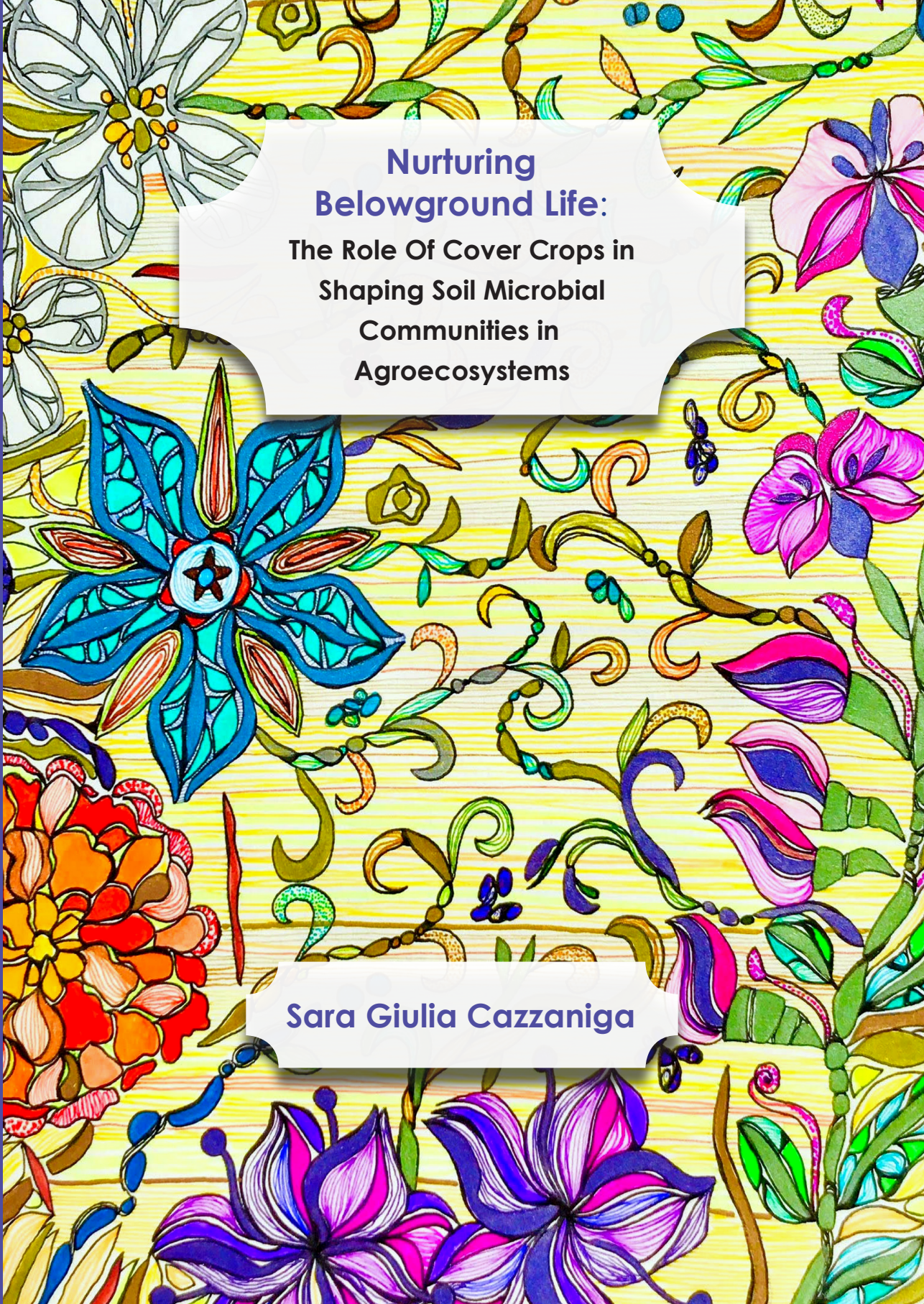

Nurturing Belowground Life:

The Role Of Cover Crops in Shaping Soil Microbial Communities in Agroecosystems

Sara Giulia Cazzaniga

Propositions

1. Cover crop choice matters as both species and cultivars have specific effects on the native soil microbiome.
   (this thesis)
2. Abundance and activity of nematophagous microorganisms can be steered with cover crops.
   (this thesis)
3. Experimental sustainability studies should follow environmental sustainability principles.
4. The emphasis on positive results in scientific publishing hinders scientific integrity and progress.
5. Providing legal rights to the soil as *'res communis'* is crucial for combating climate change.
6. To allow for work-life balance, it is necessary to normalize working in non-working hours.

Propositions belonging to the thesis, entitled
Nurturing Belowground Life: The Role Of Cover Crops in Shaping Soil Microbial Communities in Agroecosystems.

Sara Giulia Cazzaniga
Wageningen, 06 December 2023

Nurturing Belowground Life: The Role Of Cover Crops in Shaping Soil Microbial Communities in Agroecosystems

Sara Giulia Cazzaniga

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Nurturing Belowground Life: The Role Of Cover Crops in Shaping Soil Microbial Communities in Agroecosystems

Sara Giulia Cazzaniga

Thesis

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Table of Contents

Chapter 1

General Introduction

Sara Giulia Cazzaniga

1.1 Soil: The Foundation of Life

Soil is a critical component of terrestrial ecosystems, playing a vital role in several societal, economic and environmental functions (Blum, 2005; De Deyn and Kooistra, 2021). Beyond its pivotal role in food, feed and fibre production, soil also fulfils important ecosystem services such as filtering and storing water, storing and recycling nutrients, carbon sequestration and habitat provision and reservoir for biodiversity (Schulte et al., 2014). The performance of such soil functions is crucial for proper agricultural production systems and to ensure their resilience. Nevertheless, the capacity of soils to execute such functions is at risk due to degradation processes, including but not limited to, biodiversity loss, erosion, compaction, organic matter decline, and contamination (Foley et al., 2011; Nations et al., 2020). Agricultural intensification has been favouring short-term increases in food production to the detriment of soil functions, including many that are important for long-term food security (Foley, 2005; Spanner, 2015). As a result, the EU Soil Observatory has calculated that 61% of EU land is already affected by soil degradation (https://esdac.jrc.ec.europa.eu/esdacviewer/euso-dashboard/) (Foley et al., 2011; Nations et al., 2020). Looking forward, it is imperative to couple the need for agricultural production with the need of preserving, supporting and optimising soil functions and, intrinsically, soil health (UN, 2015; Bünemann et al., 2018; Strauss et al., 2023).

1.2 Healthy soils are multifunctional living systems

The term soil health is widely used to describe the general condition or quality of soils and is usually aligned with sustainability goals (Lehmann et al., 2020). The concept of soil health touches upon planetary health (Whitmee et al., 2015) as it was defined as the 'the capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health (Doran and Zeiss, 2000). Following this concept, soil health is currently being assessed based on a set of biological, chemical and physical attributes (Doran, 2002; Bünemann et al., 2018). The most traditionally used biological indicators of soil health include microbial activity, diversity, and biomass, and earthworm community, while physicochemical indicators include soil organic matter content, electrical conductivity, pH, bioavailable and mobile nutrients (N, P, K, Mg), heavy-metals, penetration resistance and water holding capacity, among others (Lehman et al., 2015; Rinot et al., 2019; Lehmann et al., 2020). Evidently, measured soil biological properties are scarcer as compared to physical-chemical ones. Although highly correlated with soil health functions, such as nutrient cycling, soil aggregate formation, moisture retention and erosion control (Lehmann et al., 2017; Creamer et al., 2022), the scarcity of biological indicators results in an overly simplistic representation of the impact of soil biology on soil health (Banerjee and Van Der Heijden, 2023). One reason for the lack of biological indicators is the difficulty in finding easy-to-measure and comprehensive indices able to capture the complexity of soil biology (Bünemann et al., 2018).

1.3 Soil communities, composition and interactions

Soil harbours a complex living system, and is home to a vast array of microbial life, including bacteria, archaea, fungi, protists, nematodes, earthworms, and other small invertebrates. These organisms feed on plant material, organic debris and or on each other, thus engaging in a complex network, called the soil food web (Moore and De Ruiter, 2012; De Vries et al., 2013). Within the food web, bacteria and fungi are dominant in terms of biomass, and comprise an overwhelming biodiversity and, as such, they constitute the base of the soil food web. They are referred to as primary decomposers: bacteria feed on relatively easily degradable fractions of organic matter, while fungi can decompose recalcitrant plant polymers as their main source of carbon and energy (Boer et al., 2005; Suleiman et al., 2019; Frąc et al., 2022). Protists are unicellular eukaryotic microorganisms that play a vital role in shaping food web structures in various ecosystems, including soil. They are an essential part of the microbial community and participate in numerous ecological interactions, including predation on bacteria and fungi (Gao et al., 2019). Nematodes, microscopic roundworms, are the main representative of metazoans in soil. They have high trophic diversity in the soil as herbivores (feeding on plants), bacterivores (feeding on bacteria), fungivores (feeding on fungi), omnivores and predators (feeding on other organisms, including other nematodes) (Puissant et al., 2021). This diverse feeding behaviour allows nematodes to influence the abundance and activities of other soil organisms, including bacteria and fungi (Mielke et al., 2022). Together, protists and nematodes contribute directly and indirectly to nutrient recycling, promotion of plant growth, and the transfer of energy to higher trophic levels in the soil food web (Gao et al., 2019; Wilschut and Geisen, 2021). Higher trophic levels include small invertebrates, such as mites, springtails, potworms and earthworms (Blouin et al., 2013) which are involved in several kinds of ecosystem engineering (Lavelle et al., 2006).

Collectively, soil (micro)organisms contribute directly and indirectly to several soil-related functions, among which nutrient cycling, decomposition of organic matter, breakdown of harmful inorganic compounds, formation of soil structure and moisture retention (Lavelle et al., 2006; Kibblewhite et al., 2008; Kowalchuk et al., 2008; Blouin et al., 2013; Bardgett and Van Der Putten, 2014; Hirsch and Mauchline, 2015; Lehman et al., 2015; Bender et al., 2016; Fierer, 2017; Gao et al., 2019; Wilschut and Geisen, 2021). Relevant to agroecosystems, soil (micro)organisms can act as pathogens and parasites of plants (Jones et al., 2013), but can also promote plant health and productivity by competition and/or inhibition of pathogenic and parasitic organisms (Latz et al., 2012; Gomez Exposito et al., 2015; Grubišić et al., 2018). For these reasons, this thesis focuses on studying microorganisms inhabiting arable soils and involved in soil health related functions.

1.4 The soil microbiome as an indicator of soil health

The composition of soil microbial communities and relationships among the different trophic groups are highly variable in time and space and reflect the physicochemical properties and processes of soils (Pereira E Silva et al., 2012; Dubey et al., 2019; Custódio et al., 2022). These characteristics make soil microbial communities potentially good indicators for soil health (Lehman et al., 2015) and could complement the very limited set of biological indicators currently used to define soil health. The complexity and diversity of microbial communities and small invertebrate fauna in the soil make their assessment and measurement challenging. Traditional, culture-dependent, methods are laborious, time-consuming, and limited in capturing the full extent of diversity.

However, high-throughput DNA sequencing technologies, such as Illumina MiSeq and NovaSeq and Oxford Nanopore sequencing, provide powerful tools for studying the composition and diversity of microbial communities (De Corato, 2020). These technologies enable a culture-independent approach, analysing the genetic material of organisms present in the soil, the microbiome. The increasing affordability and reliability of these sequencing technologies make the microbiome a potentially powerful indicator of soil health (Wilhelm et al., 2022). By targeting different nucleic acids, sequencing technologies also allow to get an insight into the different fractions of the microbiome. In fact, DNA analyses provide a complete overview of the resident, or total, microbiome, which includes dead, dormant and active microbes (Blagodatskaya and Kuzyakov, 2013). On the other hand, RNA analyses portray the (potentially) active fraction of the microbiome (Blazewicz et al., 2013). In this thesis DNA and RNA analyses are carried out in parallel as when combined they can provide insight into the full microbiome potential to participate in processes which could result in enhanced soil health (Ofek et al., 2014; Harkes et al., 2019; Vieira et al., 2019).

1.5 Influence of agricultural management on the soil microbiome

Microbial communities are highly sensitive to agricultural management practices (Bastida et al., 2008; Francioli et al., 2016). For this reason, one of the principles of soil health management is to enhance soil biology through sustainable land management (Lehmann et al., 2020). Ideally, sustainable management embraces those agricultural practices that enhance the physical and chemical soil properties while preserving and stimulating soil microbial communities.

Among others, organic land management, which relies on the use of organic fertilisers and natural processes to manage soil fertility, rather than synthetic fertilisers (Mäder et al., 2002), has been associated with several beneficial effects on the soil microbiome. These include increased microbial biomass and catabolic activity (Martínez-García et al., 2018), increased microbial taxonomic and phylogenetic richness and diversity (Lupatini et al., 2017), increased

diversity of plant-beneficial microorganisms (Verbruggen et al., 2010), and increased microbial activity (Harkes et al., 2019).

Conservation tillage, which uses minimum or no- tillage, is a practice introduced in regenerative agriculture to minimise soil disturbance, allowing for more efficient nutrient cycling, fungal proliferation and carbon sequestration (Khangura et al., 2023). Conservation tillage practices have been linked to increased stability, diversity and enzymatic activity of soil bacterial, fungal and nematode communities as compared to conventional tilling (Zhang et al., 2014; Bongiorno et al., 2019).

The incorporation of organic amendments (compost, plant residues, chitin, biochar, etc.) can improve soil fertility and structure by increasing soil nutrient status and organic matter content (Mäder et al., 2002). In some agroecological settings, organic amendments were shown to have positive implications on soil-borne pathogen control (Cretoiu et al., 2013; Bonanomi et al., 2018), to affect microorganisms associated with nutrient cycles, and to increase the complexity of microbial–microfaunal ecological associations (Suleiman et al., 2019).

Spatial and temporal crop diversification (*i.e.*, intercropping, crop rotation, and cover cropping), provides multiple ecosystem services. Increased plant diversity, as opposed to continuous monoculture, has positive effects on the enrichment and diversification of soil C, P and N content, which in turn increase microbial biomass, diversity and activity (McDaniel et al., 2014; Kim et al., 2020; Wang et al., 2022). Among crop diversification strategies, the use of cover crops to reduce seasonal fallow has a substantial influence on soil biology and can be a valuable tool to enhance soil health (Vukicevich et al., 2016; Kim et al., 2020).

