Nematode Vectoring of Bacteriophages in a Spatially Structured Habitat

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Table of Contents

Abstract

Bacteriophages, viruses which infect bacteria, influence ecosystem dynamics at multiple levels through top-down control of bacterial hosts. Yet, little is known about soil phage ecology, where they are thought to be important drivers of bacterial death. Bacterial populations are typically patchy in the soil, making phage dispersal between them critical for phage survival and replication. The porous structure of soil presents a challenge to phages, which must disperse despite the relatively large distances between susceptible host populations, lack of pore connectivity, and adsorption to the soil matrix or non-host biota. Many viruses take advantage of vectors to overcome dispersal challenges both in the soil and elsewhere. For bacteriophages, nematodes may be a suitable vector, as they could provide active and directed transport between bacterial colonies as they forage. Here, we tested the hypothesis that bacterivorous nematodes are vectors for phages, overcoming the barriers of a structured environment to provide active transport between patches of host bacteria. We further hypothesized that spatial structure in resource availability would impact vectoring, with nematodes preferring high-resource bacteria. Finally, we hypothesized that nematodes also disperse hosts to start novel colonies throughout the microcosm. This was tested in two systems: a proof-of-concept on agar, and a compost microcosm as a realistic structured habitat. We inoculated agar and compost microcosms with a wild strain of nematodes (*C. elegans* WN2002) and a soil host-phage pair (*P. putida* and Phi ppu-W11). Nematode vectoring mediated host-phage interactions on agar. Resource enrichment and physical structure in the system modulated this three-way interaction further. Nematodes foraged on high-resource bacteria preferentially, bringing phage with them. While vectoring occurred consistently in agar, it was inconclusive in compost. Overall, this study demonstrates the importance of spatial heterogeneity for species interactions in soil.

General Introduction

Bacteriophages, viruses which infect bacterial hosts, are ubiquitous in the environment, and are thought to be key drivers of bacterial death in systems as diverse as the gut, water, and soil (Williamson et al., 2017; Clokie et al., 2011; Braga et al., 2020). Through top-down controls on bacterial populations, they participate in structuring ecological communities, for example, limiting the spread of various fish and coral diseases (Figure 1) (Danovaro et al., 2008; Hassan et al., 2020). While advances in aquatic phage ecology have led to an increased understanding of ecological interactions in this system, such species interactions and their consequences in other systems are not as well understood (Doss et al., 2017; Kalatzis et al., 2018).

Interest in soil phages has therefore been increasing in recent years. Phage abundance is expected to be even higher in soils than in marine systems, with up to 10^{10} phages/g of soil (Chevallereau et al., 2022; Williamson et al., 2017). The ubiquity of phages in the soil suggests a high rate of phage-driven bacterial death, comparable or higher to rates seen in marine systems (Ashelford et al., 2003; Kuzyakov & Mason-Jones, 2018; Suttle, 2007).

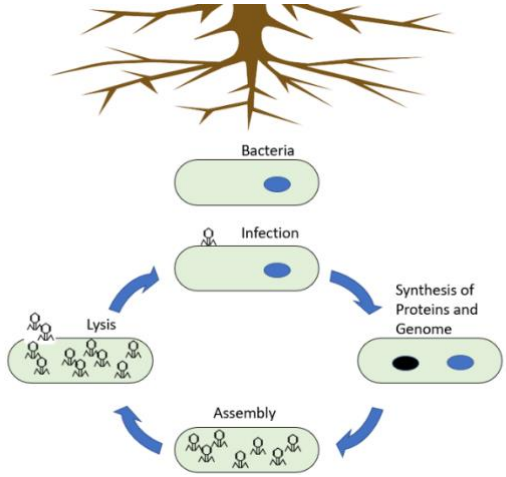

Figure 1. The lytic phage lifecycle. A phage which encounters a susceptible host infects it by injecting its genetic material into the host cell. Then, the host synthesizes many copies of the phage genome and proteins. These assemble inside the host cell into new virion particles until the cell lyses (bursts open) releasing new phages into the surroundings (Doss et al., 2017). Schematic diagrams are not to scale.

However, the soil presents an immediate challenge to free phages, which must overcome a number of barriers to disperse between new host bacteria to avoid degradation and replicate. As phages play a large role in controlling bacterial populations, information about how phages disperse should be crucial to understanding their role in soil microbial communities. For example, in other systems, predator dispersal patterns can greatly impact trophic webs through changing oscillations in predator-prey ratios, which in turn can impact ecological community composition (Houte et al., 2020; Kang et al., 2017; Vogt & Beisner, 2011). For viruses, dispersal can help explain spatial patterns of host colonization and extinction (Johnson et al., 2015). All in all, knowledge about phage dispersal is expected to reveal not only information about phage distributions, but also their interactions with host populations.

Phages are assumed to primarily disperse via random diffusion, or through mass flow during wetting events (Dennehy, 2014). However, a free phage must cross relatively large distances between susceptible hosts to replicate, which is exacerbated by the highly structured and often poorly connected aqueous phase (Dennehy, 2014; Nimmo, 2013; Young et al., 2008). By random diffusion alone, a phage must often traverse distances multiple orders of magnitude larger than itself in the right direction to encounter a susceptible host (Baveye et al., 2018; Dennehy, 2014). In the air and water, mixing from currents allow phages to traverse these distances easily, but spatial structure vastly reduces this potential in soil

(Dennehy, 2014; Lindsley et al., 2012; Srinivasiah, 2008; Young et al., 2008; Baveye et al., 2018; Bernard et al., 2019; Erktan et al., 2020; Bull et al., 2018) (Figure 2). Furthermore, increased spatial structure, like that seen in soil, tends to reduce phage populations, while offering refugia to host populations, allowing them to escape phage infection altogether, amplifying these challenges (Berngruber et al., 2013; Bull et al., 2018; Li et al., 2020; Erktan et al., 2020; Houte et al., 2020). Phages also experience a high rate of adsorption to soil minerals (Kimura et al., 2008). By binding to soil grains or non-host biota, phages are likely to be less available for infection to even nearby hosts. In fact, sorption is a commonly used method for removing unwanted viruses from various environments, such as wastewater (Bixby & O'Brien, 1979; Maat et al., 2019; Moore et al., 1981; Pratama & Elsas, 2018; You et al., 2022; Zhao et al., 2008). All of this together suggests an immense challenge for free phages to randomly encounter a susceptible host in soil.

