middens in the field were regularly distributed across the soil surface (Figure 3.11). Regular distributions of earthworm middens at small scales may result from a balance of competition for resources in overlapping foraging areas and mating opportunities (Grigoropoulou and Butt, 2010). Middens were typically circular and did not overlap (Figure 3.12), resulting in the aggregation of large amounts of mulch into small discrete piles separated by largely bare soil. The amount of mulch also varied between middens, with larger and heavier middens generally containing more mulch (Table 3.3). Notably, the

proportional amount of mulch in middens (by weight) was positively correlated with midden weight but not midden area, which may indicate cumulative collection of mulch over time as middens are built upwards more than outwards by additional castings. Midden distributions at larger scales were not measured in this study, but earthworms tend to be spatially aggregated across larger areas, possibly due to associations with underlying variations in soil properties (Jiménez et al., 2001; Nuutinen et al., 1998; Rossi and Nuutinen, 2004). For large mulch applications (e.g., agricultural fields), the intensity of the smaller-scale impacts noted in this study may themselves be spatially aggregated at larger scales based on underlying earthworm densities.

As an observational study, these results do not indicate what the distribution of natural mulches in the field would have been in the absence of earthworms. Shuster et al. (2000), however, used manipulations of earthworm density in agricultural fields and found a similar reduction in mulch cover that occurred only when earthworms were present. By changing the distribution of mulch across the soil surface, earthworms can increase the spatial heterogeneity of soil properties (Subler and Kirsch, 1998). Aggregation of organic material like mulch into middens can increase microarthropod density (Hamilton and Sillman, 1989), nutrient concentrations, and microbial activity (Subler and Kirsch, 1998). This mulch-enrichment may further contribute to the role of middens as regeneration niches for plant seedlings (Milcu et al., 2006). The conditions within middens likely also contribute to accelerated mulch decomposition (Mackay and Kladivko, 1985; Teotia et al., 1950; Tian et al., 1997, 1995; Wolfarth et al., 2011). Additionally, the collection of mulch away from inter-midden soil will limit the benefits of mulch amendment to those surfaces, potentially exposing soils to increased water loss, erosion, or plant invasion (Bakker et al., 2003; Shuster et al., 2000). Overall, by aggregating large quantities of mulch into relatively small patches of a larger area, anecic earthworms will likely limit the effectiveness of mulch amendments when the goal is to maintain even and complete coverage.

3.4.4 Implications of earthworm-mulch interactions for restoration

While mulch has been used to manage earthworm communities and facilitate the recolonization of degraded sites (Lal, 1978), earthworm interactions with mulch in restoration have received minimal study. Earthworms can potentially improve mulch effectiveness,

preventing excessive residue build-up (Mackay and Kladivko, 1985), incorporating mulches into the soil to improve soil fertility (Tian et al., 1997), and helping decompose particularly woody residues (Fraser et al., 2003). Other potential novel benefits include burying mulches inoculated with biocontrol agents to control root pathogens (Stephens et al., 1994, 1993) and burying and accelerating decomposition of biodegradable plastic mulches (Zhang et al., 2018). To supplement these beneficial effects, this study highlights how earthworms can also detrimentally aggregate mulch in the short and longer term, reducing mulch cover and evenness within weeks of amendment and potentially reducing its ability to protect developing seedlings, reduce soil erosion, limit plant invasion, and improve soil conditions (Shuster et al., 2000). Crucially, both the positive and negative impacts may be self-reinforcing as earthworm communities generally benefit from mulch and may increase in size and subsequent impact following amendment. Learning to better understand and plan for both beneficial and detrimental earthworm-mulch interactions could help improve the effectiveness of mulch amendment in earthworm-inhabited systems.

Managing these earthworm-mulch interactions would require an initial earthworm community assessment of the site being managed. The proportional representation of anecic species such as *L. terrestris* may be particularly important to consider, as this functional group is uniquely responsible for the mulch collection and burial. Following this assessment, a project manager might control factors including mulch type and quantity to best meet different restoration goals. For example, earthworms may offer the most benefits to 'low quality' mulches with higher C:N ratios and lower decomposition rates (e.g., straw, wheat). While 'high quality' mulches (e.g., clover) may be selectively consumed by earthworms (Buck et al., 2000, 1999) and lead to faster and greater increases in earthworm density and biomass (Chen et al., 2017; Fraser et al., 2003; Tian et al., 1997), gradual earthworm-assisted incorporation and breakdown of 'low quality' mulches can provide longer lasting soil cover and a slow-release of nutrients better synchronized to plant uptake (Abail and Whalen, 2018; Tian et al., 1997).

The addition of more mulch – either through larger applications or repeated applications over time – may be the best way to mitigate the adverse impacts of earthworms observed in this study. Thicker layers of mulch will be slower to lose coverage due to earthworm-assisted aggregation, burial, or decomposition and may offer fewer benefits to earthworm communities than lighter mulch applications. Although amendment rates used in many land management

applications would likely be higher than the 'low' rate used in this study (i.e., 2.5 USG·m^{-2}, the minimum amount required to fully cover plot surfaces), these findings caution against using particularly light mulch applications in soils containing earthworms. However, higher mulch amendment rates also have potential drawbacks including higher costs, restriction of other organic inputs to the soil, and reduced water infiltration due to shallower earthworm burrowing under heavy mulch (Blanco-Canqui and Lal, 2007a; Teotia et al., 1950). Overall, better integrating earthworm-mulch interactions into restoration planning would likely require continuing experimental investigation of both short and long-term interactions and case-by-base consideration of earthworm impacts and restoration goals.

Chapter 4: Earthworm behaviour and populations respond to different wood ash amendments and anecic earthworms (*Lumbricus terrestris* L.) facilitate the burial of surface-applied wood ash

Abstract

Wood ash is a by-product of energy produced by burning wood residues that is often landfilled but may be more sustainably used for restoration as a liming agent and low-grade fertilizer. As sensitive soil organisms, earthworms may respond to wood ash amendment and be useful in comparing biotic impacts of different wood ashes, and certain species such as the deep-burrowing anecic earthworm *Lumbricus terrestris* L. may alter the incorporation of wood ash into the soil. In this study, I investigated how different wood ashes affect earthworms and how earthworms may bury surface-applied wood ash by testing: how wood ash affects the short-term growth and survivourship of *L. terrestris* (Experiment 1); whether *L. terrestris* avoids or prefers ash amended soils (Experiment 2); how wood ash affects the surface behaviour of *L. terrestris* (Experiment 3); how wood ash affects whole earthworm communities in the field three weeks and one year post amendment (Experiment 4); and how surface-applied wood ash is buried by *L. terrestris* (Experiment 5). The impacts of wood ash on earthworms in both the laboratory and field were generally adverse, but minimal in impact and short lasting. Wood ash had no effects on short-term earthworm growth or survivourship, but earthworms did respond behaviourally to certain wood ashes by avoidance and reduced surface activity. Wood ash generally decreased earthworm densities in the field in the first three weeks following application but had neutral or positive effects after one year. In burial microcosms, *L. terrestris* increased both the total amount of buried wood ash and the maximum depth to which it was buried, likely by burying wood ash-covered leaf litter and increasing soil water infiltration. Earthworm-wood ash interactions are likely influenced by highly scenario-specific circumstances, including wood ash type (e.g., fly or bottom ash), boiler properties, timing of wood ash application, earthworm community composition, and soil characteristics. Overall, this study suggests that land management using wood ash could benefit from integrating how ash interacts with earthworms, considering how earthworms respond to different wood ashes and how earthworms may help bury surface-applied wood ash when manual mixing is not an option.

4.1 Introduction

Wood ash is a by-product of energy produced by burning wood residues. In Canada, the pulp and paper industry has been a primary source of wood ash and is expected to be supplemented by a growing interest in forest bioenergy (Hannam et al., 2018). Some of this wood ash is used to produce cement and other construction materials (Pitman, 2006), but in Canada much of it is treated as waste that is landfilled at additional cost (Elliot and Mahmood, 2006). As a more sustainable alternative, wood ash can be used as a soil amendment (Hannam et al., 2018). Although wood ash is currently applied to agricultural soils and some forest soils, particularly in Europe (Augusto et al., 2008), its use in Canada has been limited by various regulatory and practical barriers related to the cost-effectiveness of landfilling, variable ash quality, and uncertainty regarding longer-term effects on ecosystems (Hannam et al., 2018).

Burning wood residues in biomass boilers produces two types of wood ash: fly ash and bottom ash. Fly ash is collected from the flue gas stream and is generally smaller, lighter and has higher concentrations of nutrients and heavy metals. Bottom ash is collected from the bottoms of boilers and is generally larger, heavier, and more variably sized (Pitman, 2006). Although amendment is not universally beneficial, wood ash typically has a pH in the range of 8 to 13 and can be used as a liming agent to help offset soil acidification and is a low grade fertilizer containing plant-essential macronutrients (e.g., Ca, Mg, K, and P) and micronutrients (e.g., Fe, Mn, Zn, B, Cu, and Mo) (Augusto et al., 2008; Pitman, 2006; Reid and Watmough, 2014). Ash physical and chemical properties can vary widely depending on feedstock form (e.g., bark, wood chips, slash), wood type (e.g., hardwood or softwood), and boiler temperature (Pitman, 2006). Wood ash is related in form and application to other materials produced from complete incineration (coal ash) or pyrolysis (biochar). Compared to wood ash, coal ash generally has lower nutrient concentrations (Hytönen, 2003) while biochar has higher processing costs and produces less energy, but may have stronger benefits for soil fertility (Reed et al., 2017).

The broader use of ash as a soil amendment is limited by the potential toxic effects of ash on the environment and human health (Hannam et al., 2018). One means of assessing these risks is studying the effects of ash amendment on species of interest (Maity et al., 2009). There have been relatively few studies of wood ash impacts on soil fauna (Aronsson and Ekelund, 2004), with minimal existing research on the responses of salamanders (Gorgolewski et al., 2016) and microarthropods and enchytraeid worms (Haimi et al., 2000; Huhta et al., 1986; Liiri et al., 2007,

2002). Earthworms are understudied but promising species for examining wood ash impacts on the soil environment as they live and feed both belowground and at the soil surface, are soft-bodied with high chemical sensitivity, and generally respond quickly to environmental stress (Edwards and Bohlen, 1996; Laverack, 1961, 1960; Markad et al., 2016).

Wood ash can have direct and indirect effects on earthworms and other soil fauna. Direct effects include mutagenic or genotoxic effects of heavy metals and other toxins such as polyaromatic hydrocarbons (Eijsackers, 2010; Grumiaux et al., 2015; Gupta et al., 2005; Markad et al., 2016), desiccation from the high water holding capacity of ash (Gorgolewski et al., 2016; D. Li et al., 2011), changes in habitat suitability from increased soil pH, or caustic burns. While most of the direct effects are likely to decrease earthworm populations, increasing pH could also reduce metal mobility and improve the habitat suitability of particularly acidic soils (Grumiaux et al., 2015; Homan, 2015; McCallum et al., 2016). Potential indirect effects of ash include changes in microbial biomass and decomposition and subsequent food availability to earthworms in response to elevated soil pH or nutrient enrichment (Huhta et al., 1986; Nieminen, 2008). How wood ash affects soil biota may be further influenced by the seasonal timing of ash amendment (Grumiaux et al., 2015), for which there has been minimal research and few management guidelines (Hannam et al., 2016; Risse and Gaskin, 2013).

How ash affects earthworms can be measured in the field by monitoring whole communities and in the lab using acute (e.g., survivourship), sublethal (e.g., weight loss), or behavioural tests (e.g., avoidance). Behavioural tests have been used less widely but can detect responses to lower levels of stress and capture non-lethal changes in behaviour that may be ecologically significant (e.g., reduced burrowing or foraging) (Yeardley et al., 1996).

