ABIOTIC AND BIOTIC INFLUENCES ON ENTOMOPATHOGENIC NEMATODE EFFICACY

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ABSTRACT

Entomopathogenic nematodes (EPNs), microscopic roundworms that infect and kill insects, have gained importance as biological control agents for the safe, environmentally friendly control of insect pests. However, their adoption by managers is limited by variable field performance, to which variability in abiotic and biotic soil properties is a likely contributor. Abiotic soil properties such as texture, moisture, and porosity can affect the ability of EPNs to survive, move, and locate hosts. Also, many soil organisms can interact with EPNs in helpful or harmful ways, though their effects on EPN infectivity remain poorly quantified. Increased knowledge of the extent and relevance of abiotic and biotic soil properties' effects on EPN performance will help managers better utilize EPNs against pests, both through increased ability to predict whether or not EPNs will be effective in a given soil and through soil management practices to increase their suitability for EPNs. My research is focused on examining the effects of biotic interactions on the ability of EPNs to infect hosts, and also to relate biotic and abiotic soil properties to EPN control of a pest insect in field soils.

In laboratory trials, I evaluated the effect of soil microarthropod communities on EPN infectivity against the model host *Galleria mellonella*. Individuals of the EPN *Heterorhabditis bacteriophora* were applied into loam soil mesocosms containing or lacking a microarthropod community. Five *G. mellonella* larvae were introduced to each mesocosm sixteen days later and removed after an additional five days. The cadavers were dissected and average number of invading EPNs determined for each replicate, as well as the overall percentage of infection. Percent EPN infection of *G. mellonella* and average EPN establishment was significantly lower in soils with fauna than in soils without fauna. In addition, linear regression analysis suggested a

significant negative correlation between the abundance of predatory mesostigmatid mites and EPN establishment within hosts. These results indicate negative effects of soil animals on EPN infectivity, likely due to predation.

In the field, the relationship between EPN efficacy and biotic as well as abiotic soil properties was tested in turfgrass athletic fields. A series of field bioassays with the EPNs *H. bacteriophora* and *Steinernema feltiae* were conducted against the root-feeding turfgrass pest *Popillia japonica*. Sentinel *P. japonica* grubs were buried in replicated plots in two school soccer fields, in both the high-traffic areas in front of the goals and the low-traffic areas in the corners of the fields. EPNs were applied to the plots and the EPN infection of the grubs recorded. Additional measurements of abiotic soil properties and the abundances of four microarthropod groups were recorded. EPNs achieved modest control of *P. japonica* grubs at one of the two sites. Efficacy of *S. feltiae* was much higher in low-traffic soils than in high-traffic soils at one of the two tested fields in 2016 but not 2017. Sand content was the only soil property tracking EPN efficacy along a non-metric multidimensional scaling matrix, indicating abiotic properties take precedence over biotic properties in determining EPN efficacy in turfgrass field soils.

While biotic interactions may reduce EPN infectivity under laboratory conditions, in the field setting of turfgrass soccer fields, abiotic soil properties are more important influences of EPN efficacy.

BIOGRAPHICAL SKETCH

Maxwell Helmberger ~~was whelped by a she-wolf somewhere north of Great Bear Lake~~ grew up in the woods beside the small town of Tower in northern Minnesota, alongside wolves, bears, peat bogs, and winter temperatures regularly reaching -45°F. He was homeschooled in some subjects but also attended the local K-12 school, graduating from Tower-Soudan Secondary in 2011. He studied Fisheries and Wildlife Biology for one year at Vermilion Community College in Ely, MN before transferring to the University of Minnesota, Duluth, where he began work in the insect ecology laboratory of Drs. Timothy Craig and Joanne Itami. In 2014, he was awarded a Biology Undergraduate Research in Science and Technology fellowship from the Department of Biology to study insect interactions with drought-stressed host plants. Later that year, he graduated *summa cum laude* with his Bachelor of Science degree in Biology.

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to my grandparents; Patricia, Richard, Caroline, and Jerome

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CHAPTER ONE

Ecology of belowground biological control: Entomopathogenic nematode interactions with soil biota

Abstract

Entomopathogenic nematodes (EPNs) have potential to control many soil-dwelling insect pests but have been limited in their usage, partly by their unpredictable field performance. Numerous abiotic and biotic factors are thought to contribute to this poor predictability, but the exact impacts and relative importance of these factors in affecting EPN performance in the field are not well understood. Previous studies have highlighted diverse interactions between EPNs and other members of the soil community, from plants and fungi to arthropods and annelids. These interactions may help or hinder EPNs in a variety of ways. However, current research has yet to determine how many of these interactions influence EPN performance under field conditions, specifically, if they contribute to the variability limiting EPN efficacy and wide-scale adoption. Here we outline current knowledge of these interactions as well as challenges and avenues for future research, such as greater integration of EPN research with soil animal and rhizosphere ecology, that will better illustrate the potential, limitations, and proper use of EPNs in pest management.

1. Introduction

Since the discovery of entomopathogenic nematodes (EPNs) in 1923 and their first commercialization sixty years later, thirteen EPN species of the genera *Steinernema* and *Heterorhabditis* have been commercially cultivated and marketed for use in a wide array of agricultural and horticultural systems (Lacey et al., 2015). EPN infective juveniles (IJs) invade insect bodies through the mouth, anus, spiracles, or cuticle (Lewis et al., 2006; Wang and Gaugler, 1998) and release their bacterial symbionts (*Xenorhabus* spp. bacteria for *Steinernema* spp. nematodes, and *Photorhabdus* spp. bacteria for *Heterorhabditis* spp. nematodes) into the insect's hemolymph. The bacteria release toxins to kill the insect, though sometimes are aided by venom proteins and anti-immune agents produced by the nematodes themselves (Lu et al., 2017), and proliferate in the cadaver. The IJs complete their development and reproduce, feeding on the bacterial biomass. After one or more generations of nematodes inside the cadaver, new IJs leave to seek new hosts in the soil (Kaya and Gaugler, 1993; Lewis and Clarke, 2012).

When using EPNs against pests in agricultural and horticultural systems, IJs are commonly applied onto soil as an aqueous suspension sprayed through a cleaned pesticide sprayer. Many other application techniques exist, such as applying EPNs through drip irrigation systems or applying them contained within bait capsules or already-infected insect cadavers (Shapiro-Ilan and Dolinski, 2015). EPNs can effectively provide a non-toxic, environmentally benign alternative to chemical insecticides for the control of some soil-dwelling pests, including white grubs (Grewal et al., 2005), citrus root weevil (Shapiro-Ilan et al., 2005, 2002), and mole crickets (Parkman et al., 1996). However, despite these and other successes (Georgis et al., 2006), extensive use of EPNs in agriculture is limited to a few markets, including citrus orchards

(Shapiro-Ilan et al., 2005) and vegetables in greenhouses (Dolinski et al., 2012; Lacey and Georgis, 2012). Despite the safety of EPNs for humans, other vertebrates, and many non-target invertebrates (Georgis et al., 2006; Lacey and Georgis, 2012), their variable performance and often limited persistence remain important factors restricting their adoption by pest managers (Georgis et al., 2006; Georgis and Gaugler, 1991, Shapiro-Ilan et al., 2002). For instance, many studies of EPN efficacy against scarab grubs in turf and nursery crop systems report highly variable control even within the same EPN species (Georgis et al., 2006; Grewal et al., 2005). An increased knowledge of the impacts of abiotic and biotic soil characteristics on the variability of EPN performance and persistence could positively impact the utilization of EPNs in soil pest management.

In addition, while many commercially available EPNs do not persist in soil longer than a few weeks or months (Ebssa and Koppenhöfer, 2011; Gaugler et al., 1997; Smits, 1996; Susurluk and Ehlers, 2008), some native EPN populations, adapted to a particular environment, have been shown to persist in the soil over multiple years when isolated, cultured, and applied to the soil (Koppenhöfer and Fuzy, 2009; Shields et al., 1999). This interest in using persistent EPN populations underscores the need to understand the biotic and abiotic factors that limit or promote EPN persistence.

Many abiotic factors influencing the ability of EPNs to survive and locate hosts in soil have been identified, and will be covered here only briefly. Soil type (Koppenhöfer and Fuzy, 2006; Kung et al., 1990a) and moisture (Grant and Villani, 2003; Kung et al., 1991) affect EPN performance, both having been correlated with EPN performance not only in laboratory settings but also in the field over regional scales (Campos-Herrera et al., 2013b). The small pore spaces of finely textured soils restrict EPN movement, soils that are too dry lack the water films that

EPNs require for movement, and EPNs may face oxygen stress in water-saturated soils. Soil temperature is another abiotic constraint, with each EPN species possessing an optimal temperature range typically somewhere between 5-35°C (Kung et al., 1991). Salinity (Thurston et al., 1994b) and pH (Kung et al., 1990b) also affect EPN performance, though usually at levels outside what would be found in a typical agricultural soil. UV light and humidity are especially important factors when EPNs are applied to a soil surface, because UV light rapidly inactivates EPNs (Gaugler et al., 1992; Smits, 1996) and low humidity renders them vulnerable to desiccation (Smits, 1996). These vulnerabilities are the root of the common recommendation that EPNs be applied to soil at dusk (Ebssa and Koppenhöfer, 2011; Shannag and Capinera, 1994).

Despite awareness of the abiotic characteristics that limit EPN efficacy against soil-dwelling pests, the ways in which EPNs are impacted by biotic soil factors have received less attention. EPNs must share the soil with enormous biodiversity beyond the pest insect they are deployed against (Fig. 1), and numerous beneficial and detrimental interactions between EPNs and soil organisms other than their hosts have been documented (El-Borai et al., 2005; Eng et al., 2005; Rasmann et al., 2005; Timper et al., 1991; Ulug et al., 2014) (Table 1). Along with abiotic soil properties, these interactions may affect the ability of EPNs to survive, move through soil, locate hosts, and reproduce (Fig. 2). However, they have been less well studied in field settings, where their potential impacts on EPN performance are most relevant.

Whether or not interactions with the soil community can produce noticeable increases or decreases in EPN field performance remains an open question, though some studies show evidence for the primacy of abiotic soil properties over biotic characteristics. For example, Campos-Herrera et al. (2016) found that abiotic properties, such as soil moisture and pH, were stronger determinants of EPN occurrence than the abundance of fungal and nematode natural

enemies in Florida mixed forests and old fields. However, McGraw and Schlossberg (2017) found no association between soil moisture and EPN occurrence at fine spatial scales in turfgrass, and suggested that biotic factors may take precedence. Overall, the extent and impact of biotic interactions as well as their relative importance in influencing EPN survival and performance against pests remain poorly understood. Here we review the known and potential effects of soil organisms on EPNs through both direct and indirect ecological interactions. Some interactions have been studied under field conditions, and others have been characterized in laboratory settings but lack description in the field. A third class of interactions have been proposed, given the current knowledge of soil ecology but not currently observed (Table 1). In this article, we highlight key questions that are not yet fully answered and propose avenues of future research to provide clearer knowledge on the potential interactions between soil biota and EPNs.

Table 1: List of most known or potential interactions between EPNs and other soil organisms, potentially affecting the ability of EPNs to survive, move through the soil, and infect hosts. Broad taxonomic groups (*e.g.*, fungi, mites) are listed in bold with several examples of more specific groups or species below.

[F] Indicates interactions characterized in field settings.

[L] Indicates interactions characterized in laboratory settings, but not in the field.

[N] Indicates interactions characterized with free-living nematodes, but not EPNs specifically.

Interaction Type	Tested taxa (examples)	Citations
Predation/infection	**Fungi**[F]	
	Arthobotrys gephyropaga, *Catenaria* sp.	El-Borai et al., 2011
	Protists[N]	
	Cryptodifflugia operculata	Geisen et al., 2015
	Nematodes[N]	
	Family Mononchidae	Moore et al., 1988; Sayre and
	Earthworms[N]	Walter, 1991
	Lampito mauritii	Dash et al., 1980
	Mites[F]	
	Gamasellodes vermivorax, *Tyrophagus*	Epsky et al., 1988
	putrescentiae, *Pilogalumna cozadensis*	Ekmen et al., 2010b
	Sancassania polyphyllae	
	Springtails[F]	
	Folsomia candida	Gilmore and Potter, 1993
	Hypogastrura scotti	Epsky et al., 1988
Exploitation	**Bacteria**[F]	
	Paenibacillus nematophilus	Campos-Herrera et al., 2012;
		Enright and Griffin, 2005
Competition	**Bacteria**[L]	
	Bacillus thuringiensis	Kaya and Koppenhöfer, 1996
	Fungi[L]	
	Beauveria bassiana	Tarasco et al., 2011
	Nematodes[F]	
	Pellioditis sp., *Acrobeloides* spp.	Duncan et al., 2003
	Oschieus spp.	Blanco-Pérez et al., 2017
	Mites[F]	
	Sancassania polyphyllae	Ekmen et al., 2010b
	Insects[L]	
	Linepithema humile, *Formica pacifica*	Baur et al., 1998
	Tetramorium chefketi, *Labidura riparia*	Ulug et al., 2014
Amensalism	**Plants**[L]	
	Tagetes spp.	Kanagy and Kaya, 1996
Phoresy	**Earthworms**[L]	
	Eisenia fetida	Campos-Herrera et al., 2006;
	Lumbricus terrestris	Shapiro-Ilan and Brown, 2013
	Mites[L]	
	Hypoaspis sp., *Gamasellodes vermivorax*,	Epsky et al., 1988
	Pilogalumna cozadensis	
	Isopods[L]	
	Porcellio scaber	Eng et al., 2005
Mutualism	**Plants**[F]	
	Pisum sativum	Hiltpold et al., 2015
	Thuja occidentalis	Van Tol et al., 2001
	Zea mays	Rasmann et al., 2005
Facilitation	**Bacteria**[L]	
	Paenibacillus popilliae	Thurston et al., 1994a

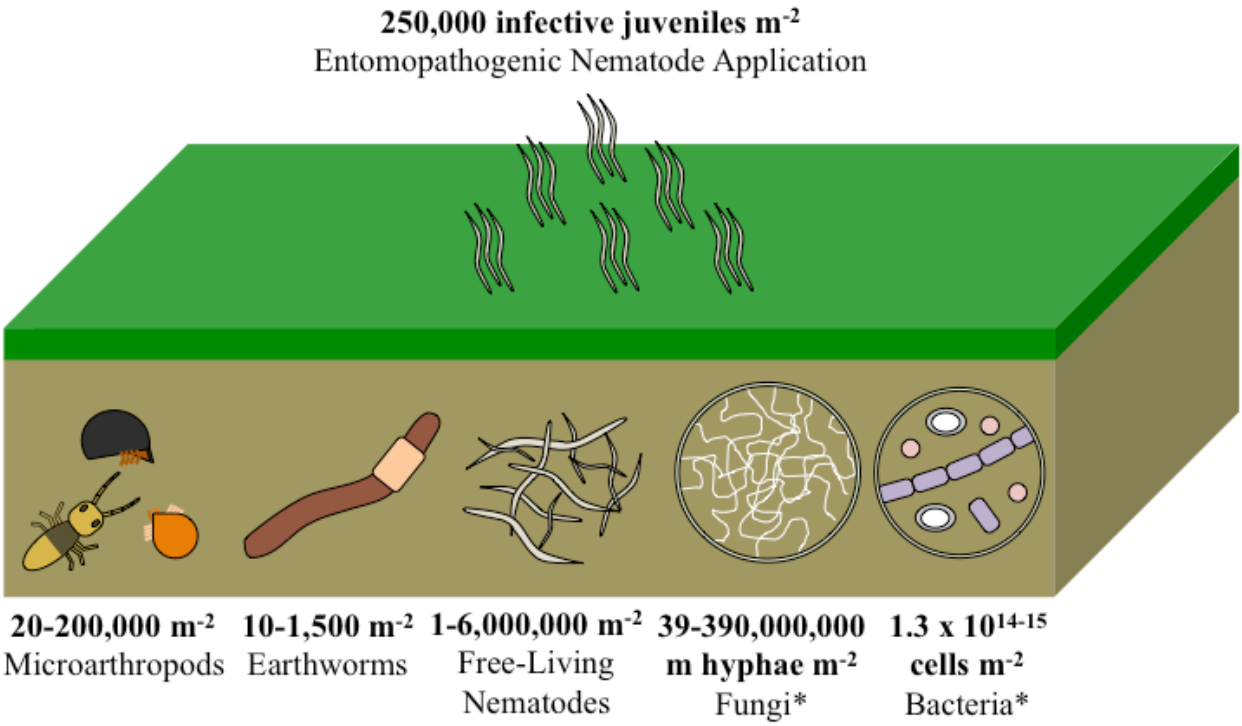

Figure 1: A selection of some of the other organisms entomopathogenic nematodes (EPNs) are exposed to when they are applied to a soil. Citations for organismal abundance are as follows: microarthropods (Giller, 1996; Koehler, 1999), earthworms (Ivask et al., 2007; Timmerman et al., 2006), fungi* (Bardgett et al., 1993; Christensen, 1989), free-living nematodes (Ruess, 1995; Yeates, 1979), bacteria* (Gelsomino et al., 1999), common EPN application rate (Koppenhöfer et al., 2015).
* Per m^2 abundances calculated from per g values to a soil depth of 10cm, assuming an average soil density of 1.30 g cm^{-3} (Beylich et. al, 2010).