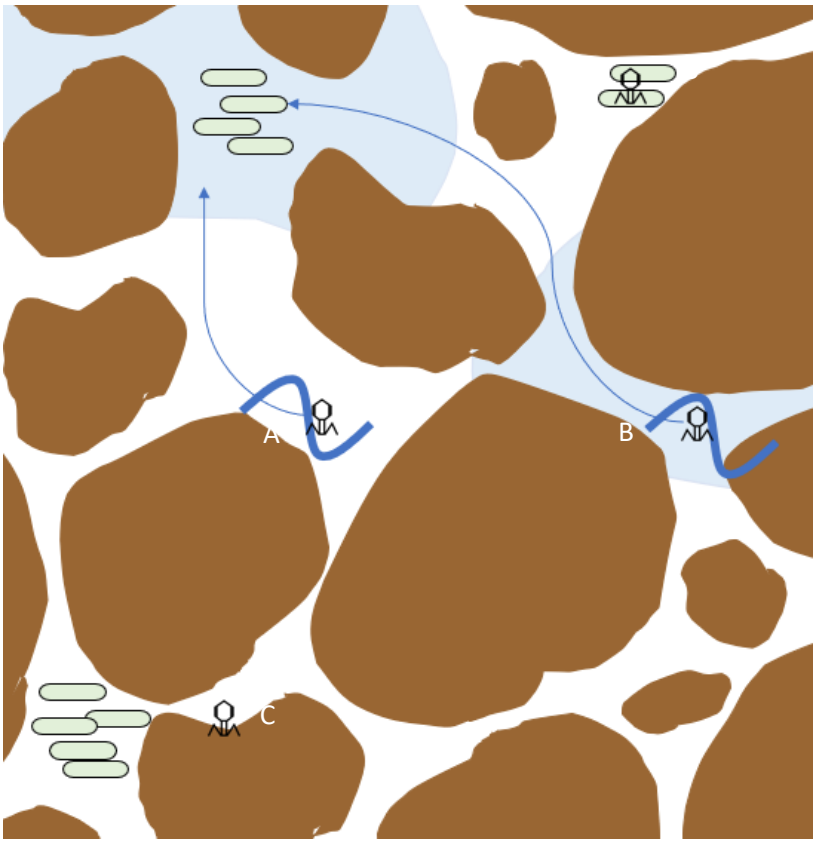

Figure 2. Soil structure presents barriers of A) distance, B) connectivity, and C) adsorption to phage dispersal. Nematodes are hypothesized to assist phage in overcoming these dispersal barriers, as they are motile and move directly toward host organisms, using chemotaxis to sense high-density, high-quality host patches (Werner et al., 2014).

Many viruses use transmission vectors to overcome dispersal challenges between distant hosts. A 2022 study saw T4 model phages transported through soil by motile non-host bacteria along a mycelium network at above 90% efficiency (You et al., 2022). Soil-borne plant viruses utilize plant-pathogenic nematodes (microscopic roundworms) to travel between roots (Dubreuil et al., 2009; MacFarlane & Neilson, 2009). In agar, nematodes are capable of introducing model phages to previously uninfected bacterial colonies (Dennehy et al., 2006; van Sluijs and Mason-Jones, personal communication). These results indicate vectoring as a direct phage dispersal mechanism

between host colonies. Still, nematode vectoring has not been proven for soil phages or in structured soil systems where dispersal barriers are increased.

In many systems spanning from microbes to mammals, impacts of resources on predation are well-established (Bohannan & Lenski, 1999; Hebblewhite & Merrill, 2009; Kang et al., 2017). In soil, dispersal-limited phage would often exhaust a bacterial colony through lysis, leaving them at risk of degradation as free phage. However, ecological modeling experiments show that predator dispersal between patches of prey under resource heterogeneity can rescue a predator population that might go extinct if it stays in a single patch (Kang et al., 2017). Through the creation of distinct niches, we expect resource heterogeneity to modulate host and phage distributions (Houte et al., 2020). In bacterial communities, high resource availability is also positively correlated to invasion potential for quickly growing microbes (Lear et al., 2022). We would similarly expect resources to impact the ability of a phage "predator" to invade a host population via nematode vectors. As nematodes tend to travel toward high density bacteria patches for forage, we would predict that hosts growing under high resource conditions face an increased predation pressure from phages via nematodes, though impacts of resource heterogeneity on spatial interactions between nematode, host, and phage is unknown (Song et al., 2013; Werner et al., 2014).

Bacterivorous nematodes are also soil microbial dispersers. As they forage, they carry bacteria on their exterior cuticle layer or within their digestive tract (Thutupalli et al., 2017). These "hitchhikers" are then dropped in new locations, where they start new populations or are introduced to existing bacterial colonies. In this way, nematodes are expected to vector hosts as well as phages, potentially providing a means of host escape from phage infection by seeding multiple host colonies throughout the soil as they forage (Thutupalli et al., 2017). Furthermore, host escape can help explain co-existence patterns of host and phage, as otherwise phage would be expected to lyse all hosts within a colony or to be eradicated by resistance development in the host population (Li et al., 2020; Schrag & Mittler, 1996). However, unknowns about the mechanisms of host dispersal must be resolved prior to testing hypotheses about escape.

In this study, we tested whether bacterivorous nematodes act as soil phage vectors and host dispersers. We hypothesized that a wild-type nematode with natural foraging behaviors in a structured environment (*Caenorhabditis elegans* strain WN2002) would vector its competitor, the soil phage Phi ppu-W11 phage and disperse the soil bacterium *Pseudomonas putida*. We

first tested this in an agar microcosm where nematodes exposed to phage were allowed to forage to two susceptible host lawns, one with hosts grown under high resource availability and one with host grown under low resource availability. We hypothesized that nematodes would prefer the high resource patch, leading to spatial heterogeneity in phage vectoring with resource heterogeneity. We also hypothesized that nematodes vector free phages, and tested this in an experiment where nematodes must cross a free-phage line to reach bacteria. We then designed an organic compost microcosm where the barriers to dispersal presented by soil physical structure were present, hypothesizing that nematodes would vector phage to a susceptible host colony in the microcosm. Through these experiments, we aimed to elucidate the role of spatial structure in mediating three-way interactions between a nematode vector, bacterial host, and phage.

General Methods

Experimental Organisms

Pseudomonas putida (DSM 291) was used as a host. *P. putida* is a gram negative, rod-shaped, bacterium with aerobic metabolism and is common to root-associated soil. It is responsible for inducing plant growth and defending plant roots against infection by pathogens (Espinosa-Urgel et al., 2000). The *Pseudomonas* phage Phi ppu-W11 (DSM 100071) was originally isolated in Germany. Its life history and morphology are unknown, but the phage is lytic under standard laboratory culture conditions. Both host and phage were obtained from the Leibniz-Institute German Collection of Microorganisms (DSMZ).

WN2002 (WBStrain00040481) is a wild isolate of the bacterivorous nematode *Caenorhabditis elegans*, collected from garden compost heaps in Wageningen, The Netherlands. This strain was chosen due to its more natural characteristics as compared to the common laboratory strain N2. WN2002 exhibits digging behavior which was expected to assist the nematode in navigating the 3D compost environment. While the N2 strain tends to spread evenly across its environment, WN2002 clumps in large groups near bacterial colonies, including *P. putida* (L. van Sluijs, personal communication, March, 2022).