In addition to being useful for comparing different wood ashes, earthworms may also influence the effectiveness of the wood ash itself. Earthworms are already used to vermicompost coal ash and other similar amendments to reduce toxicity and increase nutrient concentrations (Bhattacharya and Kim, 2016), but their effects on ash-like amendments in the field are largely unknown. Of particular interest is the potential for earthworms to facilitate the burial of wood ash. Acting as ecosystem engineers (Jones et al., 1994; Lavelle et al., 2006), earthworms can transport materials through the soil actively (e.g., picking up or consuming and defecating residues) or passively (e.g., creating burrows which act as a conduits for the burial of residues by rainfall) (Anderson, 1988). Earthworms from different functional groups – litter-dwelling

epigeics, horizontal burrowing geophagous endogeics, and deep vertical burrowing anecics (*sensu* Bouché, 1977) – may also contribute differently to wood ash incorporation. Anecic species, which form vertical burrows open to the surface and pull surface-foraged material down into the soil may have a particularly strong influence on ash burial, especially if ash is applied on top of a layer of leaf litter.

Earthworm-facilitated burial has potential applied value for management, since amendments such as biochar or wood ash generally benefit from manual incorporation into the soil. Burial helps prevent build-up of material over litter and plants, reduces aboveground losses to rainfall or wind, and accelerates ash effects deeper in the soil. Ash is disked into soils when used in agriculture but this mixing is not generally possible in systems with perennial vegetation such as forests (Elmer et al., 2015; Pitman, 2006). Although a few studies have found preliminary evidence of earthworms burying other amendments including lime (Baker et al., 1999; Chan, 2003; Springett, 1983), charcoal (Eckmeier et al., 2007; Topoliantz and Ponge, 2005, 2003), and biochar (Elmer et al., 2015), to the best of my knowledge there are no documented interactions with wood ash.

The purpose of this study was to investigate how wood ash amendments interact with earthworms. This study was guided by two primary research questions each addressed by related experiments: (1) How do different wood ashes affect earthworm growth and survivourship (Experiment 1), habitat avoidance (Experiment 2), and surface behaviour in the laboratory (Experiment 3), and community density and composition in the field (Experiment 4); and (2) Do anecic earthworms bury surface-applied wood ashes (Experiment 5)? I addressed these research questions with field studies conducted at two mixed upland forests in Ontario, Canada (Waterloo Environmental Reserve, Haliburton Forest) and with laboratory microcosm studies using the geographically-widespread and ecologically-influential anecic earthworm *Lumbricus terrestris* L. (Addison, 2009; Keller et al., 2007).

4.2 Methods

4.2.1 Sources of earthworms, soil, and ash

I purchased adult *L. terrestris* from a commercial bait vendor (Waterloo, Ontario, Canada) and used them each once for a single experiment.

I summarized the pH of the different soils and wood ashes I used in different experiments in Table 4.1. For all laboratory-based experiments (Experiments 1, 2, 3, and 5), I used an artificial soil with a neutral pH of 7.3 made by mixing three parts (by volume) Circle H Farms Black Earth potting soil with two parts screened topsoil. From past experiments I knew this soil to be suitable for earthworm survivourship, growth, and burrowing behaviour. To contrast the effects of ash amendment on a more acidic soil, I also used field-collected soil from the Haliburton field site (see *Experiment 4* for site descriptions) in the short-term growth and survivourship experiment (Experiment 1). I collected the Haliburton soil in spring 2016 near to the location of the future field application trial (see *Experiment 4*) by removing the litter layer and coarsely sieving the top 15 cm of soil.

Table 4.1: Summary of pH of soils and wood ashes used in different experiments.

Material	Type	pH	Experiments
Soil	Experimental	7.3	(1) Short-term growth and survivourship (2) Avoidance (3) Surface behaviour (5) Ash burial
	Waterloo	6.3	(4) Field application
	Haliburton	5.2	(1) Short-term growth and survivourship (4) Field application
Wood Ash	Fly A	9.3	All
	Bottom A	8.9	
	Fly B	12.7	(1) Short-term growth and survivourship (2) Avoidance
	Bottom B	8.3	

I obtained fly and bottom wood ash from two biomass boilers ('A' and 'B') from pulp and paper mills operating in eastern Canada using a combination of spruce, pine, and fir (SPF) bark feedstock. Boiler 'A' used an older vibrating-stoker grate boiler while boiler 'B' used a newer Wellons gasification boiler with a separate furnace and combustion chamber. For more detailed description of the wood ashes, see Gorgolewski (2015). Wood ash from boiler 'A' was available in plentiful supply and I used it in all laboratory and field experiments while I used wood ash from boiler 'B' only in the short-term growth and survivourship and avoidance

experiments. I applied ash at a rate of 10 t·ha^{-1} (dry) in all experiments, which is considered a 'low to moderate' application (Augusto et al., 2008) that balances beneficial soil modifications with minimal toxicity (Pitman, 2006).

4.2.2 Effects of wood ash amendment on earthworms

I conducted four experiments to assess how wood ash amendment affects earthworms: a laboratory microcosm experiment testing how wood ash affects the growth and survivourship of *L. terrestris* over two weeks (Experiment 1); a laboratory avoidance experiment testing whether *L. terrestris* avoids or prefers wood ash amended soils (Experiment 2); a microcosm experiment testing the effects of wood ash on the surface behaviour of *L. terrestris* (Experiment 3); and a field experiment monitoring whole earthworm community responses to wood ash amendment at two forested sites three weeks and one year post-amendment (Experiment 4).

Experiment 1: Short-term growth and survivourship experiment

I used a laboratory microcosm experiment to determine the short-term effects of wood ash amendment (Control, Fly A, Fly B, Bottom A, Bottom B) and soil source (neutral experimental soil, acidic Haliburton soil) on earthworm survivourship and biomass change over two weeks post amendment (n = 4 microcosms per ash amendment and soil source, total N = 40 microcosms). I filled nursery pots (20 cm diameter, 22 cm height) to a depth of 17 cm with either the artificial soil or Haliburton soil and watered them every 2-3 days with tap water to maintain an approximate moisture content of 30 % (w/w). I placed the microcosms randomly in an experimental chamber set for optimal culture parameters for *L. terrestris* (24 h dark, 18 °C, 65-70 % RH) (Lowe and Butt, 2005).

I weighed fresh pairs of healthy, adult earthworms after an 18 h fast and transferred them into microcosms at a population density of c. 64 m^{-2}, consistent with densities I have observed in local forests. I added 2.5 g (dry) of crushed maple litter (mixed *Acer saccharum* and *Acer platanoides*, collected from a woodlot on the University of Waterloo campus, air dried for five days) to the surface of each microcosm to provide an initial food source and fixed a rigid paper collar to prevent earthworm escape. I left the microcosms for 10 days to allow earthworms to burrow and acclimate. I then added ash by hand to the surface of microcosms at 10 t·ha^{-1} (dry) (Figure 4.1) and watered the microcosms with c. 100 mL tap water every 2-3 days.

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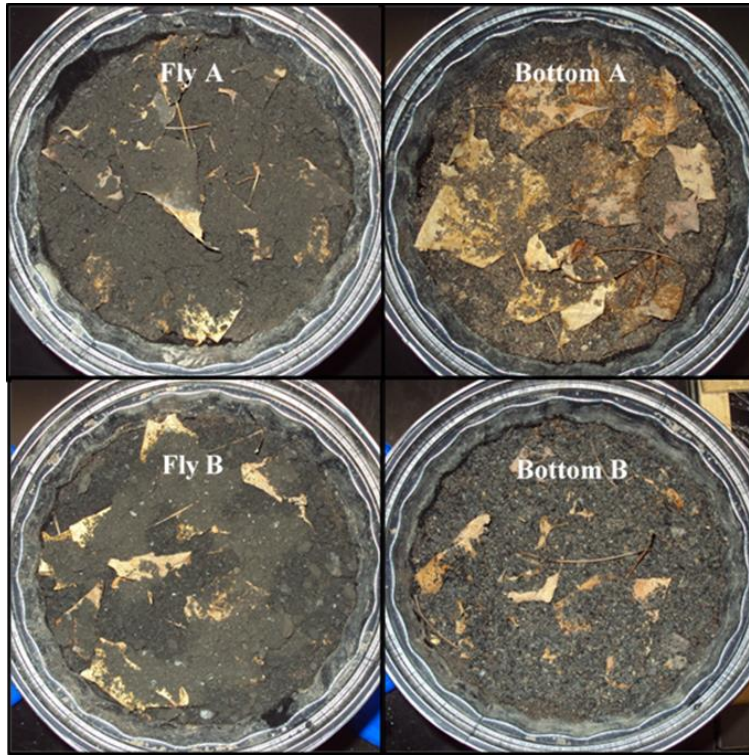
Figure 4.1: Growth and survivourship microcosms amended with different wood ash types (Fly, Bottom) from two biomass boilers (A, B) at 10 t·ha^{-1} (dry).

After two weeks, I emptied and hand-searched the microcosms. I recorded earthworms as survived or deceased/missing and fasted (18 h) and re-weighed live individuals to determine mass change. I assessed the effects of wood ash amendment (Control, Fly A, Fly B, Bottom A, Bottom B) and soil source (experimental, Haliburton) on earthworm survivourship (%) and biomass change (%) using Two-Way ANOVAs (for all statistical tests in this dissertation, see *1.4.2 Notes on statistical analyses* for general details regarding testing of assumptions, alternative tests used when assumptions were not met, and post-hoc testing).

Experiment 2: Avoidance experiment

I used a laboratory experiment to determine whether *L. terrestris* selectively burrowed in or avoided soils with different wood ash amendments (Control, Fly A, Fly B, Bottom A, Bottom B) in two-sided avoidance containers (n = 8 per ash amendment, total N = 40 containers). I filled plastic containers (38 cm length, 24 cm width, 24 cm depth) with the experimental soil to a depth

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of 9 cm and separated them into two halves with a T-shaped plastic divider inserted into the center of each container to prevent burrowing in or across the center of the container (Figure 4.2a). I shallowly covered the top panel of the divider (8 cm length, 24 cm width) with soil to create a 'neutral' middle area where earthworms could be introduced but not burrow. I randomly chose one side of each container to be amended by hand with 10 t·ha^{-1} (dry) with one of the five amendments and I used more experimental soil as a 'Control' amendment.

I removed healthy, adult *L. terrestris* from the refrigerator and transferred them to a plastic container placed in the experimental chamber for 2 h to acclimate to the conditions used for the duration of the experiment (24 h dark, 18 °C, 65-70 % RH). I transferred groups of four earthworms to each avoidance container and placed them in the center of the 'neutral' middle platform (Figure 2b). Following the recommendations of Yeardley et al. (1996), I searched the containers by hand after 48 h to determine what proportion of the four earthworms established in the ash-amended side of the container. I assessed the effects of wood ash amendment (Control, Fly A, Fly B, Bottom A, Bottom B) on the proportion of earthworms found in the ash-amended side of the container using One-Way ANOVA (see *1.4.2 Notes on statistical analyses*).
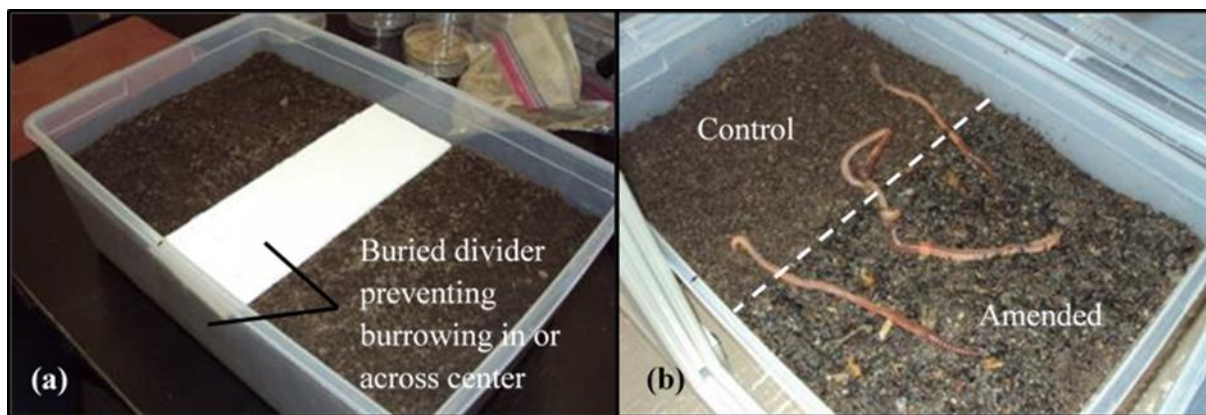
Figure 4.2: Avoidance container (a) prior to amendment, showing the buried divider, and (b) post amendment with control soil and bottom ash immediately after earthworm introduction.

