

Bioinoculants and organic soil amendments affect nematode diversity in apple orchards

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ARTICLE INFO

Keywords:

Soil biodiversity
Nematode community
Arbuscular Mycorrhiza fungi
Organic soil amendments
18S rDNA metabarcoding
Bioinoculants

ABSTRACT

Nematodes with their versatile lifestyles provide a suitable lens to decipher the conditions of agroecosystems, but less is known about how they are affected by bioinoculants and organic soil amendments. To test if treatments modify the nematode community, we studied nematode communities in two different apple orchards under organic and integrated farming. Soil was treated with products containing arbuscular mycorrhizal fungi, bio-effectors, and organic amendments. The comparison between baseline and control samples indicated an overall higher nematode richness in organic than the integrated orchard. Sampling time more than treatment had a significant effect, and higher community richness was observed during spring as compared to autumn. The variation in nematode community composition was mainly explained by sampling time followed by treatment, and orchard type. Although all treatments reduced nematode richness, their effect generally varied across treatments. In both orchards, season-dependent effects of treatment on nematode families and trophic guilds were observed, with a higher percentage of bacterivorous and lower percentage of herbivorous nematodes during spring. The effect was driven by a few families, i.e. Rhabditidae and Tylenchidae. Our study provides insights about the effect of soil treatment on nematodes with implications for the development and modification of bioinoculants.

1. Introduction

Nematodes are microscopic unsegmented worms that are key indicators of soil ecosystem conditions, also essential in providing insight into the structure and functioning of the soil food web (Bongers, 1990; Bongers and Ferris, 1999; Yeates and Bongers, 1999). They are considered the most abundant, diverse, and highly specialized animals on Earth and are characterized by versatile trophic guilds (van den Hoogen et al., 2019, 2020). Based on anatomical and physiological properties, nematodes are categorized by their feeding habits as: bacterivorous, fungivorous, herbivorous, carnivorous, omnivorous, as well as vertebrate and invertebrate parasites (Yeates et al., 1993; Yeates and Bongers, 1999). The trophic group categorization acknowledges that nematode taxa from monophyletic families are similarly adapted to specific environmental conditions and food resources (Ferris et al., 2001).

Nonetheless, taxa within the same feeding guild may display considerable variation influenced by interspecific interactions (Dos Santos et al., 2009), and differences in life-history characteristics (Bongers, 1990). Several attributes of nematodes favour their use as bioindicators of environmental health: (i) easy recovery from the soil matrix; (ii) sensitivity to changes in the soil environment due to their permeable cuticle; (iii) ability to withstand anaerobic conditions and desiccation (thus detectable in all seasons); (iv) a wide range of generation times (i.e. days to years) (Bongers, 1990; Ferris et al., 2001). Therefore, nematodes provide a suitable lens to study soil ecosystem services (Yeates, 1979; Sohlenius and Sohlenius, 1980; Ferris, 2010) and deduce soil food web structures (Ferris et al., 2001; Du Preez et al., 2022).

Accurate nematode identification relies on morphological and molecular methods for taxonomic assignment to family, genus, and species levels. For morphological identification, a high level of expertise is

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<https://doi.org/10.1016/j.apsoil.2023.105004>

Received 15 February 2023; Received in revised form 1 May 2023; Accepted 6 June 2023

Available online 15 June 2023

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needed to resolve nematode taxonomy, even at the family level (Waeyenberge et al., 2019). DNA-based methods offer alternatives for characterizing soil nematode composition in the face of the declining availability of expertise in morphological species identification and the limited number of distinguishing morphological features (Derycke et al., 2005, 2007, 2010). The decreasing costs for sequencing and the increasing availability of curated reference databases, such as SILVA138 (Quast et al., 2013; Yilmaz et al., 2014) promote the use of molecular approaches like metabarcoding; and also facilitate the generation of large datasets of soil biodiversity (Kageyama and Toju, 2022). However, morphological expertise will remain critical in the curation of molecular nematode data, as well as gathering knowledge about nematode ecology and biology (Geisen et al., 2018).

The soil biota, including nematodes, provides critical ecosystem services that are necessary for the sustainable functioning of natural and managed ecosystems (Barrios, 2007; De Vries et al., 2013). The diversity and abundance of soil biota are important indicators of soil health, and are vital for determining the state of ecosystem services (Ciobanu et al., 2015; Ferris and Tuomisto, 2015). Anthropogenic activities, which create soil disturbance, such as ploughing and soil amendment practices, like fertilizer enrichment, pesticide, and herbicide application influence soil biodiversity (Beare et al., 1992; Frey et al., 1999; Porazinska et al., 2003; Treonis et al., 2010; Thiele-Bruhn et al., 2012; Zhang et al., 2017; Berg and Cernava, 2022). The manipulation of farm conditions, especially those involving the application of fertilizers and pesticides, has supported major improvements in crop production to enable humankind to feed an ever-increasing population. This has not been without its detrimental impact on soil biodiversity and soil health (Thiele-Bruhn et al., 2012). Reducing the application of mineral fertilizers, and replacing these with organic soil amendments, can help boost soil biodiversity and eventually promote self-regulating systems (Thiele-Bruhn et al., 2012; Bender et al., 2016; Herren et al., 2020). Another approach towards soil “ecological engineering” (Bender et al., 2016; Machado et al., 2017) involves replacing synthetic chemical pesticides with biologically-based crop protectants and growth promoters. The application of bioextract from compost and biodynamic preparations, which are central components of biodynamic agriculture, was also shown to influence soil biodiversity (Olimi et al., 2022). Similarly, microbiota-based products (“bioinoculants”) are used to complement conventional farming practices and increase farm productivity, while supporting a healthy environment (Singh and Trivedi, 2017; Korsen, 2018). Bioinoculants are formulations containing microorganisms, which are responsible for resource availability, plant health, and resilience to biotic and abiotic stresses (Singh et al., 2020). Notably, bioinoculants can have a direct effect on plants and modulate the local microbiome to exert positive functions (Berg et al., 2021).

Currently, agricultural bioinoculants including products using arbuscular mycorrhizal fungi (AMF) constitute the fastest-growing bioproducts industry, with an annual rate of 17 %, and an anticipated market value of approximately 12 billion US dollars (Singh et al., 2020). The symbiotic associations of AMF and plants are widespread, evidenced in many non-/agricultural crops, and contribute to various ecosystem functions (Gosling et al., 2006). The interaction of AMF (commonly part of bioinoculant treatments) with plant parasitic nematodes, in particular, has been investigated (Hol and Cook, 2005; Bintarti et al., 2020). Further evidence about the action of AMF against nematodes was found by observing a reduction in the population of plant parasitic nematodes (*Pratylenchus coffeae* and *Radopholus similis*) in bananas (Elsen et al., 2008; Omolara Olaniyi, 2014; Schoutedden et al., 2015). Similarly, for ecological intensification, a strategy that seeks to integrate conventional farming and extensive systems, the use of organic manures is common practice (Bender et al., 2016; Machado et al., 2017; Huss et al., 2022). The application of organic manures such as compost is associated with enhanced soil biodiversity and crop yield (Mäder et al., 2002; D’Hose et al., 2014). Furthermore, traditional organic farming systems such as biodynamic farming use fermented manures and their extracts,

including the observation of weather and seasons to guide various activities such as planting, weeding, and harvesting. Biodynamic preparations can be applied as solid manure or liquid extracts into soil or by foliar spraying to enhance soil quality and plant health (Reganold, 1995; Scheuerell and Mahaffee, 2002; Carlo Ponzio and Ramesh Gangatharan, 2013). Often, bioextracts and bioinoculants are applied together with sugar-rich amendments (e.g., from molasses) to enhance microbial activity. The use of such “bioeffectors” has become a common practice in farming systems (Van Oosten et al., 2017), although, little is known about their role for soil ecosystem functioning.

