Disease-decreasing diversity: evidence for lowered parasite prevalence in microscopic nematode communities with higher species numbers

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Abstract

High host biodiversity can reduce parasite prevalence, a phenomenon widely studied in macroscopic organisms and termed the 'dilution effect of disease'. Data from host-parasite dynamics in microbial communities is lacking but is essential to assess the stability of microbial communities under global change. Here, we study diversity-disease effects in wild nematode communities by profiting from the molecular tools available in the well-studied model nematode *Caenorhabditis elegans.* Nanopore sequencing was used to characterize nematode community diversity and composition whereas parasites were identified using experimental assays based on fluorescent staining or fluorescent reporter strains. Our results indicate biotic stress is abundant in wild nematode communities. Dilution-disease effects were observed based on the presence of microsporidian parasites and by activation of the Intracellular Pathogen Response. Together, this provides the first demonstration of diversity-disease effects in microbial communities and establishes the use of nematode communities as model systems for disease dilution.

Introduction

Biodiversity of microorganisms is vital for ecosystem functioning and changes host-parasite relationships (Wagg *et al.* 2014). In macroscopic species, high biodiversity can restrain parasite presence: the so-called 'dilution effect of disease' (Keesing & Ostfeld 2021; Ostfeld 2009, 2017). The dilution effect results from reduced parasite reproduction because parasites experience difficulties finding a high-quality host amongst all species present. In contrast, parasites that replicate in a low-biodiversity population spread easily the because chances that the next organism they encounter is also a highly suitable host, are high. Despite the necessity of well-functioning microbial communities for ecosystem stability, host-parasite interactions in microbial communities are little understood. In case dilution effects exist in these communities this helps predict ecosystem functioning under changing conditions that alter biodiversity (Berlinches de Gea *et al.* 2022; Collins *et al.* 2020) .

Dilution effects occur in communities with species that vary in host-quality for the parasite of interest and species that lower transmission between high-quality hosts. An example of dilution effects was observed in ponds amphibians. In these managed high biodiversity ponds the number of parasite-infected Pacific tree frog tadpoles was halved compared to when these co-occurred with just three other species (Johnson *et al.* 2013b). The finding of this dilution effect was strengthened by the observation that high biodiversity in natural ponds even reduced parasite spread in these tadpoles by about 78% (Johnson *et al.* 2013b). However, observation of dilution effects has not always been straightforward, because they depend on complex interactions within the natural community. Dilution effects can be obscured or enhanced by parasite encounters, susceptibility and transmission of the species in the community, the order, timeframe and magnitude in which species enter or disappear from the community, life history traits of the host and host range of the parasite (Chen *et al.* 2022; Civitello *et al.* 2015; Collins *et al.* 2020; Faust *et al.* 2017; Ferraguti *et al.* 2021; Gandy *et al.* 2022; Halliday *et al.* 2020; Johnson *et al.* 2008, 2013b, a; Keesing & Ostfeld 2021; Linske *et al.* 2018; Liu *et al.* 2020, 2023; Okamoto *et al.* 2019; Su *et al.* 2022). Interestingly, microorganisms that are relatively easy to grow and manipulate would present excellent models for studying dilution effects, however, dilution effects have not yet been studied in microbial communities.

Nematodes present one of the most diverse and abundant group of microorganisms worldwide (van den Hoogen *et al.* 2019) and some species possess characteristics that make them excellent model microorganisms. Of all nematodes, the free-living bacterivorous nematode *Caenorhabditis elegans* is a key model organism in many biological fields, ranging from developmental biology to neuroscience (Gaertner & Phillips 2010; Hahnel *et al.* 2020; Link 2006; Marsh & May 2012). Today's science not only focuses on the molecular and genetic understanding of this 1mm-sized organism in the laboratory, but considers its natural ecology too which led to the discovery of many naturally-infecting parasites (Schulenburg & Félix 2017). Parasites infecting *C. elegans* and related bacterivorous species comprise opportunistic bacteria and obligatory parasites including oomycetes, microsporidia and viruses many of which cause deadly infections (Frézal *et al.* 2019; Grover *et al.* 2021; Osman *et al.* 2018; Richaud *et al.* 2019; Schulenburg & Félix 2017; Zhang *et al.* 2016). However, whether nematode community diversity affect the presence of these parasites is currently unknown. To study this, it would be essential to accurately characterize the diversity and composition of these nematode communities. Current practices to characterize nematode communities mostly use molecular techniques like qPCR and short-read metabarcoding (Geisen *et al.* 2018). These techniques

are feasible for studying trophic groups of nematodes, but do not suffice when a lower taxonomic resolution (i.e. genus or species) is required. Alternatively, light microscopy could be used to precisely identify nematode species, but this requires extensive expertise and time (Van Megen *et al.* 2009). Aforementioned techniques are thus not suitable to accurately determine nematode community diversity and composition in large-scale experiments, however the long-read technology of Nanopore sequencing now allows to sequence the complete 18S gene. Use of this long 18S fragment often enables species-level metabarcoding of nematode communities (van Himbeeck *et al.* 2023).

In this study, we initially assess the (a)biotic environment where *C. elegans* was detected and thereafter, parasite presence was linked to the species diversity in nematode communities to investigate if dilution effects occur in microorganisms. During the autumn season natural nematode communities were collected from decaying organic matter. Sampling locations were chosen based on where *C. elegans* was previously found (Cook *et al.* 2017), so that molecular tools in this species could be used for efficient discovery of stress induced by bacterial, microsporidian and viral parasites. Morphological and Nanopore 18S gene amplicon sequencing reconstructed the nematode diversity and community networks which were connected to parasite observations. Altogether, our results provide the first indication of disease-diversity relationships within microbial communities and prospect a new experimental model system.