Experiment 3: Surface behaviour experiment

I used a laboratory microcosm experiment to assess the effects of wood ash amendment (Control, Fly A, Bottom A) on the aboveground surface behaviour of *L. terrestris* over eight sequential nights (n = 6 microcosms per ash amendment, total N = 18 microcosms). I filled

nursery pots (20 cm diameter, 22 cm height) with the experimental soil and communally fasted and weighed healthy adult *L. terrestris*. I added four earthworms to each microcosm at a higher density of c. 128 m^{-2} to increase the frequency of nightly aboveground foraging events. I placed a total of nine 3 cm $\times$ 3 cm squares of dried maple leaf litter (see methods for Experiment 1 for litter details) on the surface of each microcosm, with eight spread evenly around the inner circumference and one in the middle. I then added wood ash by hand to the surface of microcosms at 10 t·ha^{-1} (dry), covering the litter squares. I placed the microcosms randomly in an experimental chamber (14 h light: 10 h dark, 18 °C, 65-70 % RH) and watered them with c. 100 mL of tap water every 2-3 days. I monitored the microcosms using an infrared video camera set to record from 30 minutes before to 30 minutes after the 10 h dark 'night' period (Figure 4.3). After eight days, I hand searched the microcosms, recorded earthworms as survived or deceased/missing, and fasted (18 h) and re-weighed live individuals to determine relative mass change.
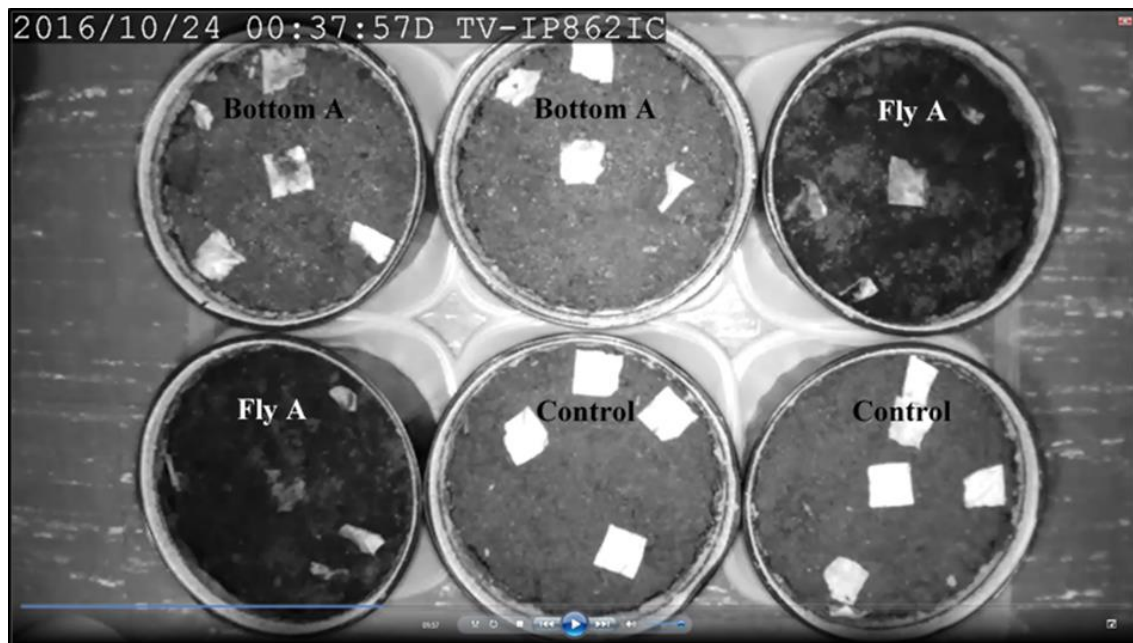
Figure 4.3: Video still of surface behaviour microcosms initially seeded with nine leaf fragments after addition of different wood ash amendments (Control, Fly A, Bottom A).

I manually reviewed the first four 10 h dark 'night' periods in the video recordings for aboveground earthworm events. I recorded an aboveground event when $\geq$ 1 cm of an earthworm

was visible at the soil surface and ended it when no part of the earthworm was visible aboveground. I recorded the timing and duration of each event in addition to whether the event included any handling of leaf litter by earthworms with their prostomium. I estimated aboveground event frequency per earthworm using the mean number of surviving earthworms I found at the end of the experiment for each microcosm. I measured cumulative leaf burial (% of the initial nine leaf squares pulled belowground) at the end of each of the eight 'night' periods. I assessed the effects of wood ash amendment (Control, Fly A, Bottom A) and observation night (1, 2, 3, 4) on aboveground event frequency per night per earthworm, individual event duration, litter handling events (% of total aboveground events), and cumulative leaf burial using Mixed ANOVAs with microcosm identity as a random factor nested within ash amendment (see *1.4.2 Notes on statistical analyses*).

Experiment 4: Field application experiment

I conducted a field plot experiment at two forest sites (Waterloo, Haliburton) to determine the effects of wood ash amendment (Fly A, Bottom A) applied at two application times (pre-litterfall, post-litterfall) on earthworm density over two years (n = 9 sets of paired ash-control plots per unique combination of site, ash type, and applicating timing, total N = 72 pairs of plots).

I established the first study site in the University of Waterloo's Environmental Reserve, a forest on the border of the deciduous forest and Great Lakes-St. Lawrence forest regions of southern Ontario dominated by sugar maple (*Acer saccharum*) with loamy, slightly acidic soils (pH = 6.3). I established the second study site in Haliburton Forest and Wildlife Reserve, a forest in the Great Lakes-St. Lawrence forest region of central Ontario dominated by sugar maple (*A. saccharum*) and American beech (*Fagus grandifolia*) with shallow, rocky, and acidic soils (pH = 5.2). Based on control plots sampled in November 2016, the Waterloo site had eight identified species at a total density of 166 ± 41 m^{-2} dominated by endogeic taxa. The Haliburton site had seven identified species at a total density of 120 ± 56 m^{-2} dominated by a mix of epigeic taxa and epigeic/anecic *Lumbricus* juveniles (Figure 4.4).
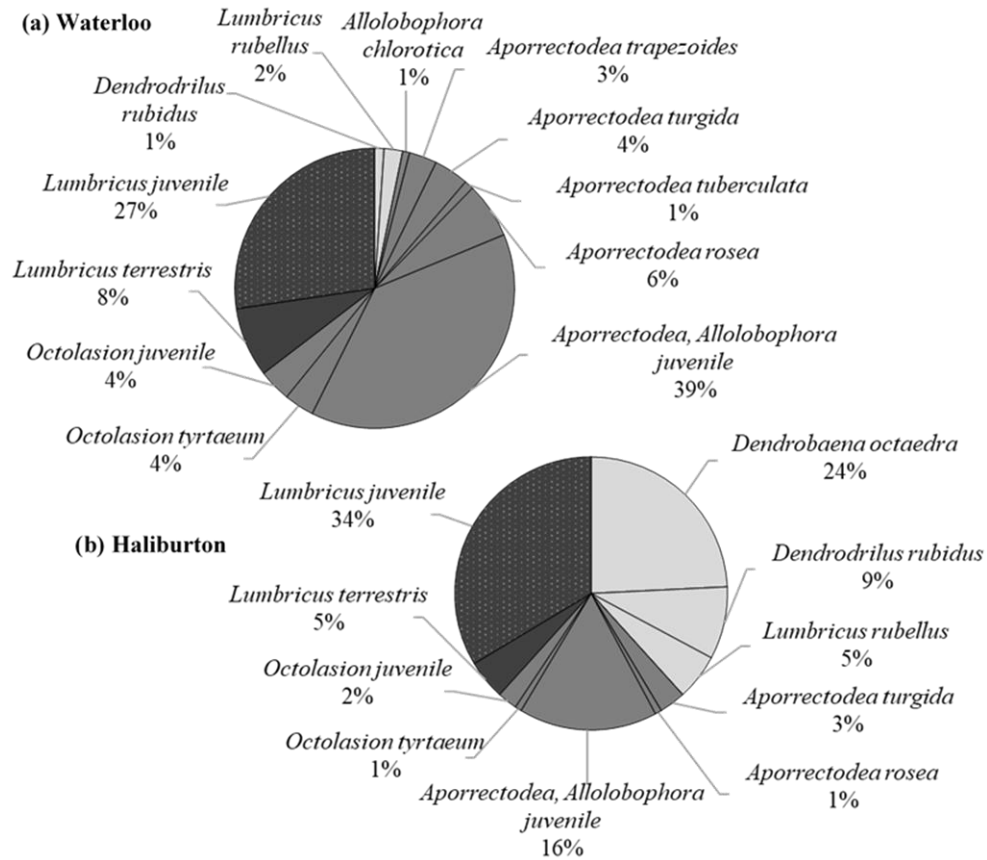
Figure 4.4: Summary of background earthworm communities at (a) Waterloo and (b) Haliburton sites based on proportional population density (% of total earthworms) of species collected from ash-free control plots sampled in November 2016 (Year 1, post-application timing) (n = 9 plots per site). Wedge colour denotes functional group: epigeic (light grey), endogeic (medium grey), anecic (dark grey), epigeic/anecic (dotted dark grey).

At each site, I established 36 pairs of 1 m × 1 m plots, leaving 1 m between paired plots and 2 m between pairs of plots. I randomly assigned half of the paired plots for either fly or bottom ash amendment. Within each ash type, I randomly assigned half of the paired plots for ash application either pre-litterfall or post-litterfall. Within each pair of plots, I randomly selected one plot for amendment with ash at 10 t·ha^{-1} (dry). I applied ash by hand in mid-September 2016 for the pre-litterfall application timing (before trees had begun to drop their leaves) and in mid-October 2016 for the post-litterfall application timing (when trees were visually estimated to have dropped c. 50 % of their leaves) (Figure 4.5). Basic weather data for the pre- and post-litterfall application periods are summarized in Table 4.2.

Figure 4.5: 1 m × 1 m plots at the Waterloo field site immediately after wood ash amendment (Fly A, Bottom A) in two application periods (Pre-Litterfall, Post-Litterfall).

Table 4.2: Summary of mean daily temperature and total precipitation over the three weeks between ash amendment and plot sampling for the pre-litterfall application (September 16-October 6, 2016) and post-litterfall application (October 19-November 8, 2016) at the Waterloo and Haliburton field sites. Weather data from the Government of Canada's historical weather database (Government of Canada, 2018).

Weather Station (Climate ID)	Application Timing	Mean Daily Temperature (°C)	Total Precipitation (mm)
KITCHENER/WATERLOO (6144239)	Pre-Litterfall	15.3	45
	Post-Litterfall	7.2	48
HALIBURTON 3 (6163171)	Pre-Litterfall	14.6	37
	Post-Litterfall	5.4	27

I sampled the plots for earthworms three weeks after ash amendment (early October for pre-litterfall plots, early November for post-litterfall plots) and then again one year later. I

collected earthworms by clearing two 25 cm × 25 cm quadrats placed in corners of the plot (0.125 m^2 total area) of litter and ash and driving earthworms to the surface by applying 2.5 L of mustard solution (10 g dry mustard powder to 1 L tap water) over 15-minutes (Lawrence and Bowers, 2002; Singh et al., 2016). I euthanized earthworms in isopropyl, transferred them to a 10 % formalin solution for 24-48 h for fixation, and transferred them back to isopropyl for storage (Hale, 2013). I identified earthworms identified to genus, species, and functional group where possible (see *1.4.1 Earthworm taxonomy and identification*).

