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A full-length SSU rRNA-based workflow for high-resolution monitoring of nematode communities reveals direct and indirect responses to plant-based manipulations

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ABSTRACT

Agricultural intensification has resulted in a decline in soil biodiversity, and concerns about the deterioration of the biological condition of soils prompted the development of measures to restore soil life. Due to the overwhelming biodiversity of soils, evaluation of such measures is not straightforward, and proxies are used to assess soil health. Because of their trophic diversity, high abundance, and relatively well-characterized ecologies, nematodes are often used as soil health indicators. However, the scarcity of informative morphological characters hampers the upscaling of this proxy. Here we present a community analysis approach that uses nanopore sequencing to generate full-length sequences of small subunit ribosomal DNAs (SSU rDNA). Cover cropping is a common agricultural practice that stimulates soil life, and we mapped the effects of ten cover crop treatments on nematode communities in a field experiment. These analyses included the monitoring of a high-impact plant-parasite, *Meloidogyne chitwoodi*. In total, 132 nematode samples were analyzed, and 65 nematode taxa were detected, mostly at the species level, including representatives of all trophic groups. As a validation, all samples were analyzed microscopically for *M. chitwoodi,* and a comparison of count and DNA read data revealed highly similar results. Treatments did not only affect plant-parasitic nematodes but also free-living nematodes in a cover crop-specific manner. Free-living nematodes from the same trophic group, and even congeneric species, responded differentially to plant-mediated manipulations of the soil microbiome. Hence, nanopore-based SSU rDNA sequencing could facilitate a substantial refinement of the use of nematodes as indicators for soil health.

1. Introduction

Soils belong to the most densely inhabited and biodiverse habitats on Earth. Microbiota in terrestrial soils are pivotal to major ecosystem functions such as carbon, nitrogen and phosphorous cycling, the generation of plant-available forms of macro and micronutrients, and soil aggregate formation (Bahram et al., 2018). Current agricultural intensification practices have been shown to result in a decline in soil biodiversity (Tsiafouli et al., 2014), and this may threaten the ecological functioning of soils. Currently, there is an urgent need for management practices that could contribute to the restoration of these ecosystem services.

Mapping and monitoring the effectiveness of sustainable soil management measures is non-trivial as soils harbour overwhelming biodiversity. In terms of biomass and biodiversity, bacteria and fungi are the dominant organismal groups in terrestrial ecosystems, but using these communities as soil health indicators is unpractical due to their extreme diversity and our limited understanding of the ecological roles of many taxa. Among the major soil organismal groups, nematodes are considered an informative group for soil health assessment (Puissant et al., 2021) due to their trophic diversity (Holtkamp et al., 2008; Biswal, 2022), well-known response to environmental disturbance (Bongers, 1990), and ease of extraction from the soil (Verschoor and de Goede, 2000). Although nematodes meet some major requirements to serve as a proxy for the soil's biological condition, their routine use is hampered by their morphological uniformity.

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Currently, nematode communities are characterized by either microscopic analyses or by DNA-based methods such as RT-PCR and short-read metabarcoding. Microscopic analysis of nematode communities has a few intrinsic limitations. Microscopic nematode identification is labour intensive, requires ample training, and typically only the first 100 to 150 individuals or 10% of the individuals, are taken into consideration (Ewald et al., 2022; Quist et al., 2016). Moreover, for many nematode taxa, only adult life stages can be identified, implying that juveniles often are not taken into consideration. Phylum-wide molecular phylogenetic studies clearly demonstrate that numerous morphology-based nematode families are para- and/or polyphyletic and often harbour representatives with distinct ecological characteristics (see, e.g., Bik et al., 2010; Meldal et al., 2007; Van Megen et al., 2009). Hence, it is desirable to have a taxonomic resolution beyond the family level. So, the use of nematode communities as a proxy for the soil biological condition (1) would require the analysis of a representative part of the nematode community (typically thousands of individuals), (2) should consider individuals of all developmental stages, and (3) should offer a high taxonomic resolution (typically genus or species level). These criteria could be met by using a DNA-based community analysis approach.

DNA-based characterization of nematode communities requires a versatile molecular framework. Various molecular markers have been proposed for such a framework, and the small subunit of the ribosomal DNA (SSU rDNA, also referred to as 18S rDNA) is currently, by far, the most used molecular marker for nematodes. NCBI (https://www.ncbi.nlm.nih.gov), for example, harbours about 30,000 partial or complete nematode SSU rDNA sequences. SSU rDNA is known as a conserved gene, and probably because of the ancient nature of the phylum Nematoda, this gene (≈1700 bp) offers a remarkably good taxonomic resolution (e.g., Holterman et al., 2006; Meldal et al., 2007). Short-read metabarcoding to characterize (artificial) nematode communities was first used by Porazinska et al. (2009). Later on, Illumina MiSeq sequencing of the V4 or the V5–V7 region of SSU rDNA was applied to map nematode communities. However, the resolution offered by either of these regions is often limited to family or order level (Harkes et al., 2020; Jorna et al., 2023; Shepherd et al., 2023), or - with a substantially decreased confidence threshold - to genus level (Du et al., 2020; Kitagami and Matsuda, 2022).

SSU rDNA harbours nine variable regions (V1–V9), and ideally, the informative signals present in all nine variable regions should be exploited. This is not possible with second-generation sequencing platforms (e.g., Illumina or IonTorrent). Long-read nanopore sequencing by platforms of Oxford Nanopore Technologies allow to sequence the complete SSU rDNA gene, which harbours the potential for species-level metabarcoding of nematode communities (Van Megen et al., 2009). Initially, the main challenge regarding nanopore sequencing was its relatively high error-rate (~10%) (see, e.g., Rang et al., 2018). However, nanopore sequencing chemistry and bio-informatics tools are subjected to constant improvement. Introduction of the Q20 chemistry resulted in a current modal raw read accuracy of 99% (Oxford Nanopore Technologies, 2023), and consequently the impact of reads errors on microbial analyses is limited (Ciuffreda et al., 2021). Next-generation sequencing technologies develop fast and for chronological platform comparisons including nanopore sequencing we refer to reviews by Hu et al. (2021), Pervez et al. (2022), and Satam et al. (2023). Full-length SSU rDNA nanopore sequencing has been used before to test DNA barcoding on an artificial community of four different nematode species (Knot et al., 2020), but – to our best knowledge – this has not been used for nematode community metabarcoding.

To test the potential of nanopore sequencing-based nematode community analyses, we used full-length SSU rDNA sequencing to map the impact of cover cropping, a practice that is frequently used in the framework of sustainable soil management. Cover crops prevent nutrient leaching and elevate the soil organic matter content, but they are also known to stimulate the soil microbiome (Blanco-Canqui et al., 2015). This stimulation during plant growth is triggered by the passive as well as active release of primary and secondary metabolites (Canarini et al., 2019). The plant species-specific release of secondary metabolites in the rhizosphere allows plants to promote selected fractions of the microbial community present in the bulk soil. Currently used cover crops belong to various plant families that are characterized - among others - by the below-ground release of family-specific categories of allelochemicals (see, e.g., Bressan et al., 2009; Hu et al., 2018). At the end of the growing season, cover crops are terminated and incorporated in the topsoil, and residues give rise to another shift in the soil microbiome in a manner that depends on the chemical composition of these residues (Liu et al., 2021).