Methods

Sampling procedure and site description

Sampling was performed at the following three locations in the Netherlands: private garden (Heelsum), private vegetable garden (Wageningen), and patch of green surrounding a ditch (Renkum) (Supplementary Table S1). These locations were chosen, because these stably contained *Caenorhabditis* species for the last two to seven years in autumn (Cook *et al.* 2017). The locations were sampled five times every three weeks starting on August 30th 2021. At least 10 samples of decaying organic matter that appeared suitable habitats for bacterivorous nematodes (typically plants in a late stage of decay) were collected at each location. A similar sampling procedure was used for all trips (opportunistic selection of available decaying organic matter), but when possible previous finding locations of *C. elegans* or parasites of bacterivorous nematodes were resampled. Because of the perishable nature of the samples, resampling was not always possible. Additionally, on-site humidity and temperature were recorded, and samples were documented in five categories: fruits, vegetables, nuts, soil and (other) plant materials. A schematic overview of the order in which collected samples were processed can be found in Supplementary Figure S1.

The Heelsum private garden has a partly forest-like nature with oak trees (*Quercus robur*) and undergrowth of dead-nettles (*Lamium* spp.) and green alkanet (*Pentaglottis sempervirens*) plants. Another part of the garden consists of a grass lawn with elevated vegetable garden boxes and a compost heap. The Wageningen vegetable garden contained about 15 different vegetable species and two compost heaps that are located adjacent to the vegetable patches. The edges of the garden contain several trees and fruit scrubs. The Renkum patch of green mostly contains butterbur (*Perasites hybridus*) plants and common nettle (*Urtica dioica*) surrounding a ditch.

Sample processing and nematode extraction

The active nematode community was extracted from the plant samples as previously described (Van Bezooijen 2006). In short, each sample (1-20g) was homogenized in a ~100mL of water using a kitchen blender (Princess, model 2005) at full speed for 5-10s. The pH of 2mL homogenized blender solution was measured at room temperature (Jenway pH electrode 924 904). Moreover, 200µL was placed on each of the three 6cm NGM plates containing either the *C. elegans* reporter strain AGD926, SJ4100 or AU133 (see bacterial reporter strain assay below). The remaining blender solution was placed on a custom-made nematode filter consisting of a Universal Hygia Milk filter (928157-56) placed on top of a Lekko (S475-30) filter clamped in a nematode extraction sieve wherein nematodes were extracted overnight in ~100mL water at room temperature (~20ºC). For each sample, dry weight and substrate moisture were determined after drying a subsample (1-10g) for 48h at 60ºC.

After nematode extraction, the solution containing the nematodes was put in a glass beaker and left to settle for 1h. Next, two-thirds of the supernatant was discarded and the remaining solution was transferred to a glass centrifuge tube. After the solution settled for 1h, a 1mL pellet (containing the nematodes) was transferred to a 9cm nematode growth medium (NGM) plate without bacteria. Then the number of nematode species in the population was determined morphologically under a Leitz Wetzlar stereo microscope (based on adult female characteristics including size, vulval placement, mouth and tail structure) and any remarking population characteristics were noted (e.g. presence of unhealthy looking nematodes). A maximum of three potential *C. elegans* nematodes per plate were

isolated for further propagation and species-specific PCR test (Barrière & Félix 2005). Half of the isolated wild nematode community was flash-frozen in liquid nitrogen 48h after the start of the nematode extraction for sequencing purposes. The remaining 50% of this isolated community of nematodes was split in two samples of 25% and placed on 9cm plates containing *E. coli* OP50. These samples were subsequently used for microsporidian and virus identification (see protocols below). Some populations containing few nematodes were not split and the full sample was flash frozen for sequencing instead. These samples were not considered in further analyses of parasite presence.

Samples were kept at 4ºC from sampling onwards, except for the nematode extraction, species diversity check and selection of nematodes for propagation that were performed at room temperature (~20ºC). Nematode extraction took 22h on average and screening and selection of the nematodes added about another 4h at room temperature.

Microsporidian identification in extracted nematode communities

Filtered nematodes were propagated for ~5 days on a 6cm NGM plate seeded with 10x *E. coli* OP50. Nematode communities that perished before this procedure or contained mites were not considered in subsequent analyses. Once the population was composed of a large quantity of non-starved adults, a portion of the plate was chunked onto a new seeded 6cm NGM plate to continually propagate potential infections. The remainder of the plate was then washed with 700 µl of M9 media and placed in a microcentrifuge tube. Samples were allowed to gravity settle for ~1-5 minutes, to remove contaminating bacteria and fungi through supernatant removal. 700 µl of acetone was added to the nematode pellet prior to spinning down in a centrifuge for 30 seconds at 8,000 rcf. The supernatant was discarded, and 500 µl of the chitin binding Direct Yellow 96 (DY96) solution (1x PBST, 0.1% SDS, 20 ug/ml DY96) was added. The samples were incubated for 30 minutes in the dark at 20°C prior to spinning down in a centrifuge for 30 seconds at 8,000 rcf. The supernatant was removed, and 20 µl of EverBrite Mounting Medium (Biotium) was added to the samples prior to mounting on glass slides for imaging using an Axio Imager.M2 (Zeiss). Z-stacks were captured at 63x using an apotome unit with maximum projection. Samples were considered infected with microsporidia if spore clusters were visible in a population. Spore sizes were assessed by measuring the length and width of at least 45 spores using Zen software.