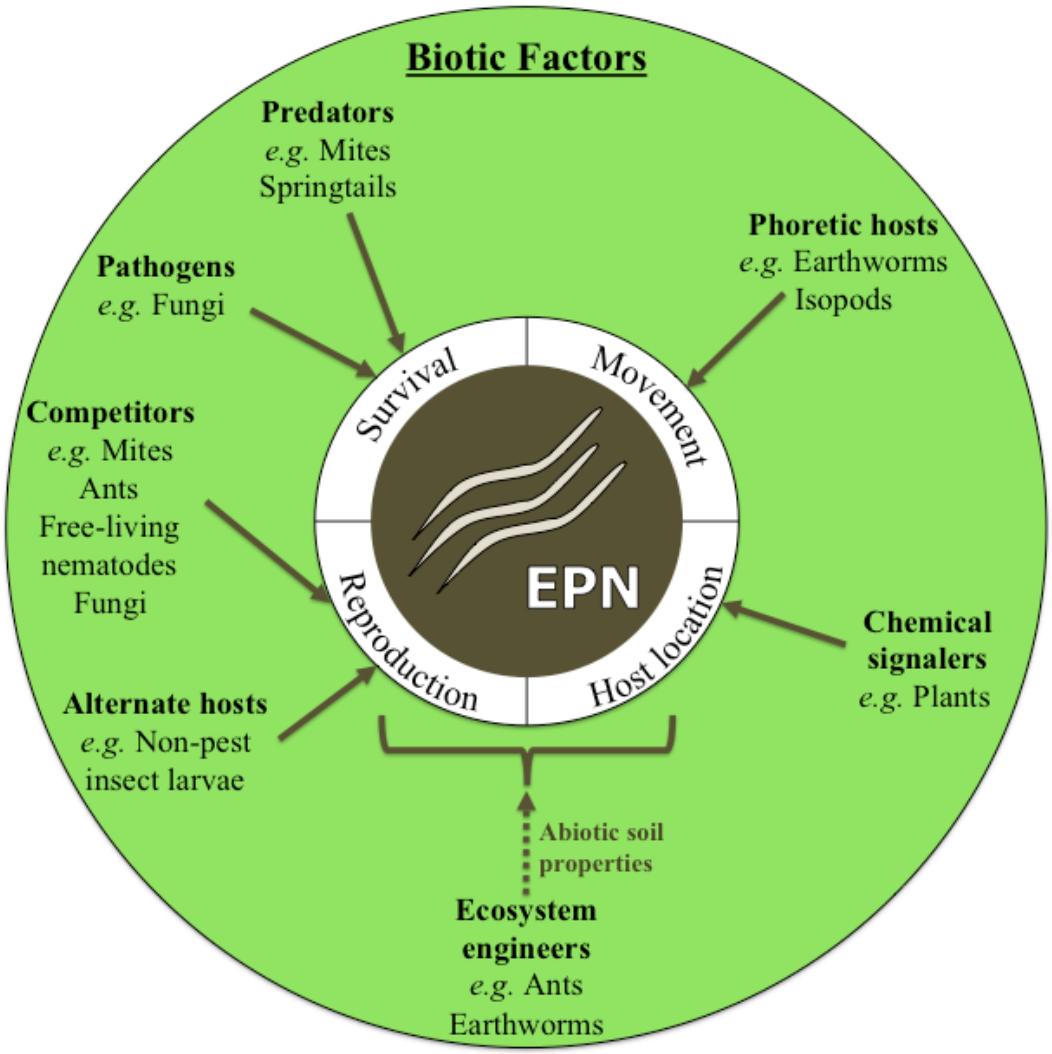

Figure 2: Diagram of the many different types of soil organisms and their effects on different points in the EPN life/host infection cycle. Note that organisms can fit into multiple categories and thus affect EPNs in different ways. Ecosystem engineers may affect EPNs indirectly through their effects on soil abiotic properties.

2. Antagonism

2.1. Predation

Predation is one of the most extensively studied interactions between EPNs and the existing soil community. In laboratory settings, many springtail and mite species consume EPNs (Epsky et al., 1988; Gilmore and Potter, 1993; Ulug et al., 2014), often to the point of reducing infection of the model host insect *Galleria mellonella* (L.) (Epsky et al., 1988; Gilmore and Potter, 1993). Although these studies provide insight into predation effects, few of them have evaluated the effects under natural conditions. Under field soil conditions, many factors could influence the strength of predation by soil animals on EPNs. Field soils possess greater structural complexity than laboratory arenas, which has been shown to reduce predation pressure by providing refuges for prey animals in soil (Hohberg and Traunspurger, 2005) and aquatic systems (Grabowski, 2004; Humphries et al., 2011). In addition, field soils possess a wide array of alternate animal and microbial food sources for predators to consume. Even strict nematophages have free-living and plant-parasitic nematode prey available. Total nematode abundance in soil lies in the millions of individuals per square meter (Ruess, 1995; Yeates, 2003, 1979) (Fig. 1), of which EPNs are only a small fraction under natural conditions (Park et al., 2014). EPN application rates in published studies vary widely, ranging between 7,400 and 1,500,000 infective juveniles (IJs) per square meter (Forschler and Gardner, 1991; Shields et al., 1999; Shields and Testa, 2015; Susurluk and Ehlers, 2008), with 250,000 IJs per square meter being a common recommended rate for commercial applications (Koppenhöfer et al., 2015). Thus, even after an inundative application, EPNs are unlikely to comprise more than even half of a soil's total nematode

community, and may not be preyed upon as intensively as has been observed in simplified laboratory settings where they comprise the entire nematode community.

Nevertheless, many studies indicate that EPNs are fed upon to some extent following inundative applications in the field, meaning that field predation still likely has some relevance. Surveys of field soil communities following EPN application reveal increases in the abundance of springtails, predatory mites, and higher predators like spiders and staphylinid beetles (Greenwood et al., 2011; Hodson et al., 2012; Jabbour and Barbercheck, 2011). These observations suggest that the addition of EPNs as a food resource for lower-level consumers induces cascading effects upward through the food web. In another field study, EPN DNA was detected in the guts of several oribatid mite species following an aqueous application of the nematodes (Heidemann et al., 2011), indicating that they can be found and consumed in field soils, even amidst the welter of other nematodes. These findings underscore the importance of studying predation and other interactions of soil organisms and EPNs in natural or semi-natural conditions.

The wider body of research surrounding predation on nematodes in general provides additional understanding of where EPNs fit into the broader soil food web. Some predatory nematodes and mesostigmatid mites specialize on nematodes (Moore et al., 1988), with some, such as the mite *Gamasellodes vermivorax* Walter, requiring nematode prey to reproduce (Walter, 1988). In addition, many generalist soil predators and supposed detritivores will consume nematodes when available (Heidemann et al., 2014a, 2011; Muraoka and Ishibashi, 1976; Walter et al., 1986). In laboratory tests, predatory mites often prefer nematodes to arthropod prey (Walter et al., 1987) and the springtail *Folsomia candida* Willem prefers nematodes to fungus (Lee and Widden, 1996). Protozoa, flatworms, and tardigrades are

additional predators of nematodes (Geisen et al., 2015; Sánchez-Moreno et al., 2008; Sayre and Walter, 1991). Although these non-arthropod predators are often mentioned as predators of EPNs (Ekmen et al., 2010b; Raja et al., 2015; Read et al., 2006), they are rarely studied in that specific context. Earthworms can also passively consume nematodes, though calling this 'predation' is questionable, as EPNs can pass through the gut of earthworms without necessarily being harmed (Shapiro et al., 1995, 1993), though reductions in viability can occur (Campos-Herrera et al., 2006). However, other studies have shown reductions in (overall) nematode abundance in soils containing earthworms (Dash et al., 1980).

Despite the parallels that can be drawn between free-living nematodes and EPNs, two aspects of EPN biology, their symbiosis with pathogenic bacteria and their association with insect cadavers, distinguish them from most other nematodes and may affect their interactions with and use by predatory organisms. EPNs and their symbionts can kill a wide variety of arthropod taxa, including ticks (Samish et al., 1999; Samish and Glazer, 2001), prostigmatid mites (Bussaman et al., 2006), isopods (Bathon, 1996; Sicard et al., 2014), and millipedes (Bathon, 1996), so it is clear that the EPN bacterial symbionts, *Xenorhabdus* and *Photorhabdus*, are toxic to more than just insects. Epsky et al. (1988) saw poor survival of the nematophagous *G. vermivorax* feeding on *Heterorhabditis heliothidis* (=*bacteriophora*) Poinar IJs and total inability of eight other mite species to fully develop on *Steinernema feltiae* Filipjev, a notable finding given that the mites originated from colonies raised on bacterial-feeding nematodes. EPNs have only rarely been observed to infect microarthropods (Epsky et al., 1988), but their bacterial symbiont may nevertheless render EPNs harmful to microarthropod predators. Thus, in some cases, microarthropods may prefer to consume non-entomopathogenic nematodes, such as free-living bacterivores, plant parasites, and predatory nematodes. Heidemann et al. (2011) noted

that oribatid mites were less likely to consume the EPN *S. feltiae* than the snail and slug parasite *Phasmarhabditis hermaphrodita* Schneider, with 3 of the 7 tested mite species consuming live *S. feltiae* and 5 of the 7 consuming dead *S. feltiae*, whereas all mites consumed live and dead *P. hermaphrodita*. In contrast, *G. vermivorax* shows no preference between *S. feltiae* and nematodes of the bacterial-feeding genus *Acrobeloides* (Walter and Ikonen, 1989). This indicates that feeding response may vary between predator taxa, or between specialist predators and opportunistic omnivores. Feeding response may also differ depending on the type of EPN being preyed upon, as *Steinernema* spp. and *Heterorhabditis* spp. differ both in the species of their bacterial symbionts and those symbionts' placement within the EPN gut (Goodrich-Blair and Clarke, 2007). However, more research is needed to confirm any repellent or toxic effects of the EPN symbiont on animals consuming IJs.

The association of EPNs with insect cadavers also sets them apart from most other nematodes. The spatial distribution of EPNs in soil varies from nearly uniform immediately following an aqueous application to patchy, aggregated swarms of IJs (Campbell et al., 1998; Shapiro-Ilan et al., 2014) to the extreme aggregation of IJs within an insect cadaver. Cadavers may attract scavenging animals that would not be significant threats to IJs in the soil, but when drawn to a cadaver may consume the resident or emerging IJs. Ekmen et al. (2010b) found that while just ten individuals of the astigmatid mite *Sancassania polyphyllae* Zachvatkin could consume 96% of IJs emerging from cadavers, they were less able to find and prey upon IJs occurring freely in the soil, as the mite is thought to be drawn primarily to cadavers and not IJs (Ekmen et al., 2010a).

2.2. Fungal pathogenesis

Nematophagous fungi (NF) are perhaps the most prominent and well-studied pathogens affecting EPNs. A few instances of microsporidian pathogens of EPNs and phages attacking their symbiotic bacteria have been noted (Kaya, 2002), but have not been extensively studied. NF can be divided into two categories: endoparasitic fungi that attach as conidia to the nematode cuticle and grow into the body cavity; or nematode-trapping fungi that catch nematodes in specially structured hyphae (Kaya and Koppenhöfer, 1996). EPN species differ in their morphological and behavioral defenses against NF. For example, IJs of the genus *Heterorhabditis* retain the cuticle of their previous juvenile stage more readily than do those of the genus *Steinernema*, which sometimes (but see El-Borai et al, 2009) provides greater protection from endoparasitic NF, suggesting that heterorhabditid EPNs would be the best species to employ in soils high in these pathogens (El-Borai et al., 2009; Timper and Kaya, 1989). Also, EPN species differ in their responses to different species of NF, with EPNs potentially being repelled, attracted, or unaffected depending on fungal species (El-Borai et al., 2011). Nematode-trapping species in particular exhibit variable lifestyles, alternating between predatory and saprotrophic behavior (Cooke, 1963; Pathak et al., 2012) in response to competition from other saprotrophic fungi (Quinn, 1987). Therefore, the danger posed by trapping NF to EPNs may vary depending on soil fungal community structure, as well as the specific NF species present and EPNs employed at a given site (El-Borai et al., 2009). Understanding characteristics of soil fungal communities that control this switch to trapping behavior in NF would again aid in assessing a soil's suitability for EPN application, and conversely, in efforts to employ NF against plant-parasitic nematodes (Kerry, 2000).

Both trapping and endoparasitic NF have been studied as EPN antagonists in field settings (Campos-Herrera et al., 2016; Jaffee et al., 1996; Pathak et al., 2012). However, whereas early field studies (Jaffee et al., 1996), did not confirm the reduced EPN infection of hosts found in soils with NF in laboratory studies (Koppenhöfer et al., 1996b), modern molecular methods have allowed for more accurate detection and measurement of NF populations in relation to EPNs in field settings. These methods are able to detect NF in samples of nematodes or bulk soil (Campos-Herrera et al., 2016; Pathak et al., 2012) and have shown spatial associations between EPNs and NF (Jaffuel et al., 2016; Pathak et al., 2017), though not always the negative associations that would be expected if NF substantially reduced EPN populations (Duncan et al., 2013; Pathak et al., 2017). Therefore, NF may not always be major limiters of EPN performance. However, Duncan et al. (2007) found higher EPN infection of the citrus root weevil *Diaprepes abbreviatus* in soils mulched with animal manure, a treatment that also decreased populations of trapping NF, indicating that NF may still be a worthwhile target for management programs seeking to increase EPN efficacy.

2.3. Plant amensalism

In addition to their prominence aboveground, plants are pivotal members of the soil community in most terrestrial (and all agricultural) ecosystems. Plant roots alter soil structure and chemistry, provide resources for soil animals and microbes, and produce a wide variety of secondary metabolites to sculpt the surrounding community for their own benefit (Hinsinger et al., 2009; Philippot et al., 2013). Although plants usually benefit from the presence of EPNs and some (discussed below in section 3.1) secrete EPN-attracting chemicals into the rhizosphere (Rasmann

et al., 2005), other root exudates can act as deterrents or toxins (Kaya and Koppenhöfer, 1996). The thiophene α-terthienyl, an extract of marigold (Asteraceae) roots, has long been known to suppress plant-parasitic nematodes, and marigolds have even been incorporated into some agricultural systems as cover crops or green manures for this exact purpose (Chitwood, 2002; Hooks et al., 2010). However, α-terthienyl also displays toxicity to EPNs at high concentrations, causing decreased survival and lower numbers of nematodes penetrating hosts (Kanagy and Kaya, 1996). Interestingly, EPNs did not perform better or worse in the presence of marigold roots than in their absence, perhaps indicating that natural concentrations of the exudate suppress plant-parasitic nematodes but not EPNs. However, this interaction has not been extensively studied with marigold or any other plant. Many plants, including other asters, brassicas, and sorghum grasses, also produce nematicidal compounds to combat plant-parasitic nematodes (Chitwood, 2002), which may also reduce EPN infectivity against insect pests when incorporated into management practices. For example, mustard green manures tilled into a potato field reduced infection of *Galleria mellonella* larvae by a wide range of EPN species (Ramirez et al., 2009). However, the use of mustard as a cover crop did not significantly reduce EPN abundance compared to other cover crops or bare soil (Jaffuel et al., 2017). These data suggest that the potential for plant antibiosis should be studied in more detail and in a wider variety of crop systems. In some cases, the benefits of plant-parasitic nematode suppression by toxic root exudates may outweigh any detriments of reduced EPN infectivity against insect pests. Attempts to reduce the presence or effects of these exudates would perhaps be best suited for cropping systems in which plant-parasitic nematodes are not economically important pests, or else for situations in which the plants producing the exudates toxic to EPNs are cover crops or weeds rather than the main crop.

2.4. Competition

An EPN-killed insect, burgeoning with bacterial biomass, is an excellent food resource for many organisms outside of the EPN-bacteria partnership. *Xenorhabdus* and *Photorhabdus* bacteria produce compounds that deter a wide range of scavengers, such as beetles (Foltan and Puza, 2009; Jones et al., 2015), ants (Baur et al., 1998), and even vertebrates (Fenton et al., 2011; Jones et al., 2017; Raja et al., 2017). However, ants, cockroaches, mites, and earwigs have still been found to feed on EPN-infected cadavers (Baur et al., 1998; Ulug et al., 2014). Scavengers can directly consume the developing and/or emerging EPNs within (Ekmen et al., 2010b) or simply break open the cadaver and leave the EPNs vulnerable to desiccation (Baur et al., 1998). Free-living and necromenic nematodes can also compete for the bacterial resources within an EPN-killed cadaver, substantially reducing the number of IJs that eventually emerge (Blanco-Pérez et al., 2017; Duncan et al., 2003). However, susceptibility to bacterivore competitors can vary between EPN species (Blanco-Pérez et al., 2017; Campos-Herrera et al., 2015). EPNs can face similar conflicts with saprotrophic microbes that compete for cadaver resources and impede the growth of both EPN and symbiotic bacteria (Navarro et al., 2014a).

EPNs can also compete with other insect pathogens sharing their host. Competition with entomopathogenic viruses, bacteria, and fungi has been reviewed in depth by Kaya and Koppenhöfer (1996) and will be discussed only briefly here. Co-infection of EPNs and many bacterial and viral entomopathogens usually results in reduced viability of the next EPN generation, due either to resource competition (Kaya and Brayton, 1978) or to the other pathogen disintegrating the insect cadaver's cuticle and desiccating the developing EPNs (Kaya and

Burlando, 1989). In contrast, effects of co-infecting entomopathogenic fungi (EPF) on EPNs are timing- and temperature-dependent, and in favorable circumstances, EPNs can exclude the fungi and develop normally (Barbercheck and Kaya, 1990). Compounds produced by EPF and EPN symbiotic bacteria are generally antagonistic to one another, although this can vary by individual EPF or symbiont species (Ansari et al., 2005; Tarasco et al., 2011). In rare instances, EPNs and EPF can colonize different parts of the same cadaver (Tarasco et al., 2011), though this would still decrease the amount of resources available to the EPNs. Interestingly, combined applications of EPNs and other entomopathogens (in management contexts) sometimes have additive or synergistic effects on pest mortality (Abdolmaleki et al., 2017; Ansari et al., 2006; Jabbour et al., 2011). However, the impact of these combined applications sometimes depend on the relative timing of both applications (Abdolmaleki et al., 2017). These data indicate that the relationship between EPNs and other entomopathogens may be less adversarial when considering an entire population of hosts rather than their interactions within a single host. Though outside the scope of the present review, different EPN species can also compete with one another within the soil profile (Koppenhöfer et al., 1996a) and within single host cadavers (Koppenhöfer et al., 1995), with the victorious species usually being the one with faster development time or less specific bacterial symbiont association (Koppenhöfer et al., 1995).

Unlike predation, competition for cadaver resources can only reduce EPN recycling and thus their potential to maintain consistent levels of pest control over time, not the initial infectivity of an aqueous EPN application. However, the practice of applying EPNs within infected insects (Raja et al., 2015; Shapiro-Ilan et al., 2006), despite its advantages of increased EPN infectivity under typical circumstances (Shapiro-Ilan et al., 2003; Shapiro and Lewis, 1999), may not be suitable for soils unusually high in potential scavengers.

2.5. Bacterial encumbrance

There is also evidence that EPNs can be negatively affected by soil microbes that exploit IJs for their own dispersal. The bacterium *Paenibacillus nematophilus* can attach itself to many *Heteorhabditis* EPN species and use them as phoretic hosts (Enright and Griffin, 2004), and an unidentified congener, *Paenibacillus* sp., attaches to *Steinernema diaprepesi* (El-Borai et al., 2005). Unlike many other *Paenibacillus* species, these bacteria are not entomopathogenic, but instead will proliferate inside of EPN-killed insects after reaching them as spores on the IJs. *Paenibacillus* presence does not significantly impact EPN reproduction or development inside of cadavers, but spores can reduce EPN mobility and ability to infect hosts in laboratory settings (El-Borai et al., 2005; Enright and Griffin, 2005). From an evolutionary perspective, bacteria that benefit from transportation to a host would not be expected to commonly overburden their source of transportation, as that would decrease their own fitness as well as the fitness of the EPN (Enright and Griffin, 2005). However, the *Paenibacillus*-EPN phoretic association itself has been repeatedly confirmed in natural soils via real time quantitative PCR (Campos-Herrera et al., 2016, Campos-Herrera et al., 2011; Campos-Herrera et al., 2012b), and increases in *Paenibacillus* spore encumbrance have been linked to reduced EPN abundance (Campos-Herrera et al., 2013a).