1.6 Cover crops have the potential to improve soil health

Cover cropping is the practice of growing fast-growing, noncash plants, usually in the off-season, leaving their biomass on the field to provide various benefits for the agroecosystem, including erosion reduction, soil organic matter build-up, weed and pathogen control, and nutrient management (Blanco-Canqui et al., 2015; Elhakeem et al., 2023). Cover crops have been used in agriculture since ancient times. Their use dates back to early civilizations in China, Greece, and Rome, where leguminous plants were grown to improve soil fertility and crop yields (Ingels and Klonsky, 1998). Today, cover cropping has been adopted globally by farmers as a sustainable and environmentally friendly agricultural practice to complement a variety of agricultural systems, including conventional and organic agriculture, conservation tillage and no-tillage systems. The effect of cover crops on soil health indicators has been explored and generally yielded positive scores for physicochemical soil properties (Wick et al., 2017; Rivière et al., 2022), although some variation exists between studies (Hao et al., 2022). Cover crops generally alter the physical soil structure by improving soil aggregation, microporosity, water content, and water quality

and can improve the soil chemical composition, by increasing organic matter, total organic carbon, microbial biomass carbon and total nitrogen (Blanco-Canqui et al., 2015; Kaye and Quemada, 2017; Wick et al., 2017; Hao et al., 2022). The ecosystem functions cover crops bring about are partly dependent on the cover crop species and the plant family they belong to. Currently, the most used cover crops belong to the plant families Poaceae, Brassicaceae and Fabaceae (Vukicevich et al., 2016; Griffiths et al., 2022), and a trend towards further diversification has been observed including, but not limited to, members of the families Boraginaceae and Asteraceae (e.g. (Elhakeem et al., 2021). Cover crops of the same plant family tend to have similar ecosystem functions, e.g., grasses typically decrease soil density, brassicas increase microporosity, and legumes promote aggregate stability (Tribouillois et al., 2015; Hudek et al., 2022). Furthermore, cover crops are characterised by species-specific traits such as root-to-shoot ratio, root length density, root diameter and canopy cover (Tribouillois et al., 2015; Hansen et al., 2021; Griffiths et al., 2022; Hudek et al., 2022). Only lately, increasing attention has been given to the potential of cover crops to manipulate the soil microbiome to enhance soil health (Finney et al., 2017), but this field is largely unexplored. This thesis addresses the existing knowledge gap by examining cover crop effects on the key components of the soil microbiome and contributes to grow the knowledge base on the use of cover crops to enhance soil health through the microbiome.

1.7 Cover crops' effects on the soil microbiome

By providing diversification of carbon and nitrogen sources through root exudation and plant residue decomposition, cover crops can have a tremendous effect on the soil microbial communities (Kim et al., 2020). During their growth, cover crops are expected to exert a selective effect on soil bacteria and fungi at the root level (rhizosphere). Through the exudation of organic compounds in the rhizosphere, in the process called rhizodeposition, plants select and promote a part of the indigenous soil microbial community in a plant species-specific manner (Hartmann et al., 2009; Mendes et al., 2013; Philippot et al., 2013). The increased abundance and activity of rhizosphere microbes attract and stimulate the community of their consumers, including protists and nematodes (Gao et al., 2019; Xiong et al., 2020; Guo et al., 2022). By grazing on bacteria and fungi these secondary consumers release nutrients that become available for plants and other microorganisms. Cover crops can continue to influence the soil microbiome when they are terminated and plant residues are incorporated into the topsoil (Murrell et al., 2020; Ray et al., 2022). The quantity and elemental composition of cover crop residues determine the bulk soil microbiome composition and influence the microbiome assembly the cash crop is exposed to at the start of the growing season (Barel et al., 2018; Nevins et al., 2018; Liu et al., 2021). This implies that steering the soil microbiomes by growing cover crops and incorporating plant residue is possible, and could influence the growth and productivity of the following main crop (Larkin et al., 2010). However, cover crop-species-specific effects are largely unknown, thus need to be first assessed and then optimised for improving soil health. This thesis contributes to this goal by examining the species-specific impacts of a range of cover crops on key components of the soil microbial communities, including bacteria, fungi, protists, and nematodes,

using DNA and RNA high-throughput sequencing techniques. In **Chapter 2** the effect of living cover crops on the rhizosphere microbiome is studied, while **Chapter 3** investigates the persistence of cover crops' effects on the soil microbiome in the bulk soil beyond cover crop termination.

1.8 Cover crops can impact pathogens and pathogens' antagonists

Among the main concerns of farmers with regard to the implementation of cover cropping are the possibility that cover crops could inadvertently become weeds in the following cropping season and provide a "green bridge" for pathogens during the winter season. This can result in the preservation of, among others, populations of plant-parasitic nematodes between the time of harvest and planting of cash crops, contributing to higher initial pathogen loads for the cash crop (Timper et al., 2006; Timper, 2014; Visser and Molendijk, 2015). Plant-parasitic nematodes are among the most harmful and notoriously hard to control pests of cultivated crops causing important economic losses (Elling, 2013; Jones et al., 2013). The realistic risk of propagating plant-parasitic nematode can be mitigated by a careful selection of cover crop species that limit or even reduce specific nematode species (Briar et al., 2016; Grubišić et al., 2018; Abd-Elgawad, 2021). Using non-host cover crops in rotation with main crops can help reduce the population of plant-parasitic nematodes in the soil by reducing the number of host plants available for the nematodes to feed on (Azlay et al., 2023). Furthermore, several cover crops are known to cause a decline in the community of PPN, partly (Vervoort et al., 2014) due to the release of secondary metabolites and their toxic derivatives upon nematode attack (Ploeg, 2008; Eugui et al., 2022). Nematicidal properties have been found in brassicas, marigolds and grasses through the production and activation of glucosinolates (Ploeg, 2008; Eugui et al., 2022), thiophenes (Hooks et al., 2010) and cyanides (Dutta et al., 2019), respectively (all upon cellular piercing by the nematode and consequent decompartmentalization). Cover crops could also (indirectly) stimulate or recruit entomopathogenic nematodes (Rasmann et al., 2005), which by predating on PPN could contribute to control populations of plant-parasitic nematode (Caccia et al., 2013; Li et al., 2023).

Conceivably, cover crops may also contribute to nematode control through the stimulation of the endogenous community of nematode antagonists. Carbon and nitrogen sources exuded in the rhizosphere or released during plant residue decomposition can stimulate the microbial community that collectively produces a general antagonistic effect towards the pathogen (Mazzola, 2002). Alternatively, the pool of secondary metabolites exuded and released by plants may also enhance populations of specific microorganisms with antagonistic activity toward the nematodes (Pascale et al., 2020; Liu et al., 2021). Of particular interest are the groups of nematophagous fungi and bacteria (Abd-Elgawad, 2020). These fungi evolved complex and diverse structures that allow them to predate or parasitise nematodes and could contribute to the control of plant-parasitic nematodes in soil (Elhady et al., 2018). However, little is known about the effect of cover crops on the population of nematophagous microorganisms in the field and their possible application to enhance the

biocontrol of plant-parasitic nematodes. This thesis aimed to advance understanding in this direction by investigating the cover crop-specific impact of a range of cover crops on plant-parasitic nematode populations (**Chapter 4**) and their antagonists (**Chapter 5**)

1.9 Thesis aims and outline

As components of environmentally sustainable agricultural practices, cover crops have the potential to contribute to the enhancement of soil health. Soil health management involves and envisions practices which can enhance the soil's biological conditions through the manipulation of the soil microbiome. Cover crops have all the prerequisites to potentially be used to engineer the soil microbiome (French et al., 2021) to assist in soil health management. However, our knowledge of the effect of cover crops on the soil microbial communities and its implication for soil health is still limited. This thesis contributes to deciphering the effects of the interactions between cover crops and soil microbiome to evaluate the contribution of this management practice to effective and sustainable microbiome engineering for soil health. A schematic representation of the thesis outline is included as Figure 1.1.

This introductory chapter (**Chapter 1** – General Introduction) presents the use of sustainable agricultural practices as tools to improve the biological condition of soils and focuses on the use of cover crops to stimulate and shape soil microbial communities.

Although cover crops have extensively been studied for their effects on the physicochemical soil properties, information on cover crops' role in the shaping of the root microbiome is still limited. **Chapter 2** aims at providing a better understanding of the species-specific effects of cover crops on the rhizosphere microbiome. In **Chapter 2**, I characterised the impact of ten commonly used cover crop species from five different plant families on the resident and potentially active fractions of the bacterial, fungal, protistan and metazoan communities in the rhizosphere through short-read Illumina MiSeq sequencing. It was hypothesised that the presence of cover crops would induce changes in the composition, activity and network complexity of the rhizosphere microbiome as compared to the fallow control. The detailed analysis of cover crops' rhizosphere microbiomes allowed me to conclude that cover crop species differ in the extent by which they manipulate the endogenous microbiome at the level of their rhizospheres. Furthermore, several microbial taxa which were promoted or repressed in a cover crop species-specific manner were identified.

The use of cover crops to generate desirable shifts in the indigenous microbial community is only sensible if these shifts would last - at least - until the onset of the main crop growing season. Following up on **Chapter 2**, **Chapter 3** evaluates the persistence of cover crop-induced changes in the soil microbiome after cover crop termination. In particular, the permanence of a cover crop effect on the bacterial, fungal and protists resident and potentially active communities was investigated two times, before the planting and after the harvest of the main crop (potato). Results of the experiment indicated that all cover crops induce species and even cultivar-specific changes in the local soil

microbiome that last at least until the onset of the main growing season. Furthermore, several microbial taxa stimulated by cover crops are related to plant-growth promotion and disease suppressiveness, illustrating the potential to improve the main crop's health.

Related to the use of cover crops for disease management, in **Chapter 4**, the focus is given to the cover crop-specific effects on the nematode community. Employing Oxford Nanopore sequencing of long DNA amplicons (≈ 1,700 bp) with MinION™, I characterized the nematode community present in the experimental field by accurately identifying nematodes from all trophic groups at the genus and species level. In the study, the dynamics of plant-parasitic nematodes and other trophic groups were among treatments and through time. Results showed that cover crops had a direct effect on the plant-parasitic nematodes and indirect effects on other trophic groups of nematodes. Both effects were shown to be cover crop and nematode species dependent.

As a potential way to control plant-parasitic nematodes, in **Chapter 5** the effect of cover crops in stimulating the native antagonistic potential of soils is investigated. Cover crop monocultures and mixtures thereof were grown on a field under different population densities of the plant-parasitic nematode *Meloidogyne chitwoodi.* This setup allowed me to assess the impact of *M. chitwoodi* density and cover crop identity, on the resident (DNA-based) and the potentially active (RNA-based) bacterial and fungal communities of antagonists. Several microbial genera described in the literature as antagonists of plant-parasitic nematodes responded to the cover crop treatments and/or the nematode densities, including facultative nematode-trapping fungi and obligate nematode-parasitic fungi.

In **Chapter 6** (General Discussion), I synthesize and incorporate results obtained from the previous chapters to discuss the contribution of cover crops to different parameters of soil health. Considerations on the use of soil microbiome as an indicator to evaluate soil health and reflections on the technical aspects of microbiome analyses are also presented.

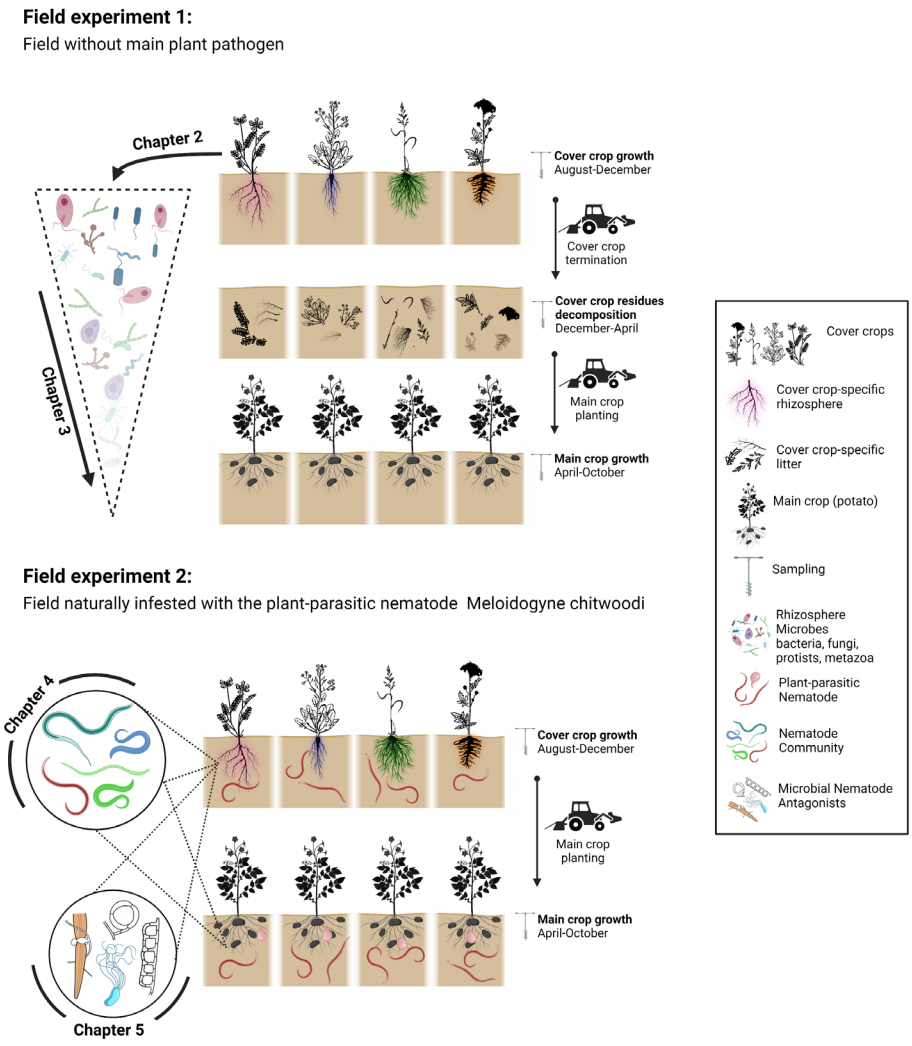

Figure 1.1 | Conceptual overview of the experimental chapters presented in this thesis. The research presented in the chapters aimed to advance understanding of cover crops' effect on the soil microbial communities by 1) identifying cover crops-species specific footprints on the rhizosphere microbial communities (**Chapter 2**) and evaluating their persistence over time in soil (**Chapter 3**), and 2) investigating the impact of distinct cover crops on plant-parasitic nematode populations (**Chapter 4**) and their antagonists (**Chapter 5**).