To help account for spatial heterogeneity across the forest floor, I calculated the difference in earthworm functional group density between paired ash and control plots (Δ = ash – control). For each unique combination of site (Waterloo, Haliburton), ash type (fly, bottom), year (1, 2), and application timing (pre-litterfall, post-litterfall), I assessed the change in earthworm functional group density using One-Sample t-Tests (H$_0$: Δ = 0) (see *1.4.2 Notes on statistical analyses*).

4.2.3 Effects of earthworms on ash burial

Experiment 5: Ash burial experiment

I used a laboratory microcosm experiment to assess the effects of different earthworm densities (none, 'low', 'high') on the burial of ash applied on top of a leaf litter layer (n = 4 microcosms per earthworm density, total N = 12 microcosms). I cut PVC pipes (15 cm diameter, 30.5 cm height) vertically into halves, taped them back together, and filled them to a depth of c. 16 cm with an experimental soil. I placed the microcosms randomly in an experimental chamber (14 h light: 10 h dark, 20 °C, 65-70 % RH) and watered them with c. 100 mL tap water every 2-3 days. I communally fasted and weighed fresh healthy adult *L. terrestris* and added them to microcosms at a rate of 0 earthworms per microcosm (none), 1 earthworm per microcosm ('low' density, 14 m^{-2}), or 3 earthworms per microcosm ('high' density, 42 m^{-2}). I added 1 g (dry) of whole leaf maple litter (see methods for *Experiment 1* for leaf litter details) to the surface of each microcosm as alternate food source.

To help find the ash in the soil, I mixed 19 g (dry) of Fly A ash with 3 g of a fluorescent powder (Glow Inc. UV Reactive Powder, Fluorescent Pink, FPPK) that glows bright pink under ultraviolet (UV) light (405 nm) (Figure 4.6a) for a combined addition rate of 10 t·ha^{-1} (dry) and applied it evenly by hand over the leaf litter in microcosms.

After 32 days, I photographed the surface of each microcosm under UV light and recovered, rinsed, air-dried, and re-weighed any leaf litter remaining on the soil surface. I then removed the tape securing the two halves of each microcosm and carefully lowered one microcosm half in 2 cm increments. At each increment, I used a sharpened metal plate to remove a 2 cm-thick soil slice (Figure 4.6b). I photographed the newly uncovered surface of the underlying soil under UV light and repeated the process to capture photographs of nine soil depths (0 cm, 2 cm…16 cm) per microcosm (Figure 4.6c). I estimated the amount of ash-tracer present at each depth (% area) by converting the images to black and white (Figure 4.6d) and determining the percentage of total pixels that were white (i.e., the high contrast fluorescent tracer against the dark soil) using ImageJ software (V1.52a).
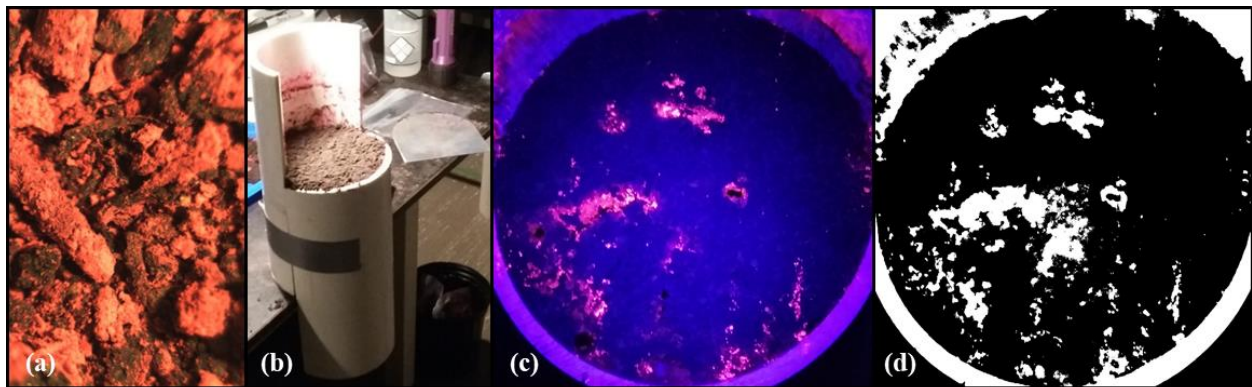
Figure 4.6: Wood ash burial microcosm preparation and deconstruction, including (a) fly ash mixed with the UV-fluorescent 'tracer' at 10× magnification, (b) removal of 2-cm thick soil slices from a microcosm, and photos of the same soil cross section under (c) UV light (ash-tracer mix appears pink) and (d) in black and white (ash-tracer appears white).

When deconstructing the burial microcosms and earthworm burrows were visible on the exposed outer edge or surface of the soil column, I used a scoopula to remove c. 20 g of soil from the burrow and c. 20 g of non-burrow soil from 5 cm distant to compare paired burrow and non-burrow soil properties. I collected approximately two samples from each microcosm for a total of n = 7 paired samples from the 'low' density microcosms and n = 8 paired samples from the 'high' density microcosms. When I encountered earthworms during deconstruction, I collected, rinsed, and weighed them fresh. I combined 5.0 g subsamples of burrow or non-

burrow soil samples and surface soil samples with 20 mL of DI water in a 1:5 ratio, mixed them by hand for 1 minute, and left them to sit for 1 h before measuring pH and electrical conductivity (EC). As a methodological test of whether pH and EC changes were attributable to ash or the tracer itself, I mixed 4 g samples of experimental soil with 1 g of different amendments (additional soil, tracer only, ash only, tracer + ash) and analyzed them as described above for pH and EC.

For the methodological test, I assessed the effects of ash presence and tracer presence on pH and EC using Two-Way ANOVAs. For the ash burial experiment, I assessed the effects of earthworm density (none, 'low', 'high') on earthworm mass change (%) and surface litter disappearance (% by weight), pH, EC, and tracer coverage (% area) using One-Way ANOVAs. I assessed the effects of earthworm density and depth on tracer coverage using a Mixed ANOVA with microcosm as a random factor nested within earthworm density. I assessed the effects of earthworm density ('low', 'high') and burrow proximity (in burrow, adjacent to burrow) on the pH and EC of paired soil samples acquired during microcosm deconstruction using Mixed ANOVA with pair identity as a random factor nested within earthworm density (see *1.4.2 Notes on statistical analyses*).

4.3 Results

4.3.1 Effects of wood ash amendment on earthworms

Experiment 1: Short-term growth and survivourship experiment

Earthworm survivourship was not statistically significantly affected by wood ash amendment (Two-Way ANOVA, $F_{4,30} = 0.50$, $p = 0.736$), soil source ($F_{1,30} = 3.57$, $p = 0.068$), or an interaction ($F_{4,30} = 1.07$, $p = 0.388$). Overall survivourship across all trials was 89 ± 21 %. Total earthworm biomass change was affected only by soil source (Two-Way ANOVA, $F_{1,30} = 25.98$, $p < 0.001$, $\omega_p^2 = 0.38$), but not by ash amendment ($F_{4,30} = 0.32$, $p = 0.865$) or an interaction ($F_{4,30} = 0.55$, $p = 0.701$). Earthworms gained mass when reared in the neutral pH experimental soil ($+ 16 \pm 9$ %) but lost mass when reared in the acidic Haliburton soil ($- 16 \pm 25$ %). Anecdotally, I observed no qualitative signs of illness (e.g., skin lesions, discolouration) on any specimens.

Experiment 2: Avoidance experiment

Ash amendment had a statistically significant effect on the proportion of earthworms found in the ash amended halves of the choice containers (One-Way ANOVA, $F_{4,35} = 3.06$, p = 0.029, $\omega^2 = 0.17$). As expected, when the ash chamber was amended with control soil, I found earthworms randomly distributed between the two chambers. I observed the same distribution for containers amended with Bottom A and intermediately lower proportions in the chambers amended with Fly A and Bottom B. I found a very low proportion of earthworms (0.03 ± 0.09) when the chamber was amended with Fly B (Figure 4.7).
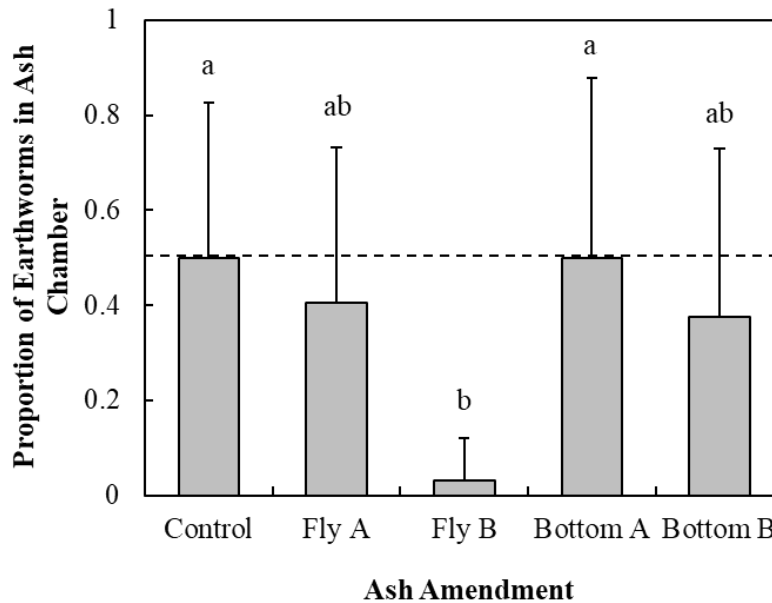
Figure 4.7: Bar chart of the proportion of earthworms (of four individuals) found in the half of an avoidance container amended with one of five wood ash amendments (n = 8 containers per ash amendment). Letters denote groupings from Tukey's HSD Test. The dotted line denotes the proportion of earthworms expected to be randomly found in the amended chamber (0.5). Error bars depict standard deviation (SD).

Experiment 3: Surface behaviour experiment

Ash amendment had no statistically significant effect on earthworm survivourship/retention (One-Way ANOVA, $F_{2,15} = 1.50$, p = 0.255) or weight change (One-Way ANOVA, $F_{2,15} = 1.56$, p = 0.242). Overall survivourship/retention was 92 ± 17 %, and individual earthworm weight increased by an average of 10 ± 9 %.

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Over the first four observation nights, ash amendment had a statistically significant effect on aboveground event frequency (Mixed ANOVA, $F_{2,15} = 15.3$, $p < 0.001$, $\omega^2 = 0.61$), individual aboveground event duration (Mixed ANOVA, $F_{2,15} = 12.9$, $p = 0.001$, $\omega^2 = 0.57$), and the percentage of litter handling events (Mixed ANOVA, $F_{2,15} = 5.24$, $p = 0.019$, $\omega^2 = 0.32$). In the absence of ash amendment, earthworms came to the surface an average of 7 times per night for 13 minutes per event. Approximately 42 % of these events involved the handling of leaf litter. Compared to ash-free controls, fly ash amendment decreased the overall frequency of aboveground events (- 63 %), the individual duration of each event (- 63 %), and the percentage of events that involved contact with litter (- 53 %). Bottom ash amendment generally had no detectable effects on aboveground activity except for an intermediate decrease on the percentage of litter handling events (- 11 % relative to controls) (Figure 4.8).