To identify the species of microsporidia present within a nematode sample, molecular characterization was performed. Briefly, 20 large adult nematodes were placed in 10 µl of lysis buffer (50mM KCl, 100mM Tris-HCl, 2.5mM MgCl2, 0.45% NP40) and placed in a thermocycler at 65°C for 60 minutes, followed by 95°C for 15 minutes. 2 µl of lysate was then used as template in a PCR reaction with the forward primer V1F [5' CACCAGGTTGATTCTGCCTGAC-3'] and the reverse primer 1492r [5'-GGTTACCTTGTTACGACTT-3'] or 18sR1492 [5'- GGAAACCTTGTTACGACTT-3'] to amplify microsporidian 18S. Sanger sequencing was performed on amplicons using the same primers listed above.

Indication of present parasites or parasites in the homogenized substrate using fluorescent reporter strains

C. elegans reporter strains AGD926 (zcIs4[*hsp-4::GFP*]), SJ4100 (zcIs13[*hsp-6p::GFP*]) and AU133 (agIs17[*myo-2p::mCherry + irg-1p::GFP*]) were obtained from the Caenorhabditis Genetic Center (CGC). These strains indicate endoplasmic reticulum (ER) stress, mitochondrial stress or bacterial virulence respectively (Supplementary Table S2) (Samuel *et al.* 2016) and were used to explore presence of harmful components in the microbiota of the homogenized substrate.

Starved populations from the reporter strains (AGD926, SJ4100, AU133) were transferred to fresh 6cm NGM plates containing *E. coli* OP50 just before nematode extraction from natural substrates. From each homogenized substrate 200µL blender solution was equally spread over the plates containing the reporter strains that were then incubated at 20ºC for 48h. After incubation, reporter nematodes exposed to blender solution were checked to investigate if GFP fluorescence was higher than GFP expressed by nematodes grown on control plates containing only *E. coli* OP50 (Samuel *et al.* 2016). Fluorescence was checked using an Olympus SZX10 microscope with a NIGHTSEA Microscope Fluorescence Adapter.

Observation of intracellular parasites using Intracellular Pathogen Response and viral stress reporter strains

Extracted nematode communities were co-cultured with each of the following four reporter strains to observe activation of antiparasitic and antiviral pathways: SOW1 and SOW6 that detect upregulation of IPR genes *eol-1* and *pals-5* and SX2790 and SX2999 that detect upregulation of the antiviral gene *lys-3* (Supplementary Table S2) (Le Pen *et al.* 2018). Fluorescence was checked once per day for 7 days using an Olympus SZX10 stereomicroscope equipped with an SZX-RFA stereo fluorescence illuminator unit and filter sets for GFP and RFP fluorescence viewing. Co-cultures were counted as positive if >10% of the reporter *C. elegans* were fluorescing.

Extraction and screening for potential viruses

Co-cultures that resulted in reporter *C. elegans* fluorescence were washed off of test plates using M9 buffer and concentrated into 1 ml total volume via centrifugation. They were then homogenized via vortexing for 4min with 30-50 1 mm diameter silicon carbide beads (BioSpec Products). Homogenate was centrifuged to pellet debris, and supernatant was passed through a 0.22 µm pore size syringe filter (GenClone). Filtrates were seeded onto plates with SOW6, SOW1, SX2790, or SX2999 reporter strains and observed for fluorescence each day for 7 days.

DNA and RNA extraction

DNA and RNA were isolated from samples that contained at least 100 nematodes at the moment of flash-freezing. From the 112 samples that met this requirement RNA and DNA was extracted as described previously by Harkes *et al.* (Harkes *et al.* 2019). In short, nematodes were bead homogenized using 3mm beads in a retch. Then RNA was isolated with a pH 4.5 phenol washing step, followed by a DNA isolation buffer washing step at pH 8.0. DNA and RNA concentrations were determined via Qubit fluorometer measurements (Thermo Scientific).

Nematode DNA amplification

After DNA/RNA extractions, 81 samples contained sufficient DNA quantity for 18S rRNA amplification. EXP-NBD196 (Oxford Nanopore Technologies ltd., UK) barcoded primers 988F (5'-

ctcaaagattaagccatgc-3′) and 2646R (5′-gctaccttgttacgactttt-3′) were used to amplify the near-complete 18S rRNA fragment (~1750bp) (van Himbeeck *et al.* 2023; Van Megen *et al.* 2009). PCR was performed in quadruplicate per sample and each reaction consisted of 12.5 µl LongAMP Taq 2x MM, 400 nM of each primer, 3 µl DNA template and 5.5 µl autoclaved Mili-Q water. After amplification all 4 PCR replicates were pooled and DNA amplification was verified on agarose gels. Finally, DNA concentrations were determined via Qubit 4 Fluorometer measurements (Thermo Scientific).

Nematode 18S sequencing, read processing and species identification

The 18S rRNA was successfully amplified for 78 samples that were used for sequencing in four batches where each library contained equimolar ratios of all samples. Unwanted small fragments (<600bp) were removed prior to sequencing using NucleoMag NGS beads (0.5:1 bead:sample ratio) (Macherey Nagel). Library preparation was performed using the SQK-LSK112 kit, following the instructions of the manufacturer. Sequencing was performed on a MinION Mk1C using R9.4.1 flow cells.

Base-calling was performed using Guppy (v 6.2.1) in super accuracy mode (https://community.nanoporetech.com). The basecalled reads were demultiplexed using Guppy barcoder (v 6.2.1.) and adapters and barcodes were removed. Three samples (LvS26, LvS81, and LvS148) were excluded from the analysis at this stage because insufficient reads were obtained. Basecalling quality was controlled using NanoPlot (v1.40.0) (mean Phred score > 15) and Decona (v0.1.3) was used to filter 1400-2600bp reads with a >Q15 quality score (De Coster *et al.* 2018; Oosterbroek *et al.* 2021). Decona then clustered reads at 95% and Medaka consensus sequences were created from clusters with at least 100 reads. NCBI BLAST was used with an in-house database for taxonomic identification of nematode species (van Himbeeck *et al.* 2023; Van Megen *et al.* 2009). Identifications with a similarity below 97% were excluded from the dataset. This data processing resulted in a OTU table containing the number of sequencing reads of each nematode taxon per sample. From this table, the number of species per sample was determined by summing the number of taxa with a read count larger than 0.