3. Facilitation

3.1. Plant root signaling

Due to the protection EPNs provide plants in both natural (Ram et al., 2008) and agricultural ecosystems (Chen et al., 2003; Toepfer et al., 2008), plants would be expected to benefit from the ability to attract EPNs to the site of belowground herbivory. As such, many plants secrete exudates in response to root feeding that can attract and mobilize EPNs against the feeding insects. Root exudates from citrus (Ali et al., 2012), pea (Hiltpold et al., 2015), conifers (Van Tol et al., 2001), and maize (Rasmann et al., 2005) all affect EPNs in this way, primarily attracting them to defend the plant from belowground attack. Pea root exudates can induce quiescence in both EPNs and plant-parasitic nematodes when present in high concentrations (Hiltpold et al., 2015; Jaffuel et al., 2015), but at low concentrations, the exudates increase EPN infectivity to *Galleria mellonella* while still subduing plant-parasitic nematodes (Hiltpold et al., 2015). Exudates from citrus roots have been found to enhance biological control even when applied to the soil in non-citrus cropping systems (Ali et al., 2012), though they also can attract free-living bacterivorous nematodes, which can compete with EPNs for cadaver resources (Ali et al., 2013). Conversely, the exudates can also modify EPN behavior in the presence of NF, reducing infection of the EPNs (Willett et al., 2017).

The interaction of EPNs with maize root exudates is the most intensively studied. Certain maize cultivars exude the sesquiterpene (*E*)-β-caryophyllene (EβC) from their roots in response to feeding damage from the western corn rootworm *Diobrotica virgifera virgifera* LeConte, thus attracting EPNs to kill the pest (Rasmann et al., 2005). EβC not only attracts EPNs, but also helps them navigate complex root architecture to locate hosts (Demarta et al., 2014). EPNs thus attracted can significantly reduce *D. v. virgifera* numbers and damage to maize roots (Hiltpold et al., 2010a; Hiltpold et al., 2010b; Hiltpold et al., 2010c). This interaction has led to extensive

research into ways to select for EPN populations more responsive to the EβC signal (Hiltpold et al., 2010a; Hiltpold et al., 2010b) and, through genetic engineering, to restore the signal to maize cultivars that have lost it (Degenhardt et al., 2009). Interestingly, selecting for enhanced response to EβC resulted in greater *D. v. virgifera* mortality and decreased root damage when *Steinernema feltiae* and *Heterorhabditis megidis* Poinar, Jackson, & Klein were applied to the soil, but selection had no effect on the insect-killing or plant-protecting ability of *H. bacteriophora* (Hiltpold et al., 2010c). This suggests that some EPN species may be less reliant on host plant chemical signals than other species, and so would be the optimal choice of EPN for use on crops and cultivars lacking those signals.

It must be noted that while these interactions have been intensively studied in a few crop plant systems, soil is an incredibly complex chemical environment, replete with volatile and solubilized compounds from plant and microbial sources, most of whose functions are unknown (Delory et al., 2016; Insam and Seewald, 2010; Leff and Fierer, 2008). Under natural conditions, the full importance of established chemical cues relative to other compounds remains to be fully understood. In addition, other physicochemical complexities of soil, properties such as moisture, texture, and pH, can affect how volatile chemicals disperse through the soil pore matrix (Chiriboga et al., 2017; Som et al., 2017), impacting the efficiency with which damaged plants can recruit EPNs (Chiriboga et al., 2017).

3.2. Phoresy on soil animals

Dispersing through the soil to locate patchily distributed insect hosts is one of the principal challenges facing EPNs (Stuart et al., 2006). Soil moisture and texture limit the ability of EPNs

to disperse independently through the soil matrix (Grant and Villani, 2003; Kung et al., 1990a), however, like many other microinvertebrates, nematodes are capable of dispersing phoretically on the surface of larger animals. EPNs can disperse phoretically both at and below the soil surface on isopods (Eng et al., 2005) and earthworms (Campos-Herrera et al., 2006; Shapiro-Ilan and Brown, 2013; Shapiro et al., 1995, 1993). Other macrofauna such as myriapods or slugs may also serve as phoretic hosts, but those taxa have not been tested. Microarthropods may also provide EPNs with transportation through the soil. Epsky et al. (1988) observed EPNs exploiting mites as phoretic agents, although the study was not conducted in soil, where EPNs may risk being abraded against mineral grains or scraped from the mite's exoskeleton as they move through the soil pore matrix. However, EPNs were seen clustering on the dorsal surface of mites, suggesting an active behavior of the nematodes that they would presumably perform in soil.

3.3. Entomopathogen commensalism

Unlike the entomopathogens discussed above in section 2.3, *Paenibacillus popillae* (formerly *Bacillus popillae*), causative agent of the milky spore disease in Japanese beetle *Popillia japonica* Newman, not only avoids conflict with co-infecting EPNs, but also enhances EPN penetration of the midgut of infected grubs (Thurston et al., 1994a). Though this 'compensatory infection' may not result in greater insect mortality following EPN application, it could potentially increase EPN recycling and their ability to persist in controlling Japanese beetle over the course of a season. However, native milky spore disease is not often a major source of *P. japonica* mortality (Cappaert and Smitley, 2002; Hanula and Andreadis, 1988), though it can be a dominant pathogen at some sites (Redmond and Potter, 2010). In addition, commercial

formulations of *P. popillae* are often ineffective (Redmond and Potter, 1995). It is therefore unlikely that exploiting the EPN-milky spore disease commensalism will lead to enhanced biological control strategies.

4. Host community interactions

Although many of the previously discussed interactions represent top-down pressures on EPN populations, some soil organisms could provide bottom-up effects on EPNs and their ability to infect pests. Obviously, characteristics of a pest population such as density (Ebssa et al., 2011), dominant life stage (Power et al., 2009), and individual pest species within a generalized group such as 'white grubs' (Koppenhöfer et al., 2006; Morales-Rodriguez et al., 2010) can affect the success of an EPN application. However, effects of other, non-target insect hosts on EPN success against a pest have received little attention. Although some EPN species, such as *Steinernema scarabaei* Stock & Koppenhöfer and *S. scapterisci* Nguyen & Smart, specialize on single or limited numbers of related host species (Koppenhöfer and Fuzy, 2003; Nguyen and Smart, 1991), others, including many commercially available species, have wide host ranges and can infect organisms beyond the targeted pests (Bathon, 1996; Peters, 1996). The impacts of EPNs on non-target hosts have been studied in the context of determining their environmental safety (Babendreier et al., 2015; Bathon, 1996), yet less well understood are the effects of alternate hosts on EPN population dynamics, persistence in soil, and especially their ability to infect pests. Abundance of insect hosts, pest or otherwise, is predictably associated with increased population levels of EPNs (Efron et al., 2001; Harvey and Griffin, 2016; Mráček et al., 2005; Mráček and Bečvář, 2000), though not in all cases (Campbell et al., 1995; Půža and Mráček, 2005). Susurluk

and Ehlers (2008) found that persistence of *Heterorhabditis bacteriophora* was nearly doubled in cropping systems with high availability of hosts throughout the course of the study compared to systems lacking viable hosts.

The effect of alternate hosts on EPN infection of pests is likely dependent on several variables, perhaps most importantly the relative timing of the alternate host and the pest life cycles. Alternate hosts present at the same time as the target pest could potentially reduce infection of the pest via apparent mutualism or increase pest infection via apparent competition, depending on a wide range of traits specific to the EPN, pest, and alternate host under consideration (Abrams et al., 1998; Holt and Lawton, 1994). In contrast, alternate hosts present at different times from target pests could enhance EPN persistence, providing a temporal stepping-stone to sustain the population through periods where pest insects are absent from the soil. This was observed by Sulistyanto and Ehlers (1996), which found that EPNs applied to control the scarab grub *Aphodius contaminatus* Herbst could control a second grub occurring later in the season, *Phyllopertha horticola* (L.), at a level high enough to suggest that recycling within the *A. contaminus* and not simple persistence of the original application had occurred.

5. Habitat modification by ecosystem engineers

Soil organisms act not only on one another, but also on the abiotic soil environment around them. Though abiotic properties may be more important controls of EPN performance than biotic properties in some systems (Campos-Herrera et al., 2016), abiotic properties can be shaped by the activities of living ecosystem engineers. Soil macrofauna, especially earthworms, can alter many of the soil properties important for EPN performance, notably soil moisture via burrowing-

induced changes in porosity, water infiltration, and water storage (Bottinelli et al., 2015; Capowiez et al., 2009; Lavelle et al., 1997). Ants and termites can also cause similar changes both in their mounds and in surrounding soils (Frouz et al., 2003; Nkem et al., 2000). Some changes may be restricted to their mounds, such as increases in deep-soil porosity in ant mounds (Nkem et al., 2000), but other changes extend throughout their range of activity. In addition, ants and termites are both capable of increasing water infiltration rates through their tunneling activities (Mando et al., 1996), to the point where excluding these organisms can decrease crop yields in arid soils (Evans et al., 2011). A similar decrease in water infiltration might also reduce EPN performance, suggesting that the presence of these ecosystem engineers would be beneficial to EPNs.

Ecosystem engineers can also influence another major abiotic control of EPN performance, soil texture. Ingestion and passage through the guts of earthworms (Carpenter et al., 2007; Suzuki et al., 2003) and scarab larvae (Suzuki et al., 2003) can erode mineral grains to the point of converting coarse and medium sand grains into fine and very fine sand (Suzuki et al., 2003), though any potential effects of scarabs on soil texture at field scales are not well characterized. Earthworms can also alter the vertical distribution of a soil's mineral constituents (Resner et al., 2011), changing the soil profile that EPNs must penetrate to reach the target pest. Ants similarly redistribute soil particles, as Nkem et al. (2000) found lower clay and higher silt and sand contents in ant-impacted soils, even five meters from the mounds and in foraging tracks extending farther. Any fauna-induced changes to soil texture would likely occur over timescales much longer than those of any direct ecological interaction such as predation. However, earthworms are prominent invasive species in certain parts of the world, such as the northern United States (Bohlen et al., 2004). Thus, their influence on soil physical properties would be a

new addition to those ecosystems, one with potential to alter soils' suitability for EPNs over time.

Whether these and other fauna-induced changes are beneficial or detrimental to EPN performance would obviously depend on a variety of factors, such as engineer species, soil characteristics, EPN foraging strategy, and target pest. For example, earthworm burrow size, depth, and structure differ considerably across species and ecological groups (Capowiez et al., 2006), as do the size and distribution of ant and termite mounds (Greenberg et al., 1985), and so the activities of different species would likely have different effects on EPNs. Thus, no single 'engineer effect' is likely to exist. To date, we are not aware of a single study seeking to characterize indirect effects of faunal ecosystem engineering on EPN performance, although Shapiro-Ilan and Brown (2013) found higher dispersal and infectivity of *Steinernema carpocapsae* in soils containing the earthworm *Lumbricus terrestris* compared to soils without it, which the authors attributed primarily to phoresy but could have also been a result of more favorable soil conditions generated by the earthworms.

6. Challenges and future goals

A common theme emerges when considering the literature surrounding ecological interactions between EPNs and other soil organisms, as does a question that should be on every pest manager's mind: do biotic interactions matter? Can the action of other soil organisms enhance or reduce the biological control potential of EPNs, either the initial infectivity of an application or the efficacy and dynamics of a persistent population? Soil organisms are well positioned to have a role in the variable field performance of EPNs. Their abundance and community composition

are highly variable in time and space (Frey, 2015; Giller, 1996; Moore and de Ruiter, 1991), and also vary with soil management practices (Chu et al., 2007; Donnison et al., 2000; Gan and Wickings, 2017; Schon et al., 2008; Wickings and Grandy, 2013). For example, springtails and mites, two key groups of soil microarthropods with multiple potential effects on EPNs, range in abundance from hundreds to hundreds of thousands of individuals per square meter (Giller, 1996; Koehler, 1999). Thus, if these and other soil organisms can influence EPN performance, then their overall effect would be variable, and variable, unpredictable EPN performance would result. The temporal variation in soil communities would perhaps be especially important for management strategies seeking to establish persistent EPN populations, as those EPNs would be exposed to many different soil communities over the course of their time in the soil, which could affect the EPNs differently at different times.

However, the question of other soil organisms' effects on EPNs remains largely unanswered, as the majority of studies so far quantifying the impact of most ecological interactions on EPN performance have been conducted in laboratory settings. In studying predation, analyses of predator gut contents and soil community response to EPN application may identify animal groups that consume EPNs in the field, but cannot determine if predators impact EPN populations strongly enough to interfere with pest control. A few studies have, directly or indirectly, shed some light on this question, such as studies of biological control practices combining EPNs and *Hypoaspis* predatory mites, which are known to consume EPNs (Epsky et al., 1988). Borgemeister and Berndt (2003) suggested intraguild predation by mites on EPNs as an explanation for the not completely additive effect of EPNs and *Hypoaspis aculeifer* Canestrini mites on thrips mortality. However, the authors did not assess EPN infection of the thrips themselves and so could not directly tie the mites to decreased EPN efficacy *per se*.

Wilson and Gaugler (2004) correlated springtail and mite abundance in turfgrass with declining EPN infection of *Galleria mellonella*, which could have been due to predation by the microarthropods, especially since the correlations were only present when EPNs were surface-applied as opposed to subsurface-applied. However, *G. mellonella* infection was assessed under laboratory conditions, not in the field. Similar knowledge gaps exist for other interactions. Duncan et al. (2003) observed reduced EPN emergence from field-collected cadavers in which free-living bacterivore competitors were also present, but the effect of this competition on future insect infection remains unknown. Phoresy of infective juveniles has never been examined in the field, nor have indirect effects of ecosystem engineers ever been explicitly tested (but see Shapiro-Ilan and Brown, 2013). To date, the only field studies assessing the effects of soil organism interactions on EPN performance against an agricultural pest have been those investigating root chemical signaling (Ali et al., 2012; Hiltpold et al., 2010b) nematophagous fungi infection (Campos-Herrera et al., 2014; Duncan et al., 2007), and *Paenibacillus* encumbrance (Campos-Herrera et al., 2014; Duncan et al., 2013). Still, previous studies under artificial conditions suggest that many other ecological interactions could have important effects on EPN performance in soil.

A concerted effort to quantify the effect of other soil organism interactions with EPNs under field conditions will help determine whether or not soil biota need to be taken into account when deciding to use EPNs as a pest control tool. Several avenues of research show promise. First, correlative studies associating biotic (and abiotic) soil properties with native EPN occurrence or applied EPN efficacy have provided understanding of which soil properties are most important (Campos-Herrera et al., 2016; Campos-Herrera et al., 2013), as well as the relative importance of biotic and abiotic factors. In addition to informing targets for soil

management, such findings could eventually help managers predict EPN performance in advance, reducing the likelihood of control failure due to an ineffectual application on a suboptimal soil. These studies should take wider ranges of soil biota into account, and be continued in a wider variety of agricultural systems and management regimes.

Soil biota could also be incorporated to a greater extent into studies examining the effects of agricultural management practices on EPNs, specifically to determine if the downstream consequences of different agronomic, cultural, and pest management practices on soil communities comprises part of those practices' overall influence on EPNs. Studies in Florida citrus orchards have already taken this approach, linking increased abundance of EPN natural enemies as a result of specific management practices to reduced EPN occurrence and increases in pest activity (Campos-Herrera et al., 2014; Campos-Herrera et al., 2013). However, more general and widespread management practices have yet to receive attention of this kind. For instance, tillage both reduces densities of earthworms and microarthropods in soils (House and Parmelee, 1985; Reeleder et al., 2006; Winter et al., 1990), and has varying effects on EPN infection rates, including positive effects on *Steinernema riobrave* Cabanillas, Poinar, & Raulston (Millar and Barbercheck, 2002). Whether the effects of tillage on EPNs are entirely due to its abiotic changes to the soil or partly mediated by tillage's effects on the soil community remains to be determined. Pesticide use is another example of a management practice with potential indirect effects on EPNs. The direct toxicity of pesticides to EPNs varies by active ingredient, with effects ranging from 100% EPN mortality to no apparent harm (Krishnayya and Grewal, 2002; Navarro et al., 2014b; Rovesti et al., 1988). EPNs can even be applied in the same tank mix as certain pesticides, such as imidacloprid, with which some EPNs have a synergistic effect on scarab grub mortality (Koppenhöfer et al., 2000a). Imidacloprid paralyzes the grubs and prevents

them from grooming themselves and removing EPNs (Koppenhöfer et al., 2000b). However, pesticides also have significant, and usually negative, effects on other soil biota, such as arthropods (Kunkel et al., 1999; Peck, 2009) and saprotrophic fungi (Gan and Wickings, 2017), effects which could again have downstream implications for EPNs.

As a potential avenue beyond correlative analysis for gauging the relative importance of abiotic soil conditions and biotic communities for EPNs, the 'reciprocal transplant' experimental design commonly used by ecologists to determine the relative importance of abiotic (*e.g.* environmental) and biotic (*e.g.* genotypic) factors in governing organism or community characteristics (Hedderson and Longton, 2008; Meola et al., 2014; Pascoal et al., 2012) could be repurposed. Sterilized soils from each of multiple sites could be placed in permeable field mesocosms at each site, left for sufficient time to allow the sites' native biota to colonize, and be inoculated with EPNs and a selected host. However, the effects of one site's community on another's soil and vice versa, separate from their effects on EPNs, would have to be accounted for, as factors such as soil pH are known to affect interactions between EPNs and other organisms (Campos-Herrera et al., 2014).

Further beyond the 'soft' manipulation of a reciprocal transplant study, experiments directly manipulating soil communities, either by excluding or augmenting different taxa, are commonplace in soil ecology and provide insight into the effects of soil organisms on community dynamics and ecosystem processes (Crowther et al., 2013; Soong et al., 2016; Uvarov and Karaban, 2015). Thus, alone and in combination with correlative studies, these approaches will likely also be useful for understanding soil organism effects on EPN performance. One limitation of this approach is its relative inability to separate the effects of individual taxa and interactions on EPN performance (Fig. 3), especially in exclusion

experiments where manipulating specific taxonomic groups instead of broad size classes is difficult. A single taxonomic group or even single species could interact with EPNs in multiple, possibly contrasting ways, and the magnitude and direction of each group's effects would likely be difficult to determine. However, the relative strength of positive interactions versus negative interactions would be determinable by the overall effect on EPN performance of a community's presence or increased abundance of specific groups or overall community diversity. For example, Khan et al. (2016) observed decreased survival of four EPN species in soils containing organisms compared with sterilized soils, suggesting that negative interactions with members of that soil community predominated, regardless of individual species-level interactions.

Future studies should also investigate differences in interactions between soil biota and EPNs that occupy different ecological niches. For instance, EPNs vary between species (and sometimes within species depending on context, see Griffin, 2015) both in their movement behavior ('ambush' versus 'cruise' foragers) and their preferred foraging depths (Ferguson et al., 1995; Kaya and Gaugler, 1993; Neumann and Shields, 2006), potentially resulting in different EPN species encountering different biotic communities, as abundance and species composition of many soil organism groups, from bacteria to springtails, vary by depth (Frey, 2015). The different sizes of each EPN species' IJ stage may also affect their vulnerability to predators of different sizes. Of the thirteen commercially available EPN species, the largest, *Steinernema longicaudum* Shen & Wang, is nearly double the length of the smallest, *Heterorhabditis indica* Poinar, Karunakar, & David (Adams and Nguyen, 2002).