Cumulative leaf burial was not statistically significantly affected by ash amendment (Mixed ANOVA, $F_{2,15} = 2.27$, $p = 0.137$) or by an interaction of ash amendment and observation night ($F_{14,105} = 1.07$, $p = 0.393$). Cumulative leaf burial was only affected by observation night, ($F_{7,105} = 37.17$, $p < 0.001$, $\varepsilon_{GG} = 0.31$, $\omega_p^2 = 0.34$), with an increase from 4 ± 7 % on night one to 48 ± 22 % by night eight.
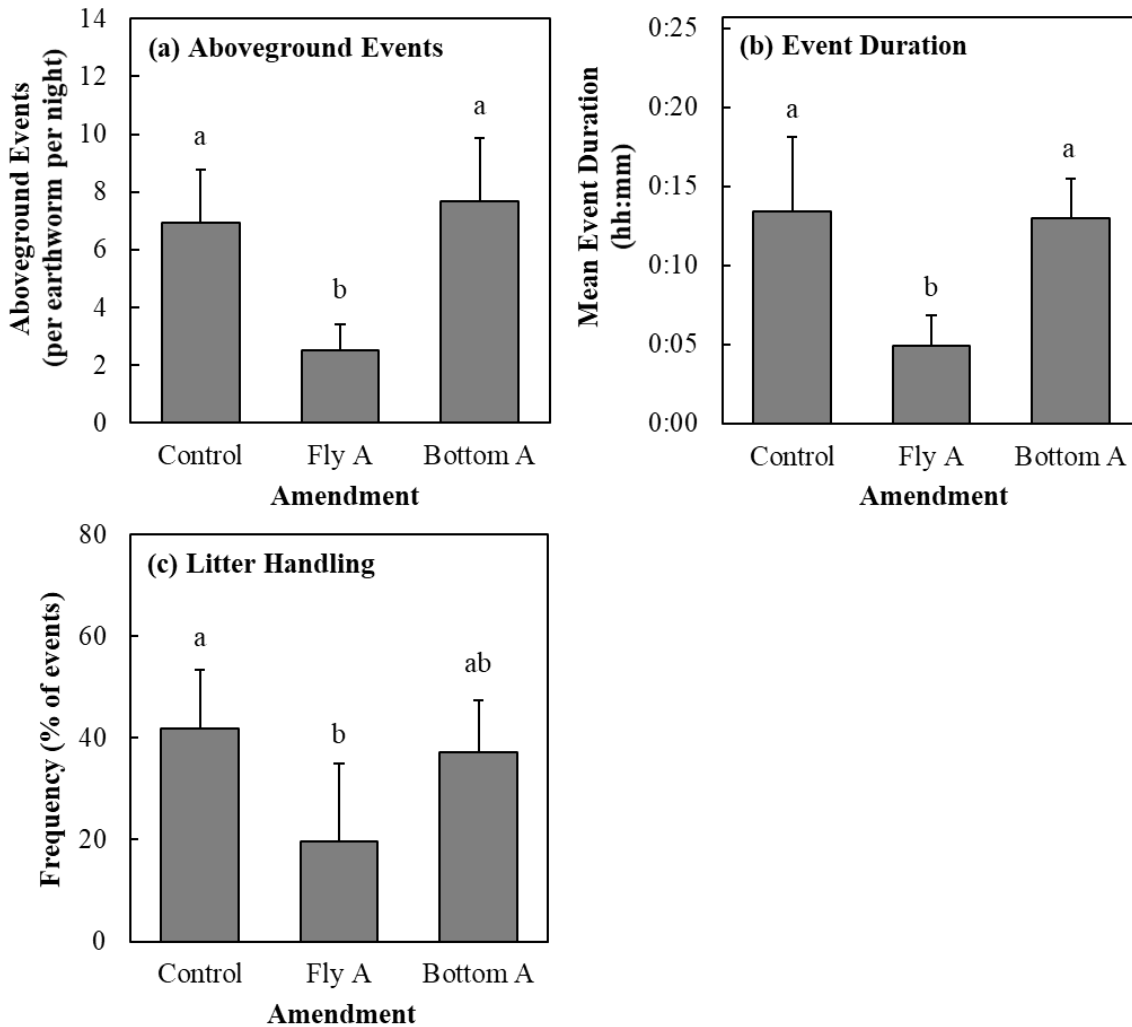
Figure 4.8: Bar charts of the main effects of ash amendment over four observation nights on (a) total frequency of aboveground events per earthworm per night, (b) average individual event duration, and (c) the percentage of total events that involved litter handling (n = 6 microcosms per ash amendment, initially four earthworms per microcosm). Letters denote groupings from Tukey's HSD Test. Error bars depict standard deviation (SD).

Experiment 4: Field application experiment

Across both the Waterloo and Haliburton sites, ash amendment generally decreased the densities of certain earthworm functional groups in the first three weeks post-application and had either no affect or increased densities in the following year, and most ash effects were observed for fly ash rather than bottom ash (paired tests summarized in Table 4.3 and Table 4.4).

Table 4.3: One-Sample t-Tests from the Waterloo site comparing the change in earthworm density (m^{-2}) between paired ash and control plots (Δ = ash – control) amended with different ash types (fly or bottom) at different times (pre-litter, post-litter) and measured 3 weeks or 1 year post amendment (n = 9 paired plots per unique treatment combination). t-values, p-values, and changes in density are in bold for statistically significant tests.

			Waterloo					
Ash Type	**Time Since Amendment**	**Application Timing**	**Δ Epigeic**			**Δ Endogeic**		
			t	**p**	**Δ**	**t**	**p**	**Δ**
Fly	3 weeks	Pre-Litter	-0.80	0.447	-	-1.34	0.217	-
		Post-Litter	-0.76	0.471	-	**-5.86**	**< 0.001**	**-52 ± 27**
	1 year	Pre-Litter	1.03	0.332	-	1.76	0.116	-
		Post-Litter	0.37	0.719	-	1.83	0.104	-
Bottom	3 weeks	Pre-Litter	1.00	0.347	-	0.88	0.403	-
		Post-Litter	0.00	1.000	-	-0.71	0.500	-
	1 year	Pre-Litter	0.11	0.916	-	-1.26	0.244	-
		Post-Litter	0.82	0.438	-	-1.40	0.198	-
Ash Type	**Time Since Amendment**	**Application Timing**	**Δ Anecic**			**Δ *Lumbricus* juv.**		
			t	**p**	**Δ**	**t**	**p**	**Δ**
Fly	3 weeks	Pre-Litter	-0.69	0.512	-	-1.71	0.126	-
		Post-Litter	-1.95	0.086	-	**-4.03**	**0.004**	**-25 ± 18**
	1 year	Pre-Litter	0.58	0.578	-	1.22	0.257	-
		Post-Litter	-2.06	0.073	-	**5.33**	**0.001**	**21 ± 12**
Bottom	3 weeks	Pre-Litter	-0.32	0.769	-	**-3.30**	**0.011**	**-19 ± 17**
		Post-Litter	-0.71	0.498	-	-0.32	0.760	-
	1 year	Pre-Litter	0.35	0.738	-	0.85	0.421	-
		Post-Litter	0.00	1.000	-	0.54	0.606	-

Table 4.4: One-Sample t-Tests from the Haliburton site comparing the change in earthworm density (m^{-2}) between paired ash and control plots (Δ = ash – control) amended with different ash types (fly or bottom) at different times (pre-litter, post-litter) and measured 3 weeks or 1 year post amendment (n = 9 paired plots per unique treatment combination). t-values, p-values, and changes in density are in bold for statistically significant tests.

			Δ Epigeic			Δ Endogeic		
Ash Type	**Time Since Amendment**	**Application Timing**	**t**	**p**	**Δ**	**t**	**p**	**Δ**
Fly	3 weeks	Pre-Litter	0.55	0.594	-	**-3.00**	**0.017**	**-32 ± 32**
		Post-Litter	-1.98	0.084	-	-0.50	0.631	-
	1 year	Pre-Litter	-0.74	0.480	-	0.96	0.366	-
		Post-Litter	**2.32**	**0.049**	**22 ± 29**	1.47	0.180	-
Bottom	3 weeks	Pre-Litter	-0.83	0.432	-	0.52	0.620	-
		Post-Litter	-1.14	0.288	-	-1.32	0.225	-
	1 year	Pre-Litter	0.94	0.373	-	0.41	0.695	-
		Post-Litter	0.49	0.636	-	0.34	0.746	-

			Δ Anecic			Δ *Lumbricus* juv.		
Ash Type	**Time Since Amendment**	**Application Timing**	**t**	**p**	**Δ**	**t**	**p**	**Δ**
Fly	3 weeks	Pre-Litter	-0.69	0.512	-	-1.33	0.219	-
		Post-Litter	2.00	0.081	-	-0.66	0.525	-
	1 year	Pre-Litter	1.79	0.111	-	-0.19	0.852	-
		Post-Litter	0.69	0.512	-	1.92	0.092	-
Bottom	3 weeks	Pre-Litter	-0.32	0.760	-	-1.23	0.255	-
		Post-Litter	0.36	0.729	-	1.18	0.273	-
	1 year	Pre-Litter	-0.43	0.681	-	0.90	0.392	-
		Post-Litter	-0.21	0.842	-	0.58	0.578	-

At Waterloo, fly ash decreased the density of endogeics after three weeks when applied post-litterfall with no effects after one year. Fly ash also decreased the density of *Lumbricus* juveniles after three weeks and increased their density after one year, both only when applied post-litterfall. Bottom ash decreased the density of *Lumbricus* juveniles after three weeks when applied pre-litterfall, with no effects after one year. At Haliburton, fly ash had no statistically significant effect on epigeics after three weeks and increased their density after one year when applied post-litterfall, and also decreased the density of endogeics after three weeks when applied pre-litterfall, with no effects after one year (Table 4.3, Table 4.4).

4.3.2 Effects of earthworms on ash burial

Experiment 5: Ash burial experiment

In the methodological test of the tracer properties, soil pH was not statistically significantly changed by the tracer (Two-Way ANOVA, $F_{1,8} = 0.94$, p = 0.36), fly ash ($F_{1,8} = 0.23$, p = 0.64), or an interaction ($F_{1,8} = 0.23$, p = 0.64). Overall soil pH was 7.21 ± 0.08 (mean $\pm$ SD). Soil EC was statistically significantly affected only by fly ash (Two-Way ANOVA, $F_{1,8} = 299.57$, p < 0.001, $\omega_p^2 = 0.96$), not by the tracer ($F_{1,8} = 1.94$, p = 0.20) or an interaction ($F_{1,8} = 1.80$, p = 0.22). Soil EC was approximately 2.4 times higher in the presence of fly ash and was unaffected by the tracer.

In the burial experiment, all earthworms survived the experiment and earthworm weight change was not affected by earthworm density (Welch's Test, $F_{1,3.2} = 4.13$, p = 0.129). Overall earthworm weight change was - 11 $\pm$ 30 %.

Examining the soil surface of microcosms, earthworms at 'low' or 'high' densities increased the amount of leaf litter removed from the surface by 47-80 % (Welch's Test, $F_{2, 4.1} = 34.67$, p = 0.003, $R^2 = 0.84$) and reduced the amount of area covered by ash-tracer by 74-89 % (One-Way ANOVA, $F_{2,9} = 146.65$, p < 0.001, $\omega^2 = 0.96$) (Table 4.5). Earthworm density also affected surface soil EC (One-Way ANOVA, $F_{2,9} = 8.20$, p = 0.009, $\omega^2 = 0.54$), which was highest in the 'low' density microcosms, lowest in the 'high' density microcosms, and intermediate when earthworms were absent (Table 4.5).

Table 4.5: Summary of the effects of earthworm density (none, 'low', 'high') on surface soil litter disappearance, pH, electrical conductivity (EC), and tracer coverage (n = 4 microcosms per earthworm density). Letters denote groupings from Tukey's HSD Test. All results shown as mean ± standard deviation (SD).