Data analysis

All data was processed and visualised using the package *tidyverse* and *ggpubr* in custom written scripts in R (v 4.2.1) (Kassambara Alboukadel 2023; Wickham *et al.* 2019). Generalized linear models (glm), Chi-square and Wilcoxon Rank Sum tests were computed with *R base*. The effect of abiotic factors on community compositions was tested after creating a 'physeq' object in the *phyloseq* package and performing a PERMANOVA based on Bray-Curtis dissimilarity index (n = 10,000) using 'adonis2' from the *vegan* package (McMurdie & Holmes 2013; Oksanen *et al.* 2020). The *vegan* package was also used for calculating diversity indices (richness and Shannon index (H')) with 'specnumber' and 'diversity' functions (Oksanen *et al.* 2020). To account for unequal sampling effort, normalized nematode richness was defined as the total number of nematode species (based on sequencing data) found in a sample, divided by the number of individuals (based on microscopy-based count data) per gram wet substrate found in that sample. Nematode co-occurrence networks were based on significant Spearman correlations (p adjust < 0.05) between species (that were collected at least three times) as calculated with the 'rcorr' function in the *Hmisc* package and adjusted by the

Holm-Bonferroni method. Networks were visualized in Cytoscape (v3.9.1) when at least 3 species were present within a network.

Data availability

C. elegans strains isolated in this study are available from CaeNDR (caendr.org) (Cook *et al.* 2017). Custom written scripts and raw data files can be found on https://git.wur.nl/published_papers/van_himbeeck_2023_nematode_diversity_and_parasites/-/tree/main. The nematode sequencing data is accessible on SRA under BioProject PRJNA1021795. Processed data is included in the Supplementary Tables of this manuscript. Microsporidia 18S sequences are available in NCBI under the following accession numbers: OR636101, OR636102 and OR636103.

Results

Nematode community networks of the nematode C. elegans and co-occurring species

To link parasite presence to interspecies diversity in microorganisms, nematode communities were collected and analyzed. Nematode communities were obtained from decaying organic matter at three locations where *Caenorhabditis elegans* has previously been identified in the Netherlands (Cook *et al.* 2017) (Supplementary Figure S2, Supplementary Table S1). Nematode communities were subsequently extracted from the substrates, counted and the number of nematode species present was microscopically and molecularly established. Moreover, molecular analysis of the samples allowed for precise determination of the species present. The presence of *C. elegans* was of specific interest in this study, as this species was used as a reporter to monitor parasite presence.

In total 160 nematode communities were collected from ephemeral substrates and 76 of these were further characterised by sequencing (Supplementary Table S3). In these samples 58 different nematode species were molecularly characterised (Supplementary Table S4, S5). For 29 nematodes species only the genus name could be noted, as the used reference database does not always provide sequences annotated to species-level. The majority of the detected taxa were either bacterial or fungal feeders but also insect-parasitic, plant-parasitic and predatory nematodes were present (Supplementary Table S6). The estimated variance in nematode community composition originated for 20% from seasonal change, sampling location, moisture and pH differences (Supplementary table S7). Remaining variance in community compositions is likely in part attributable to biotic interactions, including nematode-bacteria, nematode-parasite, and nematode-nematode interactions (Okamoto *et al.* 2019; Vandvik *et al.* 2020).Nematode-nematode interactions were examined by searching for specific cooccurrence or exclusion of certain species between samples. Most of the identified nematodes here were either opportunists or part of the basal fauna, although a few species exemplary for progressive succession (~5% of species) were also identified (Ferris *et al.* 2001). The species that formed cooccurrence networks were typically part of basal nematode fauna that is maintained over longer periods of time (Supplementary Table S4 and S8; Figure 1A). We did not observe any significant negative correlation between species. *Panagrolaimus subelongatus* (17), *Rhabditophanes sp.* (16), *Panagrolaimusrigidus* (16), *Acrostichus sp.* (15), *Rhabditoides sp.* (13), *Panagrolaimus sp.* (13), *Rhabditella axei* (12), *Pristionchus entomophagus* (10) and *Eucephalobus striatus* (10) co-occurred at least 10 times with *C. elegans* agreeing with previously found coincidences of these species (Félix and Duveau, 2012). Nevertheless, *C. elegans* nematodes did not coincide with these particular species more often than expected based on chance which underlines the opportunistic behaviour of *C. elegans* (Supplementary Table S4). Moreover, *Caenorhabditis* species are known to coexist together in some places (Félix & Duveau 2012; Schulenburg *et al.* 2014; Sloat *et al.* 2022). Here, only one sample with a second *Caenorhabditis* species was obtained. This decaying kohlrabi contained *C. briggsae* and 7 other nematode species, but not *C. elegans*, despite molecular identification of *C. elegans* on other kohlrabies from the same location. *C. elegans* was detected in more than 71 % of the samples and we found that communities where *C. elegans* is present and absent have similar nematode richness (p=0.56, Wilcoxon Rank-Sum test). These findings support our use of it as a parasite reporter species for communities that differ in nematode diversity.

