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

Robbert van Himbeeck[1], Sara Cazzaniga[1], Sven van den Elsen[1], Job Oude Vrielink[1], Semih Karst Aslan[1], Johnny Visser[2], and Johannes Helder[1]

[1]Affiliation not available
[2]Wageningen Research

May 24, 2023

Abstract

Agricultural intensification has resulted in a decline of 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 analysed, and 65 nematode taxa were detected, mostly at species level, including representatives of all trophic groups. As a validation, all samples were analysed microscopically for M. chitwoodi, and 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.

Title:

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

Robbert van Himbeeck[1,*], Sara Cazzaniga[1,*], Sven van den Elsen[1], Job Oude Vrielink[1], Semih Karst Aslan[1], Johnny Visser[2], Johannes Helder[1]

* equal contribution

[1] Laboratory of Nematology, Wageningen University, P.O. Box 8123, 6700 ES Wageningen, The Netherlands

[2] Field Crops, Wageningen Plant Research, Wageningen University & Research, Edelhertweg 1, 8219 PH Lelystad, The Netherlands

Corresponding author:

Johannes Helder

E-mail address: hans.helder@wur.nl

Abstract

Agricultural intensification has resulted in a decline of 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 analysed, and 65 nematode taxa were detected, mostly at species level, including representatives of all trophic groups. As a validation, all samples were analysed microscopically for *M. chitwoodi,* and 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.

Keywords: third generation sequencing platforms, nanopore sequencing, soil health indicators, cover crops, *Meloidogyne chitwoodi*

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 a restoration of these ecosystem services. For this, a range of practices have been proposed including the use of organic amendments, an overall reduction of nutritional inputs, the reduction of tillage intensity and/or the maintenance of a (largely) continuous living cover. The benefits and costs of these and comparable measures have been pinpointed in a number of recent meta-analyses here exemplified by Blanchy et al. (2023) and Tepes, Galarraga, Markandya, and Sánchez (2021).

Mapping and monitoring the effectiveness of sustainable soil management measures is non-trivial as soils harbor an overwhelming biodiversity. In terms of biomass and biodiversity, bacteria and fungi are the dominant organismal groups in terrestrial ecosystems. A single gram of soil typically harbors 10^2 to 10^6 phylotypes, and this diversity range characterizes in essence all major soil types (Louca, Mazel, Doebeli, & Parfrey, 2019). Keeping in mind the extreme diversity of the primary decomposer community as well as our limited understanding of the ecological roles of many of the individual constituents, the complete mapping of bacterial and/or fungal communities as indicators for the soil biological condition is currently unpractical. As an alternative, various proxies have been proposed involving soil organismal groups with a manageable level of diversity that not only mirror their own condition, but also reflect the condition of their main food source(s). In this respect, protists, single cellular eukaryotes that mainly feed on bacteria and archaea (Geisen et al., 2018), microarthropods, predominantly mites and collembolans that live as fungivores and as predators of soil microfauna (Cortet et al., 2002), and nematodes that use plant roots, bacteria, fungi, and/or microfauna as primary food source (Ewald et al., 2022) should be mentioned.

Soil food webs are a schematic way to map and analyze soil biota. Usually, three to four trophic levels are distinguished within such a food web (see for instance Holtkamp et al. (2008)). Trophic diversity, i.e., representation at multiple trophic levels (TL), is considered to be advantageous for the ecological significance of a soil health indicator (e.g., Biswal, 2022). Among the major soil organismal groups, nematodes are trophically most diverse. Plant-parasitic nematodes mainly feed on roots (TL1). Bacterivorous and fungivorous nematode taxa (TL2) will reflect the condition of their primary food sources, respectively bacteria and fungi. Omnivorous nematodes (TL2) feed mainly in protists and

other nematodes, whereas predaceous nematodes (TL3) consume nematode representatives from all trophic levels. Therefore, nematodes are considered as a valuable organismal group for soil health assessment as they reflect shifts throughout all levels of the food web (Puissant et al., 2021). An additional benefit of nematodes is the ease by which they can be separated from the soil matrix. Because of their relative uniform shape, their specific gravity, and their mobility, nematode extraction from soil samples in the range of hundreds of grams is relatively straightforward (Verschoor & de Goede, 2000). Although nematodes meet some major requirements to serve as a proxy for the soil biological condition, its routinely use is hampered by their morphological uniformity.

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 harbor representatives with distinct ecological characteristics (see e.g., Bik, Lambshead, Thomas, & Lunt, 2010; Meldal et al., 2007; Van Megen et al., 2009). Hence, it is desirable to have a taxonomic resolution beyond 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 take individuals of all developmental stages into consideration, 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 (≈ 1,700 bp) offers a remarkably good taxonomic resolution (e.g., Martijn 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 (Du, Li, Han, Ahmad, & Li, 2020; Harkes et al., 2020;

Kitagami & Matsuda, 2022). However, the resolution offered by either of these regions is in most cases limited to family or order level. 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 (e.g., MinION) allows to sequence the complete SSU rDNA gene, which harbours the potential for species-level metabarcoding of nematode communities (Van Megen et al., 2009). Full length SSU rDNA nanopore sequencing has been used before to test DNA barcoding on an artificial community of four different nematode species (Knot, Zouganelis, Weedall, Wich, & Rae, 2020), but – to our best knowledge – this has never been used for nematode community metabarcoding.

To test the potential of nanopore sequencing-based nematode community analyses, we used the 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 are fast-growing plant species without direct commercial value that are planted to keep the soil covered outside the main crop growing season. Cover crops do not only 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 applied cover crops belong to various plant families that are characterized - among others - by 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 a field experimental 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 crop. 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 by means of nanopore sequencing. This was followed by a validation step in which SSU rDNA

143 sequence reads were compared with count data from microscopic sample analysis. This was done for
144 *M. chitwoodi*, as this plant-parasitic nematode species can routinely be detected and quantified on the
145 basis of its morphological characteristics. In a next step the following soil ecological questions were
146 addressed: 1) Does a strongly increased density of the plant-parasitic nematode *M. chitwoodi* impact
147 other plant parasitic and/or free-living nematodes? 2) How do cover crop-treatments affect free-living
148 and plant-parasitic representatives of nematode communities? 3) Does the high-resolution
149 characterization of nematode communities (till genus and/or species level) have an ecological or
150 agronomical added value?
151

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 a 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 x 42 m) organized in three blocks (Supplementary Figure 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. 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 6x3 m) were defined, and after pre-crop treatment plots were exposed to ten cover crop treatments, whereas the 11th plot remained unplanted (fallow control). Cover crop treatments included six monocultures and four mixtures (Table 1). Cover crops were sown on 7th of August 2019 and grown for five months. On the 2nd of December 2019, cover crops were mechanically terminated using a rotary tiller and residues were incorporated into the topsoil. In spring 2020 soil was tilled and on the 30th of April, the main crop potato (*Solanum tuberosum,* cultivar 'Hansa') was planted. Potato was harvested on the 14th of October 2020. For further information on this experimental field is provided in Cazzaniga et al. (2023).