Chapter 2

Pinpointing the distinctive impacts of ten cover crop species on the resident and active fractions of the soil microbiome

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Abstract

Cover crops are used in agriculture to minimise soil erosion, prevent nutrient leaching and increase soil organic matter content. Cover crops can also be grown to stimulate the soil microbial community to improve soil biological conditions. Despite their widespread use, little is known about the impact of different cover crop species on the composition and activity of the soil microbiome. Here we investigate the effect of distinct cover crop species on the rhizosphere microbiome and characterise both the resident (ribosomal (r)DNA-based) and the potentially active (rRNA-based) fractions of the bacterial, fungal, protist and metazoan communities in the cover crops rhizosphere. We conducted a field experiment using 70-litre bottomless containers in which we grew ten monocultures of commonly used cover crop species belonging to five plant families, and an unplanted control treatment (fallow). The total DNA and RNA were extracted from soil and the bacterial, fungal, protistan and metazoan communities were characterized using Illumina MiSeq sequencing. We found that all cover crop species significantly impacted the resident and the potentially active microbial communities in their rhizospheres. Cover crops exerted distinct selection strengths on the native microbial communities. For individual cover crops, the impacts on the resident and the potentially active microbial communities differed while showing similar overall tendencies. Oilseed radish (Brassicaceae) was shown to provoke the strongest microbial shifts, in part attributable to a promotion of the bacterial family Pseudomonadaceae and a repression of Microascaceae in the rhizosphere. Lentil (Fabaceae) induced a widespread stimulation of fungal taxa, including Trichocomaceae and fungal members of the Glomerales order, whereas black oat and hybrid ryegrass (both Poaceae) gave rise to relatively mild changes in the soil microbial communities. Analyses of rRNA-based rhizobiome data revealed that, except for phacelia, all cover crops induced an increase in microbial network complexity as compared to the fallow control. Data presented here provide a broad baseline for the effects of cover crops on four organismal groups, which may facilitate future cover crop selection to advance soil health.

2.1 Introduction

From the 1960s onwards, agricultural intensification has led to higher and more stable crop yields with a fraction of the labour inputs previously needed (Normile and Mann, 1999). However, intensive agriculture also carries negative effects on soil health, including degradation of the physical, chemical and biological properties of soils (Banerjee et al., 2019; Tsiafouli et al., 2015). A recent report states that 60-70% of the soils within the European Union are classified as unhealthy as a result of current agricultural practices (Veerman et al., 2020). Reconsideration and adjustments of these practices are needed to reverse this undesirable phenomenon. It is noted that 'soil health' is a broad term, which has been defined as the continued capacity of soil to function as a living ecosystem that sustains plants, animals, and people (Doran and Zeiss, 2000). Here we will use this term in a narrower sense focusing on the capacity of soil biota to sustain plant growth and development.

A number of soil management practices have been shown to be effective in improving soil health while maintaining acceptable crop production levels (Eyhorn et al., 2019; Schrama et al., 2018; Vukicevich et al., 2016). Among these, cover cropping - the cultivation of fast-growing non-economic plants between the harvest of the main crop and the sowing of the next main - is implemented to minimize nutrient leaching and soil erosion, and to increase the soil organic matter content (Blanco-Canqui et al., 2015; Kaye and Quemada, 2017; Wick et al., 2017). Cover crops also may have a positive effect on the biological condition of soils as they boost and shift the activity and abundance of soil microbes (Kim et al., 2020; Vukicevich et al., 2016; Wick et al., 2017). Potential downsides of cover crops include their potential function as reservoirs for pests and pathogens as cover crops may facilitate them to bridge a non-favourable period in their life cycle (Bakker et al., 2016; Walder et al., 2017), and their action as weeds in the next cropping season (Wayman et al., 2015). However, as long as these risks are mitigated, many plant species can be considered for cover cropping.

Most of the currently used cover crops belong to the plant families Poaceae, Brassicaceae and Fabaceae (Griffiths et al., 2022; Vukicevich et al., 2016), and a trend towards further diversification has been observed including members of the families Boraginaceae and Asteraceae (e.g., Elhakeem et al., 2021). Cover crops of the same plant family tend to have similar ecosystem functions, *e.g.,* grasses typically decrease soil density, brassicas increase microporosity, and legumes promote aggregate stability (Hudek et al., 2022; Tribouillois et al., 2015), and these characteristics co-determine cover crop choice.

Plants exert a selective effect on soil bacterial and fungal communities, and the altered rhizobiome might result in improved plant nutrient uptake and increased pathogen suppression (Berendsen et al., 2012; Doornbos et al., 2012). Such a combination of increased levels of plant-absorbable nutrients as well as an improved resistance against biotic and abiotic stresses is here labelled as microbiome-mediated soil health promotion. During vegetative growth, up to 40% of the carbon fixed by plants is released into the rhizosphere through root exudates (Bais et al., 2006; Bonkowski, 2004) which directly modulates the microbial community associated with the roots (Badri and Vivanco, 2009; Berendsen et al., 2012). The steering of the local microbiome by plants is largely dictated by the composition of these exudates (Berg and Smalla, 2009; Pascale et al., 2019), which, in turn, is largely determined by plant phylogeny. Although representatives of plant families show similarities in root exudate composition, even at species and subspecies levels genotype-specific rhizodeposits have been reported (Micallef et al., 2009; Schlaeppi et al., 2014; Yeoh et al., 2017). As previously reported for several crops and model plants (Cloutier et al., 2021; Tkacz et al., 2015; Turner et al., 2013; Uksa et al., 2014), also the selection strength, *i.e.,* the extent by which plants shape their rhizosphere by promoting and/or repressing fractions of the soil microbiome, varies per plant species. To the best of our knowledge, selection strengths of individual cover crops on the soil microbiome has never been compared.

Next to bottom-up selection by plant exudates, the bacterial and fungal communities in the rhizosphere are co-shaped by the top-down selection due to the activity of major consumers of these communities, protists and metazoans (Gao et al., 2019; Mielke et al., 2022). In temperate agricultural systems, primary consumers-biomass in the top layer of arable fields is typically 40 to 100 times smaller than the bacterial and fungal biomass (Pausch et al., 2018). In the rhizosphere, the increased abundance and activity of bacteria and fungi attracts bacterivorous protists and metazoa, including bacterial- and fungal-feeding nematodes (Bonkowski, 2004). Here, trophic interactions become a driving force co-determining the microbiome assembly and activity (Gao et al., 2019). Therefore, major categories of primary consumers, protists and metazoans, should be taken along to achieve a proper understanding of the shaping of the rhizobiome.

Although rhizosphere communities have been characterized for a substantial number of plant species (Fitzpatrick et al., 2018; Yadav et al., 2018), our knowledge of the microbial signatures of cover crop species is rather crude. Previous studies, such as the ones by Bacq-Labreuil et al. (2019), Finney et al. (2017) and Gkarmiri et al. (2017) illustrate the ability of cover crop species to affect the soil microbiome assembly and activity, and in particular how different

functional groups, such as arbuscular mycorrhiza and saprophytic fungi, respond to the presence of different cover crop species. However, most studies present data at a high taxonomic level, consider one or a few cover crop species only and are seldomly focused on the rhizosphere microbiome.

A substantial part of the soil microbial community is known to be dormant (Fierer, 2017). This 'microbial seed bank' as it was referred to by Lennon and Jones (2011) may comprise up to 80% of the cells and about 50% of the taxa in bulk soils and is here referred to as the resident fraction. Taking along the active fraction of the soil microbiome is informative as this fraction is responsible for its actual ecological functioning. Ribosomal DNA (rDNA) and rRNA-based community profiling are used to characterize the resident and active microbial fractions, respectively, but regarding the latter, some caution is justified. Dormant soil biota might harbour high numbers of ribosomes, as for instance was shown for spores of several *Bacillus* species (Filion et al., 2009). Hence, it is preferred to refer to rRNA-based communities as potentially active fractions, rather than active fractions (Blazewicz et al., 2013). Recent studies underlined the relevance of including the potentially active fractions of the microbiome (Bay et al., 2021; Harkes et al., 2019; Ofek et al., 2014).

Here, we present a field experiment in which we characterized the impact of ten commonly used cover crops (including representatives from five distinct plant families) on both the resident and the potentially active fractions of the bacterial, fungal, protistan and metazoan communities in the rhizosphere. We hypothesised that the presence of cover crops would induce changes in the composition, activity and interactions of the rhizosphere microbial community as compared to the fallow control. Furthermore, we expected to see cover crop-specific, and - to a lesser extent - plant family-specific effects on the resident and potentially active microbial fractions of all four main organismal groups. The question of the overall impact of individual cover crops on the soil microbiome was addressed both quantitatively and qualitatively: (1) do cover crop species differ in the extent by which microbial taxa in the rhizosphere are promoted and/or repressed? and (2) do cover crop species differ in the kind of microbial taxa that they promote and/or repress in the rhizosphere? Lastly, we generated microbial networks on the basis of the active fractions of the organismal groups and (3) asked ourselves whether cover crops had differential effects on the level of associations between organismal groups.

A better understanding of the specific microbial signatures of cover crops will contribute to cover crop applications beyond the current, general, scope. The insights presented in this paper might be considered a first step toward the selection of cover crops to steer the soil microbial community in such a way that they contribute to the restoration of soil health.

2.2 Materials And Methods

2.2.1 Experimental design

A field experiment was carried out at the Wageningen University and Research experimental farm 'Vredepeel', located in the southeast of the Netherlands. The experiment consisted of 11 treatments, including ten cover crop species and an unplanted control (fallow), each replicated eight times. Cover crop treatments included widely used cover crop cultivars, as well as a prospective oilseed radish cultivar referred to as E1039. Bottomless containers (70L; ∅ 55 cm, height 43 cm) were randomly positioned in eight blocks, and hence the total experiment included 88 containers. These were dug into the field in such a way that there was no height difference between the soil surface in the buckets and the surrounding soil. The containers were filled with topsoil (20 cm) originating from subplots of a nearby long-running field experiment 'Soil Health Experiment' (SHE) (Korthals et al., 2014). Each block of containers was filled with the topsoil originating from one of the SHE subplots. Half of the SHE subplots were managed following organic practices and the other half following conventional practices until 2017. It is noted that all subplots received the same soil management in the two years before this field experiment (a single application of cattle slurry per year) (field management data provided in Supplementary Table S2.1). Barley was the last main crop grown on all plots between March and June 2019. Barley crop remains were incorporated into the soil at the beginning of June 2019, and the topsoil was collected from the field at the end of June 2019 to be transferred to the containers for this experiment. In each block, ten containers were sown with single cover crop cultivars (Table 2.1), and one container was kept fallow. Cover crop seeds were sown at the end of July 2019 (sowing densities shown in Table 2.1), and weeds were removed manually during the duration of the experiment. In 14 containers cover crop growth was negatively affected by drought in the late summer of 2019 (weather data provided in Supplementary Table S2.1) and excluded from the experiment. Thus, 74 of the original 88 containers were sampled at the end of the experiment (list in Supplementary Table S2.3).

2.2.2 Soil sampling

Soil samples were collected on the 3rd of October 2019, approximately two and a half months after the sowing of cover crops. For this, 2-20 plants (depending on the plant size and root system) were randomly collected from each container, uprooted and shaken to discard non-rhizosphere soil. Plant samples

were transported to the nearby laboratory, where rhizosphere soil was collected by brushing off the soil adhering to the roots (see Supplementary Table S2.2 for number of plants used, and average and total plant dry weight). Fallow soil from the control container was collected with an auger (15 mm diameter x 20 cm depth). Three cores were sampled for each fallow container, and after thorough mixing and sieving (mesh size 5 mm), a subsample of 10 g was collected. Rhizosphere and fallow soil samples were transferred to clean Ziplock plastic bags, snap-frozen in N_2 (l), kept on dry ice during transport and subsequently stored at −80 °C at the Laboratory of Nematology.

Table 2.1 | Details of the cover crop species used in this study, including taxonomic affiliation, the origin of seeds, and sowing density. A selection of the most commonly used cover crop species was made on the basis of several relevant papers including Bacq-Labreuil et al. (2019), Hooks et al. (2010), Wick et al. (2017), Zhang et al. (2022) and Zukalová and Vasak (2002).

Common Name	Family	Species	Cultivar	Company	Sowing Density Kg/Ha
Vetch	Fabaceae	*Vicia sativa*	Amelia	Joordens Zaden (NL)	125
Lentil		*Lens culinaris*	Eston	Joordens Zaden (NL)	120
Oilseed Radish	Brassicaceae	*Raphanus sativus var. Oleiformis*	Terranova E1039	Joordens Zaden (NL)	30
Oilseed Radish				Joordens Zaden (NL)	30
Black Oat	Poaceae	*Avena strigosa*	Pratex	PH Petersen (DE)	80
Tall Fescue		*Festuca hrundinacea*	Firecracker	Barenbrug (NL)	35
Hybrid Ryegrass		*Lolium hybridum*	Daboya	Vandinter Semo (NL)	45
Phacelia	Boraginaceae	*Phacelia tanacetifolia*	Beehappy	DSV-Zaden (NL)	10
Borage		*Borago officinalis*	Wild Type	Nebelung (DE)	50
Marigold	Asteraceae	*Tagetes patula*	Ground Control	Takii Europe (NL)	8

2.2.3 Nucleic acids extraction and sequencing

Soil total DNA and RNA were extracted simultaneously following a protocol optimised for 2 g soil (Harkes et al., 2019). This extraction method comprises bead beating, precipitation of humic acids with an ammonium aluminium sulphate solution, and phenol-chloroform extraction. cDNA was synthesised from the extracted RNA using a Maxima First Strand cDNA Synthesis Kit for RT-PCR (Fermentas, Thermo Fisher Scientific Inc., USA) following the manufacturer's instructions. In preparation for the first step of the library construction, DNA and cDNA samples were diluted to 1 ng μl^{-1} and 0.1 ng μl^{-1}, respectively. Following Harkes et al. (2019), the library was generated in a two-

step PCR procedure. The first step consisted of the amplification of organismal group-specific 16S and 18S rRNA regions. To this end, locus-specific primers extended with an Illumina read area and an appropriate adapter were employed that targeted the V4 region of 16S of bacteria, and the V9, V7-V8, V5-V7 of 18S of protozoa, fungi and metazoa, respectively (Supplementary Table S2.4). For PCR amplification, 3 µl of the diluted samples were used as templates. PCR was carried out with the following temperature profile: 3 s at 95 °C; followed by 39 cycles of 10 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C; and a final extension step of 5 s at 72 °C. All reactions in the first PCR step were done in triplicate, and PCR products were pooled per sample and organismal groups. The second PCR step was performed using 40x dilutions of the amplicons from the first PCR as templates. The second PCR was used to attach the sample-specific Illumina index combination, used for multiplexing the samples upon pooling, and the Illumina sequencing adapter to the amplicons of the first PCR. The following temperature profile was employed: 3 s at 95°C; followed by 10 cycles of 10 s at 95°C, 30 s at 60°C, and 30 s at 72°C; and a final extension step of 5 s at 72°C. A random selection of products of the first and second PCR steps was checked on an agarose electrophoresis gel to ensure that the amplifications were successful in producing amplicons of the expected size. Lastly, PCR products were pooled together and sent out for sequencing to Useq (Utrecht Sequencing Facility, Utrecht, The Netherlands). Illumina MiSeq sequencing was performed using a 2 x 300 bp V3 kit.