In an experimental field setting, the effect of ten cover crop treatments that are known to differentially affect the soil microbial community (Cazzaniga et al., 2023), as well as a fallow control on nematode communities, was tested. It should be noted that one trophic group, the plant-parasitic nematodes, is directly impacted by cover crops. At the onset of this research, the experimental field was found to be infected with a low density of the Columbia root-knot nematode *Meloidogyne chitwoodi*. This allowed us to investigate – next to the cover crop effects – the impact of elevated *M. chitwoodi* densities on other plant-parasitic nematodes as well as on the free-living fraction of the nematode community. First, a full overview of the nematode communities present in the field was generated employing nanopore sequencing. This was followed by a validation step in which SSU rDNA sequence reads were compared with count data from microscopic sample analysis. This was done for *M. chitwoodi*, as this plant-parasitic nematode species can routinely be detected and quantified based on its morphological characteristics. In the next step, the following soil ecological questions were addressed: 1) Does a strongly increased density of the plant-parasitic nematode *M. chitwoodi* impact other plant-parasitic and/or free-living nematodes? 2) How do cover crop treatments affect free-living and plant-parasitic representatives of nematode communities? 3) Does the high-resolution characterization of nematode communities (until genus and/or species level) have an ecological or agronomical added value?

2. Materials and methods

2.1. Experimental field set-up

The field experiment was set up at the Vredepeel experimental field station of Wageningen University and Research, Field Crops (WUR-FC). The Vredepeel farm is located in the southeastern part of the Netherlands (700–800 mm precipitation year^{-1}, mean temperatures of 11 °C) on sandy soil (93.3% sand, 4.5% silt, 2.2% clay) (Quist et al., 2016). The current experiment was embedded in a larger trial by WUR-FC aimed at assessing the host plant status of a selection of arable and cover crops in a field with a low density of the root-knot nematode (RKN) *M. chitwoodi*. The field experiment comprised six rectangular strips (each 6 × 42 m) organized in three blocks (Supplementary Fig. 1). In half of the strips, the initial RKN concentration was raised by growing an excellent host, black oat (*Avena strigosa*, cultivar Pratex). On the other half of the strips, perennial ryegrass (*Lolium perenne*, cultivar Mercedes), a poor host of *M. chitwoodi,* was grown. For the pre-crop treatments, no fallow controls were included.

Both poaceous crops were grown in the field between August 2018 and July 2019 and are referred to as “pre-crops”. Perpendicular to the longitudinal direction of these strips, 11 plots (each 6 × 3 m) were defined, and after pre-crop treatment, plots were exposed to ten cover crop treatments, whereas the 11th plot remained unplanted (fallow control). Hence, corresponding to the two pre-crop treatments, two types of fallow control are included. Cover crop treatments included six monocultures and four mixtures (Table 1). Cover crops were sown on August 7th, 2019, and grown for five months. On December 2nd, 2019, cover crops were mechanically terminated using a rotary tiller and residues were incorporated into the topsoil. In spring 2020, soil was

Table 1
Details of the cover crop species and cultivars used in this study, sowing density, and host status for *Meloidogyne chitwoodi*.

Cover crop treatment	Treatment Code	Species	Cultivar	Sowing density (kg/ha)	Plant host status for M. chitwoodi
Black oat	BLO	*Avena strigosa*	Pratex	80	Good
Oilseed radish	OSR_R	*Raphanus sativus* var. *oleiferus*	Radical	30	Poor-Moderate
Oilseed radish	OSR_A	*Raphanus sativus* var. *oleiferus*	Adios	30	Poor
Oilseed radish	OSR_T	*Raphanus sativus* var. *oleiferus*	Terranova	30	Non-host
Phacelia	PHA	*Phacelia tanacetifolia*	BeeHappy	10	Poor
Vetch	VET	*Vicia sativa*	Ameli	125	Poor
Black oat + Oilseed radish-R	BLO_OSR_R	*multiple*	Pratex + Radical	40 + 15	Good + moderate
Black oat + Oilseed radish-T	BLO-OSR_T	*multiple*	Pratex + Terranova	40 + 15	Good + non-host
Phacelia + Oilseed radish-T	PHA-OSR_T	*multiple*	BeeHappy + Terranova	7 + 15	Poor + non-host
Vetch + Oilseed radish-T	VET-OSR_T	*multiple*	Ameli + Terranova	70 + 15	Poor + non-host
Fallow	FW	-	–	–	–

tilled, and on April 30th, the main crop potato (*Solanum tuberosum,* cultivar 'Hansa', fully susceptible to *M. chitwoodi*) was planted. Potato was harvested on October 14th, 2020.

2.2. Nematode extraction and microscopic *M. chitwoodi* quantification

To assess the nematode soil community, bulk soil samples were collected at two time points: i) at cover crop termination (December 2nd, 2019, hereafter referred to as t1) and ii) after potato harvest (October 15th, 2020, hereafter referred to as t2). In both samplings, 1.5 l of topsoil was collected from the central area (1.5 × 2.7 m) of each plot with an auger (sampling depth 25 cm, ∅ = 12 mm). After mixing the soil, a subsample of 100 mL (≈120 g) was rinsed through 180 μm sieves. The organic material remaining on the sieve after rinsing was incubated on a filter in 100 mL of water for 4 weeks at 20 °C to allow nematode eggs present in the subsample to mature and hatch (='incubation fraction'). The fraction that passed the filter (particles <180 μm including most nematodes) was elutriated with an Oostenbrink funnel and collected on three stacked 45 μm sieves ('mineral fraction'). Following three-day incubation at 20 °C, the nematodes in the mineral fraction were concentrated into a 100 mL suspension. The total number of *M. chitwoodi* was determined by microscopic analysis on a Leica DMi8 (with 40× or 400× magnification) of two 10 mL subsamples from both the mineral and incubation fraction. In case fewer than 100 *M. chitwoodi* were found in the two subsamples of 10 mL, the number of *M. chitwoodi* nematodes in the remaining fraction (80 mL) was counted as well. After counting, nematode subsamples were poured back into the original suspensions. So, complete mineral and incubation fractions were used in subsequent steps.

2.3. DNA extraction, purification and amplification

Total DNA was extracted from both the incubation and mineral fractions. To this end, nematode suspensions were first concentrated to 2 mL, then dried overnight at 65 °C. The dried pellet was resuspended in a nematode-lysis buffer and incubated at 65 °C for 2 h, as described by Holterman et al. (2006) and Vervoort et al. (2012). Lysates were purified according to Ivanova et al. (2006) using glass fibre filtration plates. Purified nematode community DNA was eluted and immediately stored at −20 °C. The DNA concentration of the combined purified lysates was quantified using a Qubit Fluorometer and subsequently diluted to an end concentration of 0.1 ng/μL.

Primers 988F (5′-ctcaaagattaagccatgc-3′) and 2646R (5′-gctaccttgt-tacgactttt-3′) (Holterman et al., 2006) were used to amplify the nearly complete SSU rRNA gene, approximately 1700 bp. Primer pairs were barcoded with barcode sequences of the EXP-NBD196 kit (Oxford Nanopore Technologies plc., UK) for sample multiplexing. PCR was performed in simplex, and each reaction contained 12.5 μL LongAMP Taq 2× MasterMix, 200 nM of each primer, 7.5 μL autoclaved Mili-Q water and 0.3 ng DNA template. DNA was amplified using a thermocycler running the cycling conditions specified in Table 2. As the samples primarily consisted of nematode DNA, a reversed touchdown-PCR could be used that allows for SSU rDNA amplification even if, for some taxa, the flanking region does not perfectly match the PCR primers. After DNA amplification, 4 μL of PCR product was loaded on a 1.5% agarose gel to verify the amplification and the concentration of all PCR products was measured using a Qubit 4 Fluorometer.

2.4. Library preparation and sequencing

Four sequencing libraries were generated to cover the 132 samples, and within each library, samples were pooled in equimolar ratios. To remove unwanted small fragments (<1,000bp), each library was bead-cleaned using 0.5× NucleoMag NGS Clean-up and Size Select beads. 150 fmol of each library was prepared for sequencing by using the Ligation Sequencing Kit SQK-LSK112, following the manufacturer's protocol. For each of the final prepared libraries, 10 fmol was loaded on a R9.4.1 flow cell (FLO-MIN106D, Oxford Nanopore Technologies Plc., UK), and sequencing was performed on a MinION Mk1C (MinKNOW v22.11.2, Oxford Nanopore Technologies Plc., UK) until on average 100,000 raw reads per sample were generated.