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 (15th October 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 four 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 40x o 400x 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 fraction 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 two hours as described by Holterman et al. (2006) and Vervoort et al. (2012). Lysates were purified according to Ivanova, Dewaard, and Hebert (2006) using glass fiber 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'-gctaccttgttacgactttt-3') (Martijn Holterman et al., 2006) were used to amplify the nearly complete SSU rRNA gene, approximately 1,700 bp. Primer pairs were barcoded with barcode sequences of the EXP-NBD196 kit (Oxford Nanopore Technologies plc., UK) for samplemultiplexing. PCR was performed in simplex and each reaction contained 12.5 µl LongAMP Taq 2x 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 do 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 0.5x 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) 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 library 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 to remove adapters and barcodes. For a single sample <1,000 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: 1,400 bp, max: 2,000 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 in 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 >5,000 nearly full SSU nematode sequences (nearly all are available on GenBank, see (M. Holterman et al., 2017; M. Holterman, Schratzberger, & Helder, 2019)). Decona output files were merged into an OTU table using a custom pPython script and identifications with an ID percentage below 97% were excluded. Prior to 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 & Holmes, 2013) in R Software (v. 4.2.2) (R Core Team, 2021). An overview of the workflow is presented in Figure 1.

2.6 Statistical analysis

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

Nematode suspensions from 132 soil samples were analysed 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 was rarefied to the lowest sample read count (5,932 reads) without replacement to adjust for sequencing depth. *M. chitwoodi* reads were extracted from the rarefied dataset and were used as response variable in a generalised linear mixed model with 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 & 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 were indicated based on the output of the mixed models using the R package glmmTMB (v. 1.1.6, (Brooks et al., 2017)).

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

Sequencing counts were normalised with cumulative sum scaling (CSS) (Paulson, Stine, Bravo, & Pop, 2013) and plotted per time point in PCoA graphs based on Bray- Curtis dissimilarity. PERMANOVA (adonis2, vegan R package (v. 2.6-4) (Oksanen et al., 2013)) 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. As PERMANOVA tests terms in sequential order, from first to last in the formula, block was always added as first term to remove the variability attributed to a block effect.

ANCOM-BC (v1.4.0, default parameters) (Lin & 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 normalised nematode OTUs were inputted as a response variable in GLMM-ZINB models with cover crop as fixed factor and block as 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.

2.6.4 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 analysed 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* 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

282 block as random factor with the glmmTMB (v. 1.1.6). GLMM-ZINB mixed models were used in excess
283 of zeros.

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 nematode community level using MinION-based full length SSU rDNA sequencing. For this, a total of 132 soil samples was 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 at family, genus or species level. After filtering out nematode taxa that were detected only once, 65 nematode taxa were selected for further analyses (Table 3). Next to 13 plant-parasitic nematode species, nematode communities harboured bacterivores (28 taxa), fungivores (9 taxa), omnivores (10 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 (84%), 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 95% 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 again our own 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 therefore conclude that this is likely a species within the genus *Meloidogyne*, but the exact species cannot be determined and was labeled as *Meloidogyne cf. exigua* (Table 3).. Among the bacterivores, the broad distribution of members of 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, Reid, Podrucka, Subbotin, & Moens, 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 most widespread (present in 24% of the samples). In the taxon overview (Table 3) nematodes that were detected in most samples at both time points (on average >80% of the samples) were highlighted. The most ubiquitous nematode taxa included six bacterivores and two plant-parasites.

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 Figure 2A, B, $p \leq 0.01$ and $p \leq 0.001$ respectively) (see also Suppl. Tables 1, 2). Irrespective of the detection method and of 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 Figure 2A, B, $p \leq 0.001$ for all four combinations) (see also Suppl Tables 1, 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 Figure 2A, B) (see also Suppl. Tables 1, 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 a field experimental setting look highly similar, irrespective 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 analyses revealed that pre-crop, cover crop and position on the field (block-effect) significantly affected the composition of nematode communities (Table 4A). The strongest effect was observed for cover crops (explaining 21% of variation), followed by a significant block effect (16%), whereas pre-crop explained 7% of the observed variation. No interaction effect was detected between the variables 'pre-crop' and 'cover crop'. At t2, just after harvest of potato, the composition of the nematodes communities was characterized again. As can be seen in Table 4B, the effects of pre-crop and block were still significant (explaining respectively 18 and 15% of the observed variation), while the impact of cover crop treatment was no longer significant. For PCoA graphs of the two time points based on Bray-Curtis dissimilarity see Suppl. Figures 2A and 2B.

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 effect of perennial ryegrass, known as a poor host for this root-knot nematode, over all cover crop treatments (Figure 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* (Figure 3). Remarkably, 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, Bongers, De Goede, Freckman, & Georgieva, 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 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 estimate coefficient (from MaAsLin2) lower than -2, or above 2. When perennial ryegrass was used as pre-crop, as shown in Figure 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 (Figure 4B). Mixtures with oilseed radish cv. Terranova (OSR_T in Figure 3) all had a strong negative impact on the omnivore *Aporcelaimellus paraobtusicaudatus* (Figure 4B). Moreover, two specific treatments that both 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). With an estimate of -2.4501, *P. crenatus* was the only root lesion nematode species that was negatively affected by black oat. Another contrast was observed for *P. neglectus* that was negatively impacted by all three oilseed radish monocultures. *P. scribneri* was not stimulated nor repressed by any cover crop. Both *P. crenatus* and *P. neglectus* were negatively affected by the cover crop vetch, and the same two *Pratylenchus* species were promoted by the main crop, potato. It is noted that the other species, *P. fallax* and *P. scribneri*, were unaffected by this main crop. From this analysis we conclude that despite of 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 a potential to be used as a proxy for the soil biological condition (Ferris, Bongers, & De Goede, 2001; Ritz & Trudgill, 1999). However, microscopy-based methods for community analysis require extensive taxonomical expertise, are labor-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 (≈ 1,700 bp), by far the most popular barcoding gene for nematodes, and results in complete overviews of nematode communities either till 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 at with a high taxonomic resolution, and present data that demonstrate the ecological and agronomic relevance of high-resolution community analyses.