One particular area of research that has grown in prominence in recent years, both within general soil ecology (Oburger and Schmidt, 2016; Philippot et al., 2013; van Dam and Bouwmeester, 2016) and the study of EPNs (Ali et al., 2013; Hiltpold et al., 2015; Jaffuel et al.,

2015; Willett et al., 2017, also see section 3.1 above), is the role of root exudates and volatiles in shaping complex community interactions belowground. These rhizodeposits provide food for soil animals (Garrett et al., 2001; Pollierer et al., 2007) and shape microbial communities, likely both by releasing anti-microbial compounds and again providing food resources (Brant et al., 2006; Broeckling et al., 2008). Research over the last two decades has advanced our awareness of the chemical complexity of plant rhizodeposition (Massalha et al., 2017) and has also begun to elucidate the role of root-derived compounds as info-chemicals mediating communication among plants, free-living and symbiotic microbes, and animals including root herbivores and EPNs (Huang et al., 2014; Lareen et al., 2016; Rasmann et al., 2005). These efforts have generated interest in the potential for manipulating plant rhizosphere traits in order to enhance belowground biological control (Degenhardt et al. 2009). However, soilborne exudates, especially volatile emissions, are variable in space and time (Dessureault-Rompré et al., 2007; Peñaloza et al., 2002) and notoriously difficult to track, and our understanding of the impact of rhizodeposits on many other soil animal taxa is still limited to only a handful of studies (Eisenhauer et al., 2012; Ruf et al., 2006; Strickland et al., 2012). Thus, it will be critical to expand this knowledge base in order to fully gauge the influence of rhizodeposit-mediated interactions on the biological control capacity of EPNs. We see these potential effects as particularly interesting to investigate further and potentially applicable to a wider range of agricultural and horticultural systems and pest management scenarios. This is because such effects would be potentially relevant in situations beyond those in which EPNs are directly attracted to roots as a result of herbivory, such as cases in which roots do not secrete EPN-attracting chemicals when consumed, or in which the pest targeted by EPNs is a soil-dwelling

stage of an aboveground feeder rather than a root feeder that would trigger release of root volatiles.

Difficulties may arise in separating the effects of multiple interactions with a single organism. For example, the overall effect of an earthworm on EPNs may be a combination of phoresy, predation, and ecosystem engineering effects. In field settings, the effects of multiple species on each other as well as on EPNs further confound the issue to potentially unmanageable levels. However, inventive experimental designs and use of modern observational techniques such as molecular gut content analysis, fatty acid analysis, real-time quantitative PCR, and stable isotope probing, which have proven excellent tools for characterizing belowground trophic ecology and species interactions in a wide variety of systems (Campos-Herrera et al., 2016; Campos-Herrera et al., 2012; Heidemann et al., 2014a; Heidemann et al., 2014b; Ruess and Chamberlain, 2010), should still provide information valuable to both pest managers and soil ecologists. Analytical procedures including path analysis, canonical correspondence, and structural equation modeling may also aid in teasing the directionality and relative importance of different biotic interactions between EPNs and complex soil communities (Campos-Herrera et al., 2012a).

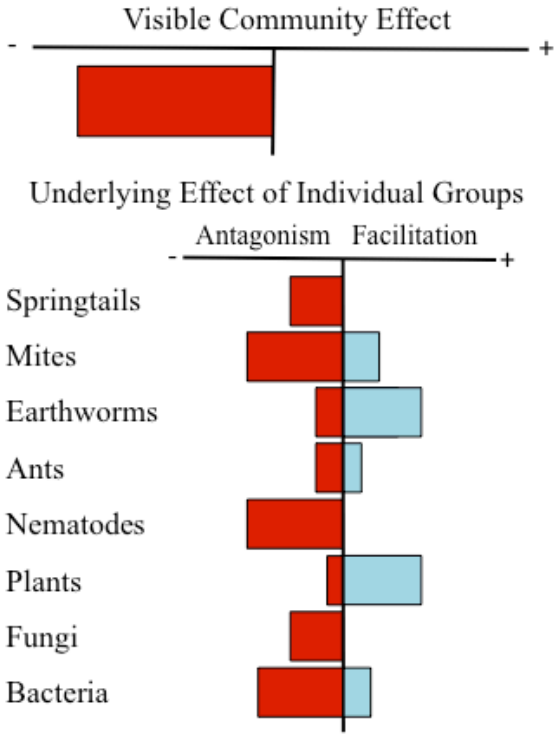

Figure 3: Hypothetical positive (blue bars) and negative (red bars) effects of eight soil organism taxa on EPN performance contributing to and (perhaps) obscured by an overall community effect. Individual species within each taxon may have positive effects, negative effects, or varying degrees of both that contribute to their group's and the overall community's effect.

7. Conclusions

EPNs interact with and are acted upon by a wide variety of soil organisms spanning the full breadth of soil's taxonomic diversity, and these interactions and their potential effects on EPN performance should be considered when studying the usefulness of EPNs against belowground pests. Despite the fact that EPNs can be applied to the soil in the same manner as a chemical pesticide, they are living organisms subject to all the biotic and abiotic pressures of the soil environment. Better understanding of these pressures, their relative importance, and the limits they may impose on EPNs will aid in successfully leveraging their pest control potential to its fullest extent.

8. References

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CHAPTER TWO

Soil microarthropod communities reduce entomopathogenic nematode efficacy

Abstract

Biological control agents applied to soil, such as entomopathogenic nematodes (EPNs), are exposed to a wide variety of organisms other than the pest they are utilized against. Interactions with these other organisms may positively or negatively affect EPN performance and may be important for managers to take into account when using EPNs to control pests. We assessed the effect of soil microarthropods on the EPN *Heterorhabditis bacteriophora*'s establishment within and infection of wax moth *Galleria mellonella* larvae in soil arenas. Presence of soil microarthropods at typical field densities significantly reduced establishment of adult *H. bacteriophora* within *G. mellonella* larvae, and reduced the percentage of host larvae infected in one of two replicated trials. In addition, adult EPN establishment was negatively correlated with the abundance of mesostigmatid mites. Our results indicate that soil microarthropods at natural abundances can reduce EPN establishment even within the structurally complex environment of soil, though this does not always lead to reductions in insect infection rates.

1. Introduction

Entomopathogenic nematodes (EPNs) are promising biological control agents and have been used successfully against a range of economically important soil-dwelling insect pests, including scarab grubs (Forschler and Gardner, 1991; Georgis et al., 2006; Grewal et al., 2005), alfalfa snout beetle (Shields *et al.*, 1999, 2009), and citrus root weevil (Duncan et al., 2013; Shapiro-Ilan et al., 2005). Third-stage EPN larvae, also known as infective juveniles (IJs), enter their host insect's body through the mouth, anus, or other body openings and expel their pathogenic bacterial symbiont into the insect hemocoel (Kaya and Gaugler, 1993; Lewis and Clarke, 2012). The bacteria (*Xenorhabdus* spp. or *Photorhabdus* spp., depending on EPN species) produce a flood of toxic metabolites, sometimes aided by venom proteins and anti-immune agents produced by the nematodes themselves (Lu *et al.*, 2017), and the insect dies of septicemia 24-48 hours later. The EPNs then feed on the bacterial biomass within the decaying cadaver, develop into adults, mate, and reproduce. When the cadaver resources are exhausted, new IJs emerge to search the soil for new hosts. EPN IJs are frequently applied directly to soil as an aqueous spray as a means of controlling belowground pests (Shapiro-Ilan *et al.*, 2006). Whether applied as a spray or emerging from a cadaver, IJs must survive and navigate through the soil environment in all its abiotic and biotic complexity to locate patchily distributed insect hosts (Campbell *et al.*, 1998).

The interactions of EPNs with their abiotic environment are relatively well characterized. Soil moisture (Grant & Villani, 2003), texture (Kung *et al.*, 1990; Koppenhöfer & Fuzy, 2006), bulk density (Portillo-Aguilar *et al.*, 1999), and other physicochemical soil properties have been shown to affect the ability of EPNs to persist, move through the soil, and infect hosts.

Knowledge of these properties and their effects on EPNs has, in specific systems, lead to management practices that increase the ability of EPNs to control pests, due to managers' ability to manipulate some soil characteristics (Duncan et al., 2013).

Also important, yet less well understood, are the effects of other soil organisms on EPN performance. Soil organisms from a wide variety of taxonomic groups can interact with EPNs in numerous beneficial and detrimental ways throughout the EPN life cycle, forming a crowd of potential friends and foes that EPNs must weave through to infect hosts. For example, chemical exudates released by plant roots can guide IJs to insect hosts (Rasmann *et al.*, 2005; Jaffuel *et al.*, 2015; Willett *et al.*, 2017), and saprotrophic microbes and free-living nematodes can compete with EPNs developing inside of a host cadaver, potentially reducing the number of IJs that eventually emerge (Duncan *et al.*, 2003; Blanco-Pérez *et al.*, 2017). For other taxa, particularly soil animals, the relationship is more complicated, as they can potentially interact with EPNs in multiple, often contrasting ways.

Prominent among the soil animals are the microarthropods, which are the most abundant and diverse arthropod taxa in most soils (Giller, 1996) and are significant drivers of key ecosystem processes (Soong *et al.*, 2016). Springtails and mites can prey upon EPN IJs (Epsky *et al.*, 1988; Gilmore & Potter, 1993; Karagoz *et al.*, 2007; Ulug *et al.*, 2014), resulting in declines in EPN-induced mortality of *Galleria mellonella* (L.) hosts of up to nearly 95% under simplified laboratory conditions (Gilmore & Potter, 1993). In a field setting, this predation could reduce the efficacy of an augmentative EPN application. At the same time, many of these and other animals can act as phoretic hosts for EPNs (Epsky *et al.*, 1988; Eng *et al.*, 2005; Shapiro-Ilan & Brown, 2013), potentially transporting IJs through the soil faster and further than they can move on their own.

Most studies characterizing animals' interactions with EPNs focus only on a single species at a time, whereas in natural settings many types of organisms can act simultaneously on an EPN population. Also, these interactions are often studied under artificial conditions (Epsky et al., 1988; Gilmore and Potter, 1993), in simplified, non-soil media lacking the structural complexity of soil or even a three-dimensional environment. Under more complex conditions, the magnitude and directionality of microarthropods' and other animals' effects may become harder to predict. Increased habitat complexity has been found to reduce predator efficacy in aquatic systems (Grabowski, 2004; Humphries *et al.*, 2011) and in soil (Hohberg and Traunspurger, 2005; see also Gilmore and Potter, 1993). Therefore, the impact of predators on EPN populations may be overestimated in simplified systems. Conversely, phoresy could be more important for EPN dispersal in more spatially complex environments. Thus, despite the effects of these interactions observed in simplified settings, the presence of multiple other animal species and interactions within complex environments adds uncertainty to the outcome.

Despite this, there is still evidence that biotic interactions can affect EPNs under natural or semi-natural soil conditions. Khan *et al.* (2016) found increased EPN survival in sterilized soil compared to soils with intact biotic communities, but did not seek to relate the difference in EPN numbers to their ability to infect hosts nor determine what other organisms were present in the soils. Their results nevertheless suggest that detrimental interactions with soil organisms predominated. In addition, Wilson and Gaugler (2004) correlated abundances of collembolans and mites with declines in EPN infectivity in laboratory assays of field-collected turfgrass soils, though the question of whether diverse soil communities can impact EPN populations to the point of reducing their ability to infect insects remains poorly explored in manipulative contexts.

We conducted two laboratory arena experiments to determine the effects of soil animal communities on the ability of the EPN *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) to infect the model host *Galleria mellonella* in semi-natural conditions created by manipulating soil microarthropod communities in field-collected soil. We hypothesized that negative interactions with the soil community would predominate, and that infection of *G. mellonella* would be lower in soils with microarthropod fauna than in soils without.

2. Methods

2.1. Arena construction and soil preparation

Experimental arenas consisted of 1 L tapered plastic pots (height, 14.0 cm; maximum diameter, 11 cm; minimum diameter, 8.5 cm) with a 2.5 cm diameter hole cut in the side wall, the hole centered 2.5 cm from the bottom of the pot. Into the hole, we inserted a 7.5 cm-long cage made from a cylinder of 1.5 mm aluminum window mesh affixed to the severed top of a screw-cap centrifuge tube (diameter 2.5 cm), securing it in place with hot glue (Fig. 1).

For the experiment we used a loam soil (32% sand, 46% silt, 22% clay, 6.4% organic matter, 60% water holding capacity) collected from a bare agricultural field in Ontario County, New York, USA. All soil was passed gently through a 6 mm screen to remove stones, large pieces of organic matter, and other debris. The soil was then defaunated by heating and drying at 65°C for 48 hours, freezing at -20°C for 24 hours, heating again for 24 hours, and freezing again for 24 hours. We chose this method to remove animal life from the soil because it is effective at

eliminating both arthropod (Huhta *et al.*, 1989) and nematode fauna (Franco *et al.*, 2017), and because its effects on soil physical and chemical properties as well as microbial function are reduced compared to other methods such as microwaving (Wright *et al.*, 1989) or autoclaving (Powlson & Jenkinson, 1976). Afterwards, the soil was remoistened to a w/w moisture content of 20% (one third of its field capacity) and allowed to incubate at room temperature for 14 days to allow for some renewal of the microbial community (Cutler & Webster, 2003). During this period, the soil was split into two bins of roughly 13 kg wet weight of soil each, one of which received live soil microarthropods heat-extracted from roughly 3.5 kg wet weight of topsoil and leaf litter collected from the forested edge of a turfgrass lawn in Ontario County, NY. The litter was selected for the ease of extracting large numbers of microarthropods from it. The extractions were carried out into small containers of water, which was transferred directly into one of the two bins of defaunated soil. Though macrofauna such as isopods and insects were extracted with the microarthropods, these were removed from the water before it was poured into the soil, so as to avoid providing alternate hosts for the EPNs. Equivalent volumes of distilled water, lacking fauna, were added to the microarthropod-free control soil. The newly-faunated soil (hereafter referred to as 'fauna soil') was gently mixed to allow the microarthropods to evenly distribute throughout the soil. Soil receiving no fauna (hereafter referred to as 'no-fauna soil') was also mixed in the same fashion. The moisture contents of both soils were continually adjusted to maintain 20% water content by mass. While this arrangement was not meant to recreate an intact and undisturbed soil profile, it did create semi-natural soil conditions retaining the important physicochemical properties present during the interactions of EPNs with hosts and other organisms in soil, such as the presence of soil micro- and macro-aggregates (preserved even

through sieving, see White, 1993), and exposure to organo-mineral complexes and their natural water retention characteristics.

2.2. Insects and EPNs

A native New York strain of *Heterorhabditis bacteriophora* ('Oswego') was obtained from an *in vivo* culture in *Galleria mellonella* maintained at Cornell University, Ithaca, New York, USA. *Galleria mellonella* for the study itself were obtained from a commercial vendor (Grubco, Inc., Fairfield, Ohio, USA) and stored at 4ºC for no longer than 1 week before being placed into arenas.

2.3. Infection trials

We filled all arenas, including the attached cage at the bottom, with approximately 750g wet weight of fauna or no-fauna soil, tamped down such that 7.5 cm of soil lay above the top of the mesh cage. Twelve hours later, 2000 infective juveniles of *Heterorhabditis bacteriophora* were applied to the soil surface of all arenas in approximately 200 µl of pipetted water, a number corresponding to a field application rate of $2.5x10^9$ IJs ha^{-1} (Shields *et al.*, 1999). The arenas were capped with vented plastic lids and allowed to incubate at 22ºC and 65% RH for 16 days before five *G. mellonella* larvae were added to the cage in the bottom of each arena. This sequence of EPN and host additions was meant to mimic an inoculative biological control scenario in which EPNs are released without the pest necessarily being present at high densities at the time of application (Shields *et al.*, 2009). The time delay between EPN inoculation and *G.*

mellonella addition also allowed for greater interaction time between EPNs and soil mesofauna before the introduction of insect hosts.

Galleria mellonella larvae remained in the cages for five days. This duration provided the EPNs adequate time to locate and infect the hosts, but was short enough to prevent that reproduction and emergence of new IJs. Dead larvae removed directly from arenas were left in Petri dishes at room temperature (22ºC) for 48 h before being frozen at -20ºC to halt further development of any EPNs inside. Live larvae were placed on moist filter paper in 12-well plates and monitored twice daily for mortality. Once dead, these larvae were left at room temperature for 72 h before freezing (Mauleon et al., 1993) to ensure that the EPNs inside had ample time to develop into adults, but not enough time to produce offspring.

We used a slight modification (Appendix A) of the pepsin digestion method (Mauleon *et al.*, 1993) to count the nematodes established within each *G. mellonella* larva. The larvae were thawed and placed in 50 mm Petri dishes containing 2 ml of a 0.8% pepsin solution. Their heads were severed, and probes used to extrude the body contents from the cuticle. The cuticle and viscera were then torn and spread around the plate to maximize exposed surface area. The plates were sealed and placed on an orbital shaker at 120 rpm for 1 hour. After this, the adult EPNs within each larva were counted under a dissecting microscope. If a larva showed clear signs of EPN infection, *i.e.*, the brick red color associated with *H. bacteriophora* infection, but no nematodes were counted, then the number of established nematodes in that larva was reported as 1. Two metrics of EPN efficacy were calculated; mean establishment of adult EPNs within *G. mellonella* larvae, and percentage of larvae infected per arena. To calculate mean establishment for each arena, we averaged the number of EPNs inside of all five larvae. The infection trial was

conducted twice, once in May 2017 with 10 replicates of each soil treatment (Trial 1) and once in July 2017 with 15 replicates of each soil treatment (Trial 2).

2.4. Arena community characterization

Immediately after filling the arenas (before the EPN experiments began), all remaining fauna and no-fauna soil was placed onto Berlese funnels and the contained fauna were extracted into 70% ethanol over a 3 day period. Relative abundances were determined for all microarthropods to characterize the two soils before the EPN addition at the start of the experiment. We were unable to determine the exact density (number per unit soil mass) of each taxon that was added to the mesocosms; however, at the conclusion of each trial, following *G. mellonella* removal from the arenas, we placed the soil from all arenas on Berlese funnels and extracted fauna in the same manner. All fauna were examined under a dissecting microscope and the number of collembolans, oribatid mites, and mesostigmatid mites (the three most abundant microarthropod taxa) were recorded. For each microarthropod group for which significant correlations with metrics of EPN efficacy were found, we identified its dominant families in Trial 1 and Trial 2 arenas from a subset of specimens collected from the richest replicates and comprising no less than half of the total specimens of that group within arenas of each trial.