Organism Culture and Enumeration

P. putida culture for experiments was prepared from active colonies grown on trypticase soy broth (TSB) agar (Appendix A.1 for all media, agar, and buffer recipes). One colony was suspended in 5 mL TSB media and was shaken on an orbital shaker for either 5 hrs (OD600 0.64) or overnight (OD600 1.4) at 160 rpm at 27 °C prior to use in experiments.

CFU plating assays were used to enumerate *P. putida* abundance using serially diluted bacterial extracts. Each bacterial sample was serially diluted in a 48-well plate with magnesium-supplemented phosphate saline buffer (mg-PBS) using either 1:10 or 1:100 dilution ratios, depending on expected bacterial abundance. 50 µL of (un)diluted bacterial sample was then spread evenly on TSB agar using a sterile spreader. Plates were left to dry briefly before inversion and incubation at 27 °C overnight. Bacterial abundance was calculated as colony forming units (CFU's) from colony counts.

Phi ppu-W11 (W11) phage lysate was prepared by infecting bacteria with a stock lysate. 50 µL of phage lysate was inoculated into 5 mL TSB media and 200 µL overnight *P. putida* culture. The infected culture was shaken on an orbital shaker for approximately 6 hrs or until lysis (clarity in the culture media) at 160 rpm at 27 °C and then centrifuged and syringe filtered at 0.22 µm. Lysate was stored at 4 °C. Fresh phage stocks were made in this way two times during the study, with each stock being used for up to 6 weeks. Phage abundance in lysates and extracts from agar or soil was quantified via soft-top plaque assays on TSB agar. The phage sample was serially diluted in a 48-well plate with Tris phage buffer using either 1:10 or 1:100 dilution ratios, depending on expected phage abundance. 100 µL (un)diluted phage samples and 100 µL *P. putida* culture were vortexed in molten TSB soft agar (0.7% bacto-agar) at 55 °C and poured on TSB bottom agar plates (1.5% bacto-agar). Plates were left to set and dry before incubating inverted at 27 °C until plaques were clearly visible (from five hrs to overnight). Phage abundance was calculated as plaque forming units (PFU's) from plaque counts.

Nematodes were cultured on 6 cm petri dishes with 8 mL standard nematode growth medium seeded with 50 µL *P. putida* overnight culture (OD_{600} 1.4). Culture strains were maintained via chunking every 3 days to 2 weeks. For all experiments, nematodes were washed from the surface of these plates in all life stages and inoculated as such. Therefore, nematodes were always added to experiments with an unknown amount of *P. putida* from the co-cultured plates. Nematodes were counted by microscopy using a Leica M205C dissecting microscope (magnification 1.75-2.5X, either on agar microcosms or using a 6 cm counting dish for compost extracts).

Experiments

Experiment 1: Vectoring in Soil Organisms Under Resource Heterogeneity

Introduction

By random dispersion, phages are unlikely to successfully reach distant host colonies in soil (Dennehy, 2014). Nematodes have been shown to assist virus dispersal by acting as vectors between host populations, but have not been tested as vectors for soil phages (Dennehy et al., 2006; Dubreuil et al., 2009; MacFarlane & Neilson, 2009). In this experiment, we test whether *C. elegans* WN2002 acts as a vector for its competitor, the soil phage W11. As nematodes prefer to forage on high density host colonies, we also predict that nematodes will disproportionately transfer phage competitors to hosts grown under high resource conditions.

Methods

The consequences of resource heterogeneity on nematode vectoring of phages were tested in microcosms where nematodes were given a foraging choice between hosts grown on high and low resource forage patches. Microcosms were constructed in 9 cm petri dishes with 20 mL nematode growth medium. Resource heterogeneity was introduced to the system by replacing 2 cm diameter plugs with either 0.1X or 1X TSB agar at 2 cm distance from each other. Approximately $2*10^7$ CFU of *P. putida* overnight culture (OD600 1.4) in 20 µL 1X TSB medium was spread on each TSB resource patch. After allowing these to dry, a 20 µL solution containing one of three treatments (Table 1) was spread on the plate at a point 2 cm equidistant from both resource patches (Figure 3). All microcosms were incubated at 20 °C without light for 40 hours after inoculation.

Table 1. Nematode and Phage Treatments for Experiments with Resource Heterogeneity

Treatment	Treatment Description	Approximate Nematodes Added	PFU's added*	CFU's added	Replicates
N	Nematodes washed from the surface of co-cultured *P. putida* plates and suspended in mg-PBS	20	0	$0^{\dagger}$	3
PN	nematodes as in "N" plus phage lysate in 1X TSB medium	20	$1*10^7$	$0^{\dagger}$	3

HPN	Nematodes as in "N", phage lysate as in "PN", and host resuspended from 1X TSB culture into mg-PBS	20	$1*10^6$	$5*10^6$	1

*PFU's added is an estimate only and has a large error margin (~1 order of magnitude) due to uncertainty about longevity of phage in storage.

† An unknown level of host was present due to nematode introduction in co-culture with host.

After incubation, bacterial density, nematode abundance, and phage titer were measured in each resource patch. Bacterial density was estimated by imaging on an aCOLyte plate imager (exposure 31; bottom light with no diffusion dish). Total nematodes were counted via microscopy in each resource patch. Phage extracts were prepared by removing resource patches with flame-sterilized spatulas into 50 mL centrifuge tubes with 8 mL bovine supplemented phage extraction buffer (PPBS). The tubes were then shaken for 2 hrs at 70 rpm at 4 °C, after which they were centrifuged for 5 min at 9,500 xg. Finally, the supernatant was syringe filtered at 0.22 µm and stored at 4 °C until soft-top assays were performed.

Firth's logistic regression (logistf package version 1.24.1), a modified binomial test, on phage presence (phage present or absent per patch) as a function of nematode presence was used to test whether nematodes vector phage on agar (R Core Team, 2021; Heinze, et. al, 2022). Firth's logistic regression was chosen as an appropriate binomial test for our data, as it accounts well for binary data with small sample sizes such as ours without reducing power. It can also analyze data with rare events (one outcome different from the rest) or perfect separation (the explanatory variable is perfectly predicted by the outcome variable), which happened in many of our experiments. Interaction effects between nematode presence and resource availability could not be evaluated due to low sample size and interdependence. Additionally, the impact of resources alone on phage dispersal could not be tested, as nematode presence is a confounding variable precluding reliable interpretation of the statistical significance. All statistics in this study were performed in R version 4.1.0 (R Core Team, 2021).