415

416 **4.2 Nematode community composition**

417 In our experimental field, we detected 65 nematode taxa with representatives from all feeding groups.
418 This nematode diversity lies in the same order of magnitude as the diversity of other agricultural fields
419 in the Netherlands (Mulder, Schouten, Hund-Rinke, & Breure, 2005) or Sweden (Sohlenius, Bostrom,
420 & Sandor, 1987). In the current community composition overview, members of the bacterivorous
421 family Cephalobidae including the genera *Acrobeles, Acrobeloides, Chiloplacus* and *Eucephalobus*, are
422 amply represented. In many studies, the abundance of the family of bacterivores has been reported in
423 both agricultural (Sohlenius et al., 1987) and natural habitats (Porazinska, Giblin-Davis, Powers, &
424 Thomas, 2012). A striking characteristic of Cephalobidae is the diversity in elaborations of body wall
425 cuticle surrounding the mouth and lips ('probolae'). *Acrobeles* is characterized by extensive, deeply
426 bifurcated probolae, whereas *Eucephalobus* members are equipped with particularly short probolae.
427 These elaborations are thought to play are role in feeding (De Ley 1992). If this assumption is correct,
428 it would imply that members of Cephalobidae differ in their feeding strategy, and apparently this
429 diversification contributed to their evolutionary success. From a soil ecological perspective, it would
430 therefore be preferable not to lump these members into a single category, as the presence of the
431 individual taxa might reflect the condition of distinct categories of soil bacteria.

432 As compared to bacterivores, fungivorous nematodes showed a patchier distribution. Most
433 widespread were members of the genera *Aphelenchus*, *Aphelenchoides,* and *Filenchus.* This might be
434 a generalizable observation for sandy arable fields in temperate climate zones; in a carrot production
435 field in Michigan, the same fungivorous nematode genera were found to be dominant (Grabau et al.,
436 2017). It should be noted that *Aphelenchus,* found in 97% of the samples by Grabau et al. (2017) was
437 considerably less prominent in our experimental field.

438 Among the predatory nematodes, *Mononchoides* was found in numerous samples. Notably
439 members of this genus can develop two stomatal morphs, and as such they can develop into
440 bacterivores or into predators (e.g., Mahboob et al., 2022). So, it is conceivable that a fraction of the
441 representatives of this genus functionally should be seen as bacterivores, and not as predators. From
442 our data we cannot assess the predatory fraction of the *Mononchoides* population.

443 Among the plant-parasitic nematodes, the stunt nematode *Tylenchorhynchus dubius* stood out
444 as it was present in nearly all samples. This observation fits well in a report by Sharma (1968) in which
445 this nematode was assessed to be the most generally occurring phytophagous nematode in lighter
446 soils in Western Europe. Its general occurrence is not limited to Europe; in a carrot field in Michigan
447 (USA) stunt nematodes were detected in 77% of the samples, with the highest relatively 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 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 in 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, Danchin, Perfus-Barbeoch, & Abad, 2013). It is expected that the DNA content of individual pre-parasitic juveniles is more-or-less a constant, and this would suggest for 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 relation 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 stage 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, also resulted in the repression of multiple other nematode taxa. This repression could be caused (in)directly by the plants as they are able to alter the soil microbiome locally (Koprivova & Kopriva, 2022). 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, a 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, Ferris, & Bayer, 1994). In this system, the lesion nematode outcompeted *M. chitwoodi.* Hence, our findings might be the resultant 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 a stimulation of *M. chitwoodi. T. dubius* belongs to the nematode family Telotylenchidae. Under field conditions in a vegetable cropping system (all vegetable were susceptible for RKNs) (Mateille et al., 2020) also observed a competition between Telotylenchidae and RKNs. As such we can conclude that difference in feeding strategy does not rule out competition between obligatory plant-parasitic nematodes.

The negative impact of *M. chitwoodi* stimulation on the omnivore *Aporcelaimellus obtusicaudatus*, a very common predaceous nematode that feeds on microdrile oligochaeta as well as on other nematodes, was unexpected. If we assume that nematodes constitute an important fraction of its overall nutritional intake, the decline of several members of the bacterivorous family Cephalobidae might be associated with the observed lower *A. obtusicaudatus* levels.

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, B). In the context of an initially low *M. chitwoodi* density, six nematode taxa including two plant parasites were strongly stimulated. In case of *M. chitwoodi,* this was associate with cover crop treatments that included black oat and vetch. Of the eight cover crop treatments associated with the stimulation of *Rhabditophanes,* seven included oilseed radish. Members of this genus are bacterivores (Yeates et al., 1993). *Rhabditophanes* sp. are unusual and basal representatives of the family Alloionematidae, the more distal members are all associated with slugs (Holovachov et al., 2016). Recently we have shown that oilseed radish strongly stimulated 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. The 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 & 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. It was remarkable to see that a member of the Rhabditidae family was promoted by numerous cover crop treatments irrespective of the pre-crop treatment. 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 condition and respond rapidly to local bacterial bloom which probably have 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., V5-7 (Capra et al., 2016)) will at most offer resolution till family level only (Harkes et al., 2019). 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. The power of this method is substantiated by the analysis of 132 soil samples from an experimental field. 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 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.

Acknowledgements

We acknowledge colleagues of WUR-FC at the Vredepeel station for their help in managing the experimental field. We thank Paul Mooijman for his contribution in the preparation of the sequencing library.

Funding

RvH was supported by the TKI project grant LWV20338. SC was supported by the TKI project grants AF18085 and TU18150 of the Dutch Topsectors Agri&Food and Tuinbouw&Uitgangsmaterialen

References

Álvarez-Ortega, S., & Peña-Santiago, R. (2013). Taxonomy of the genus *Aporcelaimellus* Heyns, 1965 (Nematoda, Dorylaimida, Aporcelaimidae). *Zootaxa, 3669*(3), 243-260. doi:10.11646/zootaxa.3669.3.3

Bahram, M., Hildebrand, F., Forslund, S. K., Anderson, J. L., Soudzilovskaia, N. A., Bodegom, P. M., . . . Bork, P. (2018). Structure and function of the global topsoil microbiome. *Nature, 560*(7717), 233-237. doi:10.1038/s41586-018-0386-6

Bik, H. M., Lambshead, P. J. D., Thomas, W. K., & Lunt, D. H. (2010). Moving towards a complete molecular framework of the Nematoda: A focus on the Enoplida and early-branching clades. *BMC Evolutionary Biology, 10*(1). doi:10.1186/1471-2148-10-353

Biswal, D. (2022). Nematodes as Ghosts of Land Use Past: Elucidating the Roles of Soil Nematode Community Studies as Indicators of Soil Health and Land Management Practices. *Applied Biochemistry and Biotechnology, 194*(5), 2357-2417. doi:10.1007/s12010-022-03808-9

Blanchy, G., Bragato, G., Di Bene, C., Jarvis, N., Larsbo, M., Meurer, K., & Garré, S. (2023). Soil and crop management practices and the water regulation functions of soils: a qualitative synthesis of meta-analyses relevant to European agriculture. *SOIL, 9*(1), 1-20. doi:10.5194/soil-9-1-2023

Blanco-Canqui, H., Shaver, T. M., Lindquist, J. L., Shapiro, C. A., Elmore, R. W., Francis, C. A., & Hergert, G. W. (2015). Cover crops and ecosystem services: Insights from studies in temperate soils. *Agronomy Journal, 107*(6), 2449-2474. doi:10.2134/agronj15.0086

Bongers, T. (1990). The maturity index - an ecological measure of environmental disturbance based on nematode species composition. *Oecologia, 83*(1), 14-19.