2.5. Statistical analyses

We used unpaired t-tests to assess differences in mean adult EPN establishment within each *G. mellonella* larva and overall percentage of larvae infected between fauna and no-fauna soil

arenas, and between the fauna soil arenas of Trials 1 and 2. Percentage data were arcsine transformed, and EPN count data were square root transformed if necessary to achieve normality before conducting t-tests. If data were not normally distributed even after square root transformation, Wilcoxon rank sum tests were used instead to test for differences. Data from Trials 1 and 2 were analyzed separately.

We also tested for differences in per kg dry soil abundance of each microarthropod group between Trial 1 and Trial 2 arenas with unpaired t-tests or Wilcoxon rank sum tests. Then, to search for relationships between microarthropod abundance and metrics of EPN efficacy, we used linear regression to relate the abundance of collembolans, mesostigmatid mites, and oribatid mites to mean EPN establishment and percentage of larvae infected in the fauna soil replicates of Trial 1 and Trial 2. This was done after confirming normality of the model errors with Shapiro-Wilk tests. All statistical analyses were performed in R (R Core Team, 2014).

Figure 1: One of the experimental arenas used in this study, shown here empty of soil. The cylindrical cage of 1.5 mm aluminum mesh at the bottom of the arena was used to confine five *Galleria mellonella* larvae.

3. Results

3.1. EPN infection and establishment

We observed significantly lower adult EPN establishment in hosts from fauna soil than in those from no-fauna soil in both trials, from a median of 6.2 adults per host with an interquartile range (IQR) of 4.2-8.5 in the no-fauna soil to a median of 1.0 adults per host with an IQR of 0.8-2.7 in the fauna soil of Trial 1 (Wilcoxon rank sum test, $W = 7.5$, $n = 48$, $p = 0.004$), a decrease of 84% (Fig. 2a). The difference was again significant in Trial 2, wherein no-fauna soil arenas saw a mean of 6.6 adults per host with an IQR of 4.0-8.8 compared to a median in fauna soil arenas of 3.8 adults per host with an IQR of 2.9-5.6 (unpaired t-test, $t = 2.391$, $df = 28$, $p = 0.024$), a decrease of 42% (Fig. 2b). A significantly lower percentage of *G. mellonella* larvae were infected with EPNs in fauna soil than in no-fauna soil in Trial 1, decreasing from a median of 100% infection with an IQR of 95-100% in the no-fauna soil to a median of 80% infection with an IQR of 45-80% in the fauna soil (Wilcoxon rank sum test, $W = 6$, $n = 18$, $p = 0.001$) (Fig. 3a). Percentage of larvae infected did not differ significantly between treatments in Trial 2 (Wilcoxon rank sum test, $W = 105$, $n = 30$, $p = 0.577$) (Fig. 3b). The fauna soil arenas of Trial 2 had significantly greater EPN establishment than those of Trial 1 (Wilcoxon rank sum test, $W = 127$, $n = 25$, $p = 0.003$), and a significantly higher percentage of larvae were infected (Wilcoxon rank sum test, $W = 140$, $n = 25$, $p < 0.001$).

3.2. Microarthropod communities

Average abundances of collembolans, mesostigmatid mites, and oribatid mites in the fauna-soil arenas at the conclusion of Trials 1 and 2 are given in Table 1. In Trial 1, oribatid mites and collembolans dominated the arenas, with median abundances of 41.7 (interquartile range 27.9-46.3) collembolans and 40 (interquartile range = 36.7-57.9) oribatid mites per kg dry soil. Mesostigmatid mites were present at a median abundance of 2.5 (interquartile range = 0.4-7.5) individuals per kg dry soil. Their relative abundances (49% Collembola, 5% Mesostigmata, and 48% Oribatida) closely matched that of the microarthropods extracted from the leftover soil at the beginning of the experiment (47% Collembola, 3% Mesostigmata, and 50% Oribatida). In Trial 2, collembolans were dominant, with a median abundance of 50.4 (interquartile range = 36.1-82.5) per kg dry soil, significantly higher than in Trial 1 arenas (unpaired t-test, $t = -2.166$, $df = 23$, $p = 0.041$). Oribatid mite abundance was much lower than in Trial 1 at a median of 3.5 (interquartile range = 1.8-10.9) per kg dry soil (Wilcoxon rank sum test, $W = 150$, $n = 25$, $p < 0.001$), and mesostigmatid mite abundance was similar at a mean of 5.0 (interquartile range = 2.6-8.8) per kg dry soil (Wilcoxon rank sum test, $W = 53$, $n = 25$, $p = 0.231$). Again, relative abundance of the three microarthropod groups in the arenas after the trial (86% Collembola, 8% Mesostigmata, and 6% Oribatida) was similar to that of the soil sampled before the trial began (78% Collembola, 9% Mesostigmata, and 13% Oribatida). Heat extraction also confirmed that no microarthropods were present in soils from the no-fauna arenas.

3.3. Microarthropod-EPN interaction

Mesostigmatid mite abundance had a significant negative correlation with mean EPN establishment in Trial 2 (linear regression, $y = -0.158x + 5.373$, $df = 13$, $r^2 = 0.223$, $p = 0.043$)

(Fig. 4), but not in Trial 1. No other significant relationships between microarthropod abundance and metrics of EPN efficacy were found. Identification to the family level revealed that the mesostigmatid community of Trial 1 soils was composed of two families, Parasitidae and Pachylaelapidae. In Trial 2 soils, only Pachylaelapidae was observed.

Microarthropod Taxon	Trial 1 Abundance	Trial 2 Abundance
Collembola	37.2 ± 5.5a	57.5 ± 6.8b
Mesostigmata	4.5 ± 1.6a	6.7 ± 1.7a
Oribatida	44.5 ± 5.5a	6.1 ± 1.4b

Table 1: Per kg dry soil abundance (mean ± S.E.) of the three most prevalent soil microarthropod taxa, collembolans, mesostigmatid mites, and oribatid mites in both Trial 1 and Trial 2 fauna-soil arenas. Different letters indicate differences in abundance between trials significant at the $p < 0.05$ level.

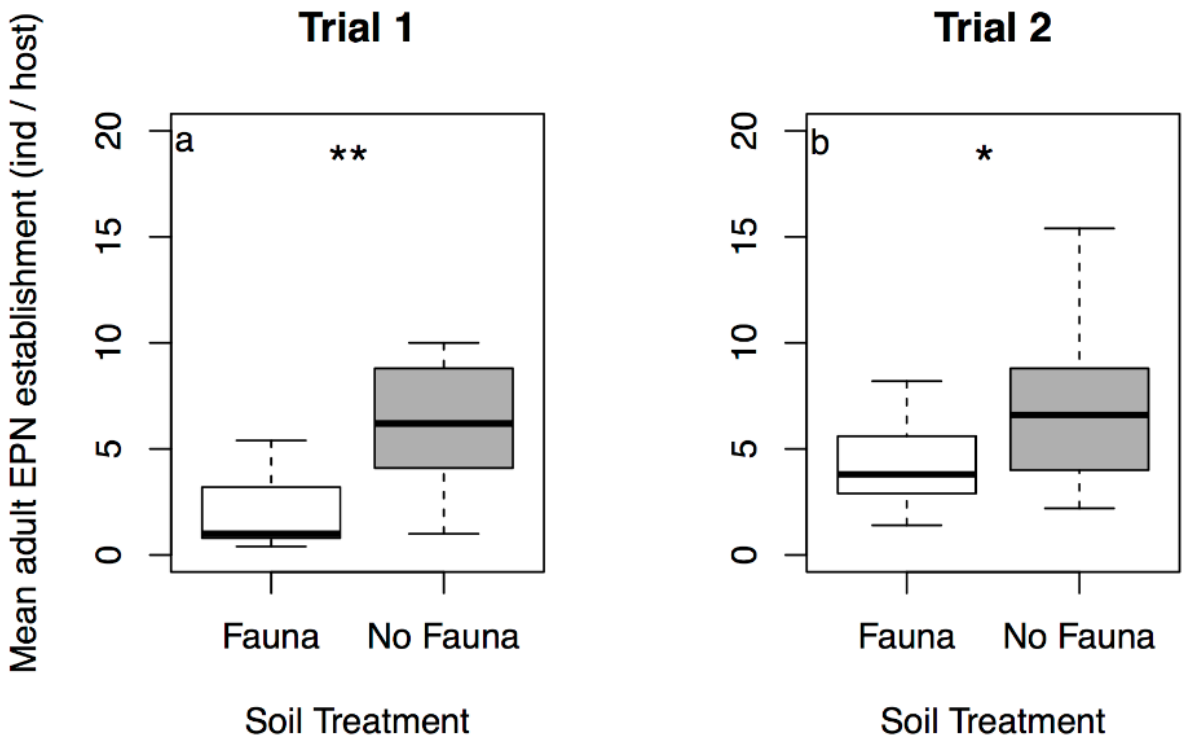

Figure 2: Boxplots showing establishment of adult EPNs per *Galleria mellonella* larva in fauna soil (white) and no-fauna soil (gray) arenas of Trial 1 (a) and Trial 2 (b). Boxes represent the third quartile (75th percentile), median (50th percentile) and first quartile (25th percentile), with upper whiskers reaching Q3 + 1.5 × interquartile range (IQR) and lower whiskers Q1 − 1.5 × IQR. Outliers beyond these limits are plotted as individual points. Asterisks indicate differences between soil treatments significant at the $p < 0.05$ (*) or $p < 0.01$ (**) levels, according to unpaired t-tests or Wilcoxon rank sum tests.

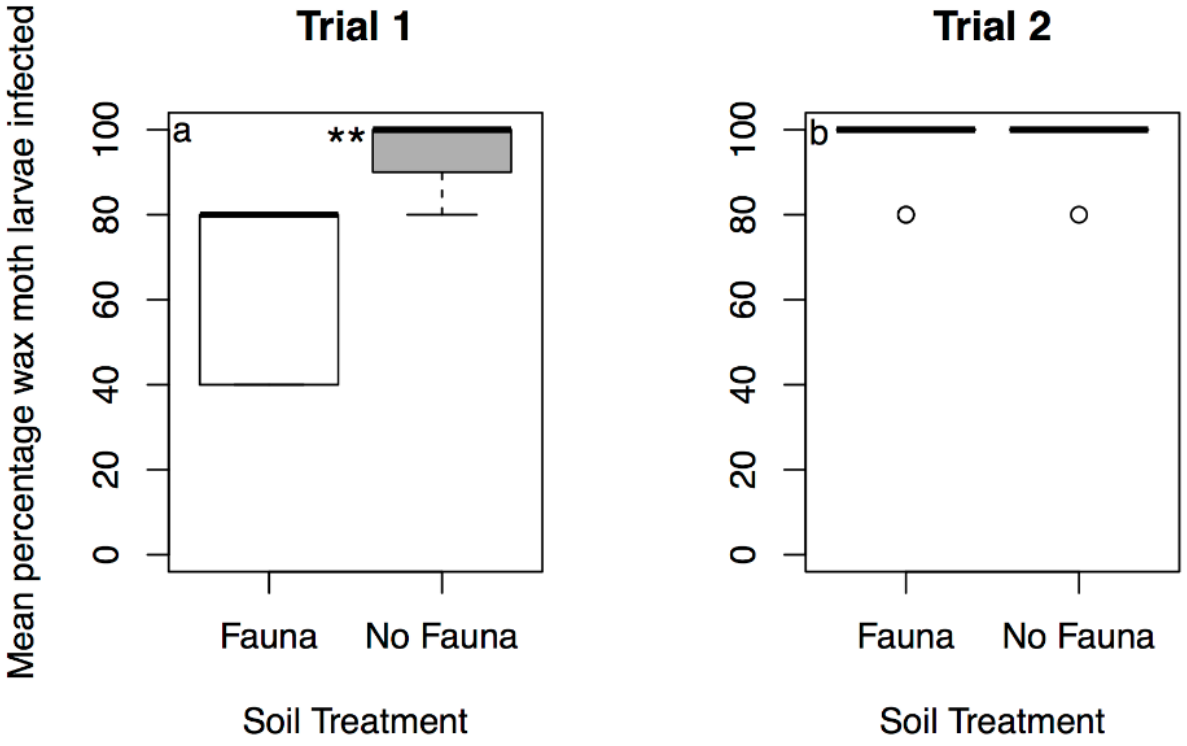

Figure 3: Boxplots showing percentages of *Galleria mellonella* larvae infected by *Heterorhabditis bacteriophora* EPNs per arena in fauna soil (white) and no-fauna soil (gray) for Trial 1 (a) and Trial 2 (b). Boxes represent the third quartile (75th percentile), median (50th percentile) and first quartile (25th percentile), with upper whiskers reaching Q3 + 1.5 × interquartile range (IQR) and lower whiskers Q1 − 1.5 × IQR. Outliers beyond these limits are plotted as individual points. Asterisks indicate differences between soil treatments significant at the $p < 0.01$ (**) levels, according to unpaired t-tests or Wilcoxon rank sum tests.

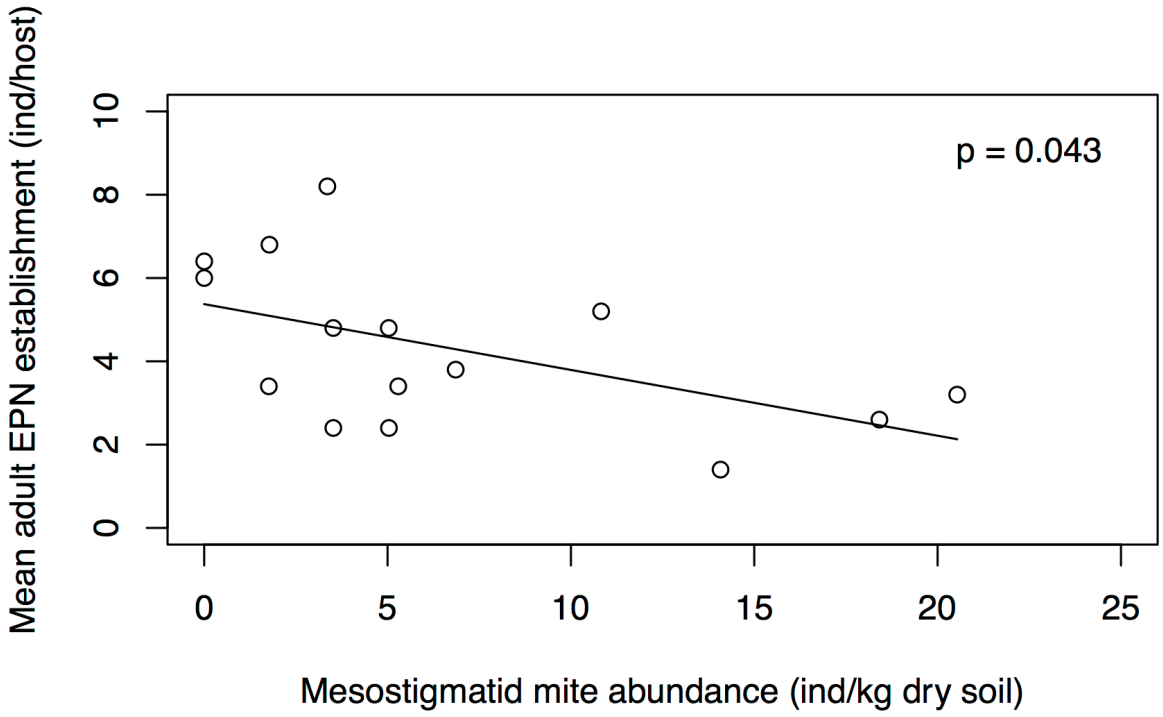

Figure 4: Scatterplot relating the per kg dry soil abundance of mesostigmatid mites to mean adult EPN establishment within larvae of the wax moth *Galleria mellonella* in Trial 2 arenas.

4. Discussion

Presence of soil microarthropods significantly reduced EPN infection of *G. mellonella* larvae, both in terms of percentage of larvae infected and number of EPN adults established within each larva. In addition, mesostigmatid mites were negatively correlated with EPN establishment during the second trial. We demonstrated an association between microarthropods and reduced infectivity of EPN populations even within environments containing some of the complexity of field soils, though not to the extent observed in previous soil-free experiments (Epsky *et al.*, 1988; Gilmore & Potter, 1993). Though the sieved soil in our arenas did not completely mimic the structural complexity and heterogeneity of a field soil, the presence of a three-dimensional soil matrix combined with a diverse assemblage of microarthropods places this study on a level between the majority of previous works in non-soil media and the manipulative field experiments potentially achievable through augmentation and/or exclusion of fauna in natural soils (Helmberger *et al.*, 2017).

Our results concur with the general findings of studies by Khan *et al.* (2016), finding lower EPN survival in soils containing a natural biotic community than in sterilized soils, and by Wilson and Gaugler (2004), finding significant negative correlations between EPN infection of *G. mellonella* and collembolan and mite abundance in field-collected turfgrass soils. Yet our study differs due to the use and characterization of diverse and variable microarthropod communities, which allow for multiple ecological interactions to potentially take place and give us some ability to tie EPN response to specific taxonomic groups. Our results are also unique in that they show that in addition to infection rates, microarthropods may also reduce the number of EPNs able to invade and establish within an insect host. While these differences in invasion and

establishment did not always result in different infection rates of the relatively susceptible host *G. mellonella* (Li *et al.*, 2007), many pests targeted by EPNs are naturally soil-dwelling and to some degree resistant to EPNs (Peters & Ehlers, 1997; Koppenhöfer & Fuzy, 2006). When attempting to manage a pest, differences in the size of the 'invading force' of EPNs may be more likely to result in hampered biological control over relatively short time scales like the one in our study.

Although it is difficult to know for certain which types of ecological interactions led to the observed decrease in EPN establishment and infection, as with Khan *et al.* (2016), the overall negative effect of the community's presence suggests that the predominant interactions between *H. bacteriophora* and the microarthropods were detrimental to the EPNs (*e.g.*, predation). Mesostigmatid mites are primarily predators (Koehler, 1999), and some specialize on nematode prey (Moore *et al.*, 1988; Walter & Ikonen, 1989). Collembolans and oribatid mites are more generalist feeders, and many species will readily consume nematodes, including EPNs (Epsky *et al.*, 1988; Gilmore & Potter, 1993; Heidemann *et al.*, 2011, 2014). In addition, both oribatid and mesostigmatid mites have also been shown to be able to phoretically disperse EPNs to hosts in simplified settings (Epsky *et al.*, 1988), though neither Epsky *et al.* (1988) nor Gilmore and Potter (1993) observed phoresy of EPNs by springtails. This is noteworthy because of the overwhelming dominance of springtails in the fauna soil arenas of Trial 2 compared to Trial 1, where this was not the case (Table 1). If phoresy by microarthropods does indeed occur in soils as well as non-soil media, as suggested by the active aggregation behavior of EPNs upon the backs of mites observed by Epsky *et al.* (1988), then the potential for phoresy in Trial 2 arenas would have been less than in Trial 1. Yet despite this, EPNs in the fauna soil of Trial 2 performed significantly better than they did in Trial 1 by both of our assessed metrics. This

suggests that phoresy is perhaps not a major influence on the ability of EPNs to locate and infect hosts, at least at the spatial and temporal scales present in our study, or when considering phoresy specifically by microarthropods. Other potential phoretic hosts, especially macrofauna such as isopods and earthworms (Eng *et al.*, 2005; Shapiro-Ilan & Brown, 2013), may be more relevant due to their larger size, faster movement, and ability to burrow through soil rather than being confined to existing pore spaces (Stork & Eggleton, 1992).