Earthworm Density	Litter Disappearance (% mass)	pH	EC (μS)	Tracer Coverage (% area)
None	$2^a \pm 2$	$7.22^{n.s.} \pm 0.06$	$602^{ab} \pm 40$	$92^a \pm 9$
'Low'	$47^b \pm 19$	$7.18^{n.s.} \pm 0.12$	$644^a \pm 55$	$10^b \pm 2$
'High'	$80^b \pm 20$	$7.23^{n.s.} \pm 0.05$	$527^b \pm 24$	$24^b \pm 9$

Using UV photography, I was able to track the burial of wood ash by earthworms (Figure 4.9). Across the 2 cm to 16 cm depths, earthworm density and depth had an interactive effect on ash-tracer coverage (Mixed ANOVA, $F_{4.3,19.5} = 2.95$, $p = 0.04$, $\varepsilon_{GG} = 0.31$, $\omega_p^2 = 0.22$). When earthworms were absent, the amount of ash-tracer below 2 cm deep was minimal and did not change with depth (simple main effect, One-Way Repeated Measures ANOVA, $F_{1.0,3.0} = 3.78$, $p = 0.15$, $\varepsilon_{GG} = 0.14$). When earthworms were present, the amount of ash-tracer found below 2 cm decreased by depth at both 'low' earthworm density (simple main effect, One-Way Repeated Measures ANOVA, $F_{1.4,4.2} = 14.90$, $p = 0.015$, $\varepsilon_{GG} = 0.20$, $\omega_p^2 = 0.77$) and the 'high' earthworm density (simple main effect, One-Way Repeated Measures ANOVA, $F_{2.0,6.1} = 5.30$, $p = 0.0046$, $\varepsilon_{GG} = 0.29$, $\omega_p^2 = 0.45$) (Figure 4.10).

(a) No Earthworms   **(b) 'Low' Density**   **(c) 'High' Density**
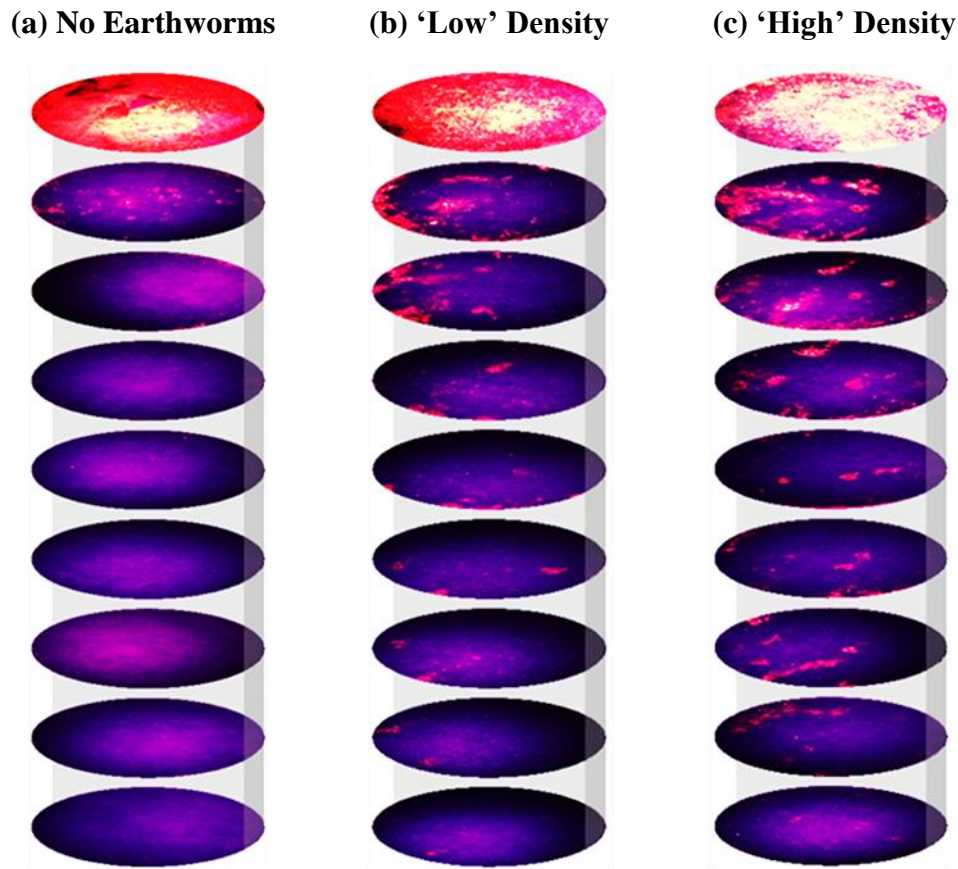
Figure 4.9: Stacks of UV photos of soil slices (15 cm diameter) at 2 cm increments (0 cm, 2 cm … 16 cm) from representative microcosms with different earthworm densities: (a) none, (b) 'low' density (1 per microcosm), and (c) 'high' density (3 per microcosm). The tracer powder mixed with the surface-applied ash appears pink in images.

At each measured depth down to 8 cm, earthworms generally increased the amount of ash-tracer found, with statistically significant earthworm effects found at depths of 2 cm (simple main effect, Welch's Test, $F_{2,4.6} = 10.81$, $p = 0.018$, $R^2 = 0.61$), 4 cm (simple main effect, Welch's Test, $F_{2,4.1} = 13.76$, $p = 0.0015$, $R^2 = 0.62$), 6 cm (simple main effect, Welch's Test, $F_{2,4.0} = 20.69$, $p = 0.008$, $R^2 = 0.55$), and 8 cm (simple main effect, One-Way ANOVA, $F_{2,9} = 54.56$, $p < 0.001$, $\omega^2 = 0.90$). Compared to earthworm-free controls, 'high' densities of earthworms generally buried the most ash-tracer, while 'low' densities had intermediate or no effect (Figure 4.10).
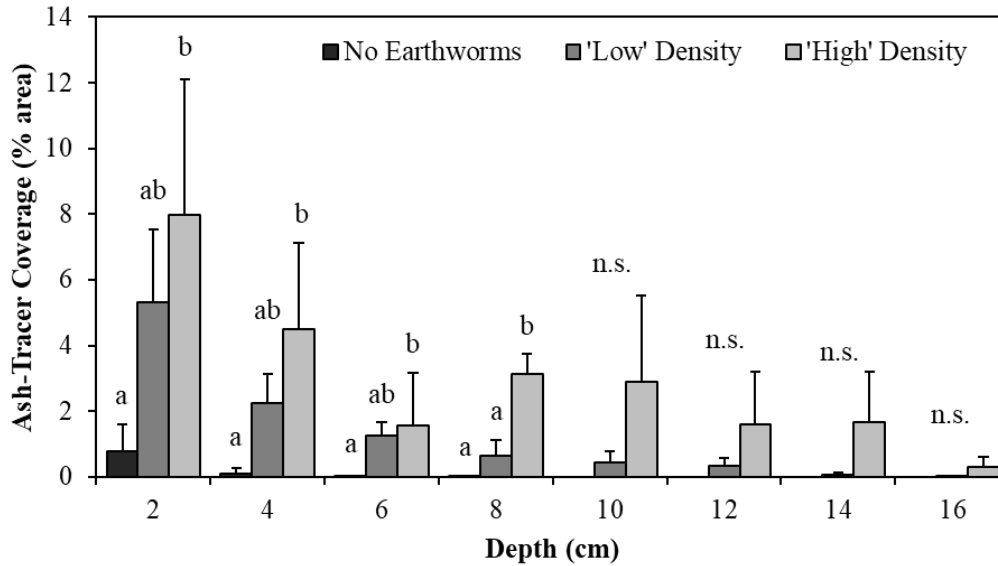
Figure 4.10: Bar chart of the simple main effects of earthworm density (Black: No earthworms, Dark grey: 'Low' Earthworm Density, Light grey: 'High' Earthworm Density) on the coverage of fluorescent tracer mixed with ash (% area) across a range of 2 cm depth increments (n = 4 per earthworm density and depth). Letters denote groupings from Games-Howell Test (2-6 cm) or Tukey's HSD Test (8+ cm) for each depth. Error bars depict standard deviation (SD).

I often observed ash-tracer powder concentrated around earthworm burrows both at the surface and belowground (Figure 4.11a) and on and around buried, partially decomposed leaf litter (Figure 4.11b). Comparing soil from earthworm burrows to adjacent soil, EC was statistically significantly affected by burrow proximity (Mixed ANOVA, $F_{1,13} = 21.75$, $p < 0.001$, $\omega_p^2 = 0.34$), but not by earthworm density ($F_{1,13} = 0.76$, $p = 0.40$) or an interaction ($F_{1,13} = 1.81$, $p = 0.15$). Overall, soil EC was 53 % higher in burrows compared to adjacent soil.
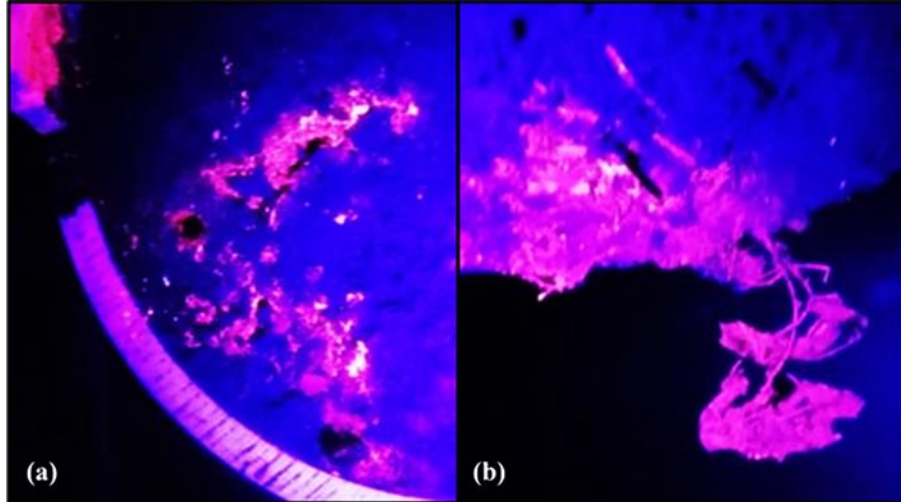
Figure 4.11: UV photographs of ash-tracer (a) concentrated in and around belowground earthworm burrows and (b) on and around partially decomposed leaf litter recovered from a depth of 16 cm.

4.4 Discussion

4.4.1 Effects of wood ash amendment on earthworms

Results of the growth and survivourship experiment (Experiment 1), avoidance test (Experiment 2), surface behaviour experiment (Experiment 3), and field application (Experiment 4) collectively suggested that the impacts of wood ash on earthworms in both microcosms and field conditions were generally adverse, but minimal in impact and short lasting. These findings are consistent with the small number of studies that have examined the impacts of wood ash and similar amendments (e.g., coal ash, biochar) on soil fauna that report generally null to moderately negative short-term impacts followed by largely null effects in the long-term (Augusto et al., 2008; Weyers and Spokas, 2011).

Although I observed no detectable effects of any of the wood ashes on short-term earthworm survivourship or growth, earthworms did respond behaviourally to some of the ashes. For example, although earthworms were not demonstrably impacted by Fly Ash B when unavoidably exposed for two weeks, when given the option they strongly avoided soils amended with that ash (Figure 4.7). Similarly, although earthworm growth and survivourship were unaffected by Fly Ash A, amendment of soil with Fly Ash A reduced the frequency and duration of surface excursions and the proportion of those events that involved handling of litter (Figure

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4.8), though earthworms still seemed able to bury the same amount of leaf litter in a shorter period of time. Overall, fly ash elicited stronger behavioural responses than bottom ash, which is consistent with the higher pH and metal concentrations of the fly ashes used in this experiment and the generally stronger impacts of fly ash on soil chemistry and biota (Gorgolewski et al., 2016; Noyce et al., 2016).