Bressan, M., Roncato, M.-A., Bellvert, F., Comte, G., Haichar, F. e. Z., Achouak, W., & Berge, O. (2009). Exogenous glucosinolate produced by Arabidopsis thaliana has an impact on microbes in the rhizosphere and plant roots. *The ISME journal, 3*(11), 1243-1257.

Brooks, M. E., Kristensen, K., Van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., . . . Bolker, B. M. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R journal, 9*(2), 378-400.

Capra, E., Giannico, R., Montagna, M., Turri, F., Cremonesi, P., Strozzi, F., . . . Pizzi, F. (2016). A new primer set for DNA metabarcoding of soil Metazoa. *European Journal of Soil Biology, 77*, 53-59. doi:10.1016/j.ejsobi.2016.10.005

Castagnone-Sereno, P., Danchin, E. G. J., Perfus-Barbeoch, L., & Abad, P. (2013) Diversity and evolution of root-knot nematodes, genus *Meloidogyne*: New insights from the genomic era. In*: Vol. 51. Annual Review of Phytopathology* (pp. 203-220).

Cazzaniga, S.G., Braat, L., van den Elsen, S., Lombaers, C., Visser, 747 J., Maciá-Vicente, J.G., Postma, J., Mommer, L. & Helder, J. (2023). Pinpointing the distinctive impacts of ten cover crop species on the resident and active fractions of the soil microbiome. Available at SSRN: https://ssrn.com/abstract=4365653 or http://dx.doi.org/10.2139/ssrn.4365653

Cortet, J., Ronce, D., Poinsot-Balaguer, N., Beaufreton, C., Chabert, A., Viaux, P., & Cancela De Fonseca, J. P. (2002). Impacts of different agricultural practices on the biodiversity of microarthropod communities in arable crop systems. *European Journal of Soil Biology, 38*(3-4), 239-244. doi:10.1016/s1164-5563(02)01152-4

De Ley, P., 1992. The nematode community of a marginal soil at Camberene, Senegal, with special attention to functional morphology and niche partitioning in the family Cephalobidae. Mededelingen van de Koninklijke Academie voor Wetenschapen, Letteren en Schone Kunsten van België - Klasse der Wetenschappen. 53, 107–153.

Doorenspleet, K., Jansen, L., Oosterbroek, S., Bos, O., Kamermans, P., Janse, M., . . . Nijland, R. (2021). High resolution species detection: accurate long read eDNA metabarcoding of North Sea fish using Oxford Nanopore sequencing. *bioRxiv*, 2021.2011. 2026.470087.

Du, X. F., Li, Y. B., Han, X., Ahmad, W., & Li, Q. (2020). Using high-throughput sequencing quantitatively to investigate soil nematode community composition in a steppe-forest ecotone. *Applied Soil Ecology, 152*. doi:10.1016/j.apsoil.2020.103562

Ewald, M., Rusch, D., Rißmann, C., Trost, B., Theuerl, S., & Ruess, L. (2022). Effects of irrigation and fertilization practice on soil nematode communities in arable land. *Applied Soil Ecology, 177*. doi:10.1016/j.apsoil.2022.104546

Ferris, H., Bongers, T., & De Goede, R. G. M. (2001). A framework for soil food web diagnostics: Extension of the nematode faunal analysis concept. *Applied Soil Ecology, 18*(1), 13-29. doi:10.1016/s0929-1393(01)00152-4

Geisen, S., Mitchell, E. A. D., Adl, S., Bonkowski, M., Dunthorn, M., Ekelund, F., . . . Lara, E. (2018). Soil protists: A fertile frontier in soil biology research. *FEMS Microbiology Reviews, 42*(3), 293-323. doi:10.1093/femsre/fuy006

Grabau, Z. J., Maung, Z. T. Z., Noyes, D. C., Baas, D. G., Werling, B. P., Brainard, D. C., & Melakeberhan, H. (2017). Effects of cover crops on pratylenchus penetrans and the nematode community in carrot production. *Journal of Nematology, 49*(1), 114-123. doi:10.21307/jofnem-2017-051

Harkes, P., Suleiman, A. K. A., van den Elsen, S. J. J., de Haan, J. J., Holterman, M., Kuramae, E. E., & Helder, J. (2019). Conventional and organic soil management as divergent drivers of resident and active fractions of major soil food web constituents. *Scientific Reports, 9*(1), 13521. doi:10.1038/s41598-019-49854-y

Harkes, P., van Steenbrugge, J. J. M., van den Elsen, S. J. J., Suleiman, A. K. A., de Haan, J. J., Holterman, M. H. M., & Helder, J. (2020). Shifts in the Active Rhizobiome Paralleling Low *Meloidogyne chitwoodi* Densities in Fields Under Prolonged Organic Soil Management. *Front Plant Sci, 10*, 1697. doi:10.3389/fpls.2019.01697

Holovachov, O., Boström, S., Tandingan De Ley, I., Mc Donnell, R. J., Alvarado, S., Paine, T. D., & De Ley, P. (2016). *Alloionema similis* n. sp., a genetically divergent sibling species of A. appendiculatum Schneider, 1859 (Rhabditida: Alloionematidae) from invasive slugs in California, USA. *Systematic Parasitology, 93*(9), 877-898. doi:10.1007/s11230-016-9668-2

Holterman, M., Karegar, A., Mooijman, P., Van Megen, H., Van Den Elsen, S., Vervoort, M. T. W., . . . Helder, J. (2017). Disparate gain and loss of parasitic abilities among nematode lineages. *PLoS ONE, 12*(9), e0185445. doi:10.1371/journal.pone.0185445

Holterman, M., Rybarczyk, K., Van den Elsen, S., Van Megen, H., Mooyman, P., Pena-Santiago, R., . . . Helder, J. (2008). A ribosomal DNA-based framework for the detection and quantification of stress-sensitive nematode families in terrestrial habitats. *Molecular Ecology Resources, 8*(1), 23-34.