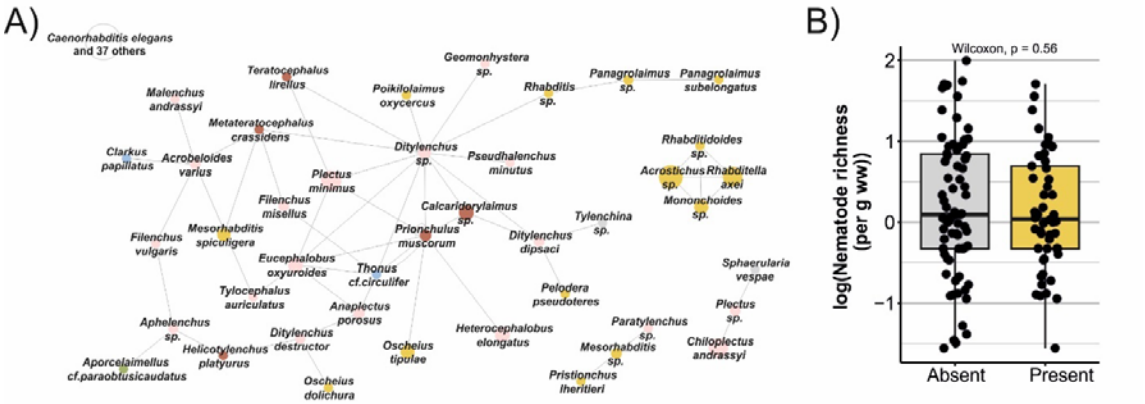

Figure 1 Nematode-nematode co-occurrences in decaying organic plants – A) Spearman-based (adjusted $p < 0.05$) co-occurrence networks of species that were present in at least 3 samples and contained at least 3 species. Colours indicate the c-p value of the species depicted (colonizer-persister value; higher values indicate species typically present in more mature nematode communities): yellow indicates c-p value 1, pink indicates c-p value 2, red indicates c-p value 3, blue indicates c-p value 4, green indicates c-p value 5, grey indicates c-p values are not known or variable for this genus (Ferris *et al.* 2001). Circle size reflects the number of samples in which a species was observed in the sequencing data. B) Nematode richness (in normalized species richness) in samples where *C. elegans* was absent or present.

Nematode diversity determines parasite presence in natural field populations of nematodes

Collected nematode communities were screened for phenotypes that indicate the presence of intracellular parasites and/or pathogenic bacteria. Nine different classes of biotic stress were observed, which were screened using four distinct experimental procedures (Figure 2A): i) microsporidia were visualised by fluorescent staining of extracted nematodes (Jarkass *et al.* 2022) ii) mitochondrial stress, ER stress and stress caused by bacterial virulence were indicated by growing fluorescent reporter strains on the homogenized substrate (Samuel *et al.* 2016), iii) IPR activation and viral stress were observed by co-culturing four different fluorescent reporter strains with the field extracted nematode community and iv) presumptive viruses were detected based on co-culturing 0.2 µm filter extracts (that activated multiple reporter strains in assay iii) with IPR or viral stress reporter strains. Some nematode communities died before they could be screened or could not be maintained successfully in the laboratory, and were excluded from further analysis (Figure 2B). All experiments harboured indications of parasite presence and 80% of the collected samples responded in at least one of the experimental assays (Figure 2C). In most cases, samples were found positive for multiple stressors (Figure 2C). Notably, these numbers likely underrepresent the total number of parasites occurring in the wild as not all may infect the reporter strains used, parasites might be lost during the extraction procedure and nematodes potentially evade parasites in some of the assays (Kaletsky *et al.* 2020; Meisel & Kim 2014; Schulenburg & Ewbank 2007). Taken together, these data suggest that biotic stress is a common burden for wild nematodes.

To answer our main research question, namely if nematode diversity lowers parasite presence, biodiversity of nematodes was expressed in richness (species number by sequencing) and a combination of richness and evenness (species number and the relatively proportion of each species; Shannon's index) (Supplementary Table S9). The nematode diversity was normalized (for substrate and community size) to facilitate cross-sample comparisons as the samples collected in this study varied because of ephemeral nature of the substrates and the consequent opportunistic sampling method that was applied. Subsequently, we compared parasite presence to nematode diversity for each of the experimental assays. For two assays, density-disease correlations were observed: 1) microsporidia were more often present in communities with lowered nematode richness (Figure 2D) and 2) activation of the *eol-1*-based IPR reporter strain was more often observed in co-cultures when communities with a lowered nematode richness (Figure 2E). In agreement with previous studies, we found that presumptive *C. elegans*-infecting viruses were rare and observed in only 2 (1.5%) of the screened communities (Frézal *et al.* 2019). Both communities contained lower than average nematode richness (Figure 2F), but the low incidence of viral infections obstructs drawing solid conclusions from this observation. The other experimental assays did not yield any disease-dilution effects (Supplementary Figure S2), which may relate to the specialization of the studied parasites as dilution effects are not expected when generalist parasites are present (Keesing & Ostfeld 2021). Importantly, these findings are backed by the morphology-based dataset (Supplementary Table S3). Finally, the observed dilution effects were observed for species richness, but not species evenness, suggesting presence of key species in the communities that either greatly spread (e.g. 'superspreaders') or dilute (e.g. 'superdiluters') parasites (Supplementary Figure S4).