2.5. Data-processing

Basecalling of raw reads was performed using Guppy (v6.2.1, Oxford Nanopore Technologies Plc., UK) in super-accuracy mode. Guppy was then used to demultiplex samples and remove adapters and barcodes. For a single sample, <1000 reads were obtained, and it was therefore excluded from further analyses. Read quality was determined using NanoPlot (v.1.40.0) (mean Phred quality score >15). Decona (v0.1.3.) (Doorenspleet et al., 2021) (https://github.com/Saskia-Oosterbroek/decona) was used to further process the sequencing data from FASTQ files to polished consensus sequences: reads were filtered on length (min: 1400 bp, max: 2000 bp) and quality (>Q15); next, reads were clustered at 97% identity and draft consensus sequences constructed with Minimap2 and Racon were subsequently polished using Medaka. Finally, the BLAST function integrated into Decona was used for taxon delineation against an in-house curated nematode SSU rRNA reference database, and the top hit was selected. This in-house reference database consists of >5000 nearly full SSU nematode sequences (nearly all are available on GenBank; see Holterman et al. (2017, 2019)). Decona output files were merged into an OTU table using a custom Python script (Suppl. Script 1) and identifications with an ID percentage below 97% were excluded. Before statistical analyses, nematode taxa that were detected only once were excluded. The OTU table and metadata were subsequently processed using phyloseq (v. 1.42.0) (McMurdie

Table 2
Temperature profile for PCR amplification of nearly full-length nematode SSU rDNA. Amplification of.

	Temperature	Time	# cycles		Temperature	Time	# cycles
	94 °C	3 min	1×				
Amplification step 1	94 °C	30 s	5×	Amplification step 3	94 °C	30 s	5×
	45 °C	30 s			57 °C	30 s	
	65 °C	4 min			65 °C	2min 30 s	
Amplification step 2	94 °C	30 s	5×	Amplification step 4	94 °C	30 s	25×
	57 °C	30 s			57 °C	30 s	
	65 °C	3 min			65 °C	2 min	
					65 °C	5 min	1×
					12 °C	Continuous	1×

and Holmes, 2013) in R Software (v. 4.2.2) (R Core Team, 2021). An overview of the workflow is presented in Fig. 1.

2.6. Statistical analysis

2.6.1. Comparison of *M. chitwoodi* microscopical counts and sequencing counts

Nematode suspensions from 132 soil samples were analyzed first microscopically and thereafter molecularly for the presence of *M. chitwoodi* after pre-treatment of a field with either black oat, a good host, or perennial ryegrass, a poor host for *M. chitwoodi* (referred to as 'pre-crops') followed by cover crop treatments as described in Table 1. The sequencing data were rarefied to the lowest sample read count (5932 reads) without replacement to adjust for sequencing depth. *M. chitwoodi* reads were extracted from the rarefied dataset and were used as the response variable in a generalized linear mixed model with a negative binomial distribution (GLMM-NB), with cover crops, pre-crops and time point as fixed factors and block as a random factor. Zero-Inflated Negative Binomial mixed models (GLMM-ZINB) (Zhang and Yi, 2020) were used in excess of zeros (zero-inflation tested with performance R package). Microscopic *M. chitwoodi* counts were used in a GLMM-NB model with interaction between cover crops and pre-crops and time as fixed factors and block as a random factor. Box plots with log-transformed reads or counts were generated in ggplot2 (v. 3.4.1) (Wickham, 2016) and statistical significance was indicated based on the output of the mixed models using the R package glmmTMB (v. 1.1.6, Brooks et al., 2017).

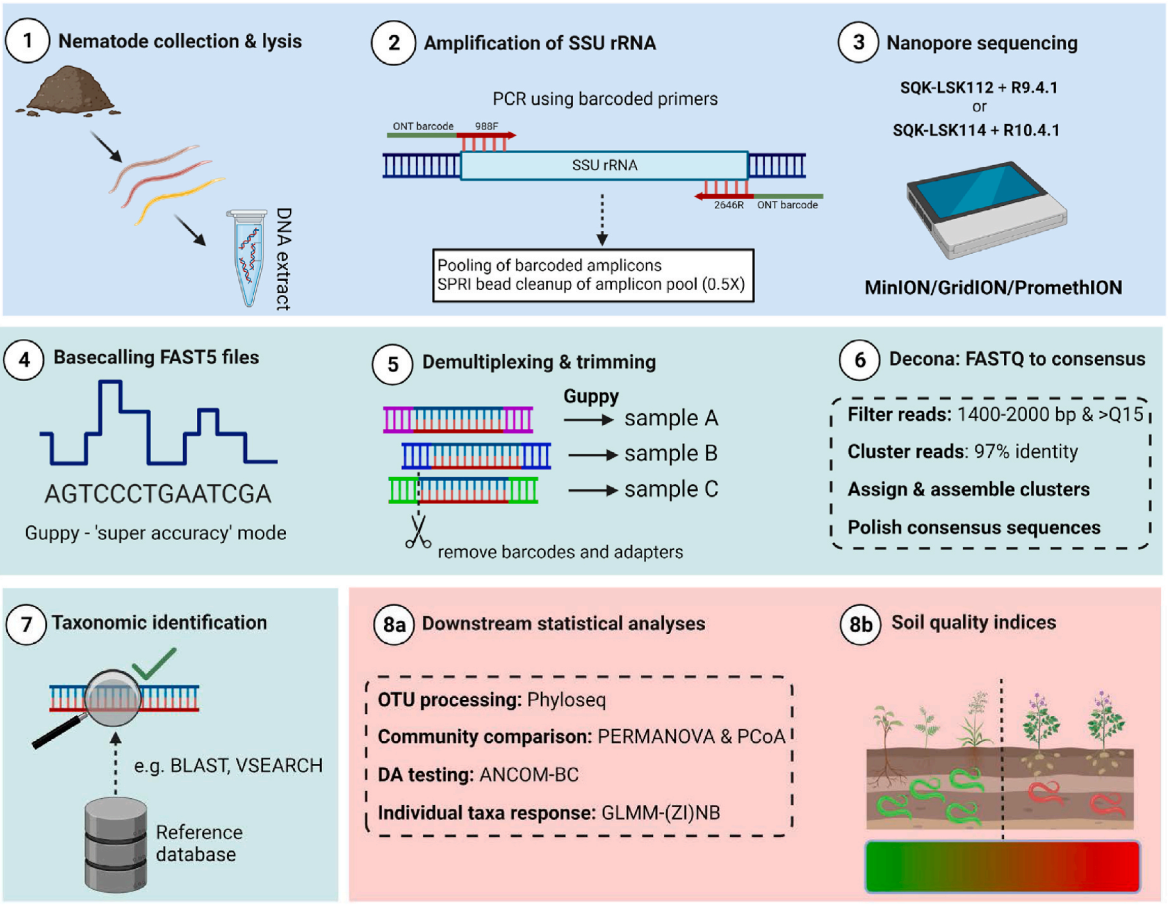

Fig. 1. Workflow for nanopore sequencing-based nematode community analysis. 1. Nematodes are separated from the soil matrix, concentrated and lysed. 2. Amplicons spanning the complete SSU rDNA gene are generated, and (3) resulting libraries ran on a nanopore sequencing device. After high-accuracy base-calling (4), demultiplexing and trimming (5), polished consensus sequences are generated (6). A curated reference database is used for nematode taxon identification (7), and resulting community composition data are statistically analyzed (8a) and, for example, used for nematode-based soil quality indices (8b).