Holterman, M., Schratzberger, M., & Helder, J. (2019). Nematodes as evolutionary commuters between marine, freshwater and terrestrial habitats. *Biological Journal of the Linnean Society, 128*(3), 756-767. doi:10.1093/biolinnean/blz107

Holterman, M., Van Der Wurff, A., Van Den Elsen, S., Van Megen, H., Bongers, T., Holovachov, O., . . . Helder, J. (2006). Phylum-Wide Analysis of SSU rDNA Reveals Deep Phylogenetic Relationships among Nematodes and Accelerated Evolution toward Crown Clades. *Molecular Biology and Evolution, 23*(9), 1792-1800. doi:10.1093/molbev/msl044

Holtkamp, R., Kardol, P., van der Wal, A., Dekker, S. C., van der Putten, W. H., & de Ruiter, P. C. (2008). Soil food web structure during ecosystem development after land abandonment. *Applied Soil Ecology, 39*(1), 23-34.

Hu, L., Robert, C. A. M., Cadot, S., Zhang, X., Ye, M., Li, B., . . . Erb, M. (2018). Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nature Communications, 9*(1). doi:10.1038/s41467-018-05122-7

Ivanova, N. V., Dewaard, J. R., & Hebert, P. D. (2006). An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular ecology notes, 6*(4), 998-1002.

Kitagami, Y., & Matsuda, Y. (2022). High-throughput sequencing covers greater nematode diversity than conventional morphotyping on natural cedar forests in Yakushima Island, Japan. *European Journal of Soil Biology, 112*. doi:10.1016/j.ejsobi.2022.103432

Knot, I. E., Zouganelis, G. D., Weedall, G. D., Wich, S. A., & Rae, R. (2020). DNA Barcoding of Nematodes Using the MinION. *Frontiers in Ecology and Evolution, 8*. doi:10.3389/fevo.2020.00100

Koprivova, A., & Kopriva, S. (2022). Plant secondary metabolites altering root microbiome composition and function. *Current Opinion in Plant Biology, 67*, 102227.

Lin, H., & Peddada, S. D. (2020). Analysis of compositions of microbiomes with bias correction. *Nature Communications, 11*(1), 1-11. doi:10.1038/s41467-020-17041-7

Liu, X., Hannula, S. E., Li, X., Hundscheid, M. P. J., klein Gunnewiek, P. J. A., Clocchiatti, A., . . . de Boer, W. (2021). Decomposing cover crops modify root-associated microbiome composition and disease tolerance of cash crop seedlings. *Soil Biology and Biochemistry, 160*. doi:10.1016/j.soilbio.2021.108343

Louca, S., Mazel, F., Doebeli, M., & Parfrey, L. W. (2019). A census-based estimate of earth's bacterial and archaeal diversity. *PLoS Biology, 17*(2). doi:10.1371/journal.pbio.3000106

Mahboob, M., Bashir, I., Asif, M., Nazir, N., Jahan, R., & Tahseen, Q. (2022). Molecular and phenotypic characterization of two cryptic species of the predatory genus *Mononchoides* Rahm, 1928 (Rhabditida: Diplogastridae) and their congeneric affinities. *Journal of Helminthology, 96*. doi:10.1017/s0022149x22000323

Mallick, H., Rahnavard, A., McIver, L. J., Ma, S., Zhang, Y., Nguyen, L. H., . . . Schwager, E. H. (2021). Multivariable association discovery in population-scale meta-omics studies. *PLoS Computational Biology, 17*(11), e1009442.

Mateille, T., Tavoillot, J., Goillon, C., Pares, L., Lefèvre, A., Védie, H., . . . Djian-Caporalino, C. (2020). Competitive interactions in plant-parasitic nematode communities affecting organic vegetable cropping systems. *Crop Protection, 135*. doi:10.1016/j.cropro.2020.105206

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One, 8*(4), e61217. doi:10.1371/journal.pone.0061217

Meldal, B. H. M., Debenham, N. J., De Ley, P., Tandingan De Ley, I., Vanfleteren, J., Vierstraete, A., . . . Lambshead, P. J. D. (2007). An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. *Molecular Phylogenetics and Evolution, 42*, 622-636.

Mulder, C., Schouten, A. J., Hund-Rinke, K., & Breure, A. M. (2005). The use of nematodes in ecological soil classification and assessment concepts. *Ecotoxicology and Environmental Safety, 62*(2), 278-289.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R., . . . Wagner, H. (2013). Package 'vegan'. *Community ecology package, version, 2*(9), 1-295.

Paulson, J. N., Stine, O. C., Bravo, H. C., & Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nature Methods, 10*(12), 1200.

Porazinska, D. L., Giblin-Davis, R. M., Faller, L., Farmerie, W., Kanzaki, N., Morris, K., . . . Thomas, W. K. (2009). Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. *Molecular Ecology Resources, 9*(6), 1439-1450. doi:10.1111/j.1755-0998.2009.02611.x

Porazinska, D. L., Giblin-Davis, R. M., Powers, T. O., & Thomas, W. K. (2012). Nematode Spatial and Ecological Patterns from Tropical and Temperate Rainforests. *PLoS ONE, 7*(9). doi:10.1371/journal.pone.0044641

Puissant, J., Villenave, C., Chauvin, C., Plassard, C., Blanchart, E., & Trap, J. (2021). Quantification of the global impact of agricultural practices on soil nematodes: A meta-analysis. *Soil Biology and Biochemistry, 161*. doi:10.1016/j.soilbio.2021.108383

Quist, C. W., Schrama, M., de Haan, J. J., Smant, G., Bakker, J., van der Putten, W. H., & Helder, J. (2016). Organic farming practices result in compositional shifts in nematode communities that exceed crop-related changes. *Applied Soil Ecology, 98*, 254-260. doi:10.1016/j.apsoil.2015.10.022

R Core Team. (2021). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Ritz, K., & Trudgill, D. L. (1999). Utility of nematode community analysis as an integrated measure of the functional state of soils: Perspectives and challenges: Discussion paper. *Plant and Soil, 212*(1), 1-11. doi:10.1023/a:1004673027625

Sharma, R. D. (1968). Host suitability of a number of plants for the nematode *Tylenchorynchus dubius*. *Netherlands Journal of Plant Pathology, 74*(4), 97-100. doi:10.1007/bf02309499

Sohlenius, B., Bostrom, S., & Sandor, A. (1987). Long-term dynamics of nematode communities in arable soil under four cropping systems. *Journal of Applied Ecology*, 131-144.

Spiridonov, S. E., Reid, A. P., Podrucka, K., Subbotin, S. A., & Moens, M. (2004). Phylogenetic relationships within the genus Steinernema (Nematoda: Rhabditida) as inferred from analyses of sequences of the ITS1-5.8S-ITS2 region of rDNA and morphological features. *Nematology, 6*(4), 547-566.

Tepes, A., Galarraga, I., Markandya, A., & Sánchez, M. J. S. (2021). Costs and benefits of soil protection and sustainable land management practices in selected European countries: Towards multidisciplinary insights. *Science of the Total Environment, 756*, 143925.