Despite the imperfect mimicry of natural soil conditions, the abundance of microarthropods we observed in our arenas (Table 1) was comparable to (and in some cases lower than) their typical abundances in agricultural and natural soils (Carter *et al.*, 2009; Soong *et al.*, 2016). Thus, interactions with soil microarthropods may be one of the factors contributing to the variability in EPN field performance often reported in the literature (Georgis & Gaugler, 1991; Georgis *et al.*, 2006), even in heavily managed ecosystems with moderate-to-low microarthropod abundance.

Microarthropod abundance was also directly correlated with our metrics of EPN efficacy in one case. Mesostigmatid mites were negatively correlated with adult EPN establishment in Trial 2 (Fig. 4), but not in Trial 1. This may be due to differences in the taxonomic composition of the mites or differences in the composition of the overall community and alternate prey available to the predatory mesostigmatids in the two trials. Pachylaelapids, which were the only family observed in Trial 2 soils and one of the two present in Trial 1, consume both nematode and arthropod prey, though many species are specialized nematophages (Walter & Ikonen, 1989). In contrast, parasitid mites, which were observed only in Trial 1 soils, have been found to prey heavily upon insects and microarthropods (Koehler, 1999), although they will also consume nematodes (Muraoka & Ishibashi, 1976; Walter, 1988). Specific data on the feeding preferences

of mites in situations where multiple foods are available at the same time are known for only a few mesostigmatid species (Walter & Ikonen, 1989), but it is still possible that the community of mesostigmatid mites in Trial 2 soils was one more inclined to consume nematodes and thus more likely to be a significant influence on EPN efficacy.

In our study, the effects of microarthropods on EPN infection rates were observed after 16 days of the EPNs lacking any insect host to infect. In trials similar to ours, Ulug et al. (2014) applied IJs of the EPN *Steinernema feltiae* to soils with and without the scavenging and predatory astigmatid mite *Sancassania polyphyllae*. Their trial also differed from ours in that the *G. mellonella* host insects were already present in the soil when the EPNs were applied, and there was no significant effect of mite presence on EPN invasion of *G. mellonella* larvae, even though *S. polyphyllae* is known to consume EPN IJs (Karagoz *et al.*, 2007), albeit most commonly whilst scavenging on an infected cadaver (Ekmen *et al.*, 2010). When hosts are readily available near where EPNs are applied, IJs may be able to successfully 'run the gauntlet' of predators and other natural enemies and escape significant negative effects (as in Willett et al., 2017). Thus, in the case of an inundative application of EPNs to control a pest already present, predation and other detrimental interactions with other soil organisms may be of lesser concern. However, for management strategies seeking to have EPNs recycle within hosts and provide control for an entire season or even over multiple years (Koppenhöfer & Fuzy, 2009; Shields *et al.*, 2009), effects of interactions between EPNs and the broader soil community may be more important for scientists and managers to consider, especially since those effects may vary depending on microarthropod taxonomic group. Thus, a more careful accounting of soil animal community composition may be warranted in some EPN management strategies.

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CHAPTER THREE

Entomopathogenic nematode performance against *Popillia japonica* (Coleoptera: Scarabaeidae) in school athletic turf: Effects of traffic and soil properties

Abstract

Entomopathogenic nematodes (EPNs) have potential as an alternate means of controlling soil-dwelling pests in settings such as school athletic fields, where use of chemical pesticides is often restricted or prohibited. Athletic fields are also unique among turfgrass systems, as their distinct pattern of foot traffic can result in compaction and other soil properties varying across different areas of the field, potentially causing variability in EPN performance even within a single field, as many abiotic and biotic soil properties are known to influence EPN efficacy. We tested the efficacy of the EPNs *Steinernema feltiae* and *Heterorhabditis bacteriophora* against third-instar grubs of the Japanese beetle *Popillia japonica* in high-traffic and low-traffic areas of two New York soccer fields, one grown atop loam soil and the other atop loamy sand. Efficacy was low in the loam soil but modest for both species in the loamy sand. In addition, contrasting effects of traffic were sometimes observed, with *S. feltiae* performing better in low-traffic areas than in high-traffic areas and vice versa for *H. bacteriophora*. Non-metric multidimensional scaling revealed positive associations between efficacy of both EPN species and soil sand content, suggesting that sandy soils may be the most optimal for curative applications against turfgrass pests. These results will aid turfgrass managers with the challenges of the school grounds system.

1. Introduction

School athletic fields pose a unique challenge for turfgrass managers. The need for an even, attractive, and playable surface must be balanced with public pressure and legal requirements to reduce or eliminate pesticide use in public areas used by children (Potter, 2008). In some cases, laws such as New York State's Child Safe Playing Fields Act (Grant, 2011) restrict pesticide use to the point of prohibiting any product registered with and labeled by the United States Environmental Protection Agency (EPA), meaning that even some proven safe products are illegal to use. New York is one of the 39 US states (in addition to the District of Columbia) that impose some level of restriction on pesticide use on school grounds (Koppenhöfer et al., 2015). Whatever the level of restriction, alternative pest management tools must be considered and evaluated for use so that managers are fully informed of the options available to them.

Entomopathogenic nematodes (EPNs), minute roundworms that seek out soil-dwelling pests and kill them with the aid of their symbiotic bacterial pathogens (Lewis and Clarke, 2012), are one such alternative. They are exempted from EPA registration, which combined with their safety for humans and other vertebrates (Lacey and Georgis, 2012) makes them legal to use in almost any management situation, including on school grounds (Koppenhöfer et al., 2015; Potter, 2008). EPNs have proven effective in controlling some turfgrass pests, such as mole crickets (Dolinski et al., 2012; Parkman et al., 1996), and scarab grubs including the Japanese beetle, *Popillia japonica* (Grewal at al., 2005; Lacey and Georgis, 2012). *P. japonica* is a major pest of turfgrass throughout much of the eastern United States (Potter and Held, 2002). Its larvae hatch in midsummer and feed on grass roots throughout the summer, fall, and following spring after overwintering. Severing plant roots causes grass mortality and leads to areas of unsightly,

unstable turf, especially during dry conditions. Grubs also attract nuisance wildlife such as crows, raccoons, and skunks to turfgrass areas, which further damage turf as they peck and dig for grubs (Potter, 2008; Potter and Held, 2002). In typical turfgrass systems, *P. japonica* is commonly controlled through preventative applications of chemical insecticides such as imidacloprid (Potter and Held, 2002). In school turf or other systems where pesticide use is heavily restricted or forbidden, cultural control practices are available to potentially ameliorate grub damage (Crutchfield et al., 1995; Ladd and Buriff, 1979), but EPNs are one of the only options available for curative control. EPNs are especially promising for the control of *P. japonica*, as it is especially susceptible to EPN infection compared to other soil-dwelling turfgrass pests such as other scarab grubs (Koppenhöfer et al., 2006) and late-instar crane fly larvae (Oestergaard et al., 2006).

However, EPN performance is often variable and likely dependent on a wide array of abiotic and biotic soil properties. Soil texture and bulk density influence soil porosity and thus the ability of EPNs to move through soil and locate hosts (Grant and Villani, 2003; Koppenhöfer and Fuzy, 2006; Kung et al., 1990; Portillo-Aguilar et al., 1999), with sandier, more porous soils generally being more conducive to EPN performance. Other abiotic factors, such as temperature (Kung et al., 1991), pH (Kung et al., 1990), and salinity (Thurston et al., 1994) can also affect EPN performance. Biotic factors affecting EPNs are less well understood, though a wide variety of interactions with other soil organisms have been documented, such as predation and phoretic dispersal by soil microarthropods (Epsky et al., 1988; Ulug et al., 2014; Wilson and Gaugler, 2004), minute arthropods that are highly abundant in turfgrass and other soils (Potter et al., 1985; Rochefort et al., 2006). Many of these interactions have been shown to or have potential to enhance or reduce EPN efficacy (Helmberger et al., 2017). This makes EPNs more complicated

to employ than chemical insecticides, which combined with their high cost has limited their adoption on wide scales (Lacey et al., 2015). An understanding of how soil properties affect EPN performance is therefore critical for informing sound management decisions and increasing the adoption of EPNs as a management tool. This is perhaps especially true on athletic fields, on which the uneven distribution of foot traffic across the turf surface may produce within-field variability in soil properties (Carrow and Petrovic, 1992), most likely in compaction but potentially in other abiotic and biotic properties as well.

We conducted field bioassays over two consecutive years to evaluate the efficacy of commercial isolates of the EPNs *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) and *Steinernema feltiae* (Rhabditida: Steinernematidae) against third-instar *P. japonica* grubs in the high- and low-traffic areas of two turfgrass soccer fields. In addition, we sought to relate EPN efficacy to a variety of abiotic and biotic soil properties, including texture, compaction, and abundance of several common soil microarthropod groups, to determine which properties were most strongly associated with EPN efficacy. We predicted that EPN efficacy would vary between differently trafficked areas, and that associations between EPN efficacy and soil properties, such as texture, compaction, and microarthropod abundances, would be found.

2. Methods

2.1. *Site descriptions*

We conducted this experiment at two mixed soccer-lacrosse fields in Geneva, NY. One field lay atop a loam soil (hereafter referred to as ‘the loam field’). The other lay atop a silt loam, though

the play surface itself had been heavily amended with sand for several years prior to this study, turning the soil into loamy sand (hereafter referred to as 'the loamy sand field'). Soil properties of both fields can be found in Table 1. The loam field is one of three adjacent practice fields of a public school district and irrigated two to three times per week as needed. The loamy sand field is on a college campus, aerified and top-dressed with sand several times per year and irrigated up to daily as needed. Though this field was not subject to the Child Safe Playing Fields Act, it nevertheless had not received any chemical pesticide applications since the act's passage in 2010 and allowed us to carry out our experiment under very different soil conditions. Pre-experiment soil baiting with *Galleria mellonella* larvae revealed the presence of pre-existing populations of both steinernematid and heterorhabditid EPNs in the soil of both fields, with means of 40% ± 4.1% *G. mellonella* infection at the loamy sand field and 47.5% ± 4.8% infection at the loam field.

2.2. Plot layout and field bioassays

In September of 2016, we laid out nine 1 m^2 plots in 3x3 grids in front of both goals (high-traffic areas) and in both corners of the side opposite the player benches (low-traffic areas), with 2 m buffer spacing between plots, for a total of 36 plots at each field. Four soil-filled 1.5mm aluminum mesh sleeves containing one third-instar *Popillia japonica* grub each were buried in each plot. The sleeves were approximately 10cm long and installed just under the soil surface, confining grubs to the rhizosphere. To install each sleeve, a 2 cm diameter by 10 cm depth core was taken from the soil, which was then used to backfill the sleeve with the grub positioned halfway between the top and bottom of the sleeve. Grubs were collected from a golf course

fairway in Fulton, NY, and held at room temperature in boxes of sod until needed. Afterwards, three randomly selected plots of each grid received aqueous applications of the EPN *Steinernema feltiae* (BioLogic, Willow Hill, PA, USA) and three plots were sprayed with the EPN *Heterorhabditis bacteriophora* (BioLogic, Willow Hill, PA, USA). Prior to application, the EPNs were examined in water under a dissecting microscope to confirm viability. The EPNs were applied at a rate of $2.5*10^9$ IJs ha^{-1} with a handheld sprayer with three XR TeeJet 8004VS flat spray nozzle tips (Spraying Systems Co., Wheaton, IL, USA) mounted on a 1 m boom. The remaining plots received no EPNs, for a total of six *H. bacteriophora*, six *S. feltiae*, and six control plots in each traffic area of each field. At the time of application, mean volumetric soil water content was as follows: loam field, high traffic, 13.4%; loam field, low traffic, 16.6%; loamy sand field, high traffic, 42.8%; loamy sand field, low traffic, 47.7%. All plots were watered immediately after applying EPNs with 0.25cm of water from the fields' irrigation systems, to assist the EPNs with their entry into the soil profile. Sleeves were removed five days after EPN application, and dead grubs were placed onto White traps (White, 1927) to monitor for IJ emergence. Live grubs were placed onto moist filter paper in 12-well plates, monitored for mortality, and placed onto White traps if death occurred within 96 h. White traps were examined for signs of EPN infection 4, 6, 8, 10, 12, and 14 days thereafter and a percentage of infected grubs was calculated for each plot. The few grubs missing from their sleeves were counted as uninfected.

In September of 2017, we repeated the September 2016 field bioassay, except that five sleeved third-instar grubs were buried in each plot instead of four. In addition, the plots were again randomly distributed, but then adjusted to ensure that every *H. bacteriophora* plot was located adjacent to an *S. feltiae* plot. The plots were not placed in the exact same locations as

they were in 2016, as the managers of each field did not set up the goals in the exact same places. At the time of EPN application, mean volumetric soil water content was as follows: loam field, high traffic, 35.1%; loam field, low traffic, 33.2%; loamy sand field, high traffic, 54.2%; loamy sand field, low traffic, 50.6%.

2.3. *Soil analysis*

Concurrent with the EPN field bioassays, we collected five soil cores (2 cm diameter x 10 cm depth) from each of the six control plots (in 2016) in the high- and low-traffic areas of each field. The cores from each plot were combined and placed on Berlese-Tullgren funnels for five days to extract arthropod fauna. Arthropods were extracted into 70% ethanol, and the numbers of collembolans, oribatid mites, mesostigmatid mites, and astigmatid mites were counted. Uropodid mites (Mesostigmata: Uropodina) were counted separately from other Mesostigmata due to their distinct ecological role as phoretic predators and fungivores (Walter and Proctor, 2013). After microarthropod extraction, the cores were removed from the funnels, sieved, and dried at 80ºC. From each set of combined cores, 5 g of soil were used to gravimetrically assess soil water holding capacity, 5 g were used to assess soil organic matter content via loss-on-ignition analysis (Davies, 1974), and 50 g were used to determine soil texture (percent sand, silt, and clay content) with the hydrometer method (Sheldrick and Wang, 1993).

During the September 2017 grub bioassay, we took the same number of cores, though this time they were extracted from the *H. bacteriophora* plots, and extracted arthropods in the same manner. Soil texture was also determined independently in 2017 to account for movement of the plots due to the field managers placing the goals in different locations. In addition, we

took penetrometer readings to a depth of 10cm to assess soil compaction in every control plot (2016) or every plot (2017).

2.4. *Statistical analyses*

For the infection data from the September 2016 and 2017 bioassays, we used one-way ANOVA or Kruskal-Wallis rank sum tests if normality or homogeneity of variance could not be confirmed to test the effects of EPN treatment on percent infection of white grubs relative to the control in each traffic area of each field during each year (eight separate analyses). Percentage data were arcsine square root transformed before analysis. If we found significance in a given analysis, we then used Tukey's Honest Significant Difference tests to determine which treatments significantly differed from one another. Then, to determine the specific effect of traffic on each EPN species separately, we compared each one's percent infection between high- and low-traffic areas using unpaired t-tests or Wilcoxon rank sum tests when necessary. We also tested for differences in soil properties between high- and low-traffic areas of each field using unpaired t-tests or Wilcoxon rank sum tests when necessary. These statistical analyses were performed in R (R Core Team, 2014).

To link the efficacy of each EPN species in the 2017 trial to the abiotic and biotic soil properties measured above, we used non-metric multidimensional scaling (nmMDS). The ordination was run using soil properties (sand content, silt content, clay content, compaction, and abundance of collembolans, oribatid mites, mesostigmatid mites (excluding Uropodina), uropodid mites, and astigmatid mites) as main matrix values. The ordination was structured using a Sorensen distance measure, 500 model iterations, and an instability criterion setting of

0.00005. Data were relativized to the maximum value for each main matrix factor before analysis. To evaluate which factors exerted the greatest influence over the ordination, and to identify relationships between soil characteristics and entomopathogenic nematode infection, we included all original soil traits along with infection data from *H. bacteriophora* and *S. feltiae* within a secondary analysis matrix, obtaining Pearson correlation coefficients for each EPN species by relating the arcsine square root transformed EPN infection percentage data to the scores of each axis via linear regression. *H. bacteriophora* data were paired with the soil property data from within the same plot, and the data from each *S. feltiae* plot were paired with soil property data from the nearest (always adjacent) *H. bacteriophora* plot, except for compaction, which was measured in both *H. bacteriophora* and *S. feltiae* plots and then averaged to produce a single value. This analysis included data from each traffic area of both fields together, and was performed in PC-ORD (McCune and Grace 2002).

3. Results

3.1.1. EPN efficacy – Loamy sand field

In the low-traffic area of the loamy sand field, EPN treatment increased EPN infection of white grubs in 2016 (one-way ANOVA, $F_{2,15} = 4.785$, $p = 0.025$) and 2017 (one-way ANOVA, $F_{2,15} = 4.978$, $p = 0.022$). In 2016, *Steinernema feltiae* infected a mean of 54.2% of grubs in the low-traffic area, significantly more than were infected in control plots (Tukey's HSD test, $p = 0.023$) (Figure 1A). In 2017, *S. feltiae* infected a mean of 36.7% of grubs, again significantly more than in control plots (Tukey's HSD test, $p = 0.022$), and *Heterorhabditis bacteriophora* infected

33.3% of grubs, marginally more than in control plots (Tukey's HSD test, $p = 0.092$) (Figure 1C).

In the high-traffic area, only a marginal difference from the control was found for *H. bacteriophora* in 2016, with a mean infection rate of 45.8% (Tukey's HSD test, $p = 0.067$). EPN treatment had only a marginal effect on EPN infection of white grubs in 2016 (one-way ANOVA, $F_{2,15} = 3.170$, $p = 0.071$) (Figure 1B), and no significant effect in 2017 (one-way ANOVA, $F_{2,15} = 0.184$, $p = 0.834$) (Figure 1D). However, the lack of significance in 2017 was likely due to the abnormally high infection rates found in the control plots (Figure 1D).

Steinernema feltiae efficacy was significantly higher in the low-traffic area of the field than in the high-traffic area in 2016 (Wilcoxon rank sum test, $W = 2$, $n = 12$, $p = 0.009$), but not in 2017 (Wilcoxon rank sum test, $W = 13$, $n = 12$, $p = 0.451$). *H. bacteriophora* efficacy was not significantly affected by traffic in either year, though it did trend toward higher efficacy in the high-traffic area than in the low-traffic area in September 2016.

3.1.2. EPN efficacy – Loam field

In the low-traffic area of the loamy sand field, there was no significant effect of EPN treatment on EPN infection in either year of the study (Figure 2A,C), though grub infection with *S. feltiae* had a mean as high 41.2% in 2016.