2.2.4 Pre-processing of the sequencing data

Sequencing data were demultiplexed and subset into the four organismal groups based on their locus-specific primer sequences, using a custom Python script. Sequencing reads were pre-processed in QIIME2 (Bolyen et al., 2019). Read picking was carried out with the QIIME2 DADA2 denoising algorithm (Callahan et al., 2016) separately for each organismal group. For bacteria, protists and fungi, paired-end reads were merged and used to generate amplicon sequence variants (ASVs) (Callahan et al., 2017). For metazoa, only the forward reads were used to generate ASVs due to the short average read length of the reverse reads.

Taxonomic assignment of ASVs was carried out using the q2-feature-classifier plugin and *classify-sklearn* function (Pedregosa et al., 2011), with organismal-group-specific pre-trained reference databases. For bacteria, fungi and metazoa, the non-redundant SILVA database (Glöckner et al., 2017) (*silva-138-ssu-nr99-seqs-derep-uniq*, version 138, 99% identity criterion) was pre-trained to generate amplicon-region specific classifiers. For the taxonomic assignment of protists, the pr2 reference database (Guillou et al., 2012) was used to build the pre-trained amplicon-region-specific classifier. QIIME2 output

files were imported in R as 'phyloseq objects' with the function *import_qiime* of the phyloseq package (v1.34.0) (McMurdie and Holmes, 2013). The phyloseq package was also used to process the phyloseq objects prior to the statistical analysis. Unassigned ASVs and ASVs assigned to chloroplasts and mitochondria as well as to non-target organisms were filtered out using the function *subset_taxa*. Samples with an unacceptably low number of reads (<1000) were filtered out using the *prune_samples* function. In the R package metagMisc (v0.0.4) (Mikryukov, 2017) the function *phyloseq_filter_prevalence* was used to filter out singletons and ASVs with less than 10 reads in the whole dataset.

2.2.5 Microbiome diversity and composition

ASV diversity and richness of each cover crop rhizosphere and fallow were determined for each organismal group using rarefied ASV tables. Rarefying was performed in the phyloseq R package using the function *rarefy_even_depth* with the options 'without replacement' and 'to the minimum library size'. The function *alpha* of the microbiome R package (v.1.12.0) (Lahti, 2012-2019) was used to calculate Observed, Chao1 and Shannon diversity and richness metrics. The non-parametric Kruskal Wallis and Wilcoxon post-hoc tests with Bonferroni correction for multiple testing were used to pinpointing significant differences in alpha diversity scores among cover crop species.

Differences in microbial community structure across cover crops were calculated by constructing dissimilarity matrices with the Bray-Curtis Distance metric on the non-rarefied normalised ASV tables. Normalisation was carried out on the ASV tables using the Cumulative Sum Scaling (CSS) method (*cumNorm* function from R package metagenomeSeq v. 1.32.0) (Paulson et al., 2013). The results of the distance metrics were visualized in Principal Coordinate Analysis (PCoA) graphs for each organismal group, built with the function *plot_ordination* implemented in the phyloseq R package. The factors explaining the dissimilarities among the microbiomes of the different treatments were tested using PERMANOVA (*adonis2* function from the vegan package, Oksanen et al. (2013). The factors included in the PERMANOVA were 'subplot' (to account for block effects), 'nucleic acid' (to account for the difference between DNA and cDNA), and 'treatment' (to account for the effect of the cover crop treatments). To make comparisons between cover crop's rhizosphere and fallow and across cover crop's rhizospheres, pairwise PERMANOVAs (pairwise adonis) were carried out based on Bray-Curtis multivariate distances with Benjamini-Hochberg correction for multiple testing and 999 permutations. The R^2 values resulting from the pairwise contrasts of each cover crop species *versus* the fallow control, were used as a proxy for the selection strength exerted by individual cover crops on the native microbial community at the level of the rhizosphere.

The taxonomic composition of the cover crop's microbiome was visualised with stacked bar plots, generated using the function *plot_composition* of the microbiome R package. The plots show the relative abundance per cover crop treatment of the most abundant microbial families (bacteria and fungi) or orders (metazoa and protists), while taxa represented by less than 2% of all reads were grouped in the "Other" category.

2.2.6 Differential abundance analyses of rhizospheric microorganisms by ANCOM-BC

A Differential Abundance analysis (DA) of microbial taxa was performed with analysis of compositions of microbiomes with bias correction (ANCOM-BC) in R (ANCOMBC R package) (Lin and Peddada, 2020). The test aimed to pinpoint the differential effect of each cover crop on the fallow soil in the generation of each of the cover crops' rhizospheres. Cover crops species and block were the covariates of interest, while the fallow soil (control) was used as the reference level. ANCOM-BC was performed at the family level for bacteria and fungi, and at the order level for protists and metazoa. To do this, AVS tables were agglomerated to the desired taxonomic level in with *tax_glom* function of the phyloseq R package. The beta coefficients resulting from the linear regression indicated depletion (negative values) or enrichment (positive values) of the differentially abundant taxa in the rhizosphere of each cover crop compared to the fallow. Results of the ANCOM-BC test allowed us to generate rhizospheric profiling per each cover crop species. Rhizospheric profiling were plotted in heatmaps, and associated dendrograms were generated based on the Euclidean distance. Dendrograms indicated the distance between cover crop species based on the value of beta coefficients as a function of the abundance of each differentially abundant taxon.

2.2.7 Microbial networks based on potentially active rhizospheric communities.

Network analyses were performed to pinpoint the correlations among members of the active microbiome fraction in the rhizosphere of the ten cover crops and the fallow treatment. A co-occurrence analysis was carried out with SparCC (Friedman and Alm, 2012) for each treatment in R software (*sparcc* function from SpiecEasi R package (Kurtz et al., 2021). To run SparCC, the ASV tables and the taxonomy tables were agglomerated to family (bacteria and fungi) or order (protists) levels to reduce the network complexity. For each SparCC analysis, the statistical significance of the inferred correlations was assessed by computing a bootstrap value (function sparccboot of SpiecEasi R package). Statistically significant ($p < 0.05$) SparCC correlations with a value of > 0.6 or

< −0.6 were included into the network analyses. The visualization of the network was initiated in R with the igraph package (Csardi and Nepusz, 2006), and then transferred to Cytoscape SW (Shannon, 2003) with the function createNetworkFromIgraph of the RCy3 R package (Gustavsen et al., 2019). Network statistics were analysed in Cytoscape. Higher values for network characteristics such as numbers of nodes and edges and average number of neighbours were considered a proxy for network complexity. Furthermore, the number of positive and negative connections across the different organismal groups, *i.e.*, bacteria, fungi, and protists was calculated in the form of a ratio (across groups connections/total connections) for fallow and cover crops rhizosphere to infer the effect of cover crops on the multitrophic interactions alone.

2.3 Results

2.3.1 Sequencing results

The sequencing of 74 DNA and 74 cDNA rhizosphere samples from ten cover crop species and the bulk soil (fallow) treatment resulted in a total of 24,823,255 reads from four organismal groups, *i.e.*, bacteria, fungi, protists and metazoa. After pre-processing and filtering, 6,082,172 (2,886,425 DNA and 3,195,747 cDNA) reads were retained and used for further analyses, of which 3,252,176 belonged to bacteria, 868,312 to fungi, 1,475,652 to protists and 486,032 to metazoa, with a median of 30,431 reads per DNA and 26,754 per cDNA sample. Samples featuring less than 1,000 reads were filtered out from each organismal group's dataset (Table 2.2). Due to a high number of samples with a low number of reads in the metazoan cDNA dataset, the analyses of the metazoan community were based on DNA data only. 6,631 ASVs were assigned to bacterial taxa, 258 to fungal taxa, 1,812 to protistan taxa and 82 to metazoan taxa. For bacteria and fungi, the taxonomic resolution allowed for investigation of the communities up to the family level, whereas protists were mainly studied at the order level, as only a minority of ASVs were assigned to lower taxonomic levels. The metazoan community was only studied at the order level.

2.3.2 Factors affecting the rhizosphere community composition

The microbiome composition of the resident (DNA) and potentially active (cDNA) microbial communities significantly differed. PERMANOVA showed that the factor 'nucleic acid type' explained 12% of the overall variation in both the bacterial and the fungal communities ($p < 0.001$), and 13% in the protist's community ($p < 0.001$) (Supplementary Figure S2.1, Supplementary Table

S2.5). Hence, resident microbial communities as well as the potentially active fractions thereof were analysed separately. In the next coming sections, we simplified and shortened the term 'potentially active fractions of the microbial community' to 'active fractions' solely to facilitate readability. The effect of blocks was significant (PERMANOVA < 0.001) but limited as it explained only 7.2, 6.4 and 8.4% of the variation across the bacteria, fungal and protistan communities (Supplementary Table S2.5). For the metazoan community, however, the block effect was more prominent, as it accounted for 17.9% of the overall microbiome variation (Supplementary Table S2.5).

Cover crop species had a major effect on the assembly of both the resident and active fractions of the rhizosphere microbiome for all four organismal groups, explaining 33 to 51% (PERMANOVA, $p < 0.001$) of the overall variation (Table 2.2, Figure 2.1). The smallest effect was observed for the resident metazoan community, whereas the strongest impact was detected for the active fraction of the fungal community (Table 2.2).

Table 2.2 | PERMANOVA analysis with Bray-Curtis dissimilarity metric to assess the variation explained by cover crop treatment on the resident (DNA) and active (cDNA) communities separately, and on each of the four organismal groups.

	RESIDENT COMMUNITY (DNA)			**ACTIVE COMMUNITY (CDNA)**		
Organismal Group	R^{2} [a]	n [b]	p-value	R^{2} [a]	n [b]	p-value
Bacteria	0.40	73 (−1)	0.001	0.40	74	0.001
Fungi	0.44	74	0.001	0.51	70 (−4)	0.001
Protists	0.37	73 (−1)	0.001	0.37	74	0.001
Metazoa	0.33	68 (−6)	0.001	ND [c]		

a R^2 = fraction of the variation explained by the experimental factor cover crop,
b n = the number of samples included for the test. Between brackets, the number of samples removed due to low sequencing coverage is given. Dissimilarity is significant for p-values < 0.01.
c ND = not determined, metazoa community was only assessed at the DNA level (resident community).

Both oilseed radish cultivars showed the strongest effect on microbial communities across all cover crop species, as shown by clearly separate data clusters in PCoA ordinations (Figure 2.1). In contrast, black oat, hybrid ryegrass, and marigold induced relatively mild shifts, as indicated by the proximity of these samples to the fallow control in the PCoAs (Figure 2.1). These patterns were supported by the pairwise PERMANOVA analyses (Table 2.3). Table 2.3 shows levels of contrast between resident and active fractions of the bacterial and fungal communities in the rhizosphere of individual cover crops as compared to the microbial community assembly in the fallow control. As can be seen in Figure 2.1 and based on the R^2 values presented in Table

2.3, the extent by which plants shape their rhizobiome, hereafter referred to as selection strength, is cover crop species-specific. Oilseed radish-E and -T (Brassicaceae) showed the highest selection strength on all three organismal groups (R^2 values: 0.36-0.52, Table 2.3). Hybrid ryegrass and black oat generally had the lowest selection strength on all organismal groups (R^2 values: 0.12-0.23). It is noted that tall fescue, another representative of the Poaceae, had a selection strength close to the one of phacelia (Table 2.3). On average, the impact of cover crops on the active fraction of the microbial communities was stronger than on the resident community. This was most prominent for the fungal community with average (for all cover crop species studied) R^2 values of 0.32 and 0.38 for the resident and the active communities respectively.

2.3.3 Impact of cover crops on the microbial richness and diversity in the rhizosphere

To assess the effects of individual cover crops on the ASV richness and diversity, three diversity indices were used (Observed, Shannon and Chao1). All three indices identified vetch, tall fescue, lentil, phacelia and borage as cover crops associated with resident and active bacterial diversities that exceeded the diversity in the fallow controls. The two oilseed radish cultivars showed alpha diversities lower than the fallow controls (Supplementary Table S2.7 A, B). As compared to the bacterial communities, cover crops had a milder effect on the fungal richness and diversity indices. The four representatives of the families Fabaceae and Boraginaceae, vetch, lentil, borage and phacelia, as well as tall fescue (Poaceae), were associated with more diverse resident and active fungal communities compared to fallow by the indices Observed and Chao1 (Wilcoxon post-hoc with Bonferroni correction $p<0.05$). Both for the resident and the active fractions, the Shannon index did not reveal significant differences between the fallow control and the aforementioned cover crops (Supplementary Table S2.7 C, D).

Also for the protists, vetch, lentil, borage and phacelia, as well as tall fescue were often associated with microbiomes showing a higher diversity than the fallow control. However, the Shannon index pointed to the absence of significant differences in the resident protistan communities between most cover crop species (except for black oat and the two oilseed radish cultivars) and the fallow control. Regarding the active protist fractions, the Shannon index did not reveal a significant difference between the fallow control and any of the cover crops (Supplementary Table S2.7 E, F). Concerning the resident metazoan community, borage and vetch were associated with the most diverse resident community (Supplementary Table S2.7 G).