Diversity-disease relationships can be driven by host density, frequency or a combination of both (Collins *et al.* 2020; Faust *et al.* 2017; Keesing & Ostfeld 2021; Su *et al.* 2022). To explore which of these may determine diversity-disease relationships in microbial nematode communities, the data gained in the microsporidian assay was explored further. We identified microsporidia in 15 out of 76 screened samples (16%) (Supplementary figure S5, S6). Almost half of these microsporidia isolates were found in *C. elegans* (Supplementary Table S10). The microsporidian isolates included the previously described species *Nematocida parisii* (Troemel *et al.* 2008; Zhang *et al.* 2016), but not all microsporidia could be molecularly determined and may represent novel species. Moreover, microsporidia were identified in *Pristionchus uniformus*, *Panagrolaimus rigidus* and in other nematodes of which the species could not be determined. Here, host density was defined as the number of host species per wet gram of substrate and host frequency was defined as the proportion of the host amongst the different nematode species in the sample. Analysis by a generalized linear model suggested a trend that host density ($p = 0.058$) as well as the interaction between host density and frequency ($p = 0.072$) might affect microsporidian presence in *C. elegans* (Supplementary Table S11), but additional data would be essential to observe if the same trend remains. As indicated above, key species may affect spread of parasites, but we could not pinpoint presence of key species in our relatively small dataset containing only 3 identified microsporidian hosts species (chi-square $p > 0.05$ for expected versus observed microsporidia infections per species). Microsporidia spores are known to be resistant to environmental conditions, including pH and desiccation (Fenoy *et al.* 2009; Santillana-Hayat *et al.* 2002). Furthermore, pH has been shown to induce germination is thought influence the infectivity of microsporidia (Willis & Reinke 2022). Here, microsporidia were more often observed on alkaline substrates (Supplementary Figure S7), whereas substrate moisture varied (Supplementary

Figure S7. In conclusion, because pH and nematode diversity were not correlated (glm, p = 0.46 (Supplementary Table S12), microsporidian presence in our samples was more likely to be observed in nematode communities with lower richness (possibly dependent on relatively higher host density- and frequency within the community) and an alkaline environment.

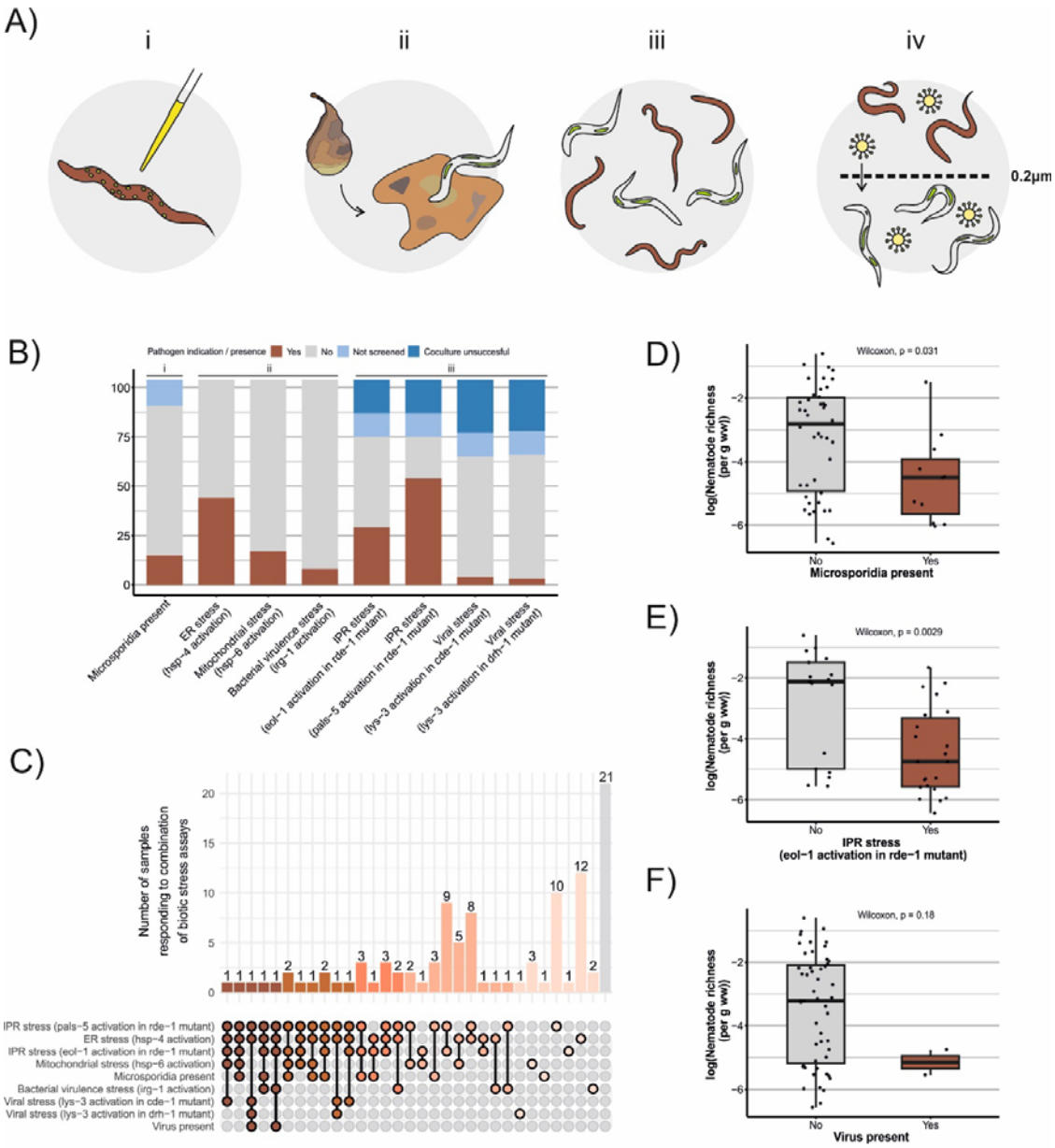

Figure 2 Biotic stress in nematode communities using *C. elegans* as a reporter species – A) The four different experimental assays to screen for biotic stress: i) staining of extracted nematodes to visualise microsporidia, ii) screening of *C. elegans* reporter strains on homogenized sample substrate containing a mixture of microbes, iii) co-culturing *C. elegans* reporter strains with the extracted nematode community and iv) co-culturing *C. elegans* reporter strains on 0.2 µm filtrate of the extracted nematode community B) The fraction of samples that was found to respond to each of the

biotic stress indication assays. Because only a selection of samples was screened for virus presence after a 0.2 µm filter step (assay iv) these samples are not depicted here. C) Overlap of biotic stress indications within each of the samples. The plot shows how many samples (count y-axis) responded to specific combinations of biotic stress assays (x-axis). Colours illustrate the number of stress assays a sample was found positive for ranging from five (red) to none (grey). D) Normalized nematode richness for samples in which microsporidia were present or absent. E) Normalized nematode richness for samples which did or did not indicated mitochondrial stress on upregulation of the *C. elegans* IPR gene *eol-1* upon coculture. F) Normalized nematode richness for samples which did or did not contain *C. elegans* viruses.