In the high-traffic area, we found a marginal effect of EPN treatment in 2017 (one-way ANOVA, $F_{2,15} = 3.117$, $p = 0.074$), with *H. bacteriophora* efficacy marginally higher than that of the control with a mean of 20.0% (Tukey's HSD test, $p = 0.066$) (Figure 2D), but not in 2016 (Kruskal-Wallis rank sum test, $\chi^2 = 0.386$, $df = 2$, $p = 0.824$) (Figure 2B).

H. bacteriophora efficacy was marginally higher in the high-traffic area of the field than in the low-traffic area in 2017 (Wilcoxon rank sum test, W = 28, n = 12, p = 0.091). No other significant effects of traffic on EPN efficacy were found for the loam field.

3.2. Soil properties

Most of the measured abiotic soil properties differed between the two fields but did not vary between high- and low-traffic areas of single fields (Tables 1, 2), with the lone exception of soil compaction in 2016, which was higher in the high-traffic areas of both the loam field (Wilcoxon rank sum test, W = 31.5, n = 12, p = 0.034) and loamy sand field (unpaired t-test, t = 3.0138, df = 6.955, p = 0.020), as well as organic matter content, which was higher in the low-traffic area of the loamy sand field (unpaired t-test, t = -2.601, df = 10, p = 0.026). Soil compaction did not significantly differ between traffic areas of either field in 2017. Soil texture, particularly sand content, did not significantly differ within fields though was obviously distinct between the loam and loamy sand field. The abundance of microarthropods varied between the two fields, and, depending on the taxonomic group, was often lower in the high-traffic areas, with the exception of astigmatid mites, which were more abundant in high-traffic areas in 2016 at both fields and in 2017 at the loam field (Tables 1,2).

3.3. Linking EPN efficacy to soil properties

NMS ordination resulted in a significant 3-dimensional solution with axes 1 and 3 explaining approximately 51 and 24 percent of the soil property variation in ordination space, respectively.

The model had a final stress value of 0.076, and an instability value of 0.00004. Along the first ordination axis, samples separated primarily by field, with all samples from the loamy sand field falling to the right of those from the loam field. These differences between fields were associated primarily with shifts in compaction, sand, silt, and clay content, and oribatid mite abundance. Additionally, within the loam field, samples from high and low-traffic field areas separated along the same axis. Separation along ordination axis 3 (vertical axis) also captured differences among fields and traffic areas of the loam field, however the differences were not as strong as for axis 1. Factors driving separation along this axis also included soil mineralogical traits and compaction as well as the abundance of astigmatid mites. Significant correlations were also found between EPN efficacy and ordination scores. Specifically, infection rates of *S. feltiae* (Pearson R = 0.193, p = 0.018) and *H. bacteriophora* (Pearson R = 0.309, p = 0.003) correlated positively with scores of axis 1, but not axis 3 (Figure 3). Infection rates of both species most closely tracked with increased sand content, and tracked most directly opposite to silt and clay content and soil compaction (Figure 3).

Property	High	Low	t (W)	df (n)	p
Site Classification					
Sand content (%)	86.7 ± 2.2	82.7 ± 1.7	1.518	10	0.160
Silt content (%)	9.0 ± 0.9	12.0 ± 1.2	-2.074	10	0.065
Clay content (%)	4.3 ± 1.5	5.3 ± 0.8	-0.865	10	0.407
WHC (%)	51.3 ± 2.5	54.6 ± 3.0	-0.828	10	0.427
OM content (%)	3.5 ± 0.3	4.3 ± 0.2	-2.601	10	**0.026**
2016					
Soil compaction (psi)	228.8 ± 9.0	162.9 ± 20.0	3.014	10	**0.013**
Collembolans	45.8 ± 16.2	81.2 ± 19.6	-1.393	10	0.194
Oribatid mites	5.9 ± 2.9	17.1 ± 7.6	(9)	(12)	0.171
Mesostigmatid mites (excluding Uropodina)	39.5 ± 14.4	48.65 ± 7.1	-0.567	10	0.583
Uropodid mites	0 ± 0	2.0 ± 2.0	(15)	(12)	0.405
Astigmatid mites	21.5 ± 8.7	2.85 ± 2.0	(34)	(12)	**0.012**
2017					
Soil compaction (psi)	224.4 ± 11.0	204.7 ± 13.0	1.270	34	0.213
Collembolans	65.2 ± 18.7	122.4 ± 36.6	-1.395	10	0.193
Oribatid mites	1.5 ± 1.5	13.9 ± 11.2	(14)	(12)	0.462
Mesostigmatid mites (excluding Uropodina)	50.3 ± 13.4	61.4 ± 9.1	-0.684	10	0.510
Uropodid mites	0.99 ± 0.99	2.4 ± 1.5	(14)	(12)	0.462
Astigmatid mites	21.8 ± 9.4	38.7 ± 12.5	-1.084	10	0.304

Table 1: Soil properties across traffic areas of the loamy sand field. All values are given as means ± SE. Properties assessed in both 2016 and 2017 listed under headers of both years. All microarthropod abundances are given as individuals per kg dry soil. Test statistics refer to differences between high- and low-traffic areas, and were determined with unpaired t-tests or Wilcoxon rank sum tests when necessary. P-values lower than 0.05 are noted in bold.

Property	High	Low	t (W)	df (n)	p
Site Classification					
Sand content (%)	31.6 ± 1.4	37.0 ± 4.6	-1.118	10	0.290
Silt content (%)	48.0 ± 0.9	44.7 ± 3.1	1.040	5.79	0.340
Clay content (%)	20.3 ± 0.7	18.3 ± 1.6	(24.5)	(12)	0.282
WHC (%)	74.0 ± 3.1	73.5 ± 3.3	0.095	10	0.926
OM content (%)	9.2 ± 0.8	7.9 ± 0.5	1.253	10	0.239
2016					
Soil compaction (psi)	253.3 ± 11.2	218.8 ± 11.2	(31.5)	(12)	**0.034**
Collembolans	1.1 ± 1.1	33.0 ± 11.9	(0)	(12)	**0.004**
Oribatid mites	49.4 ± 15.8	88.0 ± 25.0	-1.309	10	0.220
Mesostigmatid mites (excluding Uropodina)	7.4 ± 2.7	61.8 ± 28.1	(2)	(12)	**0.013**
Uropodid mites	40.8 ± 9.2	4.5 ± 3.5	(34)	(12)	**0.012**
Astigmatid mites	12.0 ± 3.7	0 ± 0	(33)	(12)	**0.010**
2017					
Soil compaction (psi)	361.4 ± 8.8	337.2 ± 8.0	(214.5)	(36)	0.098
Collembolans	36.6 ± 12.3	82.2 ± 29.4	-1.43	10	0.183
Oribatid mites	6.9 ± 4.4	69.1 ± 14.3	-4.162	5.94	**0.006**
Mesostigmatid mites (excluding Uropodina)	41.0 ± 14.7	69.8 ± 10.2	-1.609	10	0.139
Uropodid mites	16.1 ± 14.8	4.4 ± 3.0	0.772	10	0.472
Astigmatid mites	220.2 ± 175.2	10.2 ± 7.4	(31)	(12)	**0.028**

Table 2: Soil properties across traffic areas of the loam field. All values are given as means ± SE. Properties assessed in both 2016 and 2017 listed under headers of both years.All microarthropod abundances are given as individuals per kg dry soil. Test statistics refer to differences between high- and low-traffic areas, and were determined with unpaired t-tests or Wilcoxon rank sum tests when necessary. P-values lower than 0.05 are noted in bold.

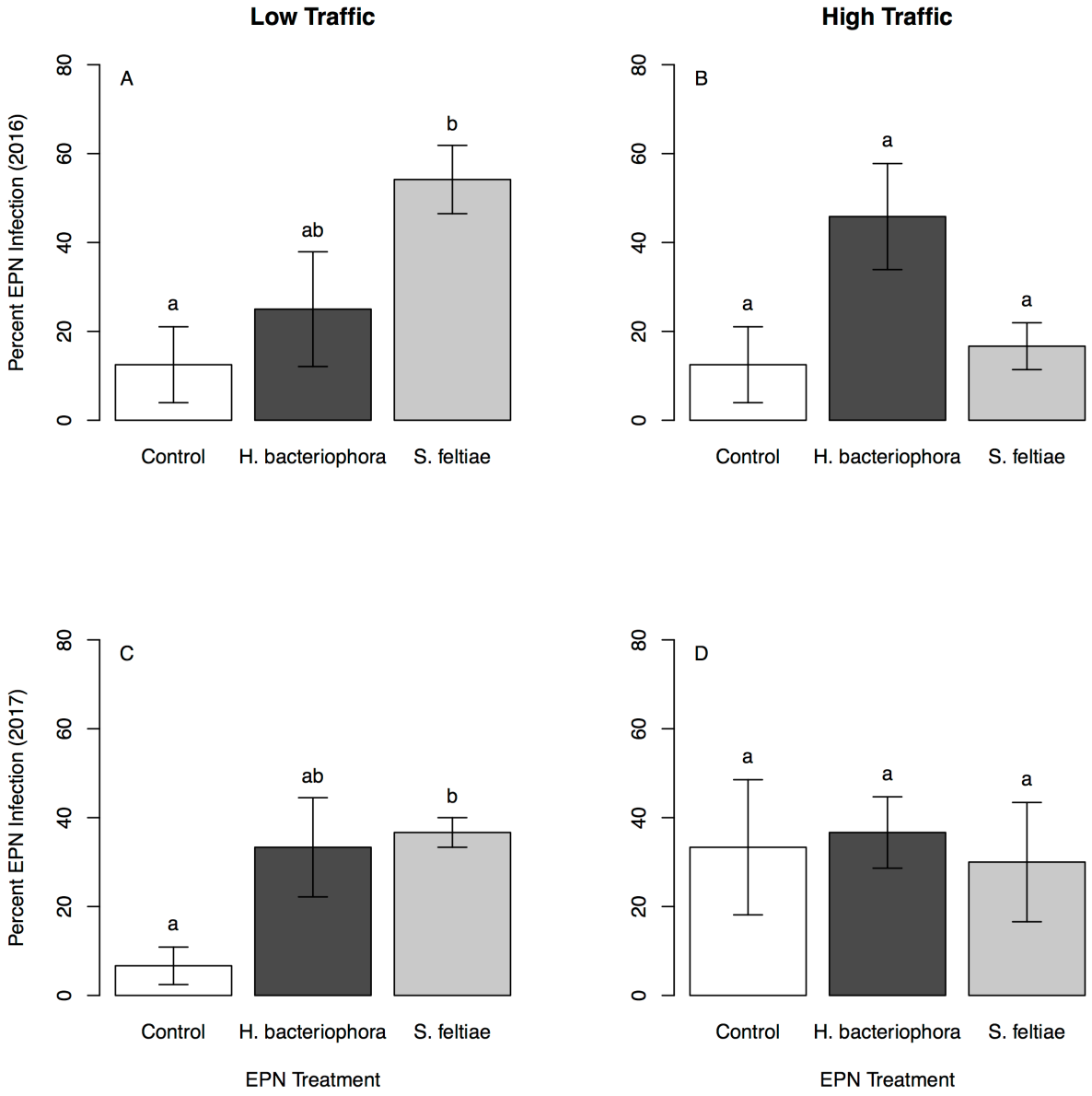

Figure 1: Mean ± S.E. percentages of EPN-infected grubs in turfgrass plots treated with *Heterorhabditis bacteriophora* (dark gray), *Steinernema feltiae* (light gray), or left as controls (white) at the loamy sand field. Data shown for 2016 (top; A,B) and 2017 (bottom; C,D), and for the low-traffic area (left; A,C) and high-traffic area (right; B,D). Differing letters indicate values significantly different from one another at the $p < 0.05$ level (Tukey's HSD test).

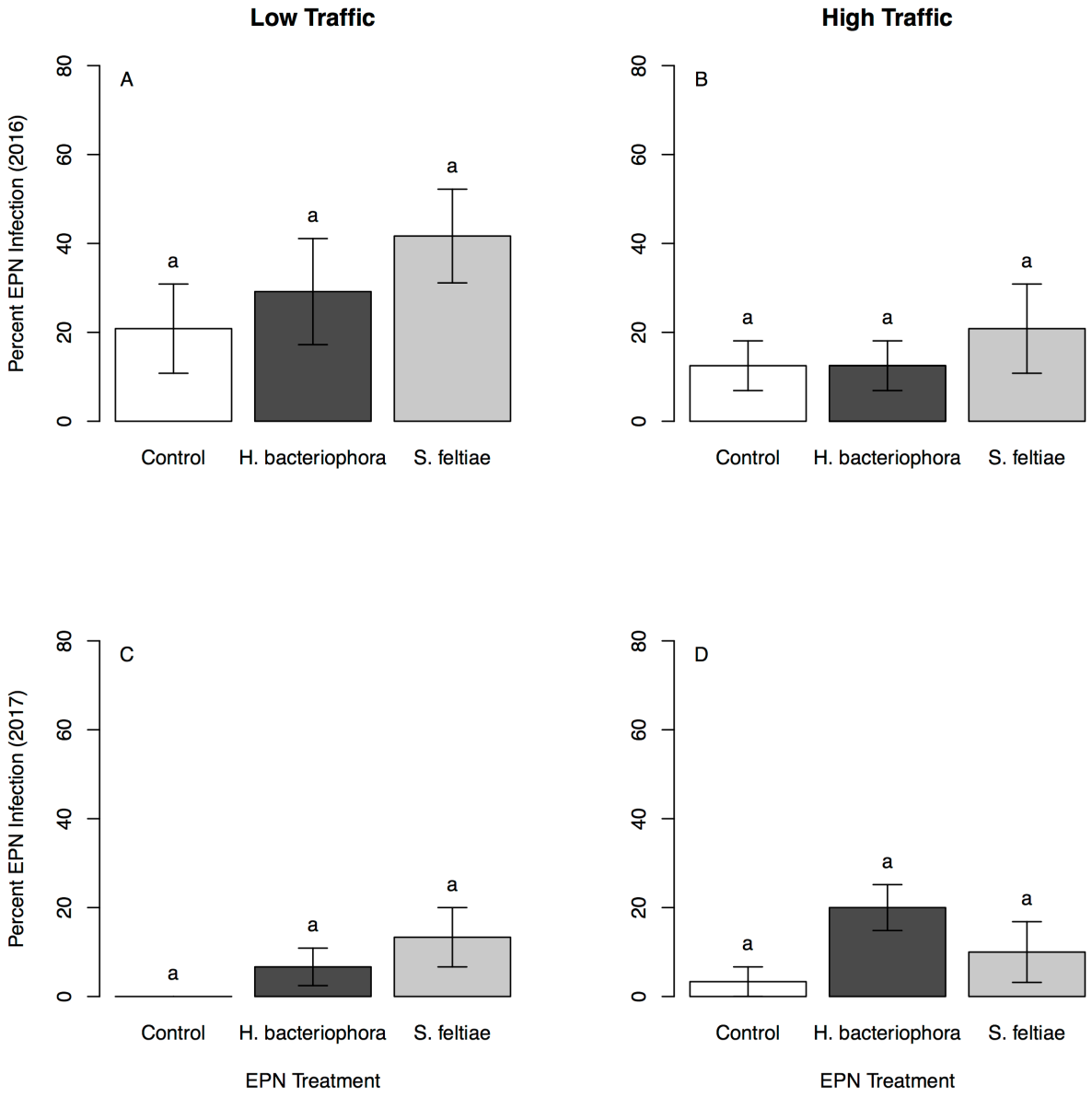

Figure 2: Mean ± S.E. percentages of EPN-infected grubs in turfgrass plots treated with *Heterorhabditis bacteriophora* (dark gray), *Steinernema feltiae* (light gray), or left as controls (white) at the loam field. Data shown for 2016 (top; A,B) and 2017 (bottom; C,D), and for the low-traffic area (left; A,C) and high-traffic area (right; B,D). Differing letters indicate values significantly different from one another at the $p < 0.05$ level (Tukey's HSD test).

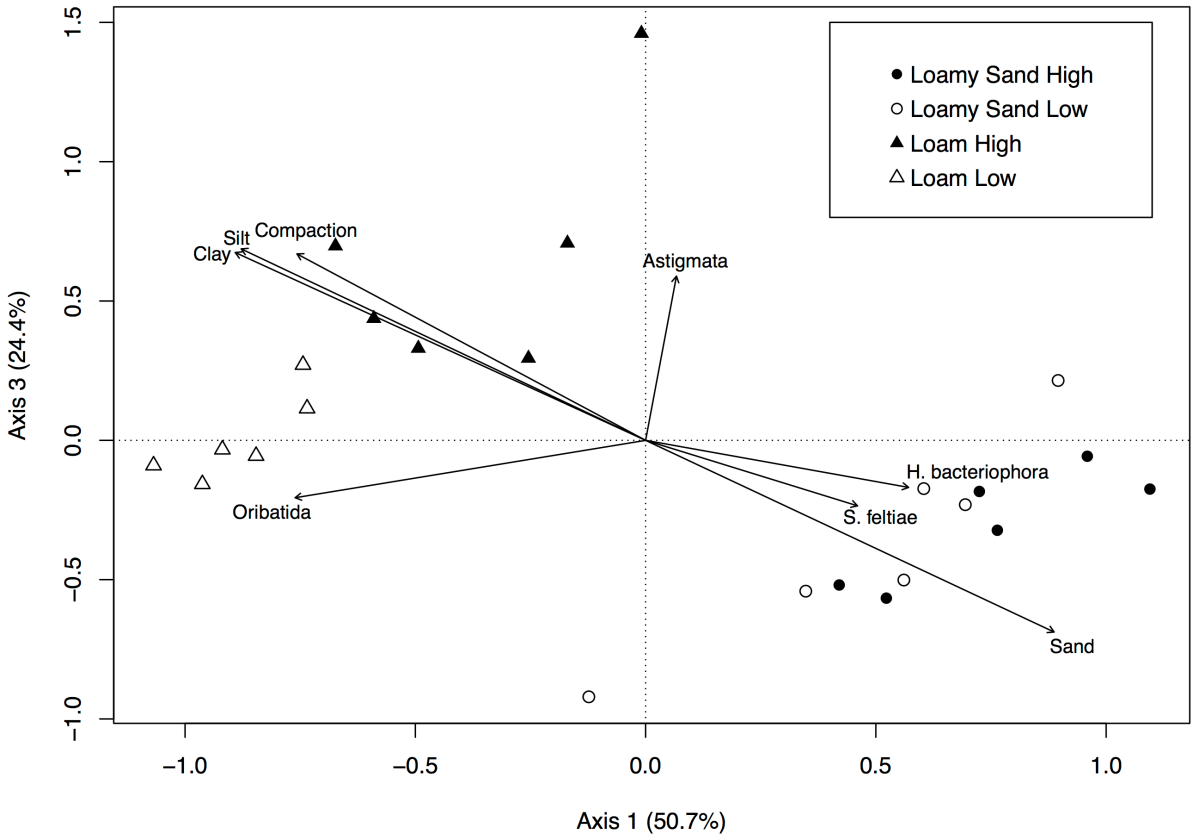

Figure 3: Non-metric multidimensional scaling (NMS) ordination of all plots in the low-traffic areas (open shapes) and high-traffic areas (closed shapes) of the loam field (triangles) and loamy sand field (circles). Vectors indicate abiotic or biotic soil properties, including EPN infection rates, significantly correlated with either axis of the ordination.