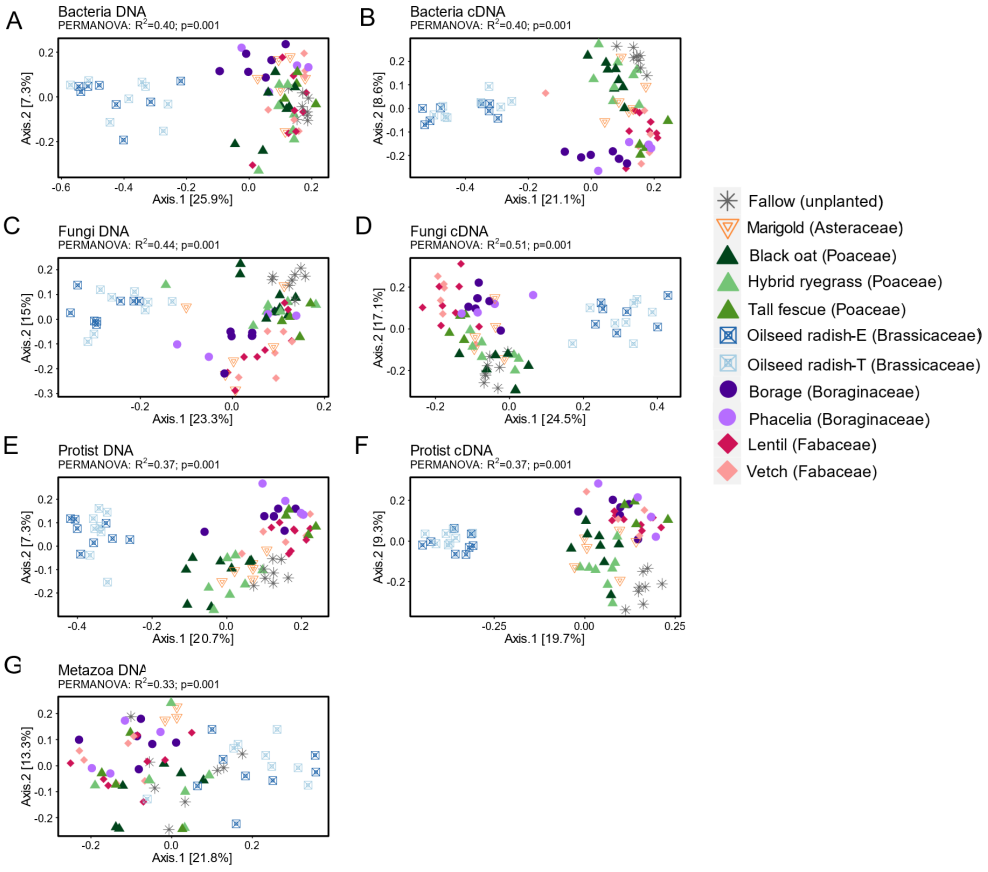

Figure 2.1 | Principal Coordinate Analysis (PCoA) of CSS normalised ASV data. Dissimilarity matrix built on Bray-Curtis metric and plotted separating ASVs based on cover crop treatment. Samples appear separated along the axes as cover crop treatment accounted for 19-26% of variation along the principal PCoA axis and explained 33-51% of the dissimilarity among sample groups (PERMANOVA, $p \leq 0.001$).

Table 2.3 | Significant (adjusted $p < 0.05$) R^2 values based on comparisons between microbial communities in cover crop rhizospheres and fallow (bulk) soil calculated by pairwise-PERMANOVA based on Bray-Curtis distance with 999 permutations and Benjamini-Hochberg (BH) correction for multiple testing. This table is an excerpt from Supplementary Table 6 where all comparisons are shown. Cover crops are presented in order of their increasing effect on the resident bacterial community.

Cover Crops	Contrast with fallow					
	Bacteria		Fungi		Protists	
	Resident	Active	Resident	Active	Resident	Active
Hybrid ryegrass	0.12	0.13	0.18	0.23	0.14	0.15
Black oat	0.13	0.16	0.12	0.17	0.16	0.20
Marigold	0.14	0.16	0.31	0.36	0.15	0.20
Lentil	0.16	0.24	0.35	0.46	0.16	0.21
Vetch	0.20	0.21	0.36	0.43	0.18	0.23
Phacelia	0.20	0.27	0.29	0.41	0.25	0.25
Tall fescue	0.21	0.29	0.25	0.32	0.26	0.26
Borage	0.27	0.34	0.35	0.44	0.23	0.25
Oilseed radish-T	0.42	0.37	0.42	0.49	0.36	0.43
Oilseed radish-E	0.44	0.41	0.52	0.51	0.35	0.40

2.3.4 Impact of cover crops on the relative abundances of microbiota in the rhizosphere

In Figure 2.2 the relative abundances of microbiota in the rhizosphere are shown for each of the ten cover crop species, as well as for the fallow control. A selected number of results will be detailed below.

Bacteria | Numerous rare bacterial taxa ('other (<2%)' in Figure 2.2) were present in the rhizosphere of all cover crops tested. In the case of hybrid ryegrass, tall fescue and lentil, this category comprised >50% of both the resident and the active bacterial community. Among the bacterial families with abundances >2%, striking differences were observed between the individual cover crop species. Pseudomonadaceae were highly stimulated in the rhizosphere of the two oilseed radish cultivars and borage (Fig 2.2 A, B). Among the 43 Pseudomonadaceae ASVs, 42 belonged to the genus *Pseudomonas*, and the remaining could not be assigned to a genus. Moreover, Moraxellaceae and Rhizobiaceae were abundantly present in the rhizospheres of the two oilseed radish cultivars. On average Moraxellaceae accounted for 8.4% of the resident and 7.6% of the active community, and Rhizobiaceae were amply represented in the resident and active communities (respectively 7.2% and 5.0%). Regarding the active fraction of the bacterial communities, Blrii41 (order Polyangiales) was most abundant in the hybrid ryegrass rhizosphere (9.8%). This bacterial family was activated by cover crops belonging to Poaceae,

Asteraceae and Fabaceae, and not by representatives of the Brassicaceae and Boraginaceae. It is noted that Oxalobacteraceae were activated by all cover crops, while the active members of this bacterial family made up <2% in the fallow control (See Supplementary Table S2.8 for details on the composition of the bacterial communities).

Fungi | As compared to the bacterial community, the fraction 'other (<2%)' is relatively small in the fungal communities (Figure 2.2 C, D). The fungal family Mortierellaceae was abundantly present in the rhizospheres of all cover crops tested, as well as in the fallow control. The relative abundances of this fungal family ranged from 9.2% (active community of borage) to 53% (resident community of fallow control). Notably, the representation of this fungal family in the resident community tended to exceed its relative presence in the active community. An opposite pattern was observed for the Microascaceae; although present within the resident community of most cover crops, they constituted a larger part of the active fraction of the fungal community. At the level of individual cover crops, the rhizosphere of borage showed the highest abundance of Plectosphaerellaceae (58.1% and 53.8% in the resident and active communities, respectively) ($p < 0.05$). The two oilseed radish cultivars stood out as their rhizospheres were highly enriched in Olpidiaceae (on average 28.7% of the resident community). It is noted that the presence of this fungal family was much lower in the active fraction (on average 5.5%). An opposite trend was observed for the Orbiliaceae, active representatives of this fungal family were amply present in the rhizosphere of the two oilseed radish cultivars (on average 8.5%), while the relative abundance in the resident community was <2% (See Supplementary Table S2.9 for details on the composition of the fungal communities).

Protists and Metazoa | The resident and active protistan communities showed a high relative abundance of rare taxa (Figure 2.2 E, F, category 'other (<2%)'). Among the protist taxa present at higher relative abundances (>2%), the Cercomonadidae and Pythiaceae were shown to be present and active in the rhizospheres of all cover crops tested. With relative abundances of 27.1% and 37.7% in the resident communities of borage and phacelia, the oomycete family Pythiaceae was dominantly present in the rhizosphere of Boraginaceae (Figure 2.2 E, F). In the case of the two oilseed radish cultivars, the Rhogostomidae (order Cryomonadida) were remarkably well represented in both the resident (on average 40.6%) and the active (on average 35.5%) fraction of the protistan community. The Vahlkampfiidae were abundant in the resident community of all cover crops rhizosphere, but they made up less than 2% of the active protist fraction. An opposite trend was observed for the Nucleariidae and Sandonidae, which were abundant in the active community of all cover crops rhizosphere but underrepresented in the resident community.

Analysis of the resident metazoan community in the rhizosphere of cover crops revealed the dominance of nematodes (seven of the most abundant orders shown in Figure 2.2 G). Rhabditida (17.7-46%), opportunistic bacterivores, were abundantly present in the rhizosphere of all cover crops. Diplogasterida (bacterivores) were present in remarkably high abundances in the rhizosphere of the two oilseed radish cultivars (on average 50%). Representatives of the orders Tylenchida and Areolaimida were commonly present in all cover crops but less so in the two oilseed radish cultivars (~3% *versus* ~20-30%) (See Supplementary Table S2.10 for details on the composition of protist and metazoan communities).

2.3.5 Differential abundance analysis of rhizosphere microbiomes

In the next step, an analysis of compositions of microbiomes with bias correction (ANCOM-BC, Lin and Peddada, 2020) was used to determine the rhizospheric profiling of the resident and active taxa of each cover crop compared to the fallow soil. Beta coefficients, a quantitative measure for differential abundances, ranged from -4.4 (Microascaceae under oilseed radish) to 7.2 (Pseudomonadaceae under oilseed radish) indicating that, overall, the stimulation of taxa in the rhizosphere by the cover crops was stronger than the repression. As this analysis concentrates on changes in abundances rather than abundances *per se,* the heatmaps generated with the beta-coefficient values include taxa present in low abundance that were lumped under the category 'other (<2%)' in Figure 2.2. On the other hand, taxa that were shown to be present in the rhizosphere of all cover crops in relative abundances comparable to fallow, such as the bacterial family Sphingomonadaceae, are not included in Figure 2.3. For all organismal groups, most taxa that were significantly affected at the DNA level (resident community) were often also influenced at the cDNA level (active community). Taking into account all bacterial taxa that were significantly stimulated or repressed, 69% was affected at both DNA and cDNA levels. For fungal and protist communities these levels of communality were respectively 78% and 78% (Supplementary Tables S2.11, S2.12, S2.13). In by far most cases, the directionality of the changes was identical; most often a taxon repressed at the DNA level was also repressed at the cDNA level, and the same holds for stimulated microbiota. Nevertheless, a few exceptions were observed: the bacterial family Iamiaceae was significantly repressed by borage at the DNA level and stimulated at the cDNA level, and Nitrosomonadaceae were stimulated by oilseed radish (cultivar E), while its activity was repressed Supplementary Table S2.11).

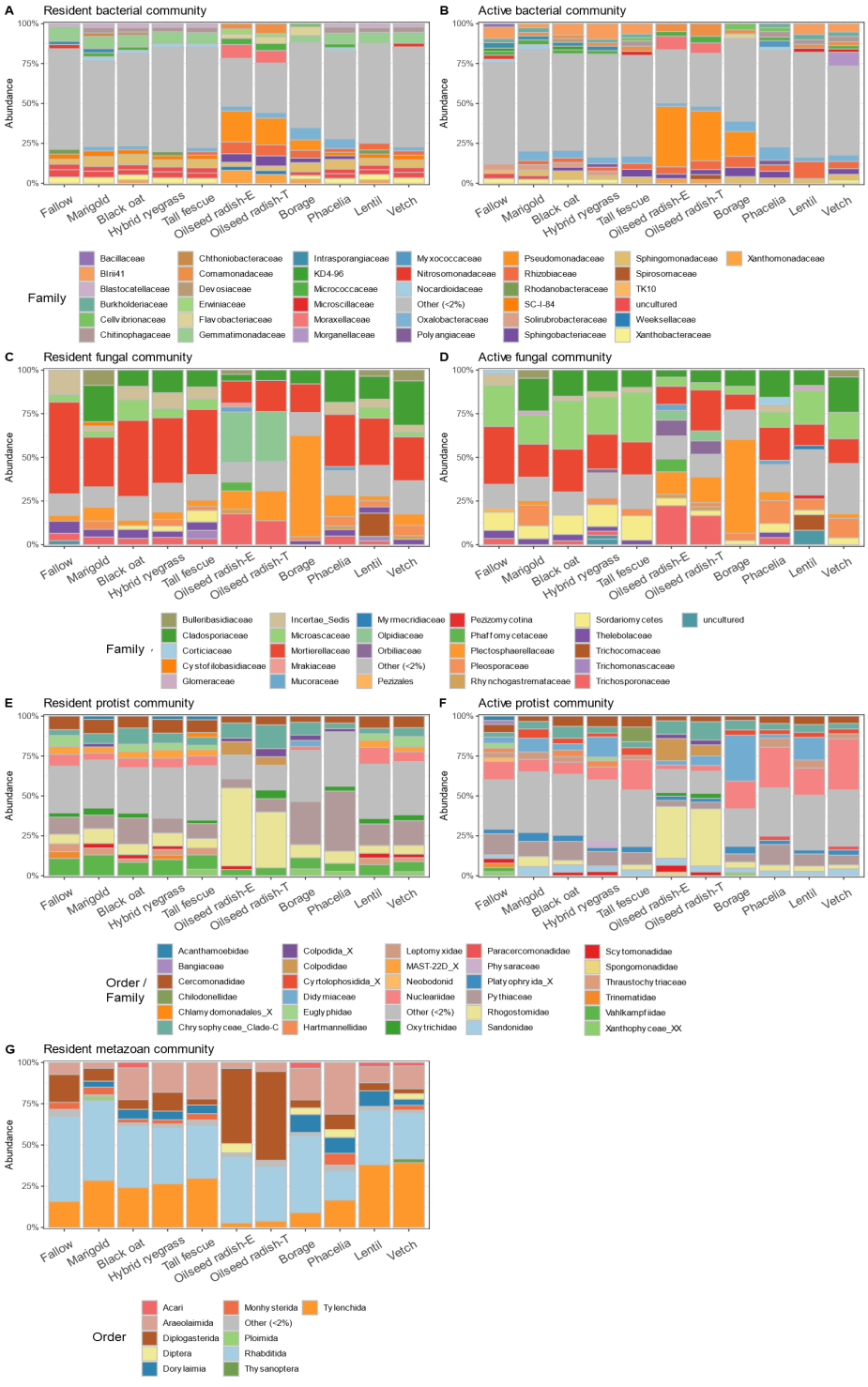

Figure 2.2 | Microbial composition at the family level of the rhizosphere of the cover

crops. A) bacterial resident community, B) bacterial active community; C) Fungal resident community; D) fungal active community; E) protists resident community; F) protists active community; G) metazoa resident community (order level). All taxa accounting for <2% or relative abundance were grouped as "Others (<2%)".