2.6.2. Effects of pre-crop and cover crop treatments on the whole nematode community

Community differences were visualized by creating ordination plots using Bray and Curtis (1957) and robust Aitchison (Gloor et al., 2017; Martino et al., 2019) dissimilarity distances. For Bray-Curtis dissimilarity, sequencing counts were normalized with cumulative sum scaling (CSS) (Paulson et al., 2013) and plotted per time point in PCoA graphs with Bray-Curtis distance using phyloseq. For the robust Aitchison distance, R package QsRutils (v 0.1.5, Quensen, 2020) was used to rclr (robust centered log-ratio) transform the data, and plotted per time point in PCA (which uses 'euclidean distance', Gloor et al., 2017; Martino et al., 2019) graphs using phyloseq. To test the effect of the factors on the communities, PERMANOVA (adonis2, vegan R package (v. 2.6–4, Oksanen et al., 2022) tests with 999 permutations were used to test the statistical significance and the variation explained by each of the variables (block, pre-crop treatment, cover crop treatment) on the nematode community at each time point for Bray-Curtis and robust Aitchison distances. As PERMANOVA tests terms in sequential order, from first to last in the formula, the block was always added as the first term to remove the variability attributed to a block effect from the variability attributable to the following factors.

ANCOM-BC (v1.4.0, default parameters) (Lin and Peddada, 2020) was used to investigate the overall impact of pre-crop and cover crop treatments on the nematode community. Non-transformed reads were used to characterize the impact of *M. chitwoodi* stimulation by black oat as pre-crop on nematode communities, as compared to the impact of perennial ryegrass as a non-host.

To study the response of nematode taxa upon the cover crop treatments after each pre-crop, CSS normalized nematode OTUs were inputted as a response variable in GLMM-ZINB models with cover crops as fixed factors and block as a random factor (in MaAsLin2 R package, v1.7.3, Mallick et al., 2021). The most affected nematode taxa were subset by selecting model coefficients higher than 2 (taxa most stimulated) and lower than −2 (taxa most repressed). Selected taxa were plotted in dot plots, one per each pre-crop.

2.6.3. Effect of cover crop treatments on four Pratylenchus species

Root lesion nematodes (*Pratylenchus* spp.) are known as a stenomorphic genus. While members of this genus are easily recognizable, species are difficult to separate. Four *Pratylenchus* species were present in the experimental field, and we analyzed whether individual *Pratylenchus* species showed distinct responses upon exposure to a range of cover crop treatments (t1) and to the potato cultivar Hansa (t2). *Pratylenchus (P. crenatus, P. fallax, P. scribneri, P. neglectus)* counts were selected from the rarefied dataset (see 2.6.1) and fitted in GLMM-NB models with cover crop treatment, time point and pre-crop treatment as fixed factors and block as a random factor with the glmmTMB (v. 1.1.6). Estimated marginal means per group and statistical differences between groups were calculated with emmeans (v. 1.8.5, Lenth, 2022) and cld (multcomp, v.1.4–23, Hothorn et al., 2008) with Benjamini-Hochberg correction for multiple testing.

3. Results

3.1. Nematode community characterization by long-read amplicon sequencing

In an experimental field setting, we aimed to map the effects of local manipulation of the density of the plant-parasitic nematode *Meloidogyne chitwoodi* at the nematode community level using MinION-based full-length SSU rDNA sequencing. For this, a total of 132 soil samples were collected at two times points, in late Autumn, just after cover crop termination ('t1 ') (66 samples) and 10.5 months later, just after the harvest of the main crop, potato ('t2') (66 samples).

Amplicons covering almost the complete SSU rDNA (≈1,7 kb) were generated for all but one of the samples, and MinION sequencing resulted in the generation of 3,013,020 filtered reads for t1 and 5,165,791 filtered reads for t2. For t1 and t2, the median number of read counts per sample was respectively 47,378 and 81,005, with a median number of OTUs of 19.50 and 20.00. Blasting OTUs against a curated nematode SSU rDNA database resulted in the identification of 86 nematode taxa. After filtering out nematode taxa that were detected only once, 65 nematode taxa at either family (5%), genus (32%), or species (63%) level, were selected for further analyses (Table 3). Next to 13 plant-parasitic nematode species, nematode communities harboured bacterivores (27 taxa), fungivores (9 taxa), omnivores (11 taxa), predators (4 taxa) and one insect parasitic taxon. Notably, we found one widespread bacterivorous taxon referred to as Rhabditidae_fam (in 97% of the samples). Full-length SSU rDNA sequences demonstrated this taxon belonged to the family Rhabditidae, but the BLAST identity was too low to assign it to a Rhabditidae genus (sequences were similar to the Rabditidae genera *Cephaloboides* and *Pellioditis* with respectively 96% and 95% identity).

As expected, *M. chitwoodi* was present in most samples (66% of samples at t1 and 100% of samples at t2), and it is worthwhile mentioning that another plant-parasitic nematode species, *Tylenchorhynchus dubius*, was even more widespread in our experimental field as it was present in respectively 91% and 100% of the samples. The presence of *Meloidogyne exigua* in 14% of the samples was unexpected as this species had been reported in Europe only from Turkey. BLAST results against our database showed an average overall identity of 97.2% with *M. exigua*. The associated consensus sequence was subsequently also identified using BLAST against the complete NCBI database, which yielded a <97% ID with a *Meloidogyne* species. We conclude this presumably is an RKN species related but not identical to *M. exigua*. For this reason, it is referred to as *Meloidogyne* cf. *exigua* (Table 3). Among the bacterivores, the broad distribution of members of the family Cephalobidae (*Acrobeles* sp., *Acrobeloides* sp., *Chiloplacus* sp., *Eucephalobus* sp., present in >75% of the samples) is noteworthy. In contrast, the distribution of fungivores was patchier; the most widespread genera, *Aphelenchus* and *Aphelenchoides,* were detected in around 20% of the samples. The entomopathogenic nematode *Steinernema affine* known to be native to The Netherlands (Spiridonov et al., 2004), was present in 3% of the samples. Among the omnivores, *Aporcelaimellus obtusicaudatus* stood out as it was present in about 70% of the samples. Predatory nematodes showed a patchy distribution in the experimental field, with *Mononchoides* being the most widespread (present in 24% of the samples).

3.2. Comparison of microscopic counts versus MinION sequence reads for Meloidogyne chitwoodi

Both sequencing- and microscopy-based analyses showed significantly higher *M. chitwoodi* densities in plots in which black oat was grown as a pre-crop as compared to perennial ryegrass (green box plots and green horizontal bars in Fig. 2A and B, $p \leq 0.01$ and $p \leq 0.001$ respectively) (see also Suppl. Tables 1 and 2). Irrespective of the detection method and pre-crop identity, cultivation of the susceptible potato cultivar Hansa resulted in a further increase in *M. chitwoodi* levels (orange box plots and black horizontal bars in Fig. 2A, B, $p \leq 0.001$ for all four combinations) (see also Suppl Tables 1 and 2). It is noted that the initial pre-crop effect on *M. chitwoodi* was still observable after exposure of the plots to potato for a full growing season (t2, after 10.5 months) (orange horizontal bars in Fig. 2A and B) (see also Suppl. Tables 1 and 2). So, although read counts cannot easily be translated into numbers of individuals for *M. chitwoodi*, the effects of treatments on *M. chitwoodi* densities in an experimental field setting look highly similar, irrespective of whether communities were analyzed microscopically or by a MinION-based DNA sequencing approach.

3.3. Main variables affecting the composition of nematode communities

At t1 (66 samples), just after cover crop termination, PERMANOVA

Table 3
Nematode biodiversity in experimental fields at the Vredepeel field station (The Netherlands). Nematodes are identified on the basis of nearly full-length SSU rDNA sequences (≈1.700 bp). Taxa are clustered according to their trophic preferences. Taxa are included only if they were detected in at least two soil samples. The percentage of samples in which individual taxa were detected at t1 (after cove crop) and t2 (after potato) is provided in separate columns. Bold: taxa on average (t1 +t2) present in >80% of the samples.