The use of organic soil amendments is reported to contribute to various parameters, such as an increase in biodiversity, improvement of the soil food web stability, and ecosystem functionality (Forge et al., 2015; Harkes et al., 2020). However, the use of organic extracts, especially those used in biodynamic farming, have shown a decrease in soil bacterial diversity (Olimi et al., 2022). Moreover, molecular approaches have revealed the effect of field management on the soil microbiota, including metazoa (e.g. nematodes) in agriculturally important crops like barley (Harkes et al., 2019a, 2019b; Suleiman et al., 2019), and wheat (Birkhofer et al., 2008). In addition, the nematode functional diversity in fields of intensively managed horticultural crops, such as bananas (tropical climate) and apples (temperate) have been shown (Lazarova et al., 2021), but less is known about the effect of bioinoculant and organic amendment application on the nematode composition of orchards. Apple is one of the most economically important horticultural crops (Vasylyeva and James, 2021), and is of particular significance in the temperate zone (Abbott, 1994; Péneau et al., 2006). In Europe, apples are mainly produced using a conventional approach, but gains from reduced pesticide usage, and an increasing consumer demand for pesticide free foods, have encouraged the adoption of organic and integrated orchard management (Weddle et al., 2009; Zalucki et al., 2009; Damos et al., 2015; Vasylyeva and James, 2021). In addition, apple replant disease (ARD) is a severe problem in apple production worldwide; here nematode-microbiota interactions are involved (Kanfra et al., 2018). Therefore, adopting sustainable and environmentally sound crop and soil protection approaches will require the development of new bioinoculants (Berg et al., 2017, 2021; Li et al., 2022).

Thus, there is a necessity to explore the potential effects of bioinoculants and organic amendments on the soil fauna of apple orchards. In contribution to this effort, the current study used 18S rDNA metabarcoding targeting the nematode fauna to assess the impact of season and treatment on the soil nematode assemblage and functional structure in two apple orchards. We hypothesized that soil supplements would modify the nematode community composition and functional groups in the different orchards, over the two sampling times. The results provide insights into the effect of potentially novel crop protection strategies for soil health with potential implications for enhancing soil ecosystem functions.

2. Material and methods

2.1. Orchard site details

This study involved two apple orchards under organic and integrated management. In the organic orchard, organic manure was topically applied, then mechanically homogenized using a tractor, especially during early spring. No synthetic pesticides or fertilizers were applied. The integrated orchard equally received manures; however, synthetic pesticides and fertilizers were also applied. Both orchards contained apple fruit trees which were approximately 10 years old. The apple trees in the different orchards varied in varieties as follows: Golden delicious in the integrated orchard and Arlet in the organic orchard. The organic, and integrated orchards were situated in similar agroecological zones, at 47.1289°N, 15.7807°E and 47.2125°N, 15.8569°E, respectively; and were approximately 8 km apart from each other. The physico-chemical analysis of the different orchard soils was performed on untreated

control (“baseline”) samples, which were taken two months prior to treatment application. High clay content in organic-, compared to integrated- orchard (i.e., organic vs integrated: 32 % vs 4 %), as well as higher organic matter content (3 % vs 2 %) was observed. The soil types in different orchards were characterized as follows: organic orchard (silt: 18 %, clay: 32 %, and sand: 50 %; pH: 6.9) and integrated orchard (silt: 19 %, clay: 4 %, and sand: 77 %; pH: 6.2). The phosphorous, potassium, total nitrogen, magnesium, and calcium content (in mg/kg) was determined, and the results are shown in Table S1.

2.2. Treatment description and application

Two arbuscular mycorrhizal fungi (AMF) products: Rhea and Mycoplant (“bioinoculants”) were used in this study. These products were respectively supplied by companies INOCULUMplus (France) and INTERMAG (Poland), in the frame of EXCALIBUR project (<https://excaliburh2020.eu/>). Each of the two AMF products contained approximately 1000 propagules/g. The AMF bioinoculants (i.e., Rhea and Mycoplant) were combined with a sugar source (i.e., “Vinasse”) to comprise the treatment combinations: Vinasse + Rhea (Rhea +) and Vinasse + Mycoplant (Mycoplant +). The bioeffector (Vinasse) is a honey-like, dark brown syrup which is produced from fermented sugar beet molasses. The Vinasse was diluted using five litres into 20 l of clean water, then applied in the plots which already received the AMF products. The “bioextract fertilizer” treatments involved extracts derived from the biodynamic manure (500P) and compost (i.e., compost tea) (Olimi et al., 2022). These “bioextract fertilizer” products were obtained from Demeter (Vienna, Austria). In biodynamic farming, the product 500P is used for field or foliar application and is based on cow horn manure amended with plant preparations (Brock et al., 2019). Water was used for 500P extraction and for field application as previously described (Olimi et al., 2022). The compost was composed of 70 % shrub cuttings, 5 % soil, and 25 % on-farm organic kitchen waste. The compost was extracted with water and mixed with molasses and stone dust to form “compost extract” that was applied to the field.

The orchards were treated with bioinoculants and bioextracts at the beginning of spring (May/2021). The bioinoculants were applied at the recommended rate (1 g/l of water-propagule suspension). For the bioinoculant and bioeffector (“Vinasse”) combination, 1 l of Vinasse was applied to the soil, just after the bioinoculants were applied. The bioextracts were applied by sprinkling the soil in vicinity of the plants, using an application rate for compost and biodynamic manure of 100 g/ha in about 35 l of water, as recommended by BioDynamie Services sarl (Château, France). For each orchard we chose a randomized plot design across six neighbouring rows consisting of 4 plots per treatment and 8–10 trees per plot (Fig. S1). To account for spatial variation, treatments were randomly distributed. All plots received the treatments for the first time.

2.3. Sampling, sample processing, and DNA extraction

Bulk soil samples in the vicinity of apple roots were randomly taken from each plot, at a depth of approximately 25 cm using an Auger with 5 cm diameter. Ten soil cores were collected in each plot ($n = 4$) and pooled to comprise a biological replicate of approximately 200 g. Soil sampling was performed one, and four months after treatment application, during spring and autumn, respectively. Samples were cooled and transported within 6 h to the laboratory at the Institute of Environmental Biotechnology (Graz University of Technology, Graz, Austria). Additionally, four samples (baseline samples) from each orchard were collected two months prior to orchard treatment. A total of 118 samples were collected from the two orchards to represent the baseline, spring, and autumn conditions. Each sample (200 g of soil) was passed through a 4mm sieve to remove coarse material, and then the finer 1mm sieve was used to refine the soil. The soil was homogenized manually by shaking and a subsample (4 g) was collected and placed in

two 2-mL tubes (Eppendorf; Hamburg-Germany). The sub-sample was then stored at -70°C until DNA was extracted. Total DNA was extracted from 500 mg of each sample, using E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Inc.; Norcross-Georgia, USA), following manufacturer instructions, and previously described in (Kawanobe et al., 2021). The DNA was quality checked using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and stored at -20°C until Polymerase Chain Reactions (PCRs) were performed to prepare the amplicon library of the nematode community.

2.4. Amplicon library preparation and high throughput sequencing

Amplicon libraries were prepared based on the nematode 18S rDNA, using a nematode-specific primer pair (F548-A and R1912) for soil DNA as previously described (Kawanobe et al., 2021). The library preparation was performed using a two-step PCR procedure, involving the amplification of 18S rDNA and the subsequent attachment of sample-specific barcodes. In the first reaction (PCR1), the primer pair F548-A (5'-TATGGTAATTGTAGAGGGCAAGTCTGGTGCC-3') and R1912 (5'-AGT-CAGCCAGGGAGAGGGCAAGTCTGGTGCC-3') was used to amplify the 18S rDNA.

Briefly, the forward primer was adapted from (Hadziavdic et al., 2014). The reverse primer R1912 was described by Holterman et al. (2006) as nematode-specific, and is known for its wider coverage of the phylum Nematoda. The upstream end of the primer sequences was modified to include pad sequences (i.e., forward-pad: TATGGTAATT, and reverse-pad: AGTCAGCCAG) and 2 base pair linkers (i.e., GT and GG attached to forward and reverse primers, respectively) inserted after the forward and reverse pad sequences. In the second reaction (PCR2), sample-specific barcode adapters were added onto the amplicons from PCR1 for multiplexed sequencing with Illumina MiSeq. All reactions were performed on a thermocycler (Bio-metra GmbH, Jena, Germany). In PCR1, 1 μl of the extracted DNA was used in each 14 μl reaction. The reaction mixture contained 6 μl (5xTaq & GO, PCR pre-mix, MP Bio-medicals), 0.5 μl (10 μM F548-A/R1912) primers, 1.5 μl (25 mM MgCl_2) and 4.5 μl of PCR grade water. The PCR program for the first amplification step included an initial denaturation (96°C , 5 min), followed by 35 cycles (94°C for 30s, 48°C for 30 s, and 72°C for 30 s), followed by final extension for 5 min at 72°C , and then cool down to 10°C .