Results and Discussion

Phage presence was perfectly predicted by nematode presence, with phage transferred in 100% of cases with nematodes present and 0 cases with nematodes absent (n=8, $p<0.001$) (Figure 3) (Appendix A.4). Across all microcosms, nematodes preferred the high resource patch, with nematodes observed in 100% of high resource patches and 25% of low resource patches (n=4).

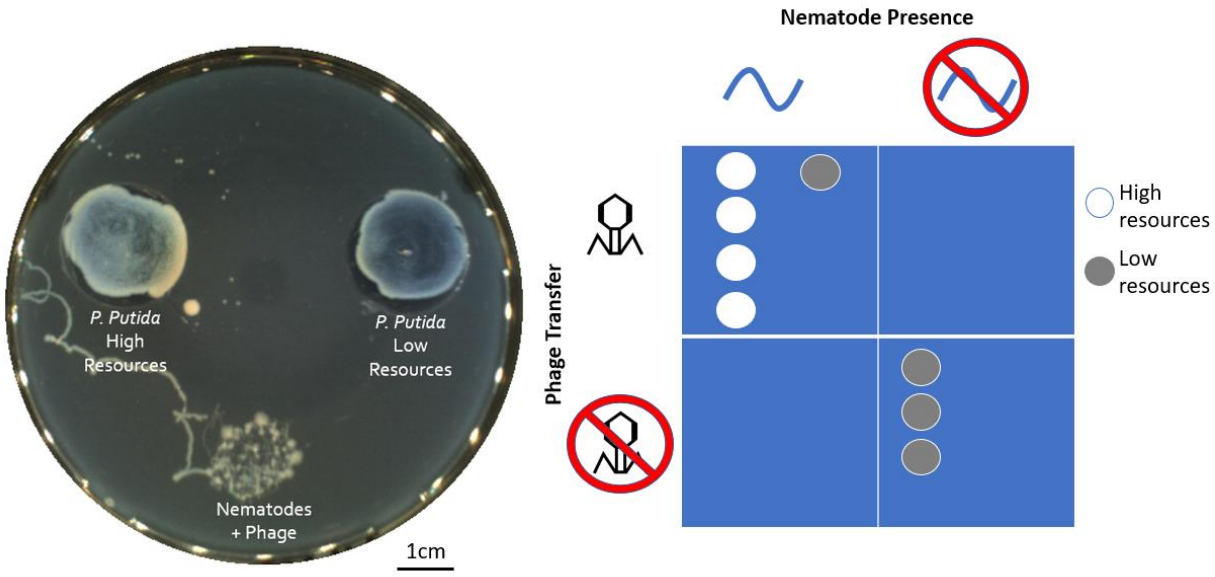

Figure 3. Left Panel: Example plate of C. elegans and Phi ppu-W11 phage introduced to NGM agar microcosms 2 cm equidistant from P. putida host grown on high resource (1X TSB) or low resource (0.1X TSB) agar plugs. Bacterial density is positively correlated with yellow color in the microcosm, and was spread as channels or single colonies by nematode dispersers. Plate image taken after 40 hrs incubation. Right panel: Graphic contingency table where each square represents a scenario where nematodes and phage are present or absent in a resource patch. Each circle represents a single replicate bacterial patch, and circle color represents resource availability.

Phage transfer to a susceptible host colony occurred in all cases where nematodes were present, demonstrating vectoring as a viable dispersal method for the W11 soil phage. This is the first time that nematode vectoring of a soil phage has been demonstrated. Additionally, nematodes preferred high resource, high density host patches. As predicted, when there was heterogeneity in resources, bacteria in high-resource patches experienced increased predation pressure from nematodes as well as increased infection from phages. As vectoring should be detrimental to the nematode while benefitting its competitor, the consistent vectoring seen in this experiment is a surprising outcome. The quickened depletion of bacteria following phage infection should force nematodes to increase foraging, resulting in more phage transport to colonies of susceptible bacteria. Still, as hosts living under high resources are preferred by nematodes, hosts under low resource conditions may escape both predation and phage infection. Following this experiment over a longer term will reveal interesting

patterns about how vectoring under spatial heterogeneity in resources impacts species interactions and distributions. For example, a future experiment could investigate a potential trade-off for growth between resource availability and predation pressure. In our current experiment, the increased predation did not seem to detract from increased growth for hosts grown under high resource availability, as seen by the density of the host patches. However, we might expect that under some threshold amount of predation, low density, low resource host colonies may have a growth advantage due to the lack of predation pressure.

Experiment 2: Vectoring Mechanisms – "Crossing the Line"

Introduction

For model organisms *E. coli* and T7, Dennehy et al. concluded that vectoring was primarily driven by nematode dispersal of infected hosts due to a negative correlation between initial phage abundance in an infected host colony and rates of phage transfer (2006). If this conclusion holds true for soil organisms, it would be expected to have important consequences for spatial patterns in species interactions. Namely, phage transfer would be negatively correlated with phage lysis of hosts, and phage that has completely lysed a host population would not be able to be transferred (Dennehy et al., 2006). In this experiment, we test whether nematodes can vector free phages as they cross a host-free phage front toward a susceptible host colony (Figure 4). Information about whether nematodes can vector free phage is a first step toward elucidating mechanisms of phage transport and developing hypotheses about spatial patterns of phage dispersal in soil.

Methods

P. putida overnight culture (OD600 1.4; 35 µL) in TSB media was plated over a semi-circle section on 1/3 of the microcosm. Thereafter, 25 µL of phage lysate ($1x10^9$ (PFU's)/ mL) in TSB media was spread down the center of the microcosm in a 1 cm wide line. A 1 cm chunk of nematodes (~100 ±50 nematodes) from 3-day old culture plates was then added on the opposite side of the phage barrier. Nematodes were therefore added along with an unknown amount of *P. putida* from the nematode culture.

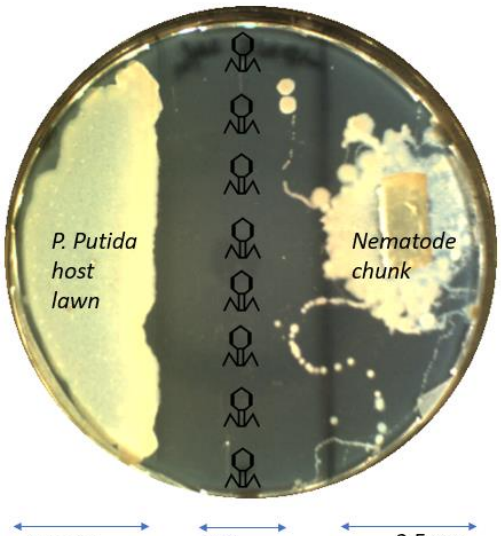

Figure 4. HPN microcosm at 65 hrs. Nematodes must cross free phage front to transfer phages to susceptible hosts in agar filled petri dishes. 35 µL hosts on 1/3 plate, phage front on middle of plate, nematode chunk on right 1/3 of plate.