Tsiafouli, M. A., Thébault, E., Sgardelis, S. P., de Ruiter, P. C., van der Putten, W. H., Birkhofer, K., . . . Hedlund, K. (2014). Intensive agriculture reduces soil biodiversity across Europe. *Global Change Biology, 21*(2), 973 -985.

Umesh, K. C., Ferris, H., & Bayer, D. E. (1994). Competition between the plant-parasitic nematodes Pratylenchus neglectus and Meloidogyne chitwoodi. *Journal of Nematology, 26*(3), 286-295.

Van Megen, H., Van Den Elsen, S., Holterman, M., Karssen, G., Mooyman, P., Bongers, T., . . . Helder, J. (2009). A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. *Nematology, 11*(6), 927-950.

Verschoor, B. C., & de Goede, R. G. M. (2000). The nematode extraction efficiency of the Oostenbrink elutriator-cottonwool filter method with special reference to nematode body size and life strategy. *Nematology, 2*, 325-342.

Vervoort, M. T., Vonk, J. A., Mooijman, P. J., Van den Elsen, S. J., Van Megen, H. H., Veenhuizen, P., . . . Helder, J. (2012). SSU ribosomal DNA-based monitoring of nematode assemblages reveals distinct seasonal fluctuations within evolutionary heterogeneous feeding guilds. *PLoS One, 7*(10), e47555.

Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York. ISBN 978-3-319-24277-4.

Yeates, G. W., Bongers, T., De Goede, R. G. M., Freckman, D. W., & Georgieva, S. S. (1993). Feeding-Habits in Soil Nematode Families and Genera - an Outline for Soil Ecologists. *Journal of Nematology, 25*(3), 315-331.

Zhang, X., & Yi, N. (2020). NBZIMM: negative binomial and zero-inflated mixed models, with application to microbiome/metagenomics data analysis. *BMC Bioinformatics, 21*(1). doi:10.1186/s12859-020-03803-z

752 **Data Accessibility and Benefit-Sharing**

753 The sequence data generated during and/or analysed during the current study are available in the
754 NCBI repositories: BioProject PRJNA971608

755

756 **Author Contributions**

757 SC, JV and JH were responsible for the field experimental design. Soil sampling, nematode extraction
758 and purification and microscopic nematode analyses were done under the supervision of JV. SC
759 generated and purified nematode lysates. SvdE, SA, JOV and RvH developed a protocol for total SSU
760 rDNA amplification and the making of nanopore sequencing libraries. RvH performed the nanopore
761 sequencing runs, and processed the primary data. Primary data analysis was mainly done by RvH, while
762 statistical data analyses were predominantly performed by SC. Data interpretation and the writing was
763 the result of a collaborative effort by RvH, SC and JH.

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

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

766

767 **Table 2.** Temperature profile for PCR amplification of nearly full length nematode SSU rDNA

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

768

769 **Table 3.** Nematode biodiversity in experimental fields at the Vredepeel field station (The Netherlands).
770 Nematodes are identified on the basis nearly full length SSU rDNA sequences (≈ 1.700 bp). Taxa are
771 clustered according to their trophic preferences. Taxa are included only if they were detected in at
772 least two soil samples. The percentage of samples in which in dividual taxa were detected at t1 (after
773 cove crop) and t2 (after potato) is provided in separate columns. Bold: taxa on average (t1 +t2) present
774 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						

775

776

777 **Table 4A**. PERMANOVA analysis with Bray-Curtis dissimilarity metric to assess the variation explained
778 by block, pre-crop, cover crop, and interaction effect between pre-crop and cover crop upon CSS
779 normalization of data after cover crop termination and incorporation in topsoil (t1).

	Df	R^2	Pr(>F)
Block	3	0.16475	0.001 (***)
Pre-crop	1	0.06517	0.001 (***)
Cover crop	10	0.20594	0.002 (**)
pre.crop:cover.crop	10	0.11007	0.475

780

781 **Table 4B**. PERMANOVA analysis with Bray-Curtis dissimilarity metric to assess the variation explained
782 by pre-crop, cover crop, or block effect upon CSS normalization of data just after the harvest of the
783 main crop, potato (t2).

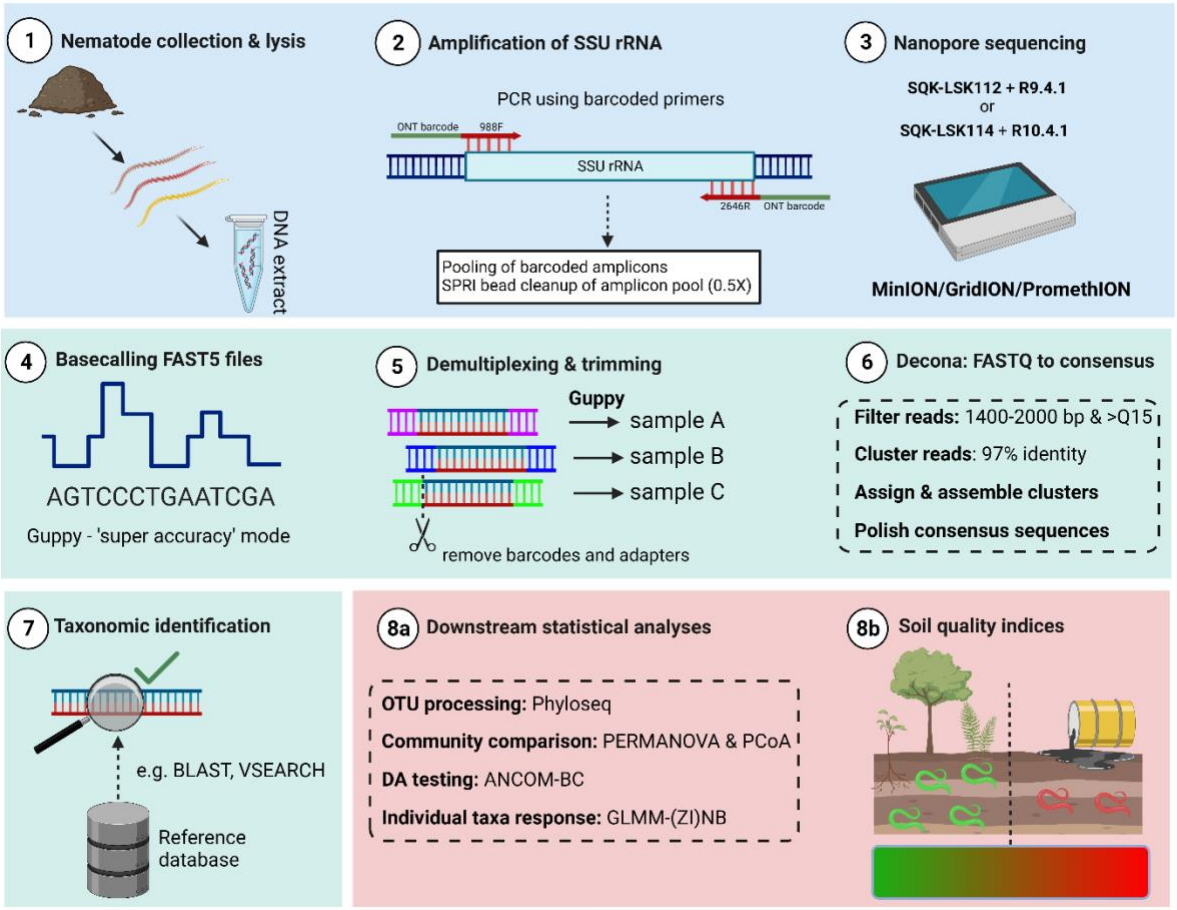
	Df	R^2	Pr(>F)
Block	3	0.14766	0.001 (***)
Pre-crop	1	0.17698	0.001 (***)
Cover crop	10	0.13123	0.141
pre.crop:cover.crop	10	0.13529	0.125