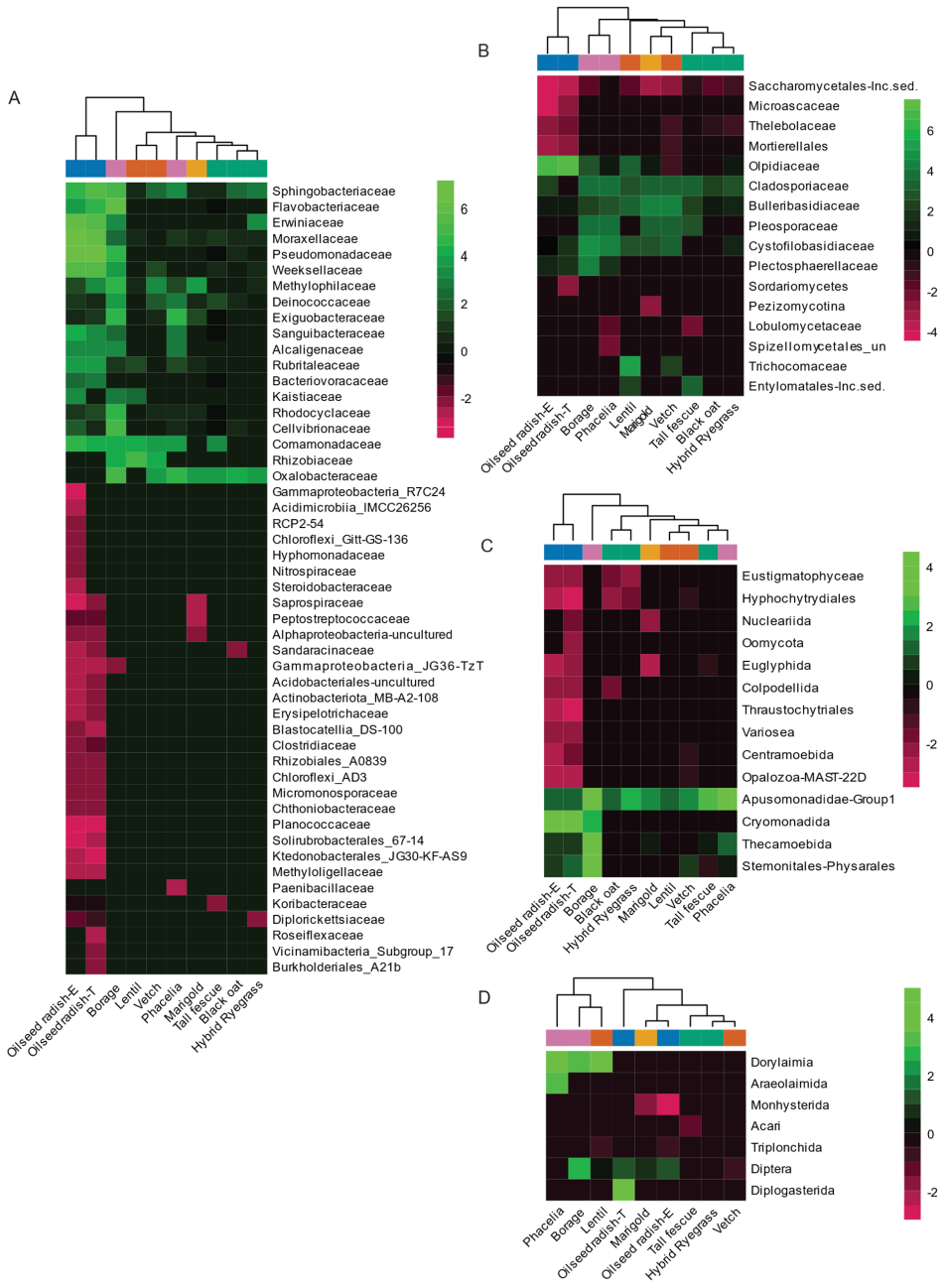

Figure 2.3 part 1, follows in the next page

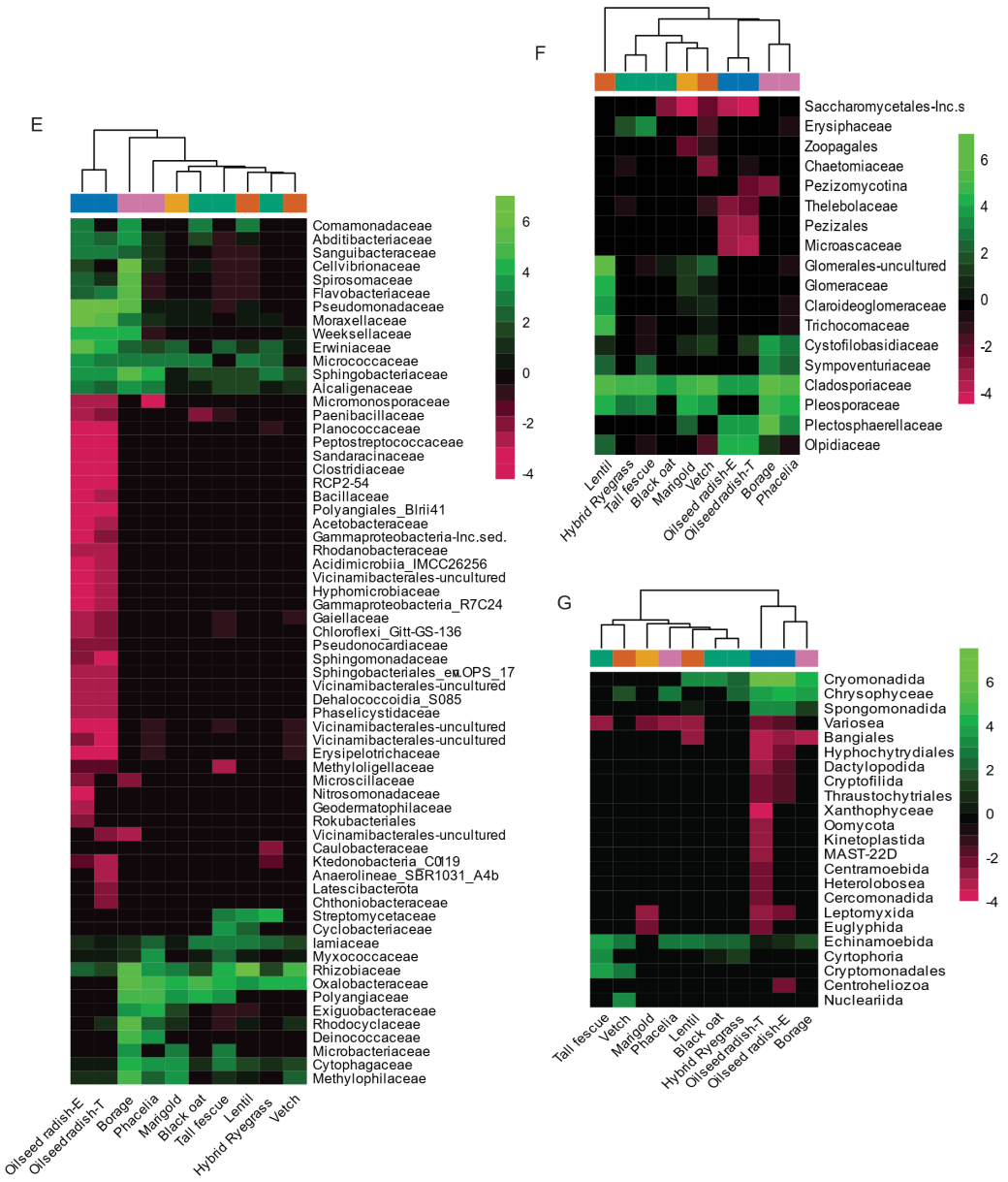

Figure 2.3 | Heatmap of the beta coefficient assigned to the differentially abundant taxa pinpointed with ANCOM-BC for A) resident bacterial families, B) resident fungal families, C) resident protist families and orders, D) resident metazoan orders, E) active bacterial families, F) active fungal families, G) active protists orders. Beta coefficients > 0 (shades of green) indicate that the average abundance of the taxon in the cover crop treatment is higher than in the reference (fallow soil). Beta coefficients < 0 (shades of red) indicate that the average abundance of the taxon in the cover crop treatment is lower than in the reference (fallow soil). The dendrograms per column and row were calculated based on

Euclidean distance. The colours above the heatmap represent the plant family of each cover crop cultivar: orange = Asteraceae, green = Poaceae, blue = Brassicaceae, purple = Boraginaceae, and red = Fabaceae. For improved figure readability, for bacteria, fungi and protists only taxa with beta coefficients higher than 3 and lower than -2 are represented. The complete heatmaps are available in Supplementary Figure S2.2. Full size figures are available at https://doi.org/10.1016/j.apsoil.2023.105012.

Bacteria | ANCOM-BC identified 175 and 177 differentially abundant families among the resident and active bacterial communities, respectively (a selection of the most enriched and most depleted families is presented in the heatmap in Figure 2.3 A, E, the complete heatmap representing all the differentially abundant families is provided as Supplementary Figure S2.2 A, D). The two oilseed radish cultivars showed bacterial profiles that were most deviant from the fallow controls. Despite a few cultivar-specific changes, the two oilseed radishes clustered together in dendrograms (Figure 2.3 A, E). In the rhizosphere of the oilseed radishes, the number of repressed bacterial taxa exceeded the number of promoted taxa for both the resident and the active bacterial communities. Planococcaceae (both in resident and active communities) and Bacillaceae (in the active community) were among the most repressed families, while Pseudomonadaceae, Moraxellaceae and Erwiniaceae resided among the most enriched families (both in resident and active communities). Borage showed a bacterial profile exceptionally distinct from the fallow soil, with a high number of differentially enriched families. Among these, the resident and active fractions of the Flavobacteriaceae, Cellvibrionaceae and Sphingobacteriaceae were strongly promoted as compared to fallow controls (in all cases beta coefficients > 5). It clustered separately from the oilseed radish and the other cover crops in the profiling of the resident rhizospheric taxa, while clustered together with phacelia in the profiling of the active taxa (Figure 2.3 A, E).

Fungi | ANCOM-BC pinpointed 44 and 46 differentially abundant fungal families in respectively the resident and active communities of the cover crop rhizospheres (see Supplementary Table S2.12 for ANCOM-BC results on the fungal community, and Supplementary Figure S2.2 B, E, F for the full heatmaps). Regarding the resident community, the two oilseed radish cultivars showed a high number of differentially abundant fungal families. Most striking is the strong repression of the representatives of the fungal family Microascaceae and the order Saccharomycetales, and the stimulation of members of the protistan family Olpidiaceae. Apart from a mild repression of Saccharomycetales in the resident rhizospheric profiling resident, the impact of lentil on members of the fungal community was invariably positive (Figure 2.3 B, F). The stimulating effect was most notable for the fungal family Trichocomaceae and the fungal order Glomerales. In Figure 2.3 F, this order is represented by the taxa called ‘Glomerales-uncultured’, ‘Glomeraceae’ and

'Claroideoglomeraceae'. For both representatives of the Boraginaceae, stimulation of the Cystofilobasidiaceae and the Pleosporaceae were observed both in the resident and the active fraction of the fungal community (Figure 2.3 B, F). The fungal family Cladosporiaceae is exceptional as its members were stimulated by almost all cover crops in both the resident and active communities (only exception oilseed radish-T) (Figure 2.3 B, F). Members of the different plant families clustered more closely together than with members of other families, with the exception of lentil and vetch which displayed a sharp distance, especially at the level of the active profiling.

Protists and metazoa | As main consumers of primary decomposers, protists and metazoans were indirectly affected by cover crops. ANCOM-BC analyses revealed that 46 and 48 protist orders were differentially abundant in the resident and active rhizosphere communities, respectively. The two oilseed radish cultivars had the broadest impact on the resident and active protistan communities, followed by borage (Supplementary Table S2.13 and Supplementary Figure S2.2 C, F for the complete heatmap of the protistan community). These cover crops clustered together in the active profiling, while they have a reduced distance in the resident profiling. Cryomonadida was relatively most enriched in the oilseed radish and borage rhizosphere. With a beta coefficient of 3.9, the resident community of the borage rhizosphere was particularly enriched in members of the Stemonitales-Physarales (plasmodial slime moulds), while for all other cover crops abundances lower than 2% were detected (Supplementary Table S2.13).

ANCOM-BC of the metazoan community identified 7 differentially abundant metazoan orders out of 79 (Supplementary Table S2.11). The highest number of differentially abundant metazoan orders was found for marigold (Figure 2.3 D). The nematode order Diplogasterida was exclusively enriched in the rhizosphere of the oilseed radish cultivar-T, and Monhysterida was depleted in oilseed radish cultivar -E and marigold, while Araeolaimida were enriched in the rhizosphere of phacelia (Figure 2.3 G). It is noted that the sample size (2 g of rhizosphere soil) is low for metazoa, and the shifts reported here require confirmation by the analysis of more and larger subsamples.

2.3.6 Potential associations within and between organismal groups in cover crop rhizospheres

Network analyses were performed to map potentially positive and negative connections within and between the active fractions of the bacterial, fungal and protist communities in the rhizosphere of cover crops, and in bulk soil for the fallow control (Figure 2.4). Metazoa could not be included in these analyses due to the low number of reads in the metazoan cDNA dataset. The network of

the fallow soil featured 59 nodes and 55 edges and 2.3 average number of neighbours (for other network characteristics see Supplementary Table S2.14). Among the cover crops tested, only phacelia's rhizosphere showed a lower network complexity (32 nodes, 26 connections, 2.17 avg neighbours) (Figure 2.4). The highest level of network complexity was induced by vetch (141 nodes, 468 edges, 7.4 avg neighbours). Generally, the highest number of connections was found between bacteria and protists, followed by connections between bacteria and fungi. A relatively low level of connectivity was detected between fungi and protists. Marigold was exceptional as the highest number of connections was found between bacteria and fungi. Fallow, phacelia, tall fescue and vetch had a majority of negative interactions between organismal groups (Figure 2.4, dotted lines), while for all other cover crops, the majority of the interactions were positive (Figure 2.4, solid lines).