Treatments included host-nematode (HN), host-phage (HP), and host-phage-nematode (HPN) (Table 2). Microcosms were incubated at 20 °C without light over 65 hrs.

Phage presence was observed by visual checks for plaque presence in the host patch every 24 hrs. Plaques appeared as discolored patches in the host lawn. Microcosms were imaged on an aCOLyte reader (exposure 31; bottom light with no diffusion dish) at 65 hrs. After approximately 65 hrs incubation, the identity of the discolorations as plaques was confirmed by soft-top plating assay. A 1/3 cm agar plug was removed from the center of the host patch of 1 microcosm of each treatment into 300 µL of Tris phage buffer and vortexed. These undiluted phage extracts were then enumerated via soft-top.

Firth's logistic regression on phage presence in the host lawn (observed as discolorations) between nematode and nematode-free treatments was used to determine whether vectoring occurred.

Results and Discussion

In all samples with nematodes added (n=5), novel discolorations (confirmed as phage plaques by soft-top assay) were observed by visual check at 65 hrs, while no plaques were seen in nematode-free microcosms (n=5). Firth's logistic regression confirmed this as significant (p=0.0017).

Table 2: Nematode Vectoring of Free Soil Phage across a Phage Front

Treatment	Phage seen visually in Host Lawn (-/+)	Replicates	Soft Top result (-/+)
HN	-	1	-
HP	-	5	-
HPN	+	5	+

Phage was consistently vectored from a free phage strip to a susceptible host lawn. As nematodes were added with residual host from nematode culture plates, phage transfer inside infected hosts cannot be ruled out. A nematode could have theoretically carried a host into the phage front to be infected and transported to the susceptible lawn before lysis. While this is a possible scenario, it is unlikely to account for all phage transfer observed within the

microcosms. WN2002 creates channels in agar as they move where they disperse *P. putida*. Observation of these channels showed the phage strip interrupting *P. putida* dispersal. In the HN microcosm, continuous channels with *P. putida* were observed crossing the agar plate, whereas host dispersal typically stopped at the phage front border in HPN microcosms from both the nematode and host lawn sides of the microcosm (Figure 4). Still, phages were transferred to the host lawn in all HPN microcosms, even though hosts were not successfully transferred much, if at all. This suggests that vectoring of free phage is more likely to explain the transfer than vectoring of infected hosts in this experiment, contrary to conclusions from Dennehy et al. (2006). Free phage vectoring cannot be conclusively confirmed however, as a host cell could have been infected in the phage front and transferred to the other side of the agar prior to lysis. In this scenario, a host channel still would not form, as transferred hosts would lyse before colony development. Further experiments can aim to verify precise mechanisms of phage transfer by using non-host bacteria or phage buffer in place of host bacteria in the microcosm. This would ensure that any phages that are vectored must be host-free.

Experiment 3: Organic Compost Microcosm Preliminary Testing

Introduction

While nematode vectoring of the soil phage W11 was shown to overcome dispersal challenges in agar, additional dispersal barriers exist in soil due to its structure (Bull et al., 2018; Erktan et al., 2020). We established an organic compost microcosm design to address the possibility of vectoring in a soil-like environment with complex spatial structure (Figure 6). Before vectoring could be reliably tested in compost, we first estimated the reliability of host and phage recovery and nematode migration rates in compost.

Methods

Organic compost (Pokon Naturado B.V., Veenendaal, Netherlands) was chosen for its resemblance to *C. elegans'* natural habitat of rotting organic material. Compost was sieved to 4 mm to remove large material. Compost gravimetric water content (WC) was then brought from 82% to 85% of dry mass via mg-PBS addition, which was below the WC at which free draining would occur. This WC was chosen to maintain necessary moisture for nematode survival. It was also expected to limit phage dispersal via mass flow by maintaining an intermediate connectivity, as 100% water filled pore space was achieved at nearly double this WC (160%) (Appendix A.2). Compost was added to 9 cm Petri dishes in a 1 cm layer at a dry bulk density of 0.28 (33.4±0.5g at 85% WC). Inoculation of experimental organisms was

achieved by replacing 0.9 cm cores from the microcosm with an equivalent volume of 82% WC compost (0.325g ±0.05) that was then inoculated with organisms via pipetting. Phages were added in TSB media. Nematodes and hosts were added in an mg-PBS suspension to bring the replaced cores to an equivalent WC with the rest of the microcosm (85%) (Appendix A.3). This was to prevent mass flow of liquid between points, and WC was kept at a constant value throughout the microcosm.

To verify our ability to recover host and phage from compost, we performed host and phage extractions immediately after inoculation into compost microcosms and after overnight incubation. In this experiment, inoculation was performed by replacing a 1.3 cm diameter plug from the microcosm center with a 0.46g (±0.05g) of 85% WC compost with an equivalent volume of 82% WC compost. Bacteria and phage densities added to the microcosm were dependent on treatment (Table 4). Inoculated organisms were added in a 12.5 µL suspension of either mg-PBS for hosts or TSB media for phages. Treatments included host (H), host incubated overnight (HO), phage (P), host-phage (HP), and host-phage incubated overnight (HPO) (Table 3).

Table 3: Treatment Groups for Tests of Host and Phage Recovery Efficiency

Treatment	CFUs added	PFUs added*	Recovery incubation time (hrs)	Replicates
H	8*10^7	-	0	2
HO	4*10^7	-	20	3
P	-	1*10^5	0	3
HP	8*10^7	6.2*10^4	0	3
HPO	1.5*10^8	3*10^7	20	1

*PFUs added is an estimate only and has a large error margin (~0.5 order of magnitude) due to uncertainty about longevity of phage in storage.

For host and phage extraction, inoculation plugs were removed from the microcosm into 50 mL polypropylene tubes with 5 mL mg-PBS buffer using a flame sterilized corer. For host extractions, 0.1g sterile silica sand was added to each sample to facilitate host release into the buffer solution. Samples were then shaken on an orbital shaker for 1 hr at 220 rpm at 4 °C. After shaking, the host samples were centrifuged for 2 min at 200 rpm at 4 °C. 1 mL of supernatant was recovered for enumeration. PPBS (2.5 mL) was then added to each sample

to extract phages. Samples were then returned to the shaker for 6 hrs at 220 rpm at 4 °C. Finally, 2 mL of liquid from each phage sample was centrifuged for 10 min at 15,000 xg. The supernatant was syringe filtered at 0.22 µm to remove cellular material and stored at 4 °C for enumeration via soft top the following day. Host and phage recovery success was defined as host or phage presence.