The subsequent reaction (i.e., PCR2) to attach sample-specific barcodes was performed in triplicates using 2 μl of PCR1 amplicons in a 30 μl reaction mixture. Each reaction mixture was composed of 6 μl (5xTaq & GO), 1.2 μl (10 μM ; Forward/Reverse barcode primers), and 19.6 μl of PCR-grade water. For the second reaction, the cycler program involved: initial denaturation (95°C , 5 min), then 15 cycles (95°C for 30 s, 53°C for 30 s, 72°C for 30 s), followed by 5 min at 72°C and cooling to 10°C . PCR products were verified (500 bp) on 1 % agarose gels (1 g agarose in 100 ml 1x TAE buffer), then stained using gel red dye, followed by visualization under UV light in a gel doc (BioRad Gel Doc XR Imaging Systems). The amplicons were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) following manufacturer instructions. Purified PCR amplicons were quantified using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and pooled in equimolar concentrations. Paired-end Illumina MiSeq 2x300 sequencing of the amplicon library was performed at GENEWIZ (AZENTA life sciences, Leipzig, Germany). The raw reads were uploaded to European Nucleotide Archive (ENA) under the study accession PRJEB5559.

2.4.1. Bioinformatics

Paired-end reads were quality checked, demultiplexed, and then primer sequences removed using Cutadapt (Martin, 2011). The forward and reverse reads were merged using PEAR v 0.9.6 (Zhang et al., 2014), and then imported for further processing using QIIME2 version 2021.11.0 (Bolyen et al., 2019). Demultiplexed and merged reads were quality filtered, denoised, and subjected to chimera removal using the DADA2 algorithm (Callahan et al., 2016). The resulting amplicon

sequence variants (ASVs) and a feature table of read counts were obtained. Taxonomic assignment of ASVs was performed in QIIME2 by comparing sequence reads against the curated SILVA138 database containing 18S rDNA sequences (Quast et al., 2013; Yilmaz et al., 2014). When taxonomic assignment was unclear, sequence reads were manually aligned with the NCBI database using blastn (Altschul et al., 1990). In addition, the nemaplex website (<http://nemaplex.ucdavis.edu/>) was used for cross-referencing nematode taxa for affirmative taxonomic assignment. Nemaplex is a specially designed website for manual reference of nematode taxonomy, and is managed by the University of California, Davis (USA). Meanwhile, the phylogenetic tree was generated using the alignment and phylogeny tools in QIIME2 as follows: de novo multiple sequence alignment with MAFFT (Kuraku et al., 2013; Katoh et al., 2019), followed by filtering unconserved and highly gapped columns from alignments; and the subsequent generation of the phylogenetic tree. The obtained ASVs counts, taxonomy table, phylogenetic tree, and experimental metadata were imported into R (version 4.0.3) using RStudio (version 1.1.432) (R Core Team, 2020) and processed using the phyloseq 1.38.0 (McMurdie and Holmes, 2013) and vegan 2.5.7 (Oksanen et al., 2020) packages. To circumvent the inflation of species richness, singleton, and doubleton ASVs across the samples were removed from the dataset.

2.4.2. Processing metabarcoding data and assignment of feeding guilds

The nematode community composition was analysed at family level, as well as by the different trophic guilds. Five trophic guilds were assigned according to Yeates et al. (1993) and included: bacterivorous, fungivorous, herbivorous, omnivorous, and carnivorous nematodes. The nematode compositional structures were assigned to different feeding guilds/categories using the Nematode Indicator Joint Analysis (NINJA) online tool (<https://shiny.wur.nl/ninja/>) (Sieriebriennikov et al., 2014).

2.5. Statistical analysis

Data were analysed with R statistical software (Version 4.0.3: <http://www.r-project.org>) using RStudio (Version 1.1.423) (R Core Team, 2020), supplemented by the web-based Microbiome Analyst tools (Dhariwal et al., 2017). Within samples, nematode diversity indices (i.e., alpha diversity) such as richness (i.e. observed ASVs), Pielou's evenness, and Faith's phylogenetic diversity (Faith's PD) were computed. Briefly, Faith's PD is a measure of biodiversity based on community phylogeny (Faith, 1992; Faith et al., 2018). The alpha diversity statistics were calculated on data that were rarefied to a minimum sampling depth of 200 reads per sample; and the rarefaction curves depicting the orchard and treatment combinations for the two sampling times were shown (Fig. S2). We used the `rarefy_even_depth` and `ggrare` functions of the `Ranacapa` package to rarefy the dataset and generate rarefaction curves (Kandlikar et al., 2018). Differences in the proportional composition of the prevalent nematode families and trophic guilds between the treatments at different sampling time, and for the two orchards were assessed. One-way ANOVA was used if data was normally distributed (i.e., Shapiro test), followed by the post hoc analysis (i.e., Tukey's HSD test) to explore the significant differences between treatments. For data that did not follow a normal distribution, the non-parametric Kruskal-Wallis's test was used, and Dunn's multiple comparisons test was performed on factors with significant overall differences. Significant differences were denoted by $P < 0.05$, and non-significant differences by $P > 0.05$.

Beta diversity analyses were performed on transformed data using the `phyloseq.transform.css` function in the `Phyloseq` package (McMurdie and Holmes, 2013). The differences in nematode community composition between treatments for two orchards and seasons were tested using permutational analysis of variance (PERMANOVA, 999 permutations) on Bray-Curtis dissimilarity matrices with the R-package `Vegan` 2.5–3 (Oksanen et al., 2020). Using the "pairwise.adonis2" function from the `pairwiseAdonis` package, comparisons between treatments and control

in the different orchards were conducted for each sampling time (Martinez Arbizu, 2020), followed by p -values adjustment using the Bonferroni method. The nematode community dissimilarities among treatments for the different orchards and seasons were visualized by principal coordinate analysis (PCoA) of Bray-Curtis distances of the nematode count data, using the `Phyloseq` package (McMurdie and Holmes, 2013).

3. Results

3.1. Description of data obtained by metabarcoding analysis

Amplicon sequencing yielded 180,765 high-quality reads, assigned to 411 amplicon sequence variants (ASVs). The ASVs were associated with eight eukaryotic phyla. The phylum Nematoda constituted the highest mean relative abundance (80.7 %) across the two orchards (Fig. S3). Other major phyla were *Arthropoda* (8.6 %), *Platyhelminthes* (9.7 %), *Annelida* 0.6 %) and other eukaryotic phyla such as *Ascomycota*, *Basidiomycota*, *Tardigrada*, and *Gracilipodida*. Filtering of nematode associated ASVs retained 143,324 high-quality reads that were assigned to 275 ASVs from 14 nematode families. Alpha rarefaction at 200 reads per sample (i.e., minimum sampling) revealed richness values ranging from 20 to 40 species (Fig. S2).

3.2. The influence of bioinoculants and bioextract on the nematode community diversity and structure

In general, a significant effect ($P < 0.0001$) of sampling time on nematode alpha-diversity indices (richness, evenness, and phylogenetic diversity) was observed. Regarding orchards, a significant difference ($P < 0.05$) in diversity indices was only seen for evenness, and no significant differences ($P > 0.05$) were observed in richness and phylogenetic diversity. Moreover, higher average values of these indices were observed during spring as compared to autumn, for both organic and integrated orchards (Table 1). There were significant differences in nematode richness (Kruskal-Wallis, $P = 0.002$), and phylogenetic diversity (ANOVA, $P = 0.0004$) observed between the different treatments, during spring in the organic and integrated orchard (Table 1). Pairwise comparison between treatments and control, showed a significantly lower richness in the biodynamic extract (500P) treatment during spring in the organic orchard ($P_{adj} < 0.05$), while no difference in richness was observed between the other treatments and control ($P_{adj} > 0.05$).