784

785 **Table 5.** Effect of monocultures and simple mixtures of cover crops and the subsequent cultivation of potato of four lesion nematode species,
786 *Pratylenchus crenatus, P. fallax, P. neglectus* and *P. scribneri.* *Pratylenchus* reads were rarefied and fitted in negative binomial mixed models
787 (GLMM-NB) with cover crop treatment, time point and pre-crop treatment as fixed and block as random factor. Estimates represent the effects of
788 the individual factors as compared to respectively fallow, t1 or black oat pre-crop. The Pr(>|z|) column represents the p-value associated with the
789 value in the z value column (not included). *** p-value < 0.001, ** p-value < 0.01, * p-value < 0.05.
790
791

	Pratylenchus crenatus			*Pratylenchus fallax*			*Pratylenchus neglectus*			*Pratylenchus scribneri*		
Cover crop treatment (t1)	Estimate	Pr(>|z|)		Estimate	Pr(>|z|)		Estimate	Pr(>|z|)		Estimate	Pr(>|z|)	
Black oat	-2.4501	0.032415	*	0.434369	0.7521		-0.1589	0.847975		-22.5946	0.99858	
Oilseed radish (cv Radical)	0.6546	0.407893		-0.61371	0.6095		-3.5844	3.36E-04	***	-22.5946	0.99858	
Oilseed radish cv Adios	-0.7675	0.355762		1.159637	0.3686		-3.6798	0.000338	***	0.6036	0.6908	
Oilseed radish cv Terranova	-0.5714	0.522252		-2.20154	0.1143		-3.0158	0.001696	**	-3.1182	0.10389	
Phacelia	0.8848	0.219207		1.484521	0.219		1.3191	0.09367		0.7268	0.61617	
Vetch	-2.3051	0.008512	**	0.826983	0.5052		-1.9881	0.026067	*	0.7455	0.62711	
Black oat / Oilseed radish (cv Radical)	-0.6889	0.385196		-0.72783	0.5453		-1.5709	0.069617		-1.6313	0.26299	
Black oat / Oilseed radish (cv Terranova)	-1.5033	0.109808		-2.02952	0.1108		-2.8702	0.002279	**	-2.1942	0.1498	
Phacelia / Oilseed radish (cv Terranova)	0.5428	0.484566		2.765724	0.0294	*	0.9318	0.257587		0.6605	0.60688	
Vetch / Oilseed radish (cv Terranova)	0.1107	0.886769		-2.343207	0.0688		-3.65	0.000282	***	-1.0942	0.44791	
Time point (t2)												
After potato cv Hansa	2.0709	0.000438	***	0.484025	0.4405		1.6431	0.000159	***	-0.3922	0.61978	
Pre-crop												
pre.crop Perennial_rye	2.4757	8.78E-06	***	0.714061	0.3678		2.8688	2.86E-09	***	2.9212	0.00527	**

792



793
794

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

802

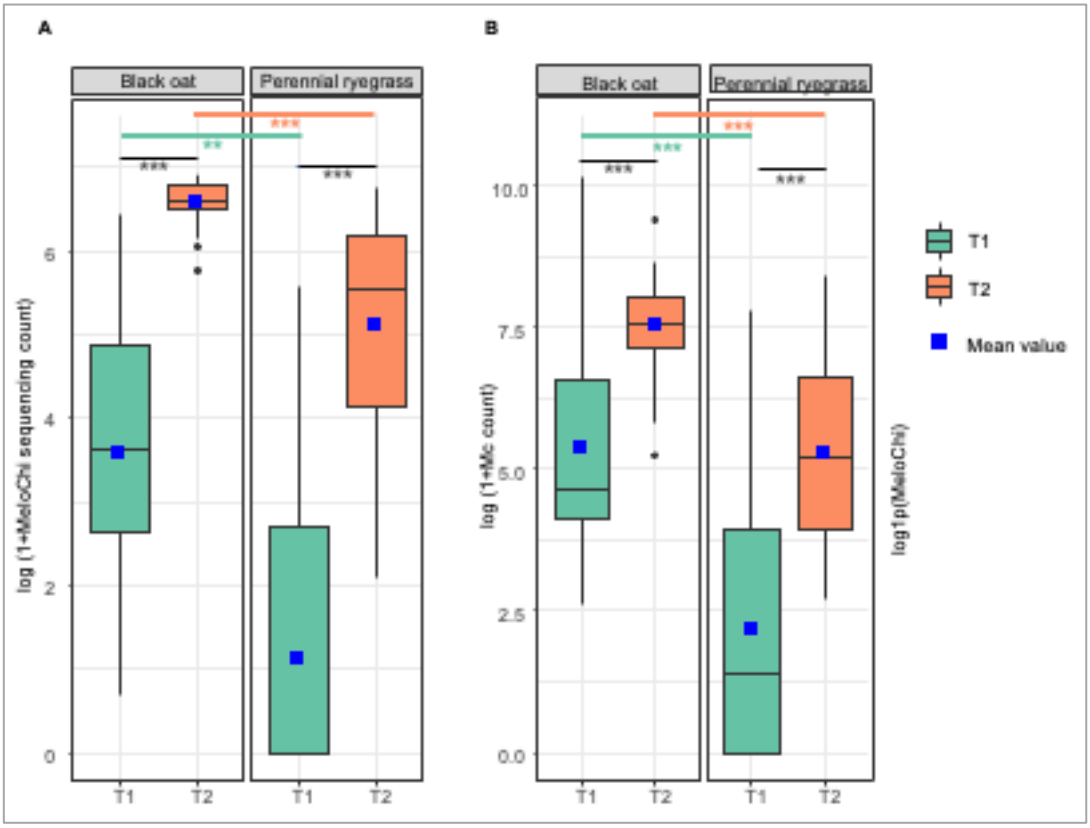


Fig. 2. Comparison of two methods to determine *Meloidogyne chitwoodi* densities at t1 (after pre-crop and cover crop treatments) and t2 (after potato). As a pre-crop either black oat or perennial ryegrass was grown (respectively a good and a poor host for *M. chitwoodi*). Subsequent, potato (a good host for *M. chitwoodi*) was grown, and soil samples were collected just after harvest. *M. chitwoodi* densities were determined using two independent approaches: nanopore sequencings reads (A) or microscopic counts (B). *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