To estimate the minimum incubation period required for a nematode vectoring experiment in compost, we tested nematode migration rate toward an infected host colony in compost. In compost microcosms, 0.9 cm diameter plugs were removed with a flame sterilized corer 2 cm apart. The first plug was inoculated with $1*10^8$ CFU's of exponential phase *P. putida* (n=9). The second plug in each microcosm was inoculated with approximately 200 non-sterile nematodes. One control microcosm was inoculated with only nematodes. Each day over the course of 3 days, three microcosms were harvested and nematodes were extracted from the host-inoculated patches, with the control microcosm harvested on day 3. The host patch was removed from the microcosm with a 0.9 cm flame sterilized corer and spread evenly across a two-layer nematode filter suspended at the surface of a 100 mL pool of demineralized water. Filters were incubated at 20 °C, and nematodes were allowed to crawl through the filter into the pool overnight. Then, the 100 mL water sample was centrifuged and the nematode pellet was resuspended in 5-10 mL demineralized water in a 6 cm Petri dish for counting. Nematode migration success (>50% recovery of nematodes in the host patch) was determined per day to estimate the migration time needed for nematodes to traverse the 2 cm distance.

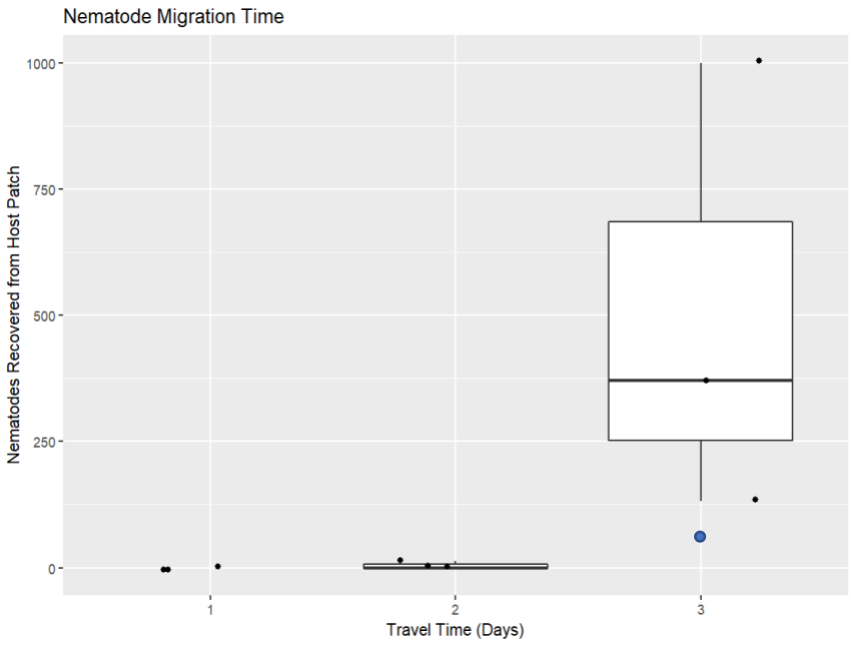

Figure 5. The number of nematodes recovered from compost microcosms 1, 2, and 3 days after inoculation 2cm from a P. putida prey colony. Blue dot represents nematodes recovered from a microcosm without P. putida inoculated.

Host and phage recovery was successful in every sample of every treatment (H, HO, P, HP, HPO). No nematodes were extracted from microcosms on day 1 (n=3). On day 2, 13 nematodes were recovered from one microcosm (n=3). Migration was successful on day 3. A minimum of 132 nematodes were recovered from each microcosm, with up to 1000 nematodes seen in the host patch, indicating nematode reproduction in the microcosm (n=3). Conversely, only 61 nematodes were recovered from the control microcosm on day 3 (Figure 5).

The successful recovery of host and phage, including after overnight incubation, demonstrates that host and phage can be reliably recovered from compost. Verification of recovery and of survival are both crucial preliminary results for a test of vectoring in compost. These results allow us to rule out an unsuitable environment for host and phage and inefficient extraction as potential reasons for lack of observed phage transfer results in later experiments.

Nematodes required a minimum incubation time of 3 days to reach the host patch, while control microcosm nematodes did not migrate in large numbers toward a point 2 cm from their inoculation patch. The consistent migration success in the presence of *P. putida* but not in its absence on day 3 shows that nematodes did navigate toward high-density host colonies, as expected. Similar to agar microcosms, we show here that nematodes are able to sense prey at these distances in compost (Song et al., 2013; Werner et al., 2014). If nematodes vector phage in soil, this sensing ability is critical to nematodes providing active transport for phage between susceptible host colonies. However, nematodes can traverse the same distance in agar in minutes, raising questions about the effect of structure on nematodes as well as phages. We expect some delay in nematode migration, as in compost there is no straight line between two points. Soil grains and air gaps present obstacles that nematodes must navigate around. Overall, however, the consistent movement of nematodes toward *P. putida* colonies allows us to test their ability as phage vectors in compost and confirms an incubation time of 3 days as appropriate for our design.

Experiment 4: Nematode Vectoring in Organic Compost

Introduction

Phage dispersal by random diffusion should be limited in soil due to complexities in soil structure (Dennehy, 2014; Erktan et al., 2020). We therefore hypothesized that nematodes are vectors for dispersal of phages in soil-like habitats. In this experiment, we tested whether WN2002 nematodes, which vector phage in agar, also act as vectors when confronted with

structure in a compost microcosm. Furthermore, we aimed to test the ability of nematodes to disperse hosts in this system as a preliminary step before testing hypotheses about nematode-mediated host escape.

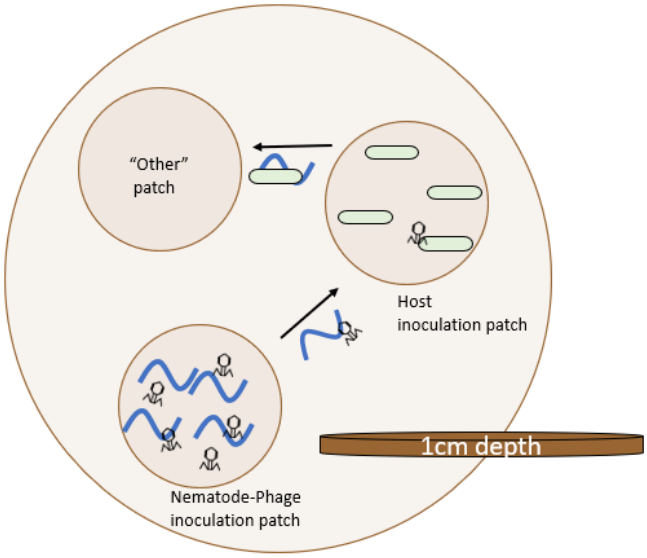

Figure 6. Schematic of compost microcosm design. 33.4g 85% WC compost was packed to a depth of 1 cm in a 9 cm petri dish. Nematodes and phages were inoculated 2 cm away from a susceptible host colony. Nematodes were allowed to move freely through the microcosm over 3 days, and were hypothesized to vector phage to the host inoculation patch. They were also expected to disperse hosts throughout the microcosm. These were sampled from an uninoculated "other" patch.