The comparison between baseline and control samples (i.e., obtained during spring and autumn, respectively) revealed a higher nematode alpha-diversity (i.e., richness, evenness, and phylogenetic diversity) in organic, as compared to the integrated orchard (Fig. S4). However, the community diversity decreased from baseline to spring and autumn samples. Nematode community evenness was consistently higher in the organic as compared to the integrated orchard. However, Faith PD was higher in the integrated orchard as compared to organic orchard, both during spring and autumn (Fig. S4). Also, no significant differences in nematode richness were observed between treatments, during spring for the integrated orchard. Furthermore, during spring we observed general significant differences in the nematode community phylogenetic diversity between treatments in organic (ANOVA: $P = 0.0004$) and integrated (ANOVA: $P = 0.0002$) orchards (Table 1). In the organic orchard and during spring, pairwise comparison revealed that 500P treatment significantly reduced the nematode phylogenetic diversity in comparison to control, while no significant differences were seen for the other treatments. Meanwhile in the integrated orchard, significantly lower phylogenetic diversity in the treatments (i.e., Compost tea and 500P extracts, Mycoplant, Mycoplant +, and Rhea +) were observed when compared to control. No significant differences ($P > 0.05$) were observed in autumn for the two orchards.

Our results based on PERMANOVA analysis suggested that the

Table 1

Diversity indices (richness, Shannon index, Pielou's evenness, and Faith's Phylogenetic diversity index). ANOVA was used for data following a normal distribution and Kruskal Wallis elsewhere. The tests show overall statistical differences between treatments, including the baseline samples for each orchard. Acronyms A and KW represent ANOVA and Kruskal-Wallis tests, employed. Values shown are mean ± standard deviation ($n = 4$).

Treatment	Diversity index	Control	Compost tea (500)	500P	Mycoplant	Mycoplant +	Rhea	Rhea +	ANOVA/Kruskal-Wallis	
Orchard	Organic									
	Spring	Richness	33.8 ± 6.1 ^{ab}	16.5 ± 4.7 ^{abc}	10.8 ± 1.0 ^c	16.0 ± 5.0 ^{ac}	21.2 ± 8.6 ^{abc}	37.5 ± 7.9 ^b	28.8 ± 7.5 ^{abc}	KW: $P = 0.002$ (KW)
	Pielou's evenness	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	KW: $P = 0.144$	
Autumn	Faith's PD	2.1 ± 0.2 ^{bc}	1.4 ± 0.2 ^{ab}	1.1 ± 0.1 ^a	1.4 ± 0.3 ^{ab}	1.6 ± 0.4 ^{abc}	2.2 ± 0.1 ^{bc}	2.3 ± 0.7 ^c	A: $P = 0.0004$	
	Richness	7.0 ± 0.1	12.0 ± 0.0	9.5 ± 0.7	11.8 ± 4.2	12.0 ± 3.0	9.0 ± 0.1	6.8 ± 4.0	KW: $P = 0.295$	
	Pielou's evenness	0.7 ± 0.1	0.9 ± 0.0	0.9 ± 0.1	0.7 ± 0.3	0.9 ± 0.1	0.4 ± 0.1	0.6 ± 0.3	KW: $P = 0.240$	
Integrated	Faith's PD	0.6 ± 0.1	1.3 ± 0.1	0.8 ± 0.3	1.2 ± 0.3	1.1 ± 0.2	0.9 ± 0.3	0.8 ± 0.5	A: $P = 0.478$	
	Spring	Richness	33.2 ± 5.7	13.2 ± 3.9	13.8 ± 1.7	15.2 ± 2.4	16.5 ± 6.5	34.0 ± 7.7	16.2 ± 8.5	KW: $P = 0.013$
	Pielou's evenness	0.9 ± 0.0	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.0	0.7 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	KW: $P = 0.729$	
Autumn	Faith's PD	2.2 ± 0.1 ^c	1.1 ± 0.2 ^a	1.3 ± 0.1 ^a	1.4 ± 0.2 ^{ab}	1.5 ± 0.3 ^{ab}	1.9 ± 0.3 ^{bc}	1.5 ± 0.4 ^{ab}	A: $P = 0.0002$	
	Richness	11.0 ± 2.7	11.0 ± 4.4	6.7 ± 2.5	13.8 ± 4.9	14.3 ± 0.6	12.8 ± 6.7	13.0 ± 3.6	KW: $P = 0.354$	
	Pielou's evenness	0.5 ± 0.3	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.2	0.8 ± 0.1	0.7 ± 0.2	0.7 ± 0.3	KW: $P = 0.815$	
Integrated	Faith's PD	1.2 ± 0.4	0.9 ± 0.3	0.9 ± 0.2	1.2 ± 0.4	1.4 ± 0.1	1.1 ± 0.5	1.3 ± 0.3	A: $P = 0.501$	

variation in nematode community composition (i.e. beta diversity) was mainly explained by sampling time ($df = 1$, $R^2 = 12\%$, $P = 0.001$), followed by treatment ($df = 6$, $R^2 = 8\%$, $P = 0.001$), and then orchards site ($df = 1$, $R^2 = 5\%$, $P = 0.001$). Furthermore, we observed a significant interaction between orchard and sampling time ($df = 1$, $R^2 = 1\%$, $P = 0.017$), as well as treatment and sampling time ($df = 6$, $R^2 = 7\%$, $P = 0.001$). During spring, treatment ($df = 6$, $R^2 = 24\%$, $P = 0.001$) as compared to orchard ($df = 1$, $R^2 = 8\%$, $P = 0.001$) had a greater effect on the nematode community composition. The orchard-specific beta diversity indicated a significant effect of treatment on the nematode community composition for organic ($df = 6$, $R^2 = 35\%$, $P = 0.001$) and

integrated ($df = 6$, $R^2 = 39\%$, $P = 0.001$) orchards. The statistical details for nematode community composition between treatments in the two fields and sampling times are presented in Table S2.

In the organic orchard, pairwise significant differences in nematode community composition were revealed between control and treatments (Mycoplant +, Compost tea (bioextract), and 500P (bioextract); pairwise PERMANOVA - $P < 0.05$). In the integrated orchard, differences were observed between control and treatments (Rhea +, Mycoplant, Mycoplant +, Compost tea, and 500P; pairwise PERMANOVA - $P < 0.05$). Similarly, during autumn, treatment ($df = 6$, $R^2 = 14\%$, $P = 0.012$) significantly influenced the nematode community composition,