Bacterivores	t1 (%)	t2 (%)	Fungivores	t1 (%)	t2 (%)	Predators	t1 (%)	t2 (%)
Achromadora ruricola	0	6	*Anomyctus xenurus*	3	3	*Clarkus papillatus*	20	18
Acrobeles ciliatus	36	57	*Aphelenchoides bicaudatus*	5	0	*Clarkus* sp.	9	8
Acrobeles complexus	**91**	**72**	*Aphelenchoides blastophthorus*	0	5	*Mononchoides* sp. (and bacterivore)	23	25
***Acrobeles* sp.**	**95**	**94**	*Aphelenchoides* sp.	15	25	*Mylonchulus hawaiiensis*	0	3
Acrobeloides apiculatus	**95**	**91**	*Aphelenchus avenae*	0	6			
Acrobeloides maximus	3	0	*Aphelenchus* sp.	2	42	**Plant parasites**		
Acrobeloides varius	**95**	**88**	*Filenchus misellus* (and plant parasite)	18	23	*Ditylenchus destructor*	33	45
Alaimus sp.	14	32	*Filenchus vulgaris* (and plant parasite)	9	14	*Ditylenchus* sp.	11	28
Anaplectus porosus	36	18	*Tylenchidae* (and plant parasite)	3	3	***Meloidogyne chitwoodi***	**68**	**100**
Chiloplacus propinquus	**100**	**98**				*Meloidogyne* cf. *exigua*	2	26
(Chilo)Plectus andrássyi	17	12	**Insect parasites**			*Meloidogyne naasi*	5	0
Cruznema sp.	45	17	*Steinernema affine*	2	5	*Paratrichodorus pachydermus*	2	3
Diploscapter sp.	33	34				*Paratrichodorus teres*	6	15
Eucephalobus oxyuroides	82	68	**Omnivores**			*Pratylenchus crenatus*	18	54
Eucephalobus striatus	**98**	**97**	*Aporcelaimellus obtusicaudatus*	65	74	*Pratylenchus fallax*	32	29
Mesorhabditis sp.	53	20	*Aporcelaimellus paraobtusicaudatus*	24	11	*Pratylenchus neglectus*	29	54
Oscheius tipulae	0	3	*Aporcelaimellus* sp.	3	23	*Pratylenchus scribneri*	21	17
Panagrolaimus sp.	2	5	*Calcaridorylaimus* sp.	14	0	*Trichodorus viruliferus*	3	8
Pelodera cylindrica	3	0	*Dorylaimoides* sp.	2	11	***Tylenchorhynchus dubius***	**91**	**100**
Pelodera teres	80	65	*Ecumenicus* sp.	8	9			
Plectus sp.	2	5	*Microdorylaimus miser*	32	58			
Rhabditidae_fam	**95**	**98**	*Microdorylaimus modestus*	3	2			
Rhabditis sp.	76	35	*Thonus circulifer*	17	17			
Rhabditis terricola	39	26	Tylencholaimellidae	36	23			
Rhabditophanes sp.	86	51	*Tylencholaimus* sp.	6	6			
Zeldia sp.	0	3						
Pristionchus uniformis	5	2						

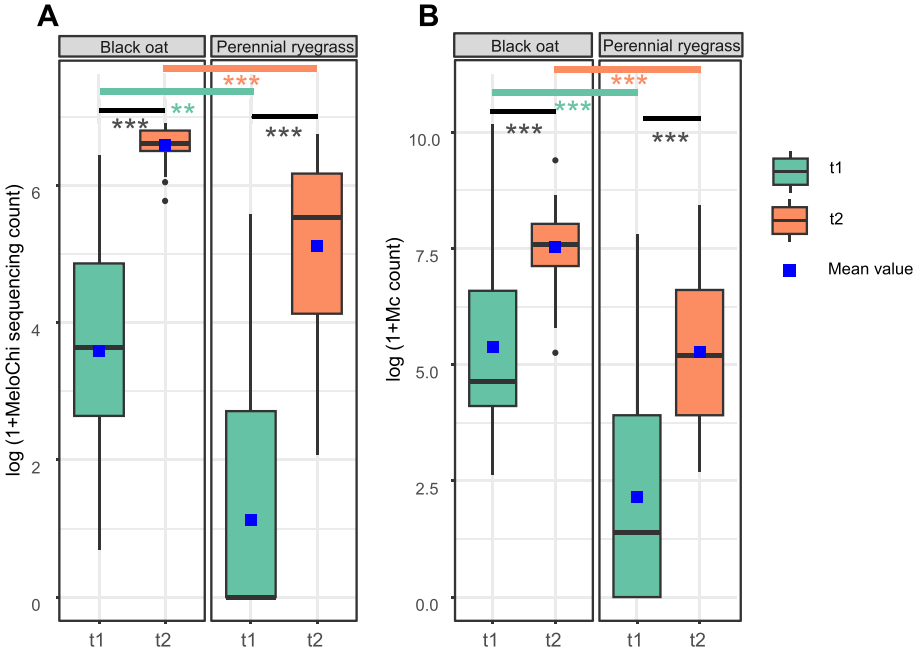

Fig. 2. Comparison of two methods to determine *Meloidogyne chitwoodi* densities at t1 (after pre-crop and cover crop treatments) and t2 (after potato): (A) Nanopore sequencings reads (rarefied to minimum library size, 5932; (B) microscopic counts. Counts were compared at t1 and t2 on the basis of the pre-crop used to create the initial two *M. chitwoodi* population densities: black oat = good host gave highest *M. chitwoodi* densities, perennial ryegrass = poor host, gave initial lowest *M. chitwoodi* densities. After pre-crop and cover crops, potato (a good host for *M. chitwoodi*) was grown, and soil samples were collected just after harvest. *** = $p \leq 0.001$, ** = $p \leq 0.01$.

analyses using both Bray-Curtis (BC) and robust Aitchison (rA) dissimilarity revealed that pre-crop, cover crop and position on the field (block-effect) significantly affected the composition of nematode communities (Table 4). The strongest effect was observed for cover crops (explaining 20% (rA) - 21% (BC) of the variation, Suppl. Fig. 2A), followed by a significant block effect (11% (rA) - 16% (BC)), whereas pre-crop explained 4% (rA) - 7% (BC) of the observed variation. No interaction effect was detected between the variables 'pre-crop' and 'cover crop'. At t2, just after the harvest of potatoes, the composition of the nematode community was characterized again. As can be seen in

Table 4
PERMANOVA analysis with Bray-Curtis and robust Aitchison dissimilarity metric to assess the variation explained by block, pre-crop, cover crop, and the interaction effect between pre-crop and cover crop after cover crop termination and incorporation in topsoil (t1) and just after the harvest of the main crop, potato (t2).

			t1		t2	
Dissimilarity metric	Factor	df	R^2	Pr (>F)	R^2	Pr (>F)
robust Aitchison (rA)	Block	3	0.11181	0.001 (***)	0.08171	0.001 (***)
	Pre-crop	1	0.03918	0.001 (***)	0.06665	0.001 (***)
	Cover crop	10	0.19784	0.001 (***)	0.16081	0.028 (*)
	Pre-crop: cover crop	10	0.12447	0.566	0.17025	0.006 (**)
Bray-Curtis (BC)	Block	3	0.16475	0.001 (***)	0.14766	0.001 (***)
	Pre-crop	1	0.06517	0.001 (***)	0.17698	0.001 (***)
	Cover crop	10	0.20594	0.002 (**)	0.13123	0.141
	Pre-crop: cover crop	10	0.11007	0.475	0.13529	0.125

Table 4, the effects of pre-crop and block were still significant (explaining respectively 7% (rA) - 18% (BC)- and 7% (rA) - 15% (BC) of the observed variation), while the impact of cover crop treatment was no longer significant using Bray-curtis dissimilarity. Only under robust Aitchison dissimilarity, cover crop and the interaction of pre-crop and cover crop were significant. For both dissimilarities, the pre-crop explained more variation in t2 than t1. PCoA graphs of the two time points based on Bray-Curtis dissimilarity (Suppl. Figs. 2A and B) and PCA graphs on robust Aitchison dissimilarity (Suppl. Figs. 3A and B) clearly show the pre-crop effects. The presence of a cover crop effect at t1 is exemplified by highlighting the effect of vetch in Supplementary Figs. 2A and B. Suppl Fig. 2B suggests that a significant effect of vetch could still be present at t2, but this is largely attributable to the substantial block effect (Table 4).