These behavioural responses suggest impacts of wood ash that could indirectly alter the soil environment by changing earthworm behaviour (Yeardley et al., 1996). For example, both avoidance of and attraction to ash-amended soils have been observed in other studies and can strongly influence long-term earthworm community composition and density in the field (Chan et al., 2008; Demuynck et al., 2014). Similarly, although not observed in surface activity experiment, longer-term changes in earthworm surface activity and foraging behaviour in the field could alter rates of litter layer persistence and the incorporation of organic matter belowground. Additional changes in earthworm behaviour resulting from wood ash and similar amendments not tested in this study but observed by other researchers include changes in the depth of earthworm activity in the soil (Huhta et al., 1986), cast production (Topoliantz and Ponge, 2005), and burrow density and volume (Yunusa et al., 2009), all of which could subsequently modify how earthworms affect various soil physical and chemical properties.

To complement these laboratory experiments, the field application experiment provided a longer-term look at how wood ash affects whole earthworm communities in two different forest soils up to one year post amendment. Generally, wood ash caused moderate decreases in density three weeks after amendment followed by null to slightly positive increases in the subsequent year (Table 4.3, Table 4.4). Decreased density measurements may indicate reduced population size (due to increased mortality, decreased reproduction, or emigration out of plots) or earthworms migrating deeper into the soil (Huhta et al., 1986; Yunusa et al., 2009) and thereby reducing the effectiveness of the mustard extraction. Based on the effects of ash observed on *L. terrestris* in the laboratory and the short period of time between ash amendment and the initial sampling (i.e., three weeks), I suspect that emigration or deeper burrowing were the leading causes of the observed density decreases.

I only observed neutral or positive effects of ash on earthworm density one year post-amendment (Table 4.3, Table 4.4), which may be due to increased population size (due to increased survivourship, increased reproduction, or immigration into plots) or earthworm activity

closer to the surface making them more responsive to sampling. The wood ash might be increasing the habitat suitability of the soils by increasing pH (with the initial pH of the sites both being slightly to moderately acidic, Table 4.1) or some other effect such as microbial stimulation or increased moisture retention (Gorgolewski et al., 2016; Grumiaux et al., 2015; Homan, 2015; Huhta et al., 1986; McCallum et al., 2016; Nieminen, 2008). Additional experimentation would be required to determine the mechanistic causes by which wood ash both increases and decreases earthworm densities in the field.

As in the laboratory experiments, fly ash tended to have stronger effects on earthworm density than bottom ash. Wood ash also had different effects on different earthworm functional groups. For example, wood ash had no detectable negative effects on epigeic or adult anecic (*L. terrestris*) earthworms. Although litter-dwelling epigeics live at the surface and are generally vulnerable to amendments, the dominant epigeic taxa were *L. rubellus* and *D. octaedra*, both of which have relatively active calcium excretion glands which increase their tolerances of heavy metals. Anecic *L. terrestris* has a similarly high metal tolerance and can also burrow to avoid adverse surface conditions (Eijsackers, 2010; Grumiaux et al., 2015). In contrast, the negatively impacted groups included endogeic species such as *A. chlorotica*, *A. rosea*, and *A. turgida*, which are all considered particularly metal sensitive (Eijsackers, 2010; Grumiaux et al., 2015), and *Lumbricus* juveniles, with juveniles and cocoons thought to have a higher susceptibility to toxicity than adults (Pati and Sahu, 2004). Although wood ash had few detectable effects on earthworm density that persisted a year later at these sites, sustained functional or species-specific responses to ash amendment in other systems could ultimately change the composition and function of earthworm communities.

The effects of wood ash on earthworm density also depended on the field site and the timing of ash application Table 4.3, Table 4.4. For example, although endogeic density responded to ash at both sites, *Lumbricus* juvenile density only changed at the Waterloo site. I also observed a decrease in endogeic density at Waterloo only when ash was applied post-litterfall and a decrease at Haliburton only when ash was applied pre-litterfall. Conditions at the two application times differed primarily in the amount of leaf litter on the ground and daily temperatures. Application timing might alter ash impacts in many ways, such as by changing how much ash is washed away by precipitation or blown away in the wind, how much ash falls directly onto the soil, or the activity level of soil microbes. Determining how these different

conditions interact with different wood ashes and site-specific properties to impact different earthworm taxa should be a priority for future research.

4.4.2 Effects of earthworms on wood ash burial

To the best of my knowledge, the results of the ash burial experiment (Experiment 5) provide the first evidence of earthworms facilitating the burial of wood ash. While passive incorporation of wood ash belowground in the absence of earthworms was minimal (Figure 4.9a), *L. terrestris* reduced the amount of ash-tracer found at the surface (Table 4.5), increased the amount of tracer-ash area found at subsurface depths, and increased the maximum depths at which tracer-ash was observed. These effects were generally stronger when more earthworms were present (Figure 4.10).

There are four primary mechanisms by which I speculate *L. terrestris* may have facilitated burial of the wood ash. First, earthworm burrowing created additional soil macropores into which wood ash could fall or be washed downwards by water. The ability of earthworm burrows to function as conduits for the transport of material from the surface has been recognized since Darwin (1881), and can contribute to the movement of other materials such as pesticides (Springett, 1983; Worrall et al., 1997) or lime (Baker et al., 1999). I often found ash-tracer coating the inside of earthworm burrows (Figure 4.11a), which could result from water carrying ash-tracer down burrows.

Second, I applied wood ash directly on top of leaf litter that was subsequently pulled belowground for food and midden construction, potentially carrying wood ash with it. While deconstructing microcosms, I found several leaf fragments belowground that still had ash-tracer around it (Figure 4.11b). Burial of litter by *L. terrestris* is similarly suspected to transport microplastic particles (Huerta Lwanga et al., 2017).

Third, the small fly ash particles could be ingested intentionally or incidentally and subsequently deposited belowground in casts. Although I did not check casts specifically for ash-tracer and never observed direct ingestion of wood ash by earthworms, both lime and charcoal are thought to be ingested and egested by earthworms (Chan, 2003; Topoliantz and Ponge, 2005, 2003) which suggests earthworms could similarly ingest and egest smaller wood ash particles.

Fourth, wood ash may have adhered to the mucus coating of the earthworms. Chan (2003) suggests this is a dominant mechanism of lime burial by earthworms and would also be consistent with the coating of burrow walls with ash-tracer observed in this experiment.

Compared to field conditions, for this microcosm experiment I used a structurally simple soil with no horizon development or other sources of pores (e.g., plant root channels, other burrowing biota). It is likely that the natural rate of wood ash incorporation belowground in the absence of earthworms from water percolation would be higher than what I observed here. Nevertheless, I expect that earthworm burrows and foraging might still generally increase the rates of water infiltration and leaf burial of more natural soils compared to those lacking earthworms (Anderson, 1988). Additionally, this experiment used only one earthworm species rather than a multi-species earthworm community as would generally be found in a natural system. While I suspect that the anecic *L. terrestris* plays the largest role in ash incorporation since it is unique in its burial of surface materials and can create large burrows up to 10 mm in diameter (Worrall et al., 1997) and 1,264 m^{-2} in density (Pitkänen and Nuutinen, 1997), epigeic and endogeic taxa may also contribute to the lateral mixing of amendments in the field (Springett, 1983).

On a methodological note, tracking the incorporation of wood ash, coal ash, or biochar into the soil can be difficult if the material is visually or texturally similar to soil. Material can be tracked by qualitatively assessing substrate colour (Topoliantz and Ponge, 2005, 2003) or visually measuring removal from the surface (Elmer et al., 2015), but these approaches may not work for all materials, soil types, or experimental designs. The UV-fluorescent 'tracer' powder I mixed in with the wood ash was a novel and effective solution for tracking ash burial. The tracer mixed well with the wood ash and provided a high visual contrast against the soil. The tracer remained adhered to the wood ash through watering over a month-long experiment and did not 'bleed' into the surrounding soil. Since the tracer did not alter soil pH or EC, I was able to chemically confirm the presence of wood ash from samples identified visually by fluorescence based on elevated EC. I recommend that this method be used in future investigations attempting to track the physical location of wood ash or other materials through the soil.

4.4.3 Implications of earthworm-wood ash interactions for restoration

The combined results of the experiments in this study suggest that ecological restoration or other management using wood ash should consider how ash interacts with earthworms. Earthworms can be used to help assess the biological risks of wood ash application, are key soil fauna whose functional composition or density and subsequent effects on the soil can be altered by ash amendment, and may themselves facilitate the burial of surface-applied wood ash.

While earthworms are already considered valuable (if underused) bioindicators for soil conditions in their native ranges (Eijsackers, 2010; Lowe and Butt, 2007), I recommend that the communities of exotic earthworms continuing to spread throughout many North American soils also be considered in this role. Despite the exotic origins of these species, their persistence and spread across more ecosystems makes them an increasingly common element of soil communities, and they retain the same physiological and life history traits that make them useful in th. Wood ash amendment itself could even facilitate increased earthworm spread into particularly acidic soils, since earthworms are rarely found in soils below pH 4.3 and deacidification of soils can increase habitat suitability (Edwards and Bohlen, 1996; Homan, 2015; McCallum et al., 2016).

Based on the results of this study, I suggest that these soil fauna biomonitoring efforts use both laboratory and field-based experiments to assess a mix of acute, sublethal, and behavioural responses (Yeardley et al., 1996). These experiments should consider both the short-term effects resulting from the initial 'flush' of materials and pH change from recently applied ash (Augusto et al., 2008; Gorgolewski et al., 2016) and the longer-term impacts of amendment (Demuynck et al., 2014). Further research is also needed concerning the potential bioaccumulation of toxic metals in earthworms since they are sensitive to epidermal uptake of toxins, able to consume metals bound to organic matter that are normally relatively non-bioavailable, and tend to accumulate certain metals rather than excrete them (Mortensen et al., 2018). The fate of these toxins and their bioavailability through earthworm tissues (e.g., to species feeding on earthworms) (Richardson et al., 2015) or casts (Bhattacharya and Kim, 2016; Gupta et al., 2005) are uncertain.

I similarly recommend that additional investigation be devoted to understanding how earthworms can change the effectiveness of wood ash as an amendment, particularly by burying surface-applied amendments. Earthworms have been suggested as delivery systems for fungal

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biocontrol agents (Singer et al., 1999), lime (Baker et al., 1999), and biochar (Elmer et al., 2015), but little additional work seems to have been carried out in these applications. Anecic earthworms in particular may help incorporate wood ash that is desired belowground but cannot be mixed into the soil (e.g., in forests). Land managers may be able to further maximize these benefits by applying ashes that have properties such as pH or particle size suitable for earthworm burial (Elmer et al., 2015). Practical application of this will require further study, particularly under field conditions and using multiple earthworm functional groups.

Finally, the results of this study emphasize that future research and land managers should consider the potential variability between different wood ashes. I observed differences in how wood ash affected earthworms based on both ash type (fly or bottom) and the boiler that produced it. Some industries also manufacture more processed forms of 'crushed' or 'granulated' ash that may interact with soil biota differently from loose, unprocessed ash (Pitman, 2006). These interactions may be further altered by other potential factors such as amendment rate (Augusto et al., 2008), soil type (Van Zwieten et al., 2010), or the timing of amendment. Overall, the interactions of soil biota such as earthworms with wood ash amendments are likely to depend highly on site- and scenario-specific factors.

Chapter 5: Conclusions

"In time invaders become the native."

\- Roman Proverb

5.1 Exotic earthworms in North America and restoration

5.1.1 Synthesis of earthworm impacts on restoration

As organisms that spend most of their lives belowground, earthworms can be difficult to study (Butt and Grigoropoulou, 2010; Hendrix, 2006). Despite the everyday familiarity of earthworms and a legacy of research dating back to at least Darwin (1881) himself, many elements of even their basic ecology are poorly understood. By investigating the interactions of earthworms with three restoration interventions (seeds, mulch, and wood ash), this dissertation contributes to our expanding knowledge of earthworm ecology, facilitates the integration of earthworm interactions into restoration, and offers insights into the broader implications of biological invasion for conservation.