Methods

Phages were inoculated in compost microcosm either in the presence or absence of nematode vectors at a distance of 2 cm from a susceptible host colony (Figure 6). One plug in each microcosm was inoculated with $1*10^8$ CFU of exponential growth phase *P. putida* (OD600 0.64). The other plug in each microcosm was inoculated with phage lysate (approximately $1.1*10^6$ PFUs) and/or 400 non-sterile *C. elegans*. Treatments included host-phage (HP; n=5), host-nematode (HN; n=5), and host-phage-nematode (HPN; n=5). Due to pipetting error, the phage(-nematode) inoculation patches were brought to 88% WC instead of the intended 85%, however artefacts from mass flow of phages between inoculation patches were still avoided at this water content as no HP microcosms saw phage transfer. Microcosms were incubated for 3 days at 20 °C without light.

After incubation, host and phage were extracted from the host inoculation patch as well as an "other" patch 2 cm away from both inoculation patches. Host and phage presence were measured as described above. Phage presence was not measured for microcosms without phage added or for "other" patches.

Firth's logistic regression was performed on phage vectoring success (phage recovered from the host patch) per treatment to test whether nematodes acted as vectors. Nematode presence was hypothesized to increase phage presence in the host patch. Host dispersal was

considered successful if hosts were present in the "other" patch and unsuccessful if they were absent. A binomial test (stats package in R) on dispersal success in NH microcosms was performed against a null hypothesis of no host transfer in any microcosm (R Core Team, 2021). Only NH microcosms were considered for this test as phage presence is expected to have an effect on host transfer via lysis. Here, we only wanted to confirm that nematodes can vector susceptible hosts.

Results and Discussion

Phage was not transferred in any HP microcosms and in only one HPN microcosm. This single transfer event was not significant, and we could not conclude that nematodes consistently vector phage ($n=5$, $p=0.43$). *P. putida* was transferred to the "other" patch in 2 NH microcosms, confirming that nematodes do act as microbial dispersers of *P. putida* ($n=5$, $p<0.001$).

We cannot rule out a false positive for phage transfer resulting from experimental error. Still, the single occurrence of phage transfer hints that nematode vectoring might be possible, but perhaps rare over the tested distances or time-scales. Therefore, the hypothesis that nematodes can assist phages in overcoming barriers to dispersal in a soil-like environment was neither clearly supported or rejected, but remains ambiguous. From agar, there is still strong evidence that nematodes can carry phage. In compost we saw clear nematode movement across the distances tested. Finally, many soil viruses rely on some non-host vector to overcome dispersal challenges (Berngruber et al., 2015; Dietzgen et al., 2018; MacFarlane & Neilson, 2009). All of this together leads us to a reconsideration of the reliability of our experimental design before drawing ecological conclusions from these results. Specifically, we did not adjust our design to account for the changes that structure from compost may introduce to vectoring patterns as compared to agar.

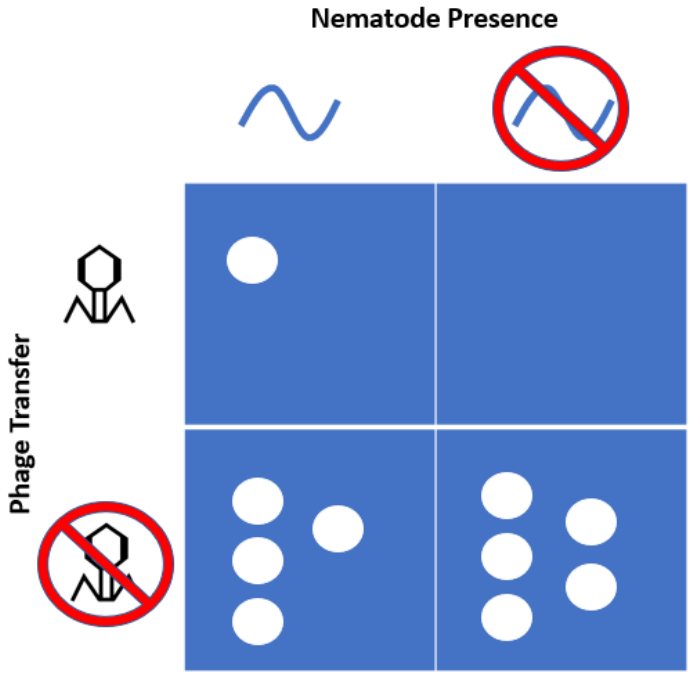

Figure 7. Phage recovery from host colonies after 3-day incubation in the presence or absence of nematode vectors inoculated into the microcosm. Phi ppu-W11 phage was scored as transferred or not when extracted from a host colony in compost microcosms. Phage was not transferred in the absence of nematode vectors, and was only transferred one time in the presence of nematode vectors (n=5).

For example, in compost, *C. elegans* travelled, and therefore carried phage, at a maximum of 1 cm per day. As nematodes molt four times during the 3-day experiment duration, externally carried phage is unlikely to make the journey to the host colony (Byerly et al., 1976). By designing a setup where nematodes reach a host colony within one day, complications arising from temporal scale can be eliminated.

Testing these hypotheses with soil nematodes in soil may also yield novel associations between nematode, host, and phage. Soil nematodes may be more likely to have existing adaptations or associations with soil phages than *C. elegans*. Small changes in affinity between nematode and phage could lead to significantly different dispersal patterns for phage. Many soil nematodes, such as *Cephalobus sp.,* are easily cultured and maintained in laboratory settings, and would be interesting candidates for future experimentation (Barrière & Félix, 2018).

Nematode-mediated host dispersal was seen in two of ten microcosms, providing evidence that *C. elegans* is a disperser of *P. putida*. By dispersing host, the nematode provides more foraging ground for itself (Thutupalli et al, 2017). While a nematode could vector a phage-infected host bacterium, spreading phage along with host, nematodes may also disperse phage-free host cells. This, combined with the host dispersal seen on agar lends stimulates hypotheses for future testing about host escape and its consequences for host-phage distributions and coexistence in structured systems (Li et al., 2020; Schrag & Mittler, 1996).