Discussion

Host-parasite relationships depend on host community biodiversity, as a high host biodiversity can reduce parasite prevalence, i.e. the 'dilution effect of disease' (Johnson *et al.* 2008, 2013b, a; Keesing & Ostfeld 2021). Although microbial communities are vital for ecosystem functioning and stability, host-parasite relationships and the dilution effect have been poorly studied in microbial communities. We argue that understanding diversity-disease effects in microbial communities is essential for predicting ecosystem functioning in a rapidly changing environment. Here we used wild nematode communities to study the effect of host diversity on the occurrence of parasites in microbial communities. Nanopore sequencing was applied for high-resolution characterization of nematodes present within each community. With this method we molecularly defined nematode communities naturally occurring with the important model organism *C. elegans*. Fluorescent reporter tools available in this model organism were used to identify bacterial-, microsporidian- and viral-induced stress inflicted on *C. elegans*. These screenings also discovered potentially new parasite species. In line with the dilution theory, specialistic parasites were more often found in communities with fewer nematode species, indicating that diversity-disease effects can be found among microbes.

Potential mechanistic bases of dilution effects in ephemeral nematode communities

The short-lived ephemeral nematode communities sampled in this study consisted mostly of bacterial-feeding *r*-strategists that could experience strong competition for food in their temporary environment. Competition between species can drive dilution effects, especially in absence of predation (Su *et al.* 2022). Although several predatory species were observed, these were often present at low density and frequency. An exception was formed for facultative predators like *Pristionchus* (Serobyan *et al.* 2014), but these may also feed on bacteria in this habitat. Together, low predation and high competition circumstances suggest possibilities for observation of dilution effects, and we indeed observed fewer parasites in communities with lower species diversity. Yet of note, potential predation by other species, such as mites or fungi was not studied here. In this study, most nematode taxa detected were either bacterial or fungal feeders, in contrast to agricultural soil communities where omnivorous, plant-parasitic and predatory nematodes are also prevalent (van Himbeeck *et al.* 2023). The more diverse life trait history strategies of nematodes in soil may reduce competition and increase mutualistic relationships between species. Under these conditions, amplification effects of disease may even be observed instead (Johnson *et al.* 2012; Su *et al.* 2022).

Nematodes on ephemeral substrates need to migrate between food sources and likely specialize on moving between these by using vectors such as isopods, slugs or snails (Chiba *et al.* 2023; Lee *et al.* 2017; Schulenburg & Félix 2017). Migration of ephemeral-specialised nematodes provides a spatial factor in the host-parasite dynamics that might be compared to that of a fragmented landscape. A systematic experiment altering host diversity of plant patches indicated dilution effects of a fungal disease became stronger on smaller spatial scale (Liu *et al.* 2023). This confirmed an earlier correlation study that demonstrated biotic interactions have more profound effects on prevalence of amphibian chytrid fungus, West-Nile virus and *Borrelia burgdorferi* (causing Lyme's disease) on a local than continent-wide scale (Cohen *et al.* 2016). Moreover, fragmentation of forests in residential areas resulted in a greater host diversity and lower encounter probability between competent hosts and

B. burgdorferi (Linske *et al.* 2018). Yet, the presence of an incompetent host like white-tailed deer within this system can cause a greater reducing disease spread than overall host richness (Linske *et al.* 2018). Thus, both the fragmentation of ephemeral nematode communities as well as the possible presence of key species ('superdilutors' or 'superspreaders') may contribute to the observation of diversity-disease effects in this study (Halliday *et al.* 2020).

Specific nematode-parasite interactions underlie dilution effects

Diversity-disease relationships within the nematode communities were tested via four different biotic stress assays examining nine classes of biotic stress. The vast majority of nematode communities in the collected samples responded in at least one of these tests, suggesting that biotic stress is frequent in wild nematode populations. Within these communities, we found a significant relationship between nematode diversity and intracellular parasite prevalence as 1) samples containing microsporidian parasites had a lower nematode diversity and 2) the *eol-1*-based Intracellular Pathogen Response reporter strain more often responded to communities with a lower diversity. The experimental assays to indicate biotic stress covered diverse possible origins of stress-inducing parasites with varying host specificities. Parasites most prone to experience dilution effects are those that have a narrow host range (Collins *et al.* 2020). Our data corresponds with this theory, as we observed dilution effects in microsporidian infected communities. Microsporidia are parasites of most types of animals with the vast majority of species only observed to display host and tissue specificity in one or two closely related hosts (Bojko *et al.* 2022; Murareanu *et al.* 2021; Willis & Reinke 2022). Microsporidia that infect nematodes also often display specialized tissue specificity, though there are examples of more generalist species(Wadi & Reinke 2020; Zhang *et al.* 2016). Microsporidian occurrence in communities with lower species richness thus enhances their chance of encountering a high-quality host. Moreover, we observed dilution effects using the *eol-1* reporter strain in *rde-1* immunocompromised *C. elegans*. *Eol-1* is an RNA decapping enzyme that is upregulated during viral and microsporidian infections and is involved in activating enhanced RNAi in response to mitochondrial stress(Chen *et al.* 2017; Han *et al.* 2019; Mao *et al.* 2020; Sowa *et al*. 2019).