4. Discussion

Our results indicate that EPNs can provide modest control of third-instar *P. japonica* grubs in optimal soil conditions, and that their efficacy can vary across the differently trafficked areas of school athletic fields. However, the levels of efficacy we observed for *Heterorhabditis bacteriophora* fell short of typical levels reported in the literature (Grewal et al., 2005), and indeed, only marginal differences were found between EPN infection rates in *H. bacteriophora* plots and control plots. Interestingly, *Steinernema feltiae* performed as well as *H. bacteriophora*, if not slightly better in some circumstances, and achieved control near to the highest levels observed in other studies (Alm et al., 1992; Kard et al., 1988), even those applying *S. feltiae* at much higher rates (Alm et al., 1992). However, in other circumstances, the application of commercial EPN products failed to increase infection rates above those resulting from the naturally occurring EPN populations of the fields (assessed in the control plots). The infection potential of these natural populations varied over space and time, and despite high infection rates observed during initial pre-sampling with *G. mellonella*, was usually low in the main field bioassays with *P. japonica* grubs, with the exception of in the high-traffic area of the loamy sand field in 2017. The cause of this spike is unclear, as is the reason why additional EPN applications failed to increase grub infection despite doing so in the low-traffic area of that field.

The unique usage of our turf system, *i.e.* the pattern of foot traffic manifesting itself in high- and low-traffic areas of the field, produced differences in EPN efficacy in some circumstances. *Steinernema feltiae* performed significantly better in the low-traffic area of the loamy sand field in 2016, though no such effects were observed in 2017. The effects of traffic on *Heterorhabditis bacteriophora* were less certain, though it tended to perform better in the high-

traffic areas, at least those of the loamy sand field in 2016 and those of the loam field in 2017, despite a negative effect of soil bulk density on *H. bacteriophora* survival observed in a laboratory study (Portillo-Aguilar et al., 1999). These differences were observed even in the loamy sand field despite the fact that sandy soils are often better at resisting the compacting effects of foot traffic than are other soils, due to stronger bridging of the mineral grains (Bingaman and Kohnke, 1970). The fact that there was no significant difference in soil compaction in 2017 between high- and low-traffic areas of either field (even with higher replication of measurements) could explain the lack of significant traffic effects on EPN efficacy in that year.

When EPN efficacy was significant along the axes of our 2017 ordinations, it was most positively associated with soil sand content, for both EPN species. EPNs often perform best in sandy soils (Duncan et al., 2013; Kung et al., 1990), though this is not always the case, even for the cruise-foraging *H. bacteriophora* (Koppenhöfer and Fuzy, 2006). In our study, EPN efficacy was distinctly higher in the sandier soils of the loamy sand field, in accordance with the common finding. Though soil compaction was much higher at the loam field than at the loamy sand field in 2017, more so than in 2016 (Tables 1,2), possibly explaining in full the negative association of EPN efficacy with compaction in our data, the topic is worth discussing in greater detail. Soil compaction and texture are somewhat interconnected, especially in relation to their effects on EPNs, as the problem EPNs face in both compacted soils and fine-grained soils is one of reduced pore size making locomotion and aeration more difficult. In addition, fine-grained soils are more susceptible to traffic-induced compaction than coarse-grained, sandy soils (Bingaman and Kohnke, 1970), making these soils a ‘double whammy’ of reduced porosity in trafficked turf. However, this does not explain the curious trend toward higher efficacy of *H. bacteriophora* in

high-traffic compared to low-traffic areas of fields. Taken together, these results linking EPN efficacy to soil properties suggest that certain turfgrass management practices may provide ways of increasing EPN efficacy in sports fields and other turfgrass systems. For example, soil aerification can decrease surface hardness and increase water infiltration rates (Atkinson et al., 2012; Brown et al., 2016; McCarty et al., 2007), and combined with repeated sand amendments could potentially increase the suitability of an athletic field soil for EPN application over time.

These results, specifically the effects of traffic, are likely generalizable to other turfgrass systems experiencing traffic and compaction, though the magnitude and distribution of compaction in other turfgrass systems may differ from that of soccer fields. It may differ on fields for other sports, such as American football or lacrosse, due to the sports' different play patterns, game frequencies, season lengths, and average player weights. Outdoor event venues and fairway margins on golf courses also experience compaction, but the effects of this compaction on EPN performance may manifest differently, or not at all, from what we observed on soccer fields.

Cost of EPN application is a final, critical factor that must be considered. Prior to the passage of the Child Safe Playing Fields Act, the average New York school system's budget for insect control was small, with a mere 0.06% of total annual expenses (less than USD \$200 yr^{-1} in 2004) spent on insecticides and biological pesticides, a percentage less than one tenth of what the average New York golf course budgeted for insect control (New York Agricultural Statistics Service, 2004). A single application over an entire high school soccer field (roughly 60 by 100m) at a rate of $2.5*10^9$ IJs ha^{-1} would be several times that amount at current EPN market costs. This has the unfortunate result of EPNs likely being too expensive, in many cases, for the type of turfgrass system that needs them most. Any management strategy incorporating inundative

releases of EPNs should thus emphasize spot treatments of the most heavily infested areas rather than whole-field applications, and further research should seek to determine if and to what extent application rates can be lowered while still retaining acceptable control. Alternately, single, inoculative applications of persistent EPN stock (Shields et al., 2009; Shields and Testa, 2017) may provide long-term plant protection over multiple years, as has been shown in other systems such as alfalfa (Neumann and Shields, 2008). Such introductions of EPNs to school athletic fields and other turfgrass areas could potentially segue into efforts to educate school grounds managers (or perhaps even students) in techniques for isolating and mass-rearing the inoculated nematodes (Testa and Shields, 2017), similar to what has been done for smallholder farmers in the developing world (Mohan et al., 2017), so as to maintain a stock for inundative spot treatments or for expanding inoculations to other turf and landscape areas. This could provide a cost-effective and educational means of pest control on school grounds.

To conclude, entomopathogenic nematodes, though perhaps not the ideal pest control solution that restricted school grounds managers are looking for, have potential as a tool in the toolbox when curative applications against Japanese beetle grubs are needed on school grounds, athletic fields or otherwise. The lack of efficacy we observed in suboptimal soil conditions may geographically restrict the regions in which commercial EPN strains will be viable and attractive to managers, but in areas with optimal soils, they may be able to substantially increase pest mortality above the level provided by native populations.

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SUMMARY

Do abiotic properties truly reign supreme?

The work described above shows that although other soil organisms can potentially reduce the efficacy of entomopathogenic nematodes (EPNs) against hosts in laboratory soil mesocosms, abiotic soil properties, particularly soil texture, are the primary drivers of field efficacy in turfgrass systems. Although an unexciting finding from an ecological perspective, the primacy of abiotic properties is perhaps ideal from a management perspective. Turfgrass managers understand many abiotic soil properties and have tools available to manage them; whereas lay knowledge of soil biodiversity is sparse, and even scientists' understanding of how to effectively manage soil communities is limited (Nielsen et al., 2015). Management strategies centering on amendment or remediation of abiotic soil properties would thus be more accessible and more likely to be adopted. However, certain considerations must be taken into account before definitively declaring abiotic factors supreme and biotic interactions irrelevant.

First, turfgrass is only one of the many agricultural, horticultural, and silvicultural systems that EPNs are utilized or studied in (Dolinski et al., 2012; Georgis et al., 2006; Lacey and Georgis, 2012). Of note, the abundance of many soil organisms such as microarthropods is often lower in turfgrass (Potter et al., 1985; Rochefort et al., 2006) than in other soil systems (Giller, 1996). Also, turfgrass species are also not currently known to release specific EPN-attracting chemical signals from their roots. Wang and Gaugler (1998) observed increased EPN attraction to wounded grass roots and posited CO_2 from increased respiration of wounded roots as the cause, though they did not measure VOCs or other exudates and thus their potential role cannot be excluded. Turfgrass in general has not been explored for belowground volatiles in

relation to EPNs to the extent of other systems such as maize (Hiltpold et al., 2010; Rasmann et al., 2005) and citrus (Ali et al., 2013; Willett et al., 2017). Thus, these other soil systems may possess communities with a greater capacity to influence EPN performance against pests.

Second, the experiments described above differed in one key respect: the relative timing of EPN and host introduction to the system. The laboratory mesocosm experiment described in Chapter 2 mimicked a preventative inoculation of EPNs against a pest not yet present, as the *Galleria mellonella* hosts were not introduced into the soil mesocosms until 16 days after the nematodes. The EPNs were thus subject to interactions with the biotic community for an appreciable span of time before they had any ability to 'escape' into the body of a host. The field plot experiment described in Chapter 3 mimicked a curative inundation of EPNs against an existent pest, as the *Popillia japonica* hosts were already in the soil when the EPNs were applied. EPNs thus had only centimeters to decimeters to travel to find a suitable, though defensive (Gaugler et al., 1994), host. The abiotic environment is omnipresent, and when EPNs are applied to a soil, it acts on them immediately. Yet soil organisms, particularly animal predators, may be slower to respond, as they must first sense the presence of the EPNs, then move through the soil toward them, and finally catch, kill, and handle their prey. Thus, while EPNs may be able to 'run the gauntlet' of natural enemies (or find aid from beneficial soil organisms unnecessary) when applied curatively, and successfully infect all the hosts that abiotic conditions allow, situations where the EPNs must persist in the soil for weeks or months between periods of high host abundance may result in greater incidence and impacts of interactions with other soil organisms, both positive and negative.

Future investigators seeking to expand study of biotic interactions affecting EPNs in field settings should thus extend their inquiry to agricultural systems other than turfgrass, and also

consider systems in which persistent EPNs are applied out of a desire for long-term pest suppression rather than curative control of outbreaks. It is in these scenarios that biotic effects are perhaps more likely to be relevant.

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APPENDIX A

Some comments on the use of pepsin digestion to count adult entomopathogenic nematodes in wax moth and scarab beetle larvae, with particular focus on laboratory mesocosm experiments

The digestive enzyme pepsin, in combination with hydrochloric acid, is used to degrade animal tissues for a variety of purposes, including assessing infection by helminths and entomopathogenic nematodes (EPNs). Mauleon et al. (1993) described a procedure to 'digest' EPN-killed insects and count the number of adult EPNs established within. Due to the original description of the method being written in French and due to certain additional insights gleaned from my own intensive use of the method, I believe it is warranted to reproduce the protocol.

100 ml of pepsin solution is made by mixing 0.8 g pepsin powder, 2.3 g NaCl, 2 ml 37% w/w HCl, and 98 ml deionized water. Store the solution at or near 4ºC for no more than one week before using. Place each dead insect in a small Petri dish, ideally one with a snap-on lid. Transfer 2 ml of pepsin solution to the dish, or enough to cover the bottom of the dish. Using probes or other dissecting tools, cut each insect into pieces and spread the viscera over the surface of its dish. This is to maximize the surface area exposed to the pepsin solution. Cover the dishes and place them on an orbital shaker at 120 rpm for 2 h. Afterwards, the guts and hemolymph of the dead insect will be reduced, but any nematodes present inside will be visible. Adults of infecting-generation *Heterorhabditis* spp. are hermaphrodites and are usually large enough to see and even count with the naked eye, though inspection and probing of the insect remains under a dissecting microscope is still useful for discerning EPNs still ensconced in or hidden under any remaining pieces of tissue. *Steinernema* spp. adults are of both sexes: large females comparable in size to heterorhabditid hermaphrodites, and small males that under low

magnification can be confused with IJs, as they are only slightly larger in size. Thus, when assessing infection by steinernematid EPNs, it is recommended to only count female adults (A.M. Koppenhöfer, personal communication).

The timing of the pepsin digestion procedure and the age of the cadavers are critical considerations for ensuring that adult EPNs will actually be present when one begins dissecting an infected cadaver and that next-generation IJs will not confuse the counts. Fortunately, cadavers can be frozen to arrest EPN development at the appropriate stage, allowing for consistent processing of large numbers of cadavers. For wax moth *Galleria mellonella* larvae, Mauleon et. al. (1993) recommends waiting three days after death, and for scarab beetle larvae, two-day-old cadavers are recommended (Koppenhöfer and Fuzy 2006).

However, in many experimental settings, such as laboratory mesocosms, the exact day of an insect's death is not always determinable. Live insects removed from a mesocosm can be placed singly on moist filter paper in a Petri dish, 12-well plate, or other container, and continuously checked for mortality, but insects that are dead when they are removed cannot be so easily aged. One solution is to monitor a mesocosm's complement of insects every day and remove only those that are dead, but this is not possible with all mesocosm designs or experimental protocols, such as when disturbance to the mesocosms must be minimized. In these cases, it is recommended that laboratory mesocosm experiments involving EPNs be preceded by small-scale but careful pilot trials assessing different timing options for insect exposure to the soil and time between insect removal from mesocosms and freezing of cadavers. Variations in soil type and condition, mesocosm design, insect and EPN species used, and relative timing of insect and EPN introduction could all potentially affect the amount of time needed for EPNs to reach and infect the insects. In addition to preliminary tests, a small number of additional

replicates can be built into a formal experiment, and the dead insects removed from these mesocosms can be frozen and dissected first, to ensure that an adequate span of time has passed before freezing the full complement of samples.

The pepsin digestion method has several advantages over the traditional White trap technique. It is usually less labor-intensive, unless hundreds or more EPNs are commonly present in each insect. It also does not require as strict a timetable as White trapping. Insect cadavers can be frozen for any length of time before performing the procedure, and at the same time, many replicates can also be examined in a very short period, especially since I have found that 1 h on the orbital shaker is sufficient time to dissolve tissue. Most importantly, the count data provided by the pepsin digestion method is a more precise and responsive metric of an EPN population's infectivity than the proportion data provided by White trapping and obtaining a simple 'yes/no' of EPN infection for each cadaver. As an aside, the proportion data provided by White trapping is also provided by pepsin digestion as a matter of course, so there is no trade-off in terms of quality of data obtained.

I cannot speak with certainty on pepsin digestion's suitability for bioassays conducted in field settings. However, I can speculate that the added abiotic and biotic complexity of field soils would create a high degree of variability in each insect's time of infection. This would be potentially problematic as the EPNs within the cadavers could be at different stages of development at any given time, which could produce misleading results. Careful vetting of the method would have to be undertaken in advance of any attempt to use it with field-laid insects.

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APPENDIX B

Results of October 2016 *Heterorhabditis bacteriophora* bioassay on school grounds

In October of 2016, 32 days after the September EPN bioassay (Chapter 3), we conducted a second, smaller field bioassay using only *Heterorhabditis bacteriophora* (Arbico Organics, Oro Valley, AZ, USA). The six control plots in each traffic area of the two soccer fields received five sleeved third-instar *Popillia japonica* larvae and were sprayed with *H. bacteriophora*. Sleeves were removed five days later and the grubs treated as in the September bioassays. This was done in response to the relatively poor performance of *H. bacteriophora* in the September bioassay and the suspicion that the commercial product obtained for that bioassay may have been of substandard viability.

We compared the percentages of EPN-infected *P. japonica* grubs in the October 2016 *H. bacteriophora* to the same plots' infection percentages as controls in the September 2016 bioassay with unpaired t-tests or Wilcoxon rank sum tests when necessary. We also compared infection percentages between the high- and low-traffic areas of the two fields. Percentage data were arcsine square root transformed before analysis.

Results of the October 2016 bioassay and comparison to the September 2016 controls are shown in Table 1. At the loamy sand field, there was significantly higher EPN infection in October following *H. bacteriophora* application in both the high- and low-traffic areas. At the loam field, there were no significant differences. *H. bacteriophora* performance did not significantly differ between traffic areas of either field.

Field	Traffic Area	Sep. 2016 Control	Oct. 2016 *H. bacteriophora*	Test statistics	p-value
Loamy Sand	High	12.5 ± 8.5%	56.7 ± 6.1%	W = 2, n = 12	0.011
Loamy Sand	Low	12.5 ± 8.5%	46.7 ± 9.9%	W = 5, n = 12	0.034
Loam	High	12.5 ± 5.6%	13.3 ± 6.7%	W = 19.5, n = 12	0.863
Loam	Low	20.8 ± 10.0%	16.7 ± 8.0%	W = 20.5, n = 12	0.731

Table 1: Percentages of infected *Popillia japonica* grubs in the September 2016 control plots compared to those in the same plots following application of *Heterorhabditis bacteriophora* in October 2016.

EPN infection percentages in the October treated plots were significantly higher than those achieved by the native population in September, and at the loamy sand field, *H. bacteriophora* performed better than it did in September 2016, though still not to the level often achieved by many applications of *H. bacteriophora* reported in the literature (Grewal et al., 2005), even in the optimal soil of the loamy sand field. Our direct use of commercial EPN products for both applications may explain the reduced efficacy compared to other studies, many of which used EPNs obtained from a commercial source and then reared once through *Galleria mellonella* hosts.

In addition, the contribution of the native EPN population cannot be ignored. Infection rates in the plots in September 2016 ranged from 12.5% to 20.8%, and these naturally occurring EPNs were in all likelihood still present in October and thus would have been part of the 13.3%-56.7% infection rates observed following the application of *H. bacteriophora*. Thus, a positive shift in the infectivity of this native EPN population could have accompanied the manual application and contributed to the increased infection of grubs at the loamy sand field. Native *Heterorhabditis bacteriophora* populations are known to perform better in October than in

September in New York State (E.J. Shields, personal communication), due to better temperature and moisture conditions. However, the October application's utter lack of effect at the loam field casts doubt on this hypothesis, at least for the less optimal soil of that field.

References

Grewal, P.S., Koppenhöfer, A.M., Choo, H.Y., 2005. Lawn, turfgrass and pasture applications. In: Grewal, P.S., Ehlers, R.-U., Shapiro-Ilan, D.I. (Eds.) Nematodes as Biocontrol Agents. CABI Publishing, Wallingford, UK. pp. 115-146.

APPENDIX C

The Garden of Her Youth: A poem

The garden of her youth was sweet,
an Elysium hers to tend.
She ate the fruits of mother's toil,
as she grieved its bitter end.

For her garden was a fleeting thing,
doomed too soon to wane,
and the world beyond her garden
loomed a black and dreadful scene
of empty voids and tow'ring cliffs,
and shifting seas between.

Yet her garden now stood dead and done,
claimed by rot and mold.
So she went to brave the unknown world,
with a single seed to hold.

Her quest was long with her garden gone,
and fell creatures stalked the night,
skitt'ring shades with daggers drawn,
and ghosts in deadly flight.

But then, one day, the scent of home
did blossom on the damp Styx air,
And to the scent she did direct
her course and des'prate dare.

There she found a fertile plain
as bright and pure as truth,
and with her seed she grew again
the garden of her youth.