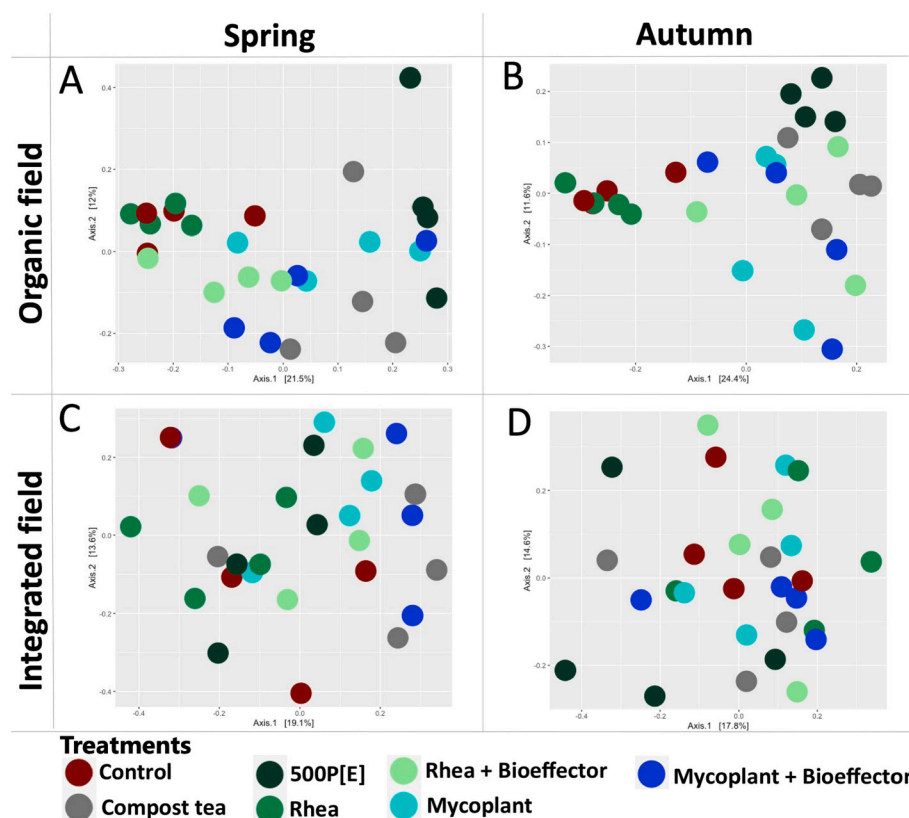


Fig. 1. Bray-Curtis based principal coordinate analysis (PCoA) plot showing the nematode community composition in the different treatments for two apple orchards during spring and autumn. Figure (A) and (B) are PCoA representation of the nematode beta-diversity in organic apple orchards during spring and autumn, while (C) and (D) represent the same for the integrated orchard. The coloured circles represent the different treatments.

more than orchard site ($df = 1$, $R^2 = 6\%$, $P = 0.001$). When the data from each orchard was analysed separately, significant effects of treatment on the nematode community composition in the different orchards were observed; organic ($df = 6$, $R^2 = 26\%$, $P = 0.001$), and integrated ($df = 6$, $R^2 = 25\%$, $P = 0.001$). However, no pairwise significant differences in the nematode community composition were observed between treatments and control, during autumn both for organic and integrated orchards (pairwise PERMANOVA: $P > 0.05$). Our results based on principal coordinate analysis (PCoA) revealed no clear separation in nematode community among treatments in the two orchards, during spring and autumn. However, some degree of separation between treatment and control was observed in the organic orchard for the two sampling times (Fig. 1). The details showing pairwise PERMANOVA statistical differences are shown in Table S2.

3.3. Composition of the nematode community as related to orchard type and sampling time

The nematode taxonomic composition was visualized using stacked bar plots representing the mean relative abundance of the nematode families, observed in the different orchards at different sampling times and for the different treatments (Fig. 2). The dominant families included *Tylenchidae* (mean relative abundances: organic = 39%; integrated = 43%), *Rhabditidae* (35%, 43%), *Mononchidae* (5%, 2%), *Dorylaimidae* (7%, 3%), and *Plectidae* (7%, 3%) (Fig. 2). The other taxa belonged to families *Cephalobidae*, *Mermithidae*, *Monhysteridae*, *Prismatolaimidae*, and *Alaimidae*. The nematode composition comparisons between the different treatments in the organic orchard, and during spring generally showed higher proportion of *Tylenchidae* in 500P as compared to

control. During autumn, the family *Tylenchidae* was lower in Compost tea and 500P in comparison to the control (Fig. 1 A and B). During spring and autumn, the Compost tea treatment as compared to control showed a higher composition of *Mononchidae* for the organic orchard. When comparing all treatments to the control throughout spring and autumn in the organic orchard, the family *Rhabditidae* was found have similar proportions in both seasons. In the integrated orchard, the family *Rhabditidae* was equally represented for all treatments during spring. However, during autumn we observed higher proportions of *Rhabditidae* in comparison to control plots (Fig. 1 C and D).

Moreover, the relative abundance of *Tylenchidae* (ectoparasitic root hair feeders) nematodes was higher in autumn than spring, both in organic (spring: 26% and autumn: 53%), and particularly in the integrated orchard (spring: 22% and autumn: 64%). In both orchards, *Rhabditidae* (bacterivorous nematodes) were higher in spring than autumn (organic: spring: 42% and autumn: 27%; integrated: spring: 59% and autumn: 26%). During spring, the abundance of this family was high in the integrated orchard compared with the organic orchard (Fig. 2 a and b). The families *Dorylaimidae* and *Mononchidae* (carnivorous) were more prevalent in the organic than the integrated orchard, both during spring and autumn. Meanwhile, the family *Cephalobidae* was observed in the Compost tea treatment, especially in the organic orchard. Generally, there was a significant effect of sampling time on the different nematode families and trophic guilds. However, a comparison between the two orchards showed significant differences only for families *Rhabditidae* and *Dorylaimidae*, as well as for omnivorous nematodes. Significantly higher proportions of the family *Dorylaimidae* were observed in control as compared to Compost tea, during spring for the integrated orchard (Table S3). No other significant differences in the

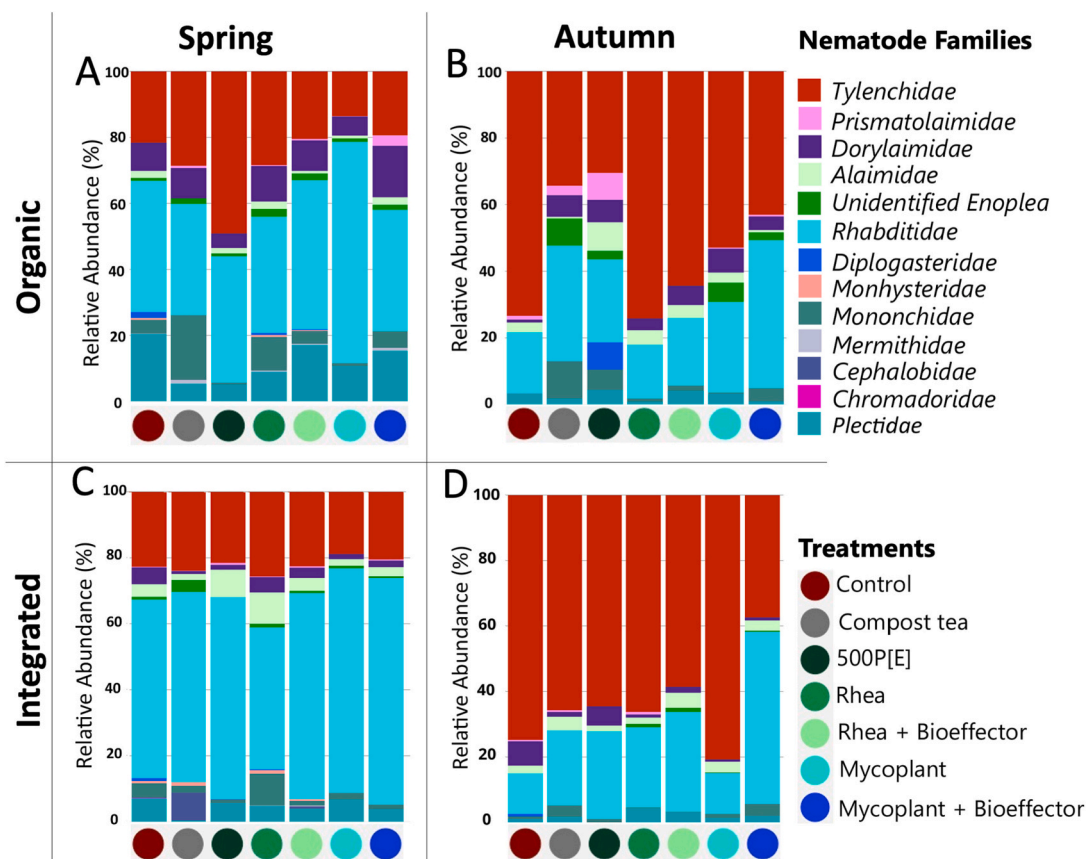


Fig. 2. Stacked bar-plot representation of the nematode composition at family level, for the different treatments in the two orchards, during spring and autumn, respectively. Figures (A) and (B) represent the family level nematode composition in organic orchard during spring and autumn respectively, while (C) and (D) show the same for the integrated orchard. Each bar represents the percentage average composition of four biological replicates ($n = 4$). The coloured circles represent treatments, while squares show nematode families.

proportion of nematode families and trophic guilds were observed between treatments in the two orchards, during spring and autumn, respectively (Table S3).