3.4. Impact of strong stimulation of *M. chitwoodi* on other nematodes

Differential abundance testing (ANCOM-BC) was used to characterize the impact of the pre-crop black oat, known as a good host for *M. chitwoodi*, as compared to the effect of perennial ryegrass, known as a poor host for this root-knot nematode, over all cover crop treatments (Fig. 3). First, it shows that the expected strong stimulation of *M. chitwoodi* by black oat was not accompanied by a stimulation of any other nematode taxon. Among the plant parasites, two lesion nematodes, *Pratylenchus crenatus* and *P. neglectus,* and the stunt nematode *Tylenchorhynchus dubius* were repressed by the pre-crop treatment that stimulated *M. chitwoodi*. Among the bacterivores, the repression of several members of the bacterivorous family Cephalobidae was detected: *Chiloplacus propinquus, Acrobeles complexus, Acrobeles* sp., and *Eucephalobus striatus* (Fig. 3). Other widespread and closely related relatives, such as *Acrobeles ciliatus* and *Eucephalobus oxyuroides* (see Table 3) were unaffected. With a β-coefficient below −3, the strongest repression was observed for *Aporcelaimellus obtusicaudatus*. Members of this widespread genus have been characterized as omnivores and as predators feeding on nematodes and enchytraeids (Yeates et al., 1993). Hence, black oat-based stimulation of *M. chitwoodi* densities was associated with a repression of other plant-parasitic as well as free-living taxa, whereas distinct responses were observed between congenerics.

3.5. Effects of cover crop treatments at the nematode community level

For each of the two pre-crops, perennial ryegrass and black oat, the impact of individual cover crop treatments upon manipulation of the *M. chitwoodi* density at t1 was analyzed taking only into consideration taxa with an estimated coefficient (from MaAsLin2) lower than −2, or above 2. When perennial ryegrass was used as pre-crop, as shown in Fig. 4A, repression of individual nematode taxa was only observed upon exposure to cover crop monocultures (five nematode taxa). For 27 nematode-cover crop combinations, a stimulation of nematode taxa was observed. It is noted that Rhabditidae_fam was stimulated by all ten cover crop treatments. *M. chitwoodi* was specifically stimulated by all cover crop mixtures (two that included black oat and two mixes that included phacelia and vetch) and by vetch as a monoculture.

When black oat was used as pre-crop, cover crop treatments predominantly resulted in the repression of nematode taxa (seven nematode taxa) (Fig. 4B). Mixtures with oilseed radish cv. Terranova (OSR_T in Fig. 3) all had a strong negative impact on the omnivore *Aporcelaimellus paraobtusicaudatus* (Fig. 4B). Moreover, two specific treatments that included oilseed radish cv. Radical negatively affected the plant parasite *T. dubius.* Only two treatments that both included black oat (black oat and oilseed radish Terranova, and black oat) resulted in a stimulation of a community member, namely non-identified member(s) of the bacterivorous family Rhabditidae.

3.6. Effect of cover crop treatments on four *Pratylenchus* species

Four *Pratylenchus* species were present in the experimental field, and in Table 5, we analyzed whether individual *Pratylenchus* species showed distinct responses upon exposure to a range of cover crop treatments (t1) and to the potato cultivar Hansa (t2). Effects of cover crops are discussed

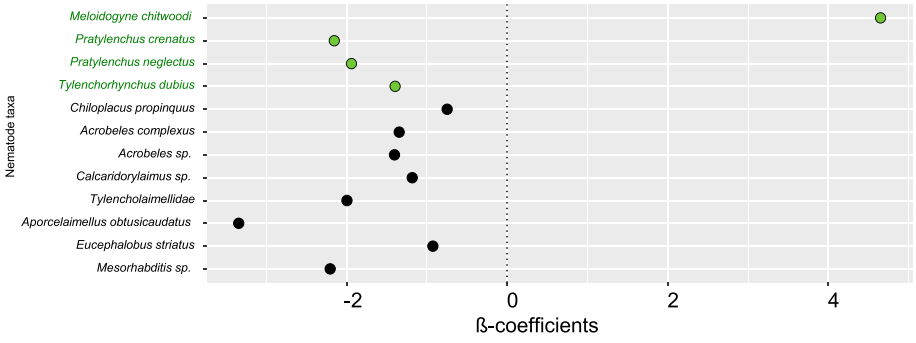

Fig. 3. Differential abundance testing (ANCOM-BC) was used to characterize the impact of the pre-crop black oat, a good host for *M. chitwoodi*, as compared to the effects of perennial ryegrass, a poor host for this root-knot nematode, on nematode communities over all cover crop treatments. The displayed taxa were differentially abundant in black oat compared to perennial ryegrass, according to the test. The ANCOM-BC beta coefficient is a measure to show the extent by which individual nematode taxa are affected by one pre-crop compared to the other. Plant-parasitic nematode taxa are highlighted in green.

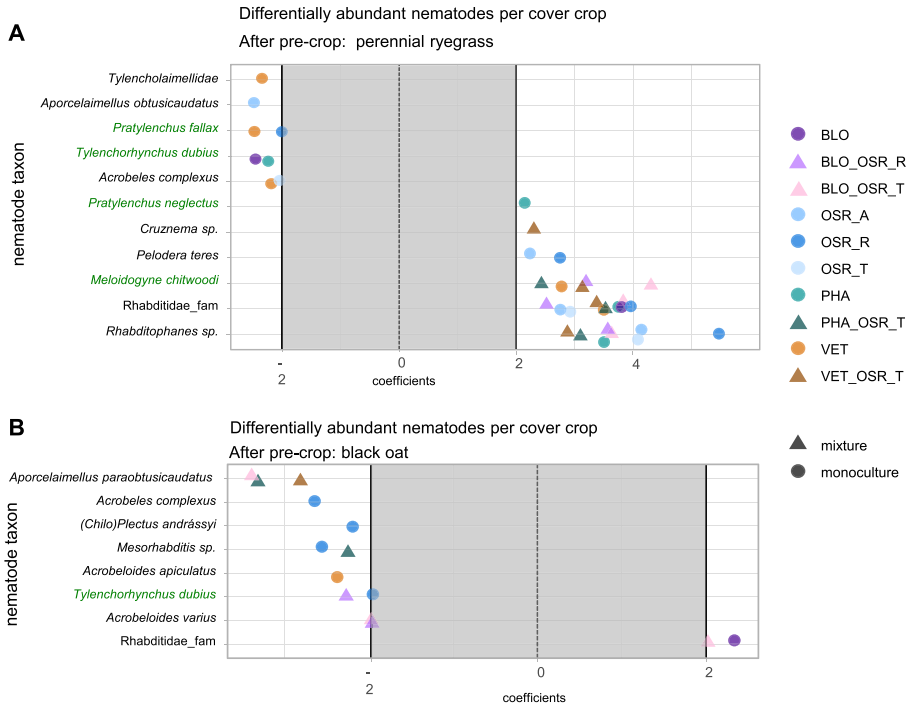

Fig. 4. Differential abundance testing (MaAsLin2) was used to characterize the impact of cover crop treatments on nematode communities in plots that were initially exposed to the pre-crop perennial rye (A) or to black oat (B). Only nematode taxa with regression coefficients lower than −2, or above 2 are shown. Plant-parasitic nematode taxa are highlighted in green.