2.4 Discussion

2.4.1 Cover crop species exert different selection strengths on the rhizosphere microbiome.

All ten cover crops characterized in this study exerted significant effects on the soil rhizobiome, and the kind of effects was shown to be plant species and - to some extent - plant family dependent. The characterization of the resident and active fractions of the rhizosphere communities of the cover crops revealed distinct levels of selection strengths by the cover crops on the rhizosphere microbiome. The two cultivars of oilseed radish affected the rhizosphere microbiome most distinctly. Both oilseed radish cultivars sharply suppressed a wide range of microbial taxa, in particular bacterial and protistan microorganisms, and, at the same time, strongly promoted a smaller subset. This trend observed on the individual taxa through ANCOM-BC is accompanied by the alpha diversity results, which indicated a lower richness and evenness in the rhizosphere (especially of) bacterial community of oilseed radish. It is noted that members of the Brassicaceae plant family, including oilseed radish, produced a category of secondary metabolites called glucosinolates. The release of these metabolites and their biocidal hydrolysis products in the rhizosphere by living roots was demonstrated for canola (Choesin and Boerner, 1991) and mustard roots (Schreiner and Koide, 1993) and directly impacted the rhizobiome (Bressan et al., 2009). We suggest that the release of glucosinolates and their breakdown products might have contributed to the observed high selection strength of both oilseed radish cultivars. Borage and phacelia (both Boraginaceae) had an overall strong effect on all organismal

groups. Borage belongs to the Boraginaceae subtribe Boraginae. Other representatives of this subtribe, namely members of the genus *Nonea,* were

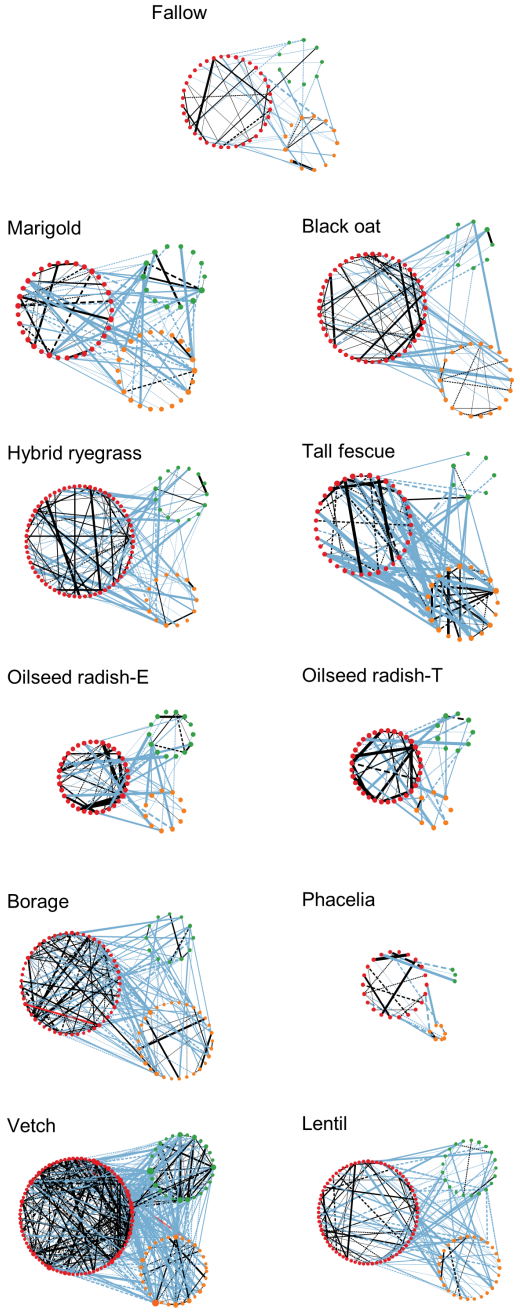

Figure 2.4 | Network co-occurrence analysis of the active rhizosphere microbiome. Each node represents a bacterial, fungal or protist taxon. The red, green and orange nodes

represent respectively bacterial families, fungal families, and protist orders. The node size is scaled based on the number of connections per node. The width of the connections (edges) represents the strength of the SPARCC correlation. Only significant ($p<0.05$) correlations with values > 0.6 (positive correlations, solid line) and <-0.6 (negative correlations, dashed line) were retained for analysis. The light blue edges indicate the interactions between different organismal groups (bacteria-fungi, fungi-protists, bacteria-protists), while black edges are used to indicate connections within members of the same organismal group.

demonstrated to secrete tricetin derivatives, a rare type of flavone (Wollenweber et al., 2002). Assuming that borage secretes similar types of flavones, it would be worth investigating whether this category of secondary metabolites is responsible for the observed effects. As compared to borage, phacelia showed an overall milder impact on the rhizobiome.

Lentil and vetch (both Fabaceae) had a remarkably strong impact on the fungal community. A stimulating effect of legumes on the fungal community abundance and diversity has been reported before and was associated with the relatively abundant release of amino acids, sugars and flavonoids in the rhizosphere (Isobe et al., 2001; Turner et al., 2013; Zhou et al., 2017). At the DNA level lentil and vetch induced comparable shifts in the microbiome, but at the RNA level substantial differences were observed between the two legumes. Although vetch and lentil are closely related plant species - both belong to the same tribe within the family Fabaceae (Fabeae) - vetch produces at least two ß-cyanoalanines that are not produced by lentil (Thavarajah et al., 2012). The more widespread repression of fungal taxa in the vetch rhizosphere might relate to these toxic substances, although it is unknown whether they are secreted in the rhizosphere.

This study included three representatives of the Poaceae, and it was remarkable to see that tall fescue had a stronger impact on the rhizobiome than black oat and hybrid ryegrass. Tall fescue showed a more widespread stimulation of rhizosphere microbiota and only a few taxa were repressed. Black oat belongs to the Poaceae subtribe Aveninae, while hybrid ryegrass and tall fescue both reside in the subtribe Loliinae, and thus are phylogenetically related. Hence, there is no phylogenetic rationale that could explain why tall fescue had a stronger impact on its rhizobiome than the two other poaceous cover crop species. However, tall fescue tends to have thicker and deeper root systems as compared to ryegrasses (Cheng et al., 2016). Because root traits impact the release of root exudates (Saleem et al., 2018), this may co-explain why tall fescue exerted a selection strength that exceeded one of the two other poaceous cover crops under investigation.

2.4.2 Compositional changes underlying differential selection strengths

Whereas an overview of all microbial taxa associated with individual cover crops was shown in Figure 2.2, the heat maps presented in Figure 2.3 focuses on microbial taxa that are significantly stimulated or repressed by one of multiple cover crop species. Generally, the absolute values of the positive beta coefficients representing the level of promotion of individual taxa, exceeded the level of repression, as represented by negative beta coefficients. So overall, the stimulating effect of cover crops on microbial life was stronger than their repressing effects. Here we highlight five cover crop-induced shifts that were particularly remarkable. Three examples involve soil microorganisms that were shown to be promoted by at least one of the cover crops investigated here, while two examples are given of cover crop-repressed microorganisms.

The increased abundance of the Pseudomonadaceae bacterial family in the rhizosphere of some cover crops was remarkable. This bacterial family constituted up to 37.9% and 15.5% of the oilseed radish and borage bacterial community in the rhizosphere and yielded beta coefficients up to 6.9. Strong plant-induced stimulation of the bacterial family Pseudomonadaceae has been reported before. In a field experiment with four crops including canola (*Brassica napus*), the *Brassica* species was shown to strongly stimulate endophytic *Pseudomonas* representatives over multiple years and locations (Cordero et al., 2020), and - to a lesser extent - Pseudomonadaceae in the rhizosphere. The bacterial family Pseudomonadaceae harbours plant pathogens, beneficial species that can act as biological control agents (Weller et al. 2002) as well as plant growth-promoting rhizobacteria (*e.g.*, Lugtenberg and Kamilova, 2009). It should be noted that levels of Pseudomonadaceae *per se* were reported not to be a good indicator of general disease suppressiveness in the transition study towards organic production (Marzano et al., 2015). Hence, more detailed monitoring is needed to assess the potential suppression-related implications of the observed stimulation of representatives of the family Pseudomonadaceae.

Two arbuscular mycorrhizal fungal (AMF) families, Glomeraceae and Claroideoglomeraceae, showed an increased presence and/or activity in the rhizosphere of some of the cover crops tested. Previously, the presence of Claroideoglomeraceae was linked to mechanically disturbed soils, while Glomeraceae were more abundant in undisturbed habitats (Moora et al., 2014). The cover crops characterized here were grown in mechanically disturbed soil. Lentil, and to a lesser extent marigold and vetch, exclusively induced enrichment of Claroideoglomeraceae. However, at the cDNA level, we observed an increased activity of both Glomeraceae and Claroideoglomeraceae for lentil,

and less prominently for vetch and marigold. Our results do not contradict the results of Moora et al. (2014) as this study focused on Glomeromycota and was performed at the DNA level only. There was no AMF signal to be expected for the Brassicaceae (non-host for AMF, Cosme et al., 2018), but we cannot explain why no increased AMF presence or activity compared to fallow soil was observed in the rhizosphere of the Poaceae included in this study.

The fungal family Cladosporiaceae showed an elevated presence in the rhizobiome in nine out of ten cover crops tested, but the most striking was the increase in its activity. This was most explicitly observed for borage, phacelia, lentil and vetch (beta coefficients > 5). The fungal family Cladosporiaceae harbours seven genera, the genera *Davidiella* and *Cladosporium* being by far the most widespread ones. *Davidiella* is most often found on aboveground tissues (Longley et al., 2020), whereas *Cladosporium* representatives are present both above- and belowground (Bensch et al., 2012). The genus *Cladosporium* harbours 189 described, mostly saprophytic, species (Sandoval-Denis et al., 2016a), and next to saprobes, this genus comprises above- and below-ground endophytes and plant pathogens. Uncharacterized *Cladosporium* members were recently detected in the rhizobiome of maize (Zhao et al., 2021) and also – in a non-agricultural setting – in the rhizosphere of giant goldenrod (Harkes et al., 2021). We hypothesize that soil-borne, saprophytic and/or pathogenic *Cladosporium* species are responsible for the observed increased presence and activity in the rhizosphere of nearly all cover crops rhizosphere compared to fallow.

The bacterial family Planococcaceae was identified as the most strongly repressed bacterial family, and the repression was almost exclusively observed in the oilseed radish rhizospheres (next to a mild repression by hybrid ryegrass). The family Caryophanaceae/Planococcaceae is a polyphyletic bacterial family with >100 species classified within 13 genera (Gupta and Patel, 2020). Recently, *Planococcus* was observed as an endophyte in sugar beet (*Beta vulgaris*) roots and in higher relative abundances in its rhizosphere (Li et al., 2020). However, no ecological explanation could be given for this shift. The absence of any known characteristics exclusive to all bacterial members of the family *Planococcaceae* (Gupta and Patel, 2020) makes it impossible to assess the ecological impact of *Planococcaceae* in the rhizosphere.

With high beta-coefficients, Microascaceae belonged to the strongest repressed fungal taxa among all cover crops, and this repression was only observed for oilseed radish. Microascaceae currently accommodate a morphologically heterogeneous group of fungi, comprising saprophytic and plant pathogenic species (Sandoval-Denis et al., 2016b). Fungal members of the Microascaceae family inhabit niches in association with different kinds of bark beetles, *Petriella*

and *Petriellopsis* are associated with soil, dung and compost (Lackner and de Hoog, 2011). We hypothesise that the toxicity of isothiocyanates associated with Brassicaceous plants (Bressan et al., 2009) may suppress this fungal family in the oilseed radish rhizosphere.

2.4.3 Active and resident fractions of the microbiome communities

The nucleic acid type was the second most relevant variable in our analyses of cover crop-affected microbiomes explaining 12-13% of the observed variation (Supplementary Table S2.5). The relevance of discriminating between the resident and the active fraction of the soil microbiome has been underlined before by, e.g., Harkes et al. (2019), Ofek et al. (2014) and Bay et al. (2021). Cover crops generally had a stronger selection strength on the active rather than of the resident soil microbiome, suggesting that RNA-based analyses may better reflect the effect of the environmental influence on the microbiome community assembly (Bay et al., 2021). DNA-based analyses allow studying microbes present in the soil in a range of states (dead, dormant, and active), while RNA analyses allow for studying the potentially active fraction of the soil microbiome (Blagodatskaya and Kuzyakov, 2013). For some taxa presented in Figure 2.2, this might be non-obvious as numerous low abundant taxa are residing in the category 'other (<2%)'. It was remarkable, however, to see that 69-78% of the microbial taxa affected at the DNA level were also affected at the cDNA level. Given that plants shape their rhizobiome to maximize their fitness (e.g., Berendsen et al., 2012), it was not surprising that manipulations observed at DNA and cDNA levels predominantly showed the same directionality. Convergence between resident and active rhizospheric communities was also reported by Bay et al. (2021), who suggested that this is the result of the strong selective environment established by plants on the indigenous soil microbiome.

2.4.4 Cover crop-induced changes in microbial networks

The network analyses presented here are based on the active fractions of the bacterial, fungal and protist communities in the rhizosphere of individual cover crops, and they allowed us to compare cover crop-induced changes with the fallow control. These co-occurrence networks could be instrumental in pinpointing potential biological interactions (Hirano and Takemoto, 2019; Lupatini et al., 2014; Shi et al., 2016). The high connectivity between bacteria and protists, for example, may reflect the feeding preferences of protists toward bacteria, rather than fungi (Gao et al., 2019). Except for phacelia, all cover crops induced microbial networks that were more complex than the one

of fallow control. This is in line with the principle of the 'rhizosphere effect', which implies that the plants' rhizosphere is a hotspot for microbial interactions (Pathan et al., 2020). Across cover crop rhizospheres, we observed great variation in the level of network complexity, as well as shifts in the interactions among organismal groups. These differences in the network complexity likely reflect the species-specific properties of the cover crops (Geisen et al., 2018; Santos et al., 2020). We hypothesised that the high complexity of the vetch microbiome might also be a result of the drought stress at the onset of this field experiment. In a ^{14}C labelling experiment, Sanaullah et al. (2012) reported that in the case of legumes, drought stress had a significantly smaller negative effect on root exudation than it had on grasses. It should, however, be noted that for the other legume in this field trial, lentil, no similar effect was observed.

Co-occurrence networks are suitable for the generation of hypotheses about the biological meaning of observed negative or positive associations. It is worth noting that high network complexity (*e.g.*, high number of nodes and edges, number of neighbours, high modularity) has been associated with soil-borne pathogen suppression (Yang et al., 2017), enhanced nutrient-cycling (Wagg et al., 2019) and higher crop productivity (Tao et al., 2018). For this reason, the value of network analyses might be even greater when stressors or pathogens are present in the study stem.

Network analyses presented here are based solely on the active fractions of the rhizosphere microbiomes, whereas most soil microbiome studies concentrate on the resident microbial community. This choice is defendable as only non-dormant taxa can actively engage in biological interactions.