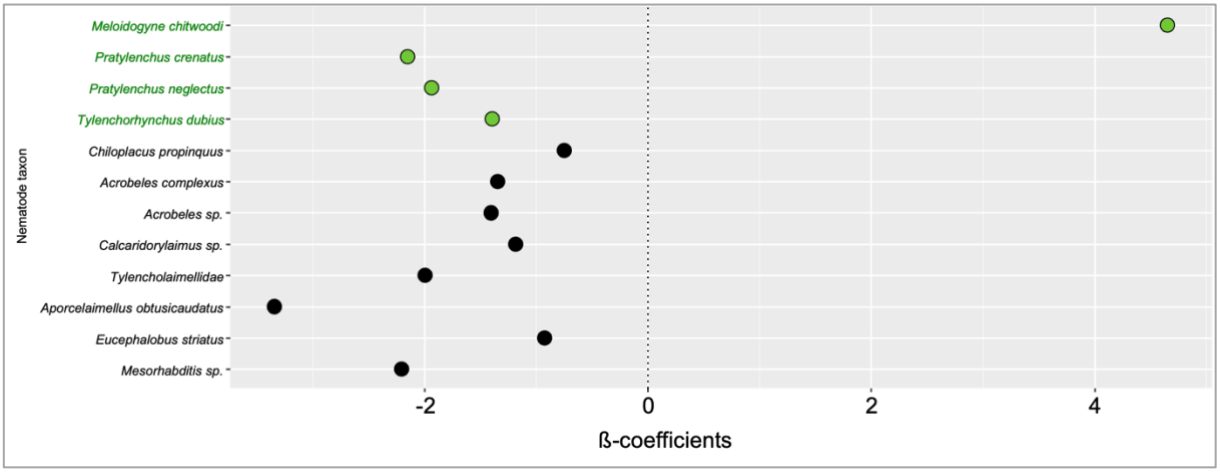


Fig. 3. Differential abundance testing (ANCOM-BC) was used to characterize the impact of the pre-crop black oat, a good host for *M. chitwoodi*, as compared to the effects of perennial ryegrass, a poor host for this root-knot nematode, on nematode communities over all cover crop treatments. The displayed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

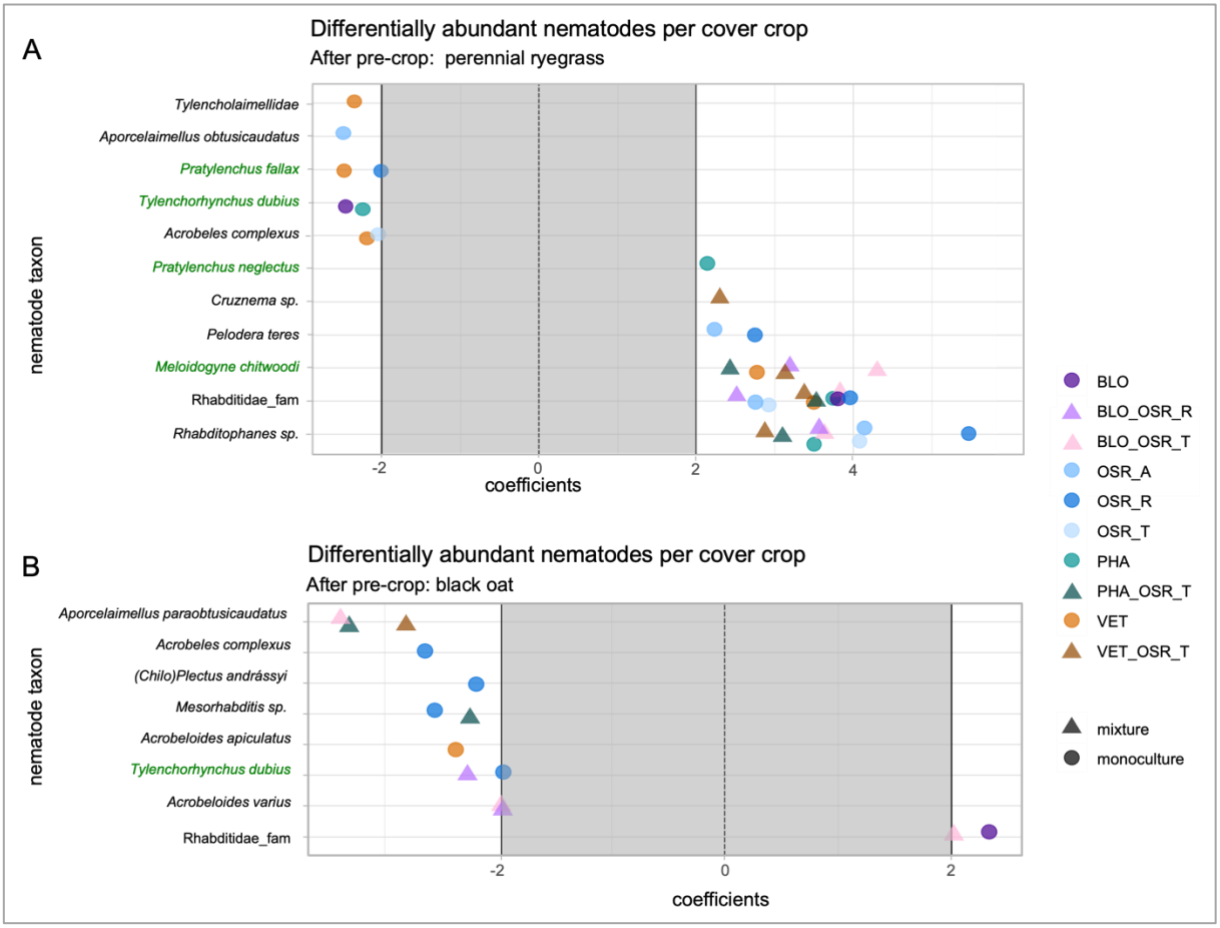


Fig. 4. Differential abundance testing (MaAsLin2) was used to characterize the impact of individual cover crops or mixtures of two cover crop species on nematode communities in fields that were initially

820 exposed to the pre-crop perennial rye (A) or to black oat (B). Only nematode taxa with regression
821 coefficients lower than -2, or above 2 are shown.