Table 5
Effect of monocultures and simple mixtures of cover crops and the subsequent cultivation of potato of four lesion nematode species, *Pratylenchus crenatus, P. fallax, P. neglectus* and *P. scribneri. Pratylenchus* reads were rarefied and fitted in negative binomial mixed models (GLMM-NB) with cover crop treatment, time point and pre-crop treatment as fixed and block as a random factor. In the table, estimated adjusted means of nematode reads per group (emm), the corresponding standard error (se.emm) and significant differences between groups (group) are indicated. Treatments within one *Pratylenchus* species that have a common letter in column 'group' are not statistically different. Cover crop groups highlighted in grey are treatments that significantly differ from the fallow control treatment.

	Pratylenchus crenatus			Pratylenchus fallax			Pratylenchus neglectus			Pratylenchus scribneri		
Cover crop treatment (t1)	emm	se.emm	group	emm	se.emm	group	emm	se.emm	group	emm	se.emm	group
Fallow	1.91	1.51	bc	2.46	2.11	abc	11.42	7.12	c	0.73	0.85	a
Black oat	0.06	0.06	**a**	1.31	1.29	abc	6.24	3.54	bc	2.18	3.79	a
Oilseed radish cv. Radical	1.85	1.53	bc	1.45	1.25	abc	0.15	0.12	**a**	0.00	0.00	a
Oilseed radish cv. Adios	0.69	0.61	ab	4.99	4.71	abc	0.24	0.18	**a**	3.54	4.30	a
Oilseed radish cv. Terranova	1.01	0.85	bc	0.25	0.27	ab	0.22	0.16	**a**	0.19	0.28	a
Phacelia	12.72	11.38	c	8.90	7.49	bc	26.18	14.17	c	6.17	6.88	a
Vetch	0.23	0.21	ab	5.37	4.83	abc	1.14	0.73	**ab**	4.17	5.00	a
Black oat/Oilseed radish (cv Radical)	0.75	0.67	ab	0.95	0.83	abc	1.32	0.81	**ab**	0.45	0.54	a
Black oat/Oilseed radish (cv Terranova)	0.23	0.21	ab	0.19	0.20	a	0.52	0.35	**a**	0.29	0.36	a
Phacelia/Oilseed radish (cv Terranova)	1.82	1.45	bc	26.23	22.88	c	22.56	13.52	c	3.93	4.20	a
Vetch/Oilseed radish (cv Terranova)	1.40	1.12	bc	0.23	0.23	ab	0.34	0.24	**a**	0.73	0.84	a
Time point												
After cover crops (t1)	0.24	0.16	a	1.34	0.63	a	0.64	0.21	a	0.18	129	a
After potato var. Hansa (t2)	3.04	1.80	b	2.11	0.99	a	3.50	0.97	b	0.19	140	a
Pre-crop												
Black oat	0.18	0.12	**a**	1.03	0.54	a	0.36	0.14	**a**	0.01	89.7	**a**
Perennial rye	4.1	2.5	**b**	2.75	1.34	a	6.24	1.65	**b**	0.05	813.4	**b**

as compared to the fallow control. *P. crenatus* was the only root lesion nematode species that was negatively affected by black oat. In contrast to the other three *Pratylenchus* species, neither the type of cover crop nor the pre-crop treatments affected *P. fallax*. *P. neglectus* was negatively impacted by all three oilseed radish monocultures and by three out of the four cover crop mixes that included oilseed radish. *P. scribneri* was not stimulated nor repressed by any cover crop. Except for *P. fallax,* all root lesion nematode species were negatively affected by the pre-crop black oat as compared to perennial rye. From this analysis, we conclude that despite their morphological resemblance, individual root lesion nematode species respond in species-specific ways upon exposure to both cover and main crops.

4. Discussion

4.1. A nanopore sequencing approach for nematode community analyses

Being abundant in virtually any soil, trophically diverse, and ecologically relatively well-characterized, nematode communities have the potential to be used as a proxy for the soil's biological condition (Bongers and Ferris, 1999). However, microscopy-based methods for community analysis require extensive taxonomical expertise, are

labour-intensive, and, most often, juvenile life stages are not taken into consideration due to a lack of informative morphological characteristics. In essence, DNA sequencing-based approaches can overcome these hurdles, but most high-throughput sequencing methods produce relatively short reads that intrinsically limits the taxonomic resolution. Here we show that Nanopore sequencing allows for the routine sequencing of full-length SSU rDNA (≈1700 bp), by far the most popular barcoding gene for nematodes, and results in complete overviews of nematode communities either until genus or (most often) to species level. Nanopore sequencing has been used before by Knot et al. (2020) to identify nematodes within an artificial community of four nematode species. Here, we present a nanopore sequencing-based workflow that allows for routine analyses of nematode communities with a high taxonomic resolution and present data that demonstrate the ecological and agronomic relevance of high-resolution community analyses.

4.2. Nematode community composition

In our experimental field, we detected 65 nematode taxa with representatives from all trophic groups, of which the majority (63%) was identified to species-level. In some instances this reflects the limitation in the resolution of the SSU rDNA gene itself, this holds for recently diverged species such as *Meloidogyne naasi* and *M. graminicola* (Holterman et al., 2009). More frequently, this is explained by reference entries in our database that have been annotated to genus or family level. Consequently, 23% and 5% of the nematode taxa could be identified till the genus and family level only. Further expanding and refining the database will lead to an even higher proportion of species-level identifications.

The nematode diversity found in our experimental field lies in the same order of magnitude as the diversity of other agricultural fields in the Netherlands (Mulder et al., 2005) or Sweden (Sohlenius et al., 1987). In the current community composition overview, members of the bacterivorous family Cephalobidae including the genera *Acrobeles, Acrobeloides, Chiloplacus* and *Eucephalobus*, are amply represented. In general, the relatively high abundance of bacterivores has been reported in both agricultural (Sohlenius et al., 1987; Van den Hoogen et al., 2019) and natural habitats (Porazinska et al., 2012; Van Den Hoogen et al., 2019).

As compared to bacterivores, fungivorous nematodes showed a patchier distribution. Most widespread were members of the genera *Aphelenchus*, *Aphelenchoides,* and *Filenchus.* This might be a generalizable observation for sandy arable fields in temperate climate zones; in a carrot production field in Michigan, the same fungivorous nematode genera were found to be dominant (Grabau et al., 2017). It should be noted that *Aphelenchus,* found in 97% of the samples by Grabau et al. (2017), was considerably less prominent in our experimental field.

Among the plant-parasitic nematodes, the stunt nematode *Tylenchorhynchus dubius* stood out as it was present in nearly all samples. This observation fits well in a report by Sharma (1968) in which this nematode was assessed to be the most generally occurring phytophagous nematode in lighter soils in Western Europe. Its general occurrence is not limited to Europe; in a carrot field in Michigan (USA), stunt nematodes were detected in 77% of the samples, with the highest relative abundance among the plant parasites present (9 genera) (Grabau et al., 2017). *T. dubius* is an ectoparasite living in upper soil layers with a wide host range (Sharma, 1968) and a high tolerance towards desiccation. These characteristics will have contributed to the proliferation of this plant parasite.

4.3. Quantification of nematode community data – sequence reads versus microscopic counts

DNA read counts cannot easily be translated into numbers of nematode individuals. Nevertheless, we made the comparison between morphology- and DNA-based analysis, and the contrasts were remarkably similar both in directionality and statistical robustness. It should be noted, however, that for *M. chitwoodi* (like for all RKNs), this comparison might be more straightforward than for most other nematode species. *M. chitwoodi* has mainly one mobile life stage in soil, the pre-parasitic second-stage juveniles. Males are the other mobile life stage, but males are only formed under stress conditions for this facultative meiotic parthenogenetic nematode species (Castagnone-Sereno et al., 2013). It is expected that the DNA content of individual pre-parasitic juveniles is more or less a constant, and this would suggest a linear relationship between numbers *M. chitwoodi* and the *M. chitwoodi*-derived DNA concentration in the community lysates. So, it should be noted that for most other nematode species, the relationship between counts and sequence reads could be less comparable. The one example that is comparable to *M. chitwoodi* in Table 1 is the entomopathogenic nematode *Steinernema affine.* Also, for this nematode, only a single mobile life stage, the Dauerlarva, is found in soil. All other life stages can be found inside their host, insect larvae. For most other nematode species mentioned in Table 1, probably multiple life stages were present in the samples under investigation.