The different nematode families were assigned to trophic guilds (i.e. omnivores, carnivores, bacterivores, fungivores, and herbivores), and their relative abundances represented by stacked bar plots (Fig. 3). Feeding guild abundance varied with sampling times (i.e. spring and autumn, respectively) in both organic and integrated orchards: bacterivores (organic: spring = 58 %, autumn = 34 %; and integrated: spring = 68 %, autumn = 36 %); herbivores (organic: 28 %, 59 %; and integrated: 25 %, 59 %); omnivores (organic: 9 %, 5 %; and integrated: 3 %, 3 %), carnivores (organic: 6 %, 3 %; and integrated: 4 %, 1 %) (Fig. 3). Bacterivorous nematodes were predominant in both orchards during spring. Herbivorous nematodes made up a large percentage during autumn and were particularly high in the integrated orchard. The carnivorous and omnivorous nematodes showed a higher percentage in the organic than the integrated orchard, both during spring and autumn. Moreover, significant differences ($P < 0.001$) attributed to sampling time were observed for all the trophic guilds. However, there were no significant differences in trophic guilds which could be attributed to treatment or orchard (Table S3). Additionally, all nematode feeding guilds, with the exception of fungivores could be assigned based on the current data.

Since nematodes exhibit distinct lifestyles (i.e., parasitic, or free-living), we excluded plant parasitic nematodes from the data and compared the proportion of free-living nematode trophic guilds in the two orchards at different sampling times (Fig. S5). Bacterivorous nematodes were most abundant in both orchards and at two sampling times,

followed by omnivorous and carnivorous nematodes, respectively. Meanwhile, the herbivorous nematodes were exclusively constituted by epidermal (root hair feeders). Our data revealed no other subcategory of herbivorous nematodes like semi-endoparasites, migratory-, and sedentary endoparasites.

3.4. Seasonal variations in nematode trophic group composition were consistent across orchards, and driven by few dominant nematode families

By comparing baseline and control samples in the two orchards (sampled during spring and autumn) we observed that the change in composition of the different trophic guilds, over the three sampling times corresponded to their dominant representative families (Fig. 4). For instance, bacterivorous nematodes and their representative dominant family *Rhabditidae* peaked during spring and had a lower proportion in autumn. Moreover, during spring, the composition of bacterivorous nematodes (*Rhabditidae*) was higher in the integrated-, in comparison to the organic orchard (Fig. 4 a and f). In contrast, herbivorous nematodes, and their associated family *Tylenchidae*, were lower during spring and increased towards autumn (Fig. 4 b and e). The percentage of carnivorous nematodes, and their dominant representative family (*Mononchidae*) were high during baseline, and decreased continuously towards spring and autumn, respectively (Fig. 4 c and g). Meanwhile, the omnivorous nematodes, which include the family *Dorylaimidae* were higher in the organic orchard, as compared to the integrated orchard, both for baseline and spring samples; however, their composition decreased below those of the integrated orchard during autumn (Fig. 4 d and h).

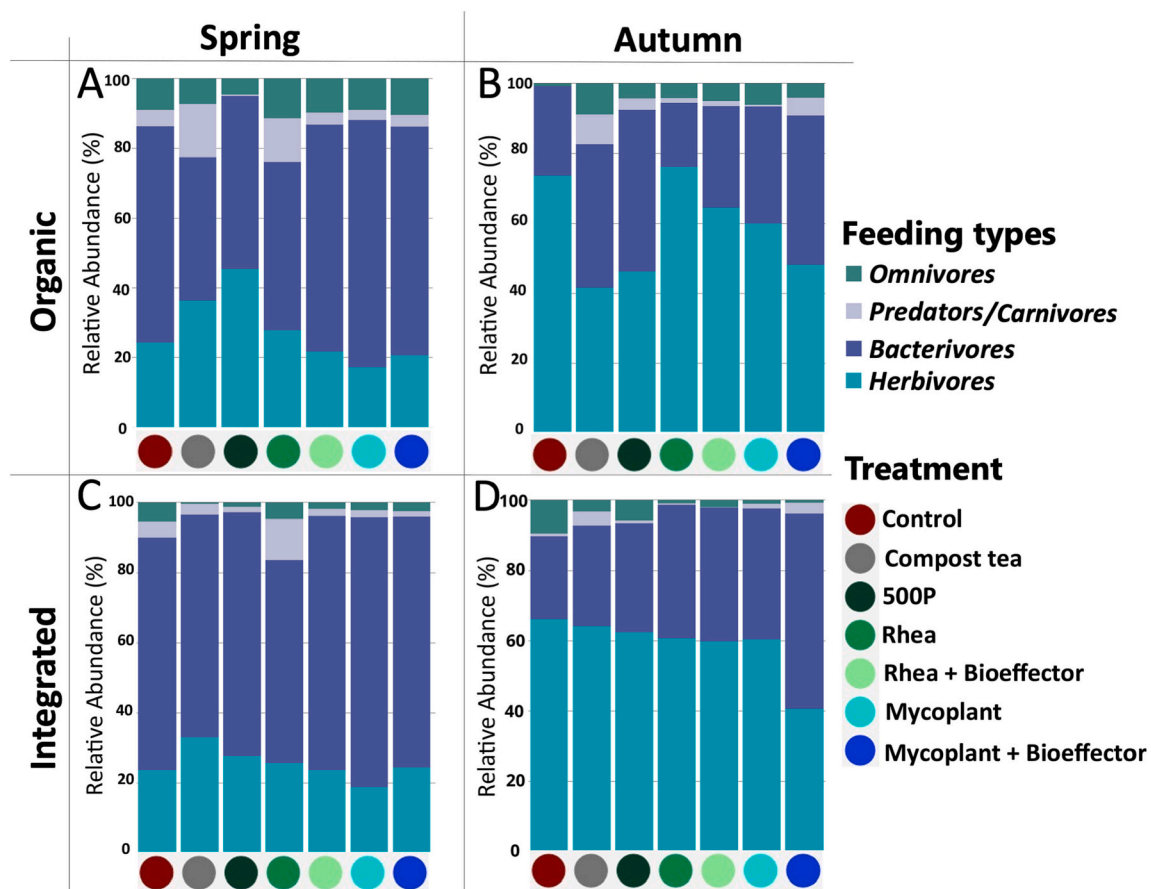


Fig. 3. Nematode community composition based on trophic guilds for the different treatments under the two orchards during spring and autumn. Figures (A) and (B) show the trophic guilds composition during spring and autumn for the organic orchard, while (C) and (D) shows the same during for the integrated orchard. Each bar represents the average percentage abundance ($n = 4$), and the stacked bars indicate different nematode feeding guilds: omnivores, carnivores, bacterivores, and herbivores.