To complement the specific discussions in each of the core data chapters, I briefly address in this concluding section three primary recurring themes across the projects. First, although most of my experiments looked at relatively isolated mechanisms, these interactions demonstrate the considerable potential of earthworms to influence overall ecosystem structure and function as ecosystem engineers. Examples include burying and aggregating seeds (Chapter 2), redistributing organic residues across the soil surface (Chapter 3), and facilitating wood ash burial (Chapter 4). The ecosystem engineering potential of earthworms is often considered primarily at longer time scales, but my experiments show how earthworms can also rapidly alter conditions immediately following management interventions. The ubiquity of these ecologically-influential impacts underscores the importance of considering the role of earthworms in restoration, regardless of their desirability or geographic origins.

Second, my results depict a mix of earthworm impacts that can be contextually beneficial or detrimental depending on the situation. For example, I found that earthworms reduced grass recruitment by seed digestion and deep seed burial, but shallowly buried seed could also be protected from predation or desiccation (Chapter 2). Similarly, I observed undesirable aggregation of mulch that exposed soils that were supposed to be protected, but also evidence of

mulch burial and accelerated decomposition that could facilitate belowground organic matter incorporation (Chapter 3). These results further challenge the expectation that any given species – native or exotic – will have exclusively 'good' or 'bad' effects (Sagoff, 2005). The challenge for restoration planning is then to recognize, understand, and navigate trade-offs between these mixed impacts (Shackelford et al., 2013).

Third, my experiments document not only impacts that earthworms had on restoration, but also impacts of restoration on earthworms. For instance, while my primary interest was in learning how earthworms affected seed, mulch, and wood ash, earthworms are also presumably benefiting nutritionally from seeds (Chapter 2) (Eisenhauer et al., 2010), and application of different amounts or kinds of mulch (Chapter 3) or wood ash (Chapter 4) changed earthworm densities or behaviour. These reciprocal effects will likely be particularly important for determining the longer-term consequences of these interactions, including potential increases, decreases, or changes in the functional composition and ecological impacts of earthworm communities over the course of restoration.

5.1.2 Recommendations for future research

The experiments in my dissertation are new investigations of unstudied or minimally studied interactions that set up many potential options for future research, and I have already addressed topic-specific recommendations in each of the data chapters. An additional general recommendation I would make for future research is to complement laboratory experiments with more field-based experiments. Although often more logistically-demanding and less useful for establishing clear mechanistic relationships, field experiments consider the complexity of conditions under which these interactions will actually matter for applied restoration and are also necessary to assess the situations in which earthworm impacts are likely to be influential compared to other factors. For instance, this has already been raised concerning the ecological importance of earthworm granivory (Cassin and Kotanen, 2016; Grant, 1983), with the results of the granivore-exclusion experiment illustrating how the ecological significance of earthworm granivory could depend in large part on the abundance of other granivores (Chapter 2). Perhaps the most useful applied experiments would occur in larger-scale restoration projects in the field that assess the consequences of modifying interventions based on expected earthworm impacts.

Despite the arguably unique impacts of anecic species such as *L. terrestris*, another general recommendation I have is to consider interactions with different earthworm species and functional groups, which are known to often produce unique outcomes (e.g., Asshoff et al., 2010; Eisenhauer et al., 2009b, 2009a, 2008) and were shown in my experiments to respond differently to mulch (Chapter 3) and wood ash amendment (Chapter 4). Understanding these community-specific responses could be useful for modifying restoration plans for areas with different or changing earthworm communities.

While this dissertation has primarily considered how general restoration interventions can interact with earthworms already present at a site, I also believe that some of the most intriguing and promising areas of research concern the specific and intentional use of earthworms as a restoration tool to achieve specific objectives. This could apply to the facilitated burial of wood ash if intentionally using an 'earthworm-friendly' amendment or inoculating anecic earthworms with the amendment (Chapter 4), but also extends to other possibilities. For example, as decomposers earthworms have already been shown to facilitate restoration by mixing organic matter into heavily degraded soils (Ganihar, 2003; Vimmerstedt and Finney, 1973; Zhang et al., 2015), preventing the buildup of organic matter in clogged constructed wetlands (Li et al., 2011), and helping break down soil-borne plastics (Huerta Lwanga et al., 2018). I observed that vertical transport by anecic earthworms was very influential in my experiments and may be particularly useful to help mix into soils amendments such as lime (Baker et al., 1999), biochar (Elmer et al., 2015), or biocontrol agents (Singer et al., 1999; Stephens et al., 1994, 1993). To date, most of these applications have only been speculative or minimally researched and seldom put into actual practice.

Finally, additional research on other earthworm topics will be needed to fully understand their implications for restoration and conservation more broadly and to make responsible management and policy decisions. A priority will be developing better survey data mapping the large-scale distribution of earthworms, particularly the currently expanding edges of the invasion such as into the northern boreal forests (Cameron et al., 2007; Tiunov et al., 2006) and the spread of the more recently arrived Asian earthworm species (e.g., *Amynthas* spp.) (Szlavecz et al., 2018). It would also be useful to have more controlled, manipulative experiments to complement the predominantly observational evidence regarding impacts on plant communities (Frelich et al., 2006; Hale et al., 2006, 2005), mycorrhizae (McLean et al., 2006), and other organisms (Ferlian

et al., 2018; Loss and Blair, 2014; Maerz et al., 2009; Migge-Kleian et al., 2006; Snyder et al., 2013), and more meta-analyses to synthesize these growing bodies of evidence (e.g., Craven et al. 2017).

5.1.3 Management of exotic earthworms and integration into restoration policy

In the introductory chapter, I argued that the earthworm case study is instructive because it limits our options; integration is perhaps more palatable when prevention or control are not practical options (Chapter 1). However, I would argue that this integration has not yet happened, as evidenced by repeated calls for control efforts acknowledged by the same proponents to be largely unrealistic (Addison, 2009; Callaham et al., 2006; Hendrix et al., 2008; Hendrix and Bohlen, 2002; Holdsworth et al., 2007a). I suspect that this failure to pursue alternative options is at least in part a result of the perhaps subconsciously native-biased, outdated conservation principles that have come to light in the ongoing invasion debate (Chapter 1).

What then is the preferred way forwards?

For exotic earthworms in North America, I believe that the best option is to embrace a novelty that most of society has already accepted as 'normal' and 'desirable' and just "learn to love 'em" (Davis, 2011). This would require an end to the calls for non-existent management options and a re-examination of the language that we use for these species, reflecting on the appropriateness of broad use of 'invasive' or even of 'exotic' for often long since established communities that are not likely to disappear anytime soon. For restoration, I believe that at a minimum this integration should consider these species as novel ecosystem elements and recognize and plan around their influential ecosystem engineering effects; this dissertation started this process for seeds, mulch, and wood ash, beginning a discussion of how these interventions could be modified for more effective use in earthworm-inhabited soils (e.g., custom seed mixes, modified mulch rates, easy-to-bury wood ash). Restoration planners could also decide more often to use earthworm interactions or earthworm inoculations as restoration tools. In time, the conservation community may even come to see today's 'invasive' earthworms as expected or desired members of a target, non-degraded soil community.

An important caveat to the "learn to love 'em" policy (Davis, 2011) means considering the subjective and contextual 'bad' along with the 'good', a standard that should arguably be upheld for all species and not just exotics (Sagoff, 2005, 1999). Many of the earthworm effects

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described in this dissertation would often make restoration more difficult, such as reducing seed recruitment (Chapter 2) or removing mulch cover (Chapter 3). Other proposed novel uses of earthworms could have similar problems, such as the potential for earthworms to concentrate and transport microplastic particles (Huerta Lwanga et al., 2017, 2016), and I think it is highly likely that exotic earthworms are at least partly responsible for some of the undesirable ecological changes that they have been charged with (Nuzzo et al., 2009). Integration is not about exonerating exotic species of any and all faults; instead, successful integration would require considering all possibilities rather than assuming a particular positive or negative effect *a priori* based primarily on geographic origin (Davis et al., 2011). The ultimate and hopeful goal of this integration is to improve the efficacy and efficiency of restoration by attempting to mitigate the undesirable impacts and take advantage of the beneficial effects.

5.2 Concluding thoughts – Reflections on invasion and restoration

Although the purpose of this dissertation is not to resolve the complex and longstanding disciplinary debates of invasion science or restoration, I believe that reflection on the earthworm invasion story can offer some brief closing insights on this broader discussion. I think that this case illustrates how all invasions are not created equal and should not be considered or managed as such (Gurevitch and Padilla, 2004). Although perhaps less conceptually appealing than general theory, I think that contextual management based on demonstrable impact and spread may be most useful for developing case-by-case policy (Davis et al., 2011; Davis and Thompson, 2001; Nackley et al., 2017). Some researchers argue that many land managers already act selectively and pragmatically because of limited resources (Kuebbing and Simberloff, 2015), but this is often seen as a compromise and is not consistently reflected in the academic literature or conservation policy (e.g., *Canada National Parks Act*, 2018; Convention on Biological Diversity (CBD), 2008; but see SERI, 2004).

The earthworm case also emphasizes a need to think further about the long-term outcomes of invasion and 'naturalization'. As a 'crisis discipline' (Chew, 2015; Soulé, 1985), invasion science may be preoccupied with the early stages of invasion and may not thoroughly address the longer-term implications of invasions, particularly when we cannot or choose not to control them. I believe that the confused policy responses to exotic earthworms illustrate the need for further consideration of when an exotic species should be re-categorized as 'naturalized'

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(Richardson et al., 2000; Valéry et al., 2013) and when 'novel ecosystems' should be embraced as valid and desirable restoration outcomes (Hobbs et al., 2006).

I think that the invasion debate is so controversial because it calls into question many of the fundamental principles and values of conservation – why do we conserve and restore? Among many possible reasons, different stakeholders may be motivated by a desire to atone for human damages, conserve key species, maintain historical fidelity, or provide ecosystem services (Clewell and Aronson, 2007, 2006). In any given scenario, certain motivations may be compatible with exotic species and others may not. Ultimately, these are fundamentally value-laden issues and no scientific discipline has the sole authority to dictate what is 'right' or 'wrong' (Brown and Sax, 2005; Larson, 2007; Larson et al., 2013), but it is also important for us to recognize this subjectivity and critically reflect on our motivations (Failing et al., 2013). By not fully addressing implicit or unconscious biases in personal values and seemingly objective criteria such as 'ecological degradation' (McDonald et al., 2016; SERI, 2004) or 'ecological integrity' (*Canada National Parks Act*, 2018), we run the risk of unintentionally misdirecting policy and compromising conservation outcomes (Sagoff, 2005, 1999; Schlaepfer et al., 2011a).

In the end, many of these debates seem to be the result of different people all trying to do what they think is right. Defenders of the conventional wisdoms of invasion biology advocate control and precaution because of a firm belief in the unique ecological threats of biological invasion (Simberloff, 2005), while critics recommend a more flexible approach that does not squander woefully limited resources and can take advantage of overlooked opportunities (Davis et al., 2011; Ewel and Putz, 2004). Proponents of emerging perspectives seek new ways to find value in novelty and set realistic targets in a changing world (Hobbs et al., 2009; Light et al., 2013), while critics caution against the allure of compromise and a 'giving up of the good fight' (Marris et al., 2013; Murcia et al., 2014; Standish et al., 2013). I believe that all are trying to navigate inevitable uncertainties and complexities in their own ways but with a shared fundamental commitment to protect global ecosystems and human society. I would like to think that in the face of a rapidly changing environment and an uncertain future, these shared good intentions will go a long way towards ultimately navigating these challenges.

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