2.4.5 The inclusion of primary consumers in the characterisation of cover crops rhizosphere

Bacterial and fungal communities in the cover crop rhizospheres are the result of the composition of the local microbial community in the soil, bottom-up control by the individual cover crops, and top-down regulation by primary consumers. The primary consumer community is here represented by protists and metazoa (mainly nematodes). It is noted that the primary consumer activity affects plant growth directly as grazing of the bacterial (and to a lesser extent fungal) biomass by protists results in the release of plant-available N (Clarholm, 1985; Xiong et al., 2020). Essentially the same holds for the impact of selective grazing by bacterivorous nematodes (Schratzberger et al., 2019). In this study, the importance of protists in the structuring of the rhizosphere microbiome assembly could be demonstrated by the high number of interactions between bacteria and protists in the network analysis and to a lesser extent between protists and fungi. In our study, the majority of

interactions across organismal groups were positive, pointing at symbiotic and cooperative interactions (Jousset et al., 2008; Rossmann et al., 2020).

Large cover crop-specific shifts were observed both in the resident and the active fractions of the protistan community. One of the most striking examples is the strong activation of the protist order Cryomonadida (Cercozoa phylum) in the rhizospheres of the two oilseed radish cultivars as well as borage. Cryomonadida are known as amoeboid eukaryvores (Fiore-Donno et al., 2022). With slightly lower beta coefficient values, the stimulation of Cryomonadida was paralleled by a clear activation of Chrysophyceae, again mainly in both oilseed radish cultivars and borage (Supplementary Table S2.13). Chrysophyceae are predominantly unicellular, golden-brown algae that commonly occur in arable soils in temperate climate zones (Lentendu et al., 2014), and we hypothesize that the observed activation of Cryomonadida could be the result of a cover crop-specific stimulation of golden-brown algae in the rhizosphere. Nevertheless, this observation was not supported by the network analysis.

Concerning Metazoa, it should be mentioned that the subsamples analysed in this study, 2 g, might have been too small to get a proper representation of the metazoan community. A more complete representation of the nematode community would require an upscaling of the DNA and RNA extraction procedure described by Harkes et al. (2019). Evidently the subsample size depends on average size of members of an organismal group, as well as their spatial distribution. For bacteria and fungi, subsamples of 0.25 – 2.0 g is the golden standard (Wydro, 2022). This also holds for protists. For nematodes, traditionally 200 g of soil is used, this figure can be reduced till 100 g is case molecular detection methods are used (Wiesel et al., 2015). Rhizosphere soil has a much higher nematode density than bulk soil. Hence, 2 g samples can be informative, but will not provide a robust nematode community overview.

2.4.6 Implication of the experimental design

A significant effect of blocks was observed for all four organismal groups, and in particular for the metazoa (17% vs ~7% in other organismal groups). This effect can largely be explained by the layout of the field experiment. Each block (≈ 4 m x 4 m) included 11 treatments, *viz.,* 10 cover crops and a fallow control. The eight blocks were positioned next to each other in a long rectangle (≈ 4 m x 60 m). This rectangle was positioned next to a maize field with a final crop height of about 3 m. As a result, there were slight differences in insolation between the individual blocks. This might have resulted in differences in soil temperature as this is mainly determined by ambient temperature and direct irradiation. However, as all treatments were represented in all blocks, this slight

insolation gradient along the blocks could not have had a systematic effect on cover crop-induced changes in the soil microbiome.

2.5 Conclusions

Here we pinpointed the differential effects of ten cover crop species on both the resident and the active fractions of bacterial, fungal and protistan communities in the rhizosphere. Our results indicated that oilseed radish cover crops had the strongest effect on the rhizospheric microbial communities, together with borage. Vetch and, most explicitly lentil, had a strong stimulating effect on the fungal rhizosphere community, and similarly marigold influenced the fungal community more than the bacterial and protistan ones. Representatives of the Poaceae - especially black oat and hybrid ryegrass - had a remarkably mild impact on the soil microbiome. Hence, it is concluded that cover crops differ in the extent by which they manipulate the native microbiome in their rhizospheres. Subsequently, we investigated whether microbial taxa were promoted or repressed in a cover crop-specific manner. Differential abundance analyses revealed a range of cover crop-specific microbial shifts, and even a differential impact of two cultivars of the same cover crop species (oilseed radish) could be pinpointed. We conclude that individual cover crops affect soil microbial taxa in a cover crop genotype-specific manner. RNA-based network analyses revealed that most cover crops induced an increase in the microbial network complexity as compared to the fallow soil. However, the level of increase was shown to be cover crop species-specific. Based on microbial network parameters reflecting the level of network complexity, we conclude that individual cover crops had distinct effects on the degree of potential associations between the three main organismal groups, bacteria, fungi, and protists.

Overall, our data suggest that poaceous and fabaceous cover crops could be suitable for a general stimulation of soil microbiota, while members of the plant families Boraginaceae, and, most explicitly, Brassicaceae leave a relatively strong mark on the microbial community by promoting and repressing specific taxa of the native soil microbiome. The current dataset should be seen as a starting point for the application of specific cover crop species or mixtures thereof to steer the soil microbiome in a predictable direction to promote soil health and sustain healthy crop growth. Further studies should be aimed at determining to what extent the effects of cover crops on the soil microbiome persist over time and thus may affect the growth and development of the main crop.

Data Availability

All sequences have been submitted to the NCBI database under BioProject ID PRJNA842568.

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2.6 Supplementary Material

Supplementary Table S2.1 | Total amount of fertilizers applied on the SHE field between 2015 and June 2019, Personal communications

Dates	Previously Conventional plots	Previously Organic plots
8-apr-15	25 m3/ha cattle manure	10 ton/ha cattle manure + 20 m3/ha cattle slurry
15-mrt-16	275 K-60 = 165 k20	35 m3/ha cattle slurry
22-mrt-16	290 kg CAN*	none
16-mrt-17	none	25.5 ton/ha cattle manure
22-mrt-17	40 ton/ha cattle manure	25 ton/ha cattle slurry
13-apr-17	200 kg CAN*	none
12-jun-17	200 kg CAN*	none
14-mrt-18	22 m3/ha cattle slurry	22 m3/ha cattle slurry
29-mrt-19	25 m3/ha cattle slurry	25 m3/ha cattle slurry
12-jun-19	40 m3/ha cattle slurry	40 m3/ha cattle slurry

*Calcium ammonium nitrate.

Supplementary Table S2.2 | Weather data of June, July, August, September and October 2019 received by Dacom Farm Intelligence.

Month	T-average (°C)	T-max (°C)	T-min (°C)	Precipitation (mm)	Irradiation (W/m2)	Relative humidity (%)
June	19,2	34,8	9,0	1,6	3,3	50,4
July	19,1	38,6	7,2	1,4	6,0	50,3
August	18,8	33,3	7,4	2,3	5,5	53,2
September	14,6	27,4	2,3	1,1	3,7	57,4
October	11,9	23,9	-1,5	3,2	1,8	73,7

Supplementary Table S2.3 | Weather data of June, July, August, September and October 2019 received by Dacom Farm Intelligence.

code	Cover crop	Previously Organic	Previously Conventional
A	Fallow	4	4
B	Black oat	4	4
C	Marigold	3	3
D	Tall fescue	2	2
E	Oilseed radish Terranova	4	4
F	Oilseed radihs E1089	4	4
G	Phacelia	2	2
H	Borago	3	3
I	Vetch	3	3
J	Lentil	4	4
K	Hybrid ryegrass	4	4
		Tot = 37	Tot = 37
			Tot = 74 samples

Supplementary Table S2.4 | Primers used in the first PCR step for amplification of organismal group-specific 16S and 18S rRNA regions, with adaptor sequence (underlined) and locus-specific sequence (bold).

Organism	rRNA region	Primer	Primer sequence	Refs
Bacteria	V4	515 F	TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG **GTGCCAGCMGCCGCGGTAA**	(Caporaso et al., 2012)
	(16S)	806 R	GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAG **GGACTACHVGGGTWTCTAAT**	
Fungi	V7-8	FF 390.1	TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG**C** **GWTAACGAACGAGACCT**	(Verbruggen et al., 2012)
	(18S)	FR1	GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAG **AICCATTCAATCGGTAIT**	
Protists	V9	1391 F	TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG **GTACACACCGCCCGTC**	(Lane, 1991)
	(18S)	EukBr	GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAG **TGATCCTTCTGCAGGTTCACCTAC**	(Medlin et al., 1988)

Metazoa	V5-7	M1041F	TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG **AGAGGTGAAATTCTTGGAYCGY**	(Capra et al., 2016)
	(18S)	M1648R	GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAG **ACATCTAAGGGCATCACAGAC**	

Supplementary Table S2.5 | PERMANOVA analysis with Bray-Curtis dissimilarity metric on the four organismal groups to assess the variation explained by the different factors influencing the microbial communities: cover crop treatment (tagetes, black oat, hybrid ryegrass, tall fescue, oilseed radish -E and -T, vetch, lentil, borage, phacelia and fallow), nucleic acid (DNA vs cDNA) and block (1-8). Df = degrees of freedom, SumsOfSqs = sum of squared, MeanSqs = mean squares, F.Model = F statistics, R^2 = fraction of the variation explained by the experimental factor. P = p-values < 0.01 indicate factors that significantly influence the microbiome.

Bacteria						
	Df	SumsOfSqs	MeanSqs	F.Model	R^2	P
Cover crop treatment	10	11.397	1.1397	7.7683	0.30526	0.001
nucleic acid	1	4.467	4.4669	30.4476	0.11965	0.001
block	7	2.692	0.3846	2.6217	0.07212	0.001
Residuals	128	18.778	0.1467		0.50298	
Total	146	37.334			1	
Fungi						
	Df	SumsOfSqs	MeanSqs	F.Model	R^2	P
Cover crop treatment	10	6.8127	0.68127	10.875	0.38018	0.001
nucleic acid	1	2.1355	2.13548	34.09	0.11917	0.001
block	7	1.1411	0.16302	2.602	0.06368	0.001
Residuals	125	7.8304	0.06264		0.43697	
Total	143	17.9197			1	
Protists						
	Df	SumsOfSqs	MeanSqs	F.Model	R^2	P
Cover crop treatment	10	9.189	0.9189	6.61	0.2673	0.001
nucleic acid	1	4.508	4.5077	32.425	0.13112	0.001
block	7	2.887	0.4124	2.966	0.08397	0.001
Residuals	128	17.794	0.139		0.51761	
Total	146	34.378			1	
Metazoa						
	Df	SumsOfSqs	MeanSqs	F.Model	R^2	P
Cover crop treatment	10	2.3268	0.232676	3.4084	0.33261	0.001
block	7	1.2555	0.179359	2.6274	0.17947	0.001
Residuals	50	3.4133	0.068266		0.48792	
Total	67	6.9955			1	

Supplementary Table S2.6 | R2 values of the Pairwise PERMANOVA based on Bray Curtis distance. Bold values are supported by a significant p value (shown in Supplementary Tables S2.7 A, B, C, D, available online).

E **Bacteria - Pairwise PERMANOVA - R2**

		Fallow	Marigold	Black oat	Hybrid ryegrass	Tall fescue	Oilseed radish E	Oilseed radish T	Borage	Phacelia	Lentil
DNA	Marigold	**0.14**									
	Black oat	**0,13**	**0,11**								
	Hybrid ryegrass	**0,12**	**0,13**	0,08							
	Tall fescue	**0,21**	**0,19**	**0,14**	**0,14**						
	Oilseed radish E	**0,44**	**0,38**	**0,34**	**0,36**	**0,41**					
	Oilseed radish T	**0,42**	**0,35**	**0,32**	**0,34**	**0,39**	0,07				
	Borage	**0,27**	**0,20**	**0,20**	**0,22**	**0,26**	**0,36**	**0,34**			
	Phacelia	**0,20**	0,13	**0,15**	**0,16**	0,19	**0,35**	**0,33**	**0,19**		
	Lentil	**0,16**	**0,16**	**0,13**	**0,10**	0,11	**0,39**	**0,37**	**0,21**	**0,15**	
	Vetch	**0,20**	**0,17**	**0,15**	**0,14**	0,12	**0,41**	**0,39**	**0,21**	0,14	0,07

cDNA	Marigold	**0,16**									
	Black oat	**0,16**	**0,12**								
	Hybrid ryegrass	**0,13**	**0,12**	**0,10**							
	Tall fescue	**0,29**	**0,25**	**0,21**	**0,19**						
	Oilseed radish E	**0,41**	**0,37**	**0,33**	**0,33**	**0,43**					
	Oilseed radish T	**0,37**	**0,32**	**0,29**	**0,29**	**0,39**	0,06				
	Borage	**0,34**	**0,25**	**0,28**	**0,26**	**0,33**	**0,38**	**0,35**			
	Phacelia	**0,27**	**0,19**	**0,22**	**0,20**	0,21	**0,38**	**0,34**	**0,23**		
	Lentil	**0,24**	**0,21**	**0,21**	**0,17**	**0,14**	**0,41**	**0,38**	**0,27**	**0,18**	
	Vetch	**0,21**	**0,16**	**0,17**	**0,14**	0,14	**0,35**	**0,31**	**0,20**	0,14	0,08

F **Fungi - Pairwise PERMANOVA - R2**

		Fallow	Marigold	Black oat	Hybrid ryegrass	Tall fescue	Oilseed radish E	Oilseed radish T	Borage	Phacelia	Lentil
DNA	Marigold	**0,31**									
	Black oat	**0,12**	**0,15**								
	Hybrid ryegrass	**0,18**	**0,14**	0,08							
	Tall fescue	**0,25**	0,22	**0,21**	**0,18**						
	Oilseed radish E	**0,52**	**0,35**	**0,43**	**0,40**	**0,53**					
	Oilseed radish T	**0,42**	**0,28**	**0,33**	**0,30**	**0,43**	0,07				
	Borage	**0,35**	**0,15**	**0,23**	**0,23**	**0,32**	**0,44**	**0,32**			
	Phacelia	**0,29**	0,13	**0,19**	**0,18**	**0,28**	**0,36**	**0,26**	0,14		
	Lentil	**0,35**	**0,19**	**0,28**	**0,24**	**0,25**	**0,45**	**0,37**	**0,25**	**0,21**	
	Vetch	**0,36**	0,16	**0,28**	**0,25**	**0,27**	**0,49**	**0,41**	**0,23**	**0,19**	0,13

		Fallow	Marigold	Black oat	Hybrid ryegrass	Tall fescue	Oilseed radish E	Oilseed radish T	Borage	Phacelia