4.4. Competition between M. chitwoodi and other parasitic and free-living nematode species

Stimulation of *M. chitwoodi* by growing the good host black oat as a pre-crop also resulted in the repression of multiple other nematode taxa. This repression could be caused (in)directly by the plants as they can alter the soil microbiome locally via the release of secondary metabolites. Rhizobiome changes can activate plant defenses (Koprivova and Kopriva, 2022), and this activation might be differentially perceived by distinct parasites. Otherwise, competition for available food sources could also explain the observed pattern, as obligatory plant-parasitic nematodes will compete with each other for the same resource, namely plant roots. Different feeding strategies, such as ecto-*versus* endo-parasitism and various types of endoparasitism, might milden this competition. Nevertheless, stimulation of the sedentary endoparasite *M. chitwoodi* had a negative effect on two migratory endoparasites, *P. crenatus* and *P. neglectus*. Competition between *Meloidogyne* and a *Pratylenchus* has been investigated before. Co-inoculation of barley with *M. chitwoodi* and *P. neglectus* revealed that the species that parasitized the roots first lowered the parasitic success of the other (Umesh et al., 1994). In the system of Umesh et al. (1994), the lesion nematode outcompeted *M. chitwoodi.* Hence, our findings might be the result of competition between lesion and root-knot nematodes, and - if correct - the nature of the interaction appears to be context-dependent. Alternatively, differences in host plant status might have contributed to the observed suppression of some root lesion nematode species (host plant status of cover crops for *Pratylenchus* species is largely unknown). Also, the ectoparasite *T. dubius* was negatively affected by the stimulation of *M. chitwoodi. T. dubius* belongs to the nematode family Telotylenchidae. Under field conditions in a vegetable cropping system (all vegetables were susceptible to RKNs) (Mateille et al., 2020) also observed a competition between Telotylenchidae and RKNs. As such, we can conclude that the difference in feeding strategy does not rule out competition between obligatory plant-parasitic nematodes.

4.5. Strong stimulatory or repressive effects of cover crops on nematode communities

After the growing of two pre-crops, perennial ryegrass and black oat (respectively a poor and a very good host for *M. chitwoodi*), the same cover crops had highly distinct effects on the nematode communities (Fig. 4A and B). In the context of an initially low M. chitwoodi density, 4 bacterivores and 2 plant parasites (including *M. chitwoodi*), were strongly stimulated. In the case of M. chitwoodi, this was associated with cover crop treatments that included black oat and vetch. An extensive meta-analysis performed by Puissant et al. (2021) regarding the impact of agricultural practices on nematode communities showed that - in

general - both bacterivores and plant parasites were stimulated by cover crops. An overall stimulation of plant parasites doesn't imply that all plant parasites are stimulated; in our study stimulation of *M. chitwoodi* was accompanied by repression of two other plant parasites, *Tylenchorhynchus dubius* and *Pratylencus fallax* (Fig. 4A). Repression of the genera *Tylenchorynchus* and *Pratylenchus*, as well as other plant-parasitic nematodes by cover crops has been observed before (Djigal et al., 2012; Berry et al., 2011), and depends on host status of the cover crop used.

Seven out of the eight cover crop treatments that resulted in a stimulation of *Rhabditophanes,* included oilseed radish. Members of this nematode genus are bacterivores (Yeates et al., 1993). Recently we have shown that oilseed radish strongly stimulates representatives of the bacterial families Pseudomonadaceae, Moraxellaceae and Erwiniaceae (all Gammaproteobacteria) both at the DNA and the RNA level (Cazzaniga et al., 2023). Therefore, it is tempting to suggest that *Rhabditophanes* sp. benefitted from the local increase in a potential food source, active Gammaproteobacteria.

After growing black oat as a pre-crop, most nematode taxa were significantly repressed. This is especially true for *Aporcelaimellus paraobtusicaudatus,* an omnivorous nematode that was repressed in three cover crop mixtures that all included oilseed radish. Although rDNA sequences support the distinction between *A. paraobtusicaudatus* (Fig. 4B) and *A. obtusicaudatus* (Fig. 4A) (Holterman et al., 2008), it is uncertain whether or not these should be considered two species (Álvarez-Ortega and Peña-Santiago, 2013). Assuming that microdrile Oligochaeta and other nematodes are also the main food source of *A. paraobtusicaudatus*, we hypothesize that these food sources were repressed or repelled by oilseed radish. A member of the Rhabditidae family (enrichment opportunistic bacterivores) was promoted by numerous cover crop treatments irrespective of the pre-crop treatment, which is in line with the findings of Puissant et al. (2021) and DuPont et al. (2009) that cover-crops have a positive effect on (enrichment opportunistic) bacterial feeding nematodes. The family Rhabditidae is characterized by a c-p value of 1 (colonizer – persister scale) (Bongers, 1990). Nematodes in this category typically do well under disturbed environmental conditions and respond rapidly to local bacterial bloom, which probably has happened upon the incorporation of terminated cover crop material into the topsoil.

4.6. Prospects of nanopore sequencing-based nematode community analyses

Due to their conserved morphology and due to ample convergent evolution of morphological characters, microscopy-based identification of nematodes at lower taxonomic levels is notoriously difficult. As informative DNA motifs are spread all over the SSU rDNA gene, the sequencing of specific variable regions (e.g., V4, or V5-7, Capra et al., 2016) will offer robust resolution until the order or family level (e.g., Harkes et al., 2020), or till genus level with a higher level of uncertainty (Du et al., 2020). So, both microscopy- and short-read DNA-based methods are unable to provide accurate, up-scalable and affordable nematode community analyses. Here, we demonstrated the potential of nanopore sequencing to characterize nematode communities at a low taxonomic level (predominantly species level) and in a semi-quantitative manner. A complete overview of the composition of the nematode community could be provided, and a comparison between microscopic counts and DNA reads for one of the constituents, *M. chitwoodi,* revealed highly similar quantitative contrasts. Analysis of nanopore sequence data allowed us to pinpoint the impact of the stimulation of a single plant-parasitic nematode on the nematode community as a whole, as well as the effect of individual cover crop treatments on nematode communities. Moreover, we showed that this long-read approach was able to distinguish species within the stenomorphic plant-parasitic genus *Pratylenchus*, and our analyses also showed that this resolution matters, also from an agronomic perspective.

The nanopore sequencing approach presented here requires a moderate investment in hardware, while the whole analysis procedure can be executed on a laboratory bench. The workflow presented here could give a boost to the use of nematodes as environmental indicators. It could also facilitate the development of more refined soil health indices that exploit the full width of ecological differentiation of these highly abundant and speciose soil inhabitants.

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Data accessibility and benefit-sharing

The sequence data generated during and/or analyzed during the current study are available in the NCBI repositories: BioProject PRJNA971608. The in-house nematode reference database is available upon request via a Data Sharing Agreement.

CRediT authorship contribution statement

Robbert van Himbeeck: Conceptualization, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Sara Giulia Cazzaniga:** Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Sven van den Elsen:** Investigation, Methodology. **Job Oude Vrielink:** Formal analysis, Investigation, Methodology. **Semih Karst Aslan:** Investigation, Methodology, Validation. **Johnny Visser:** Investigation, Methodology. **Johannes Helder:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – original draft, Writing – review & editing.

Data availability

PRJNA971608

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2023.109263.

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