General Discussion

In this study, we examined the possible role of nematodes as vectors for overcoming challenges to soil phage dispersal in a spatially structured environment and considered the consequences for three-way interactions between nematode, host, and phage. We verified that nematodes are vectors for soil phages in agar microcosms. In the resources experiment, phage was transferred in every case where nematodes were seen foraging. Nematodes also preferentially vectored phages to high-density patches of bacteria grown under resource enriched conditions. The "crossing the line" experiment demonstrated that nematodes are likely to vector free phages. An important caveat here is that nematodes are vectoring their direct competitor, likely at an immediate detriment to themselves. Often, a vector incurs some benefit from vectoring; for example, plants fighting virus infection tend to be more susceptible to root-knot nematode parasitism, making vectoring beneficial to the nematode (Walsh et al., 2017). Through processes like the viral shunt, phage infection in the rhizosphere may actually stimulate the growth of bacterial colonies by releasing nutrients for plants, which will in turn grow and provide more carbon for bacterial symbionts (Kuzyakov & Mason-Jones, 2018). Therefore, phage vectoring may have indirect benefits to all organisms in this study. However, these results did not carry over when the agar microcosm design was adapted to organic compost as a spatially structured substrate. While the compost experiment is still a simplification of nature, increasing the realism in our experimental design led to quite different results. Namely, nematode vectoring could account for 100% of phage transfer between host colonies in agar, but vectoring was inconclusive in compost. This result at the very least verifies that spatial structure presented challenges of scale and connectivity for phage transfer between susceptible host colonies, which must be considered as we test further questions about the causes, mechanisms, and outcomes of these interactions and their broader implications for soil community structure. In soil phage ecology, almost everything is still to be discovered!

Spatial heterogeneity in resource availability significantly altered three-way interactions between nematodes, bacteria, and phage. Bacteria grown under high resource conditions were disproportionately impacted by both nematode predators and virus infection, indicating that the spatial distribution of organisms was dependent upon the spatial distribution of resources throughout the microcosm. In terms of understanding broader soil ecology, this could be hypothesized to have important implications for soil microbial community structure. As nematodes preferentially predate on the highest density prey population, high density species of bacteria may be more susceptible to predation and phage infection. On the other

hand, as soil phages are typically specialists to one bacterial species, vectoring may not be as efficient a mechanism for phages which infect rare or low-density bacteria (Kasman & Porter, 2022). In our experiments, nematodes were only given the option to forage on host bacteria. However, in real soils, most bacterivorous nematodes may not be travelling to host bacteria for the phage, but rather the closest or highest density populations of edible bacteria, possibly of many species. The utility of nematode vectors for overcoming phage dispersal challenges in soil would then be hypothesized to be directly related to initial patterns of host and phage abundance and distributions in the soil, with widespread, high-density populations being more impacted by nematode vectoring, such as many hosts in the rhizosphere.

Mechanisms of phage transfer in soil also have relevance for predicting the development of host-phage interactions, as this determines which phages can be transferred and where. Here, we provided evidence that free phage could be vectored by nematodes. In the physically structured compost environment, phage transfer was greatly reduced if not eliminated. Again, spatial structure impacted the interactions between organisms and altered their distributions overall. However, if phage can be spread in the absence of host *in soil*, then phages have more opportunity to be vectored through an unsuitable colony to a host patch, dependent on their longevity as free phages. "Stickier" phages may also increase their probability of reaching rare hosts as nematodes forage through more space. However, phage transport distances could equally be limited by nematode cuticle shedding rates, as *C. elegans* sheds its cuticle layer 1-2 times per day, or by nematode travel distances (Byerly et al., 1976). We know that phage is widespread in the soil, but dispersal may still be a slow process (Kuzyakov & Mason-Jones, 2018). Future work should aim to resolve the distances various phages can travel, as this will impact spatial patterns of host-phage interactions.

Mass flow, mixing, and other vectors are also all unexplored dispersal mechanisms (Erktan et al., 2020). During wetting events, mass flow increases connectivity in soil. In doing so, mass flow is hypothesized to transport both host and phage, possibility causing a chance interaction (Dennehy, 2014). Non-competitors would also suffer no costs in transporting phage, as a nematode would. In fact, they may benefit from carrying phage. Motile non-host bacteria can use phage as a weapon by vectoring it into a high-quality habitat occupied by a competitor (You et al., 2022). Likely, there are many pathways to phage dispersal in soil. Incorporating these mechanisms of transport in spatially heterogeneous environments could therefore reveal interesting patterns of how structure influences species interactions and alters community structure.

While phage dispersal is still unclear in compost, hosts were clearly dispersed by nematodes, altering their distributions. By dropping host hitchhikers and starting novel host colonies, nematodes both benefit themselves by initiating more forage patches and the host by potentially providing a means of host escape from phage infection (Thutupalli, et al., 2017). As shown by the resources experiment, lower-density colonies of bacteria are less visited than high. Colonies started by nematodes are likely to be small especially at their inception, and therefore less visited. They then may be refuges from phage vectored by nematodes, at least for a time.

To predict emergent spatial outcomes of such highly complex interactions, we suggest modeling as a first step in hypothesis formation about spatial interactions between organisms in a structured environment like soil. Thutupalli and colleagues used modeling to successfully predict the consequences of microbial dispersal patterns for nematodes and their prey bacteria (2017). Models by Bickel and Or have investigated the role of connectivity and water content in structuring microbial communities at multiple spatial scales and in a three dimensional space, factoring in soil depth as a main driver of microbial community composition (2020). While these models do not currently consider phage, they demonstrate the utility of modeling for elucidating mechanisms and emergent properties of species interactions and soil structure on microbial communities. They also have great potential for revealing which spatial parameters are expected to be crucial drivers of these interactions, and should therefore be prioritized in designing later experiments. For example, in our microcosm design, modeling could be useful to ask how vectoring alters temporal fluctuations in host:phage ratios throughout the system or how an interaction between resource heterogeneity and physical structure would impact nematode movement patterns or host colony distributions in the soil.

Conclusions

In the soil, phages face a perilous journey between host colonies, which should limit their dispersal potential. Here we present the first evidence for nematode vectoring of soil phages as a mechanism to overcome phage dispersal challenges. Nematodes vectored free phage to a host lawn on agar. Nematode vectoring in compost was inconclusive, however. In both agar and compost, nematodes dispersed hosts as well as phages, raising interesting questions about nematode-mediated host escape. This study also adds to a body of evidence demonstrating that spatial structure impacts how organisms interact (Albright & Martiny,

2018; Bohannan & Lenski, 1997, 1999; Chevallereau et al., 2022; Erktan et al., 2020; Gómez et al., 2015; Hebblewhite & Merrill, 2009; Houte et al., 2020; Zhao et al., 2008). In a system with heterogenous resource distribution, hosts grown under high resources were more likely to experience nematode predation and phage infection, as nematodes vectored their competitor to this higher quality forage. The relationship between spatial structure and the resident organisms may have important consequences for the distributions of soil microbial and viral communities in soil. Experimental designs with spatial realism are crucial for understanding how soil microbial communities form and change over time.

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