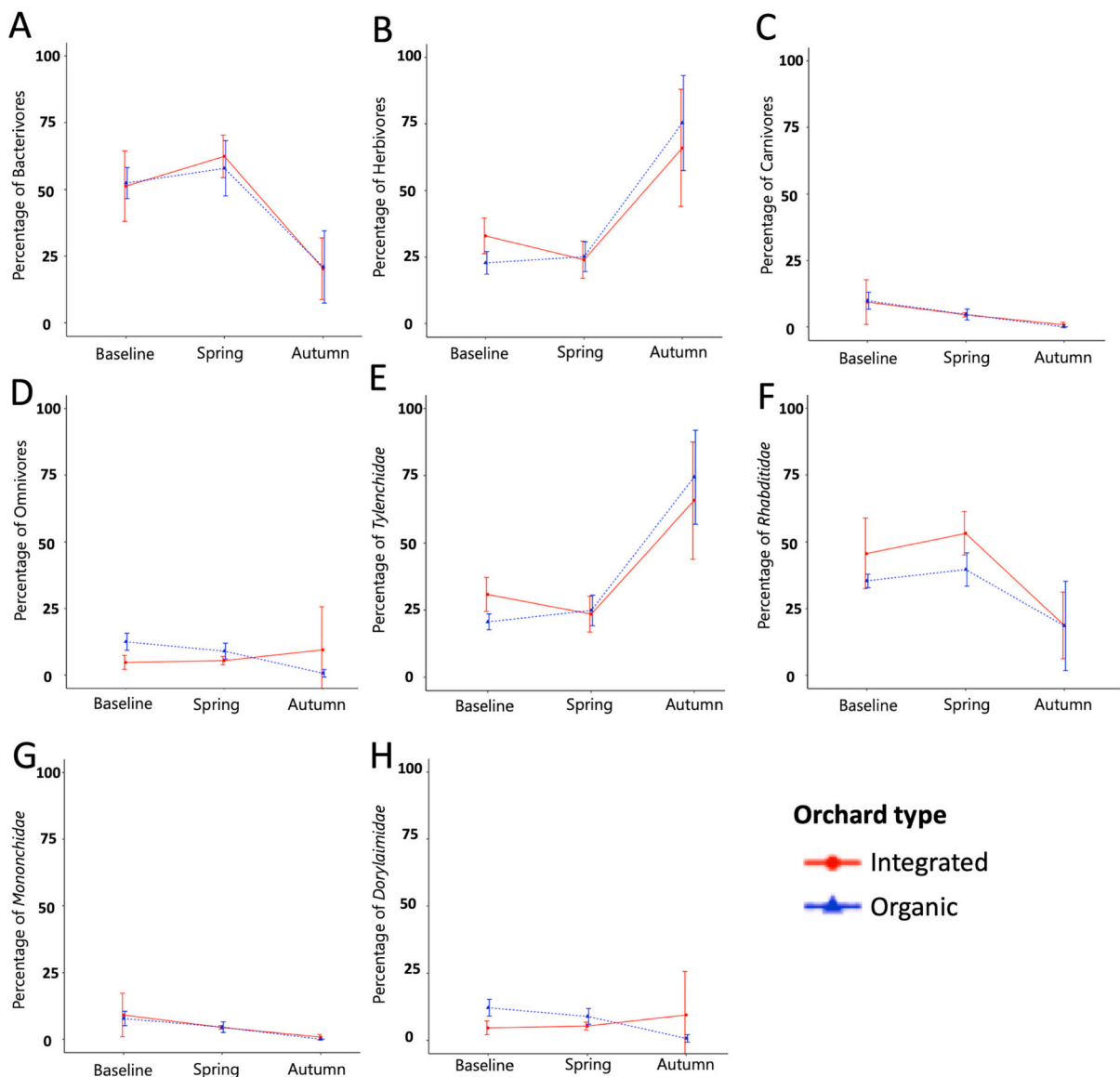


Fig. 4. Line graphs showing the average percentage proportion of nematode trophic guilds (a, b, c, and d), and family (e, f, g, and h) dynamics between baseline, and control samples during spring and autumn, respectively. The line graphs are based on $n = 4$ samples at each sampling time, both for organic, and integrated fields sampled. The different nematode families are categorized into the trophic guilds: *Tylenchidae* (herbivores), *Rhabditidae* (bacterivore), as well as *Dorylaimidae* and *Mononchidae* (omnivores and carnivores).

4. Discussion

In this study, we implemented a metabarcoding approach to investigate the potential effect of bioinoculants and organic soil amendments on nematode communities in different apple orchards. We focused on nematode composition as an indicator to assess the orchard soil conditions and the soil food web structure after soil treatment. We observed that orchard type, sampling time, and treatment significantly influenced the nematode community composition and diversity. In comparison to control plots, the samples from the biodynamic 500P treatment in the organic orchard showed a reduction in nematode richness and phylogenetic diversity during spring. Generally higher alpha diversity indices during spring as compared to autumn were observed. In both orchards, a season-dependent effect of treatment on the composition of the different nematode families and trophic guilds was observed. There were no effects on trophic group composition which were attributed to orchard or treatment application (Fig. 5).

Soil nematode communities are influenced by environmental and

agricultural management regimes (Sánchez-Moreno et al., 2006; Bongiorno et al., 2019; Talavera et al., 2019; Ferreira et al., 2020; Herren et al., 2020). Here, the impact of bioinoculants and organic soil amendments, orchard site, and season on the nematode community composition and diversity was deciphered in detail. The proportions of a several nematode families and their assigned trophic guilds varied between orchards and the time of year. The seasonal effect on nematode diversity, and density has been recorded previously, especially in agroecosystems, with emphasis on plant parasitic nematodes (McSorley and Phillips, 1993; Verschoor et al., 2001). During spring there is high precipitation, coupled with generally warm weather following the long winter period. This potentially facilitates the faster decomposition of organic matter, which potentially resulted in an increase in the proportion of bacterivorous nematodes, both in the organic and integrated orchards. However, during autumn, the weather is majorly associated with mild precipitation after the hot summer. This likely favoured the observed high presence of root feeding ectoparasitic nematodes, when compared to spring season.

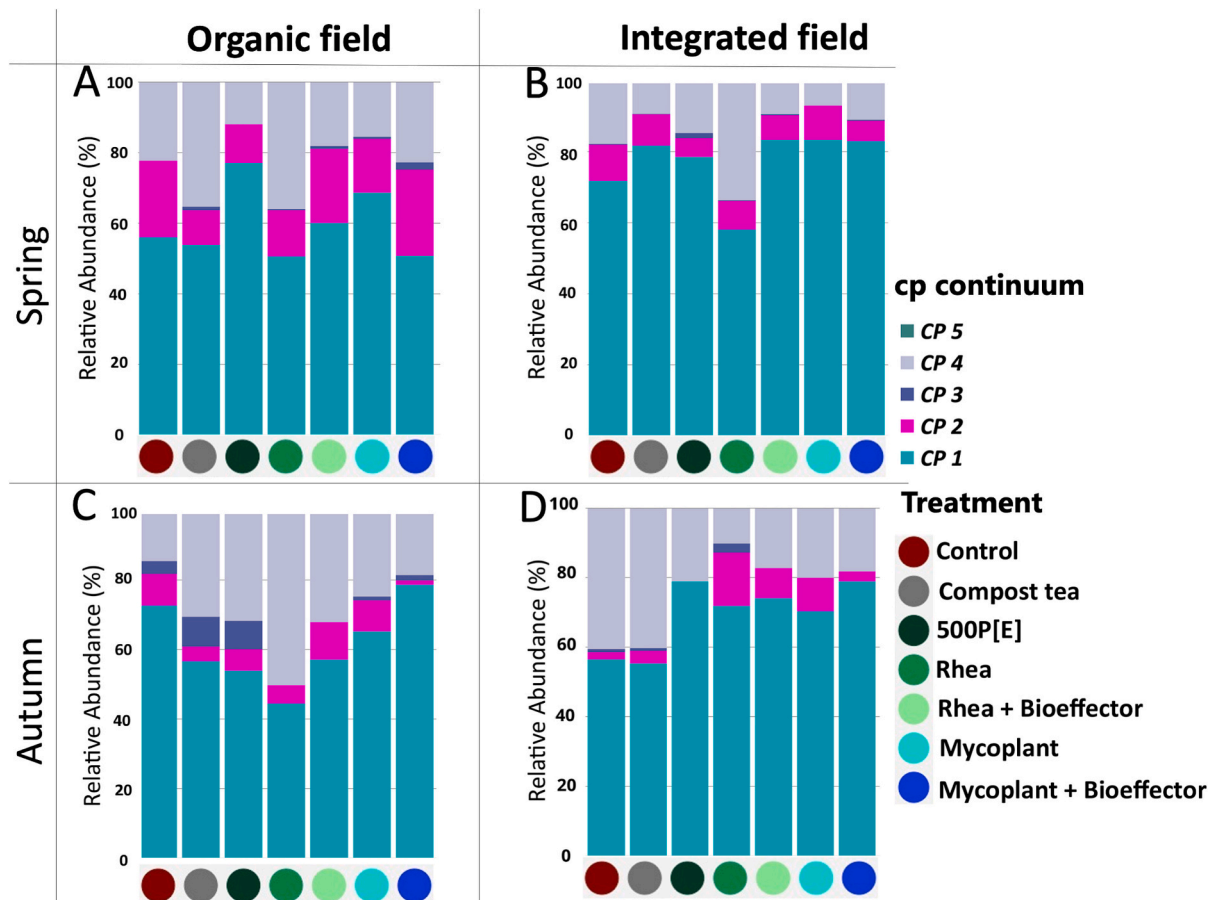


Fig. 5. The proportion of free-living nematode categorized along cp continuum as proposed (Bongers, 1990; Bongers and Bongers, 1998) for the different Orchard systems and treatment, across sampling time (spring: a and b; and autumn: c and d). Each bar represents the average percentage abundance of four replicate samples. Nematodes are classified along the cp continuum of range 1 to 5.