For other biotic stress reporter assays, we did not observe dilution effects. The *hsp-6* reporter strain reports mitochondrial stress just like *eol-1*, but this reporter strain lacked the sensitivity-increasing *rde-1* mutation which is a possible explanation for not finding a dilution effect using this assay. Also reporter strains (based on *lys-3*) that respond to viruses did not show a correlation with nematode diversity, despite the narrow host range of viruses (Félix *et al.* 2011). Likely *C. elegans* viral infections are so rare that a larger study would be necessary to draw conclusions, as we have only identified two *C. elegans* viruses in our screening (Frézal *et al.* 2019). Another of the reporter strains responding to intracellular parasites (based on *pals-5*) did not show any diversity-disease relationship. It was noted that there were relatively few sequenced communities for which expression of this reporter strain was not observed. Finally, bacteria are usually opportunistic parasites not bound to certain hosts, therefore dilution effects were not expected. Indeed, these were not observed in the reporters strain that indicate bacterial virulence and also not in the ER stress reporter strain that detects stress caused by both generalists and specialists.

Detailed insight into ephemeral nematode community compositions that include C. elegans

To date, a comprehensive overview of nematode species co-occurring with *C. elegans* in its natural habitats was lacking. Historically, most studies investigated nematode community compositions in soil and water instead (e.g. for agricultural purposes or environmental monitoring). Here long-read sequencing data was used to identify community members that occur in the same habitats as *C. elegans*. We performed (nearly) full 18S sequencing to characterize the total nematode community on a low taxonomic level (mostly species or genus) (van Himbeeck *et al.* 2023), which yielded a total of 96 taxa. Subsequent studies may further broaden knowledge about *C. elegans* community compositions by simultaneously including other microfauna such as protists and bacteria. In agreement with previous studies, we identified nematodes that are known to co-occur with *C. elegans* from the genera *Caenorhabditis (C. briggsae), Oscheius (O. tipulae* and *O. dolichura), Panagrolaimus (P. detritophagus, P. rigidus, P. subelongatus), Rhabditophanes* and *Mesorhabditis (M. spiculigera)* and *Aphelenchoides* in our communities (Félix & Duveau 2012; Schulenburg *et al.* 2014). *E. striatus* is part of the basal nematode fauna, but all of the other species are species that quickly respond to enriched food resources (Ferris *et al.* 2001). The observation that these other nematodes do not specifically co-occur suggest that *C. elegans* can be considered an opportunist whose presence may be observed across suitable ecological niches of ephemeral substrates in the Netherlands.

Conclusions

Biodiversity is important as it not only contributes to ecosystem functioning, but also to ecosystem stability, as exemplified by the dilution effect theory. Below-ground microbial communities - such as nematode communities - are vulnerable to shifts and biodiversity loss due to global change (Berlinches de Gea *et al.* 2022). When biodiversity decreases, parasites could possibly encounter a suitable host more frequently, leading to a higher parasite prevalence under (microbial) host populations and thus abolishing the dilution-effect. As microbial communities are vital for the majority of ecosystems, it is important to understand their response to a decrease in biodiversity in terms of parasite dynamics. Our study provides the first demonstration of disease dilution effects in microbial communities. Dilution effects of disease depend on dynamic multifactorial processes in communities, and as such may be strikingly difficult to understand or capture. Studying dilution effects in nematode populations poses a promising outlook for the future. In this study, we have shown that communities can be extracted from the field through the use of a small plastic bag and these could be observed and subsequently disassembled in the lab. Many collected species were successfully cultured afterwards, allowing for follow-up experiments to determine exactly which factors could cause dilution effects to occur in these communities. Under laboratory settings, one can deduct or control factors such as species presence, density and select community members (including competitors and predators) based on specific life history traits. Furthermore, nematode parasites can be examined to trace infection and transmission (even in real time) (Pirenne *et al.* 2021; Shaw & Kennedy 2022). Together, our results invite further characterization of host-parasite interactions in microbial communities, and open opportunities for using nematodes communities as a diversity-disease model system in the lab and field.

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Supplementary information

Supplementary Table S1 – Summary of sampling location information – The table indicates location details and number of collected samples at each location.

Supplementary Table S2 – *C. elegans* reporter strain genetic information

Supplementary Table S3 – Collected sample data – Contains information per sample collected including sample location, data, type, substrate weight, moisture, pH, nematode density and normalized species richness (based on morphological species identification) and whether or not *C. elegans* was isolated from the sample.

Supplementary Table S4 – Nanopore sequencing data output – The data shows reads per species per sample.

Supplementary Table S5 – Sequencing abbreviations of nematode species – The data shows reads abbreviations used for sequenced nematode species.

Supplementary Table S6 – Overview of molecularly identified nematodes in the sequenced nematode communities – The table indicates the nematode taxon, Maturity Index (according to its c-p value), feeding preference and percentage of the samples in which this taxon was discovered.

Supplementary Table S7 – Effect of abiotics on community composition based on a permutation model

Supplementary Table S8 – Co-occuring species in nematode communities – Based on Spearman correlations and Hochberg-corrected p-values.

Supplementary Table S9 – Diversity indices of sequenced samples – Shannon's H, absolute richness (number of species) and normalised species richness (species corrected for sampling effort) for each sample based on sequencing.

Supplementary Table S10 – Data microsporidian species and hosts – PCR-based host and microsporidian species identification and morphological characteristics of the nematode species identified. Some PCRs failed or nematodes were lost during the screening, here the missing data is indicated with NA.

Supplementary Table S11 – Effects of frequency and density on microsporidia of *C. elegans* – GLM testing dilution and frequency effects on microsporidian occurrence in C. elegans.

Supplementary Table S12 – Correlation of normalised richness and pH – GLM testing effect of pH on sequence-based normalised species richness.