The reduction in diversity is likely attributed to increase of the dominant members of the nematode community such as families *Rhabditidae* and *Tylenchidae*. The prevalence of the dominant families is likely enhanced following nutrient enrichment after treatment application. The influence of treatment on the nematode community composition was shown in both orchards, during spring. The influence of treatments as shown in this study, presents the potential effect of microbiota-based and organic amendments when used for ecological engineering (Bender et al., 2016). The potential effects on the soil microbiome and ecosystem functions of bioinoculants and organic soil amendments (also referred to as “microbial transplants”), including the biodynamic extracts have been previously shown (Van Der Heijden et al., 1998; Christian et al., 2019; Olimi et al., 2022). The bioextracts obtained from biodynamic manures were recently shown to increase the fungal, while lowering bacterial Shannon diversity (Olimi et al., 2022). Similar patterns were observed in the present study where these treatments showed reduced nematode diversity. However, contrasting observations have been shown by Hartmann et al. (2015), where biodynamic extracts and organic fertilizers increased soil biodiversity.

Different soil management practices affect the nematode composition (Bongers, 1990; Yeates and Bongers, 1999; Herren et al., 2020), and how these practices impact the soil conditions can be predicted by the soil nematode composition (Neher et al., 2022). For example, in agroecosystems under different fertilizer regimes, a profound effect on soil functional diversity of microflora (bacteria and fungi) and nematode populations, and consequently their composition has been reported (Sarathchandra et al., 2001). This is reflected in our study for the nematode community in two differently managed orchards in different

locations. Thus, we provide initial insights into the effect of bioinoculants and bioextract from organic compost and biodynamic manures on the nematode community composition of apple orchards. The application of arbuscular mycorrhiza fungi showed a reduction in population of plant parasitic nematodes (*Pratylenchus coffeae* and *Radopholus similis*) in bananas (Elsen et al., 2008; Omolara Olaniyi, 2014; Schouteden et al., 2015), and the current study showed a potential influence of these bioinoculants and organic amendments on the composition and diversity of free-living nematodes. While free-living nematodes have been associated with healthy soils, the contrary has been shown for apple replant disease (ARD), where the soil microbiome and free-living nematodes have been cited to play a critical role in ARD manifestation (Kanfra et al., 2018). The contributions of bioinoculants (Li et al., 2022) and organic amendments (Reganold, 1995; Scheuerell and Mahaffee, 2002; Köberl et al., 2011; Ozores-Hampton, 2021) on plant health, especially through enhancing stress resilience and nutrient mobilization, has recently been highlighted. Moreover, management practices and soil types are known to influence nematode community composition in tree orchards such as apples and grapes (Forge et al., 2015; Pokharel et al., 2015; Van Geel et al., 2015).

There were seasonal variations in nematode trophic guilds across orchards that appeared to be driven by a few dominant nematode families. For instance, relative abundance of bacterivorous nematodes and their representative family (*Rhabditidae*) were higher during spring as compared to autumn, while herbivorous nematodes and family *Tylenchidae* were most abundant during autumn, for both orchards. On the other hand, carnivorous and omnivorous nematodes like *Dorylaimidae* and *Mononchidae* were mostly observed in the organic and

only rarely in the integrated orchard. The presence of *Dorylaimidae* and *Mononchidae* in soils has been associated with maturing and healthy soils, as well as high degree of trophic linkages (Ferris et al., 2001). Despite changes in family composition, the community functioning as represented by trophic guilds was shaped by seasonal patterns. Numerous roles of soil nematodes such as an increase in plant growth, nitrogen uptake, altered bacterial populations, among other benefits have been suggested (Ingham et al., 1985). For instance, the influence on bacterial community through the top-down effects of bacterivorous nematodes, with consequences on the rate of decomposition of organic matter has been shown (De Meester et al., 2016; Martins et al., 2022).

Regarding plant parasitic nematodes (i.e. herbivores), we observed their high presence in autumn as compared to spring. This could be related to an increase in plant primary productivity during the later time of the year. The plant productivity in the form of root biomass and root exudation is essential for nematode survival in the soil environment (Cook et al., 1995; Hartmann et al., 2008; Jones et al., 2009; Dennis et al., 2010), as it provides rich carbon sources to nematodes (Bais et al., 2006). Interestingly, our study showed the predominance of the epidermal root hair feeding nematodes during autumn. The root hair feeding nematodes are a special feeding group, composed of nematode families and genera, which are dominant in soil, some exhibiting special attributes for surviving unfavourable conditions (Bongers, 1990).

Apart from extensive use in microbiome studies, the metabarcoding approach has been used to study the nematode faunal community composition, diversity, and distribution patterns (Porazinska et al., 2009; Waeyenberge et al., 2019). The current study revealed thirteen eukaryotic phyla, but with the dominance of Nematoda among other phyla like Arthropoda, Platyhelminthes, and Annelida. Treonis et al. (2010) showed a similar composition of the eukaryotic phyla despite using different primers. However, in this study, the extraction of soil DNA from 500 mg soil samples might have affected the observable nematode diversity, attributed mainly to spatial differences in nematode distribution; thus, some of the present nematode taxa could not be captured. Our data contained many singleton and doubleton ASVs, and after their removal followed by normalization (rarefaction: 200 reads per sample) resulted in 20 to 40 ASVs per sample. These values are comparable to those observed in a study by Kawanobe et al. (2021), despite using higher minimum sampling depth (i.e., 20,000 reads per sample, compared to 200 in the present study). Nonetheless, there is need for standardization of soil-DNA based nematode metabarcoding including the DNA extraction from nematode suspensions as previously reported (Geisen et al., 2018; Griffiths et al., 2018; Treonis et al., 2018; Herren et al., 2020), and corresponding these with morphology-based identification. Recently, Kageyama and Toju (2022) attempted to define the suitable soil weight (volume) necessary for soil faunal studies. Moreover, Sapkota and Nicolaisen (2015, 2018) employed a special sample homogenization by grinding and the extraction of a soil sub-sample in the conventional soil extraction kits. Therefore, the standardization of workflows including sample size, as well as kits for soil fauna studies is still work in progress.

5. Conclusion

Nematode functional guilds have been used for close to four decades to study soil conditions in various ecosystems including agroecosystems. In the present study, the composition of different nematode families and trophic guilds varied between seasons, and the composition of different trophic guilds was consistently driven by a few dominant nematode families. The dynamics in soil nematode functional guilds can be anticipated through the identification of dominant nematode taxa at family or genus level (Yeates et al., 1993). Through the lens of nematodes, and by using DNA metabarcoding approaches, we can measure the potential ecological state of our agroecosystems. Our study provides insights about the effect of bioinoculants and specific bioextracts from compost and biodynamic manures on the nematode composition, and

their implication on soil ecosystem functions.

Funding

This work was supported by EXCALIBUR research project with funding from the European Union's Horizon 2020 Research and Innovation Program under grant agreement No. 817946.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

The dataset supporting the conclusions of this article is available in the European Nucleotide Archive (ENA) (<http://www.ebi.ac.uk/ena>) under the project number PRJEB5559.

Acknowledgements

We are grateful to Robert Matzer (Gleisdorf), and Josef Singer (Gleisdorf) for offering their apple orchards for treatment application, perform sampling, as well as for maintaining the experimental plots. We also express sincere gratitude to Ron de Goede from the Soil Biology Group at Wageningen University for the critical revision of this manuscript.

Abbreviations

AMF	Arbuscular Mycorrhiza Fungi
ASV	Amplicon Sequence Variant
DNA	Deoxyribonucleic acid
18S rDNA	18 Subunit of ribosomal Ribonucleic Acid

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2023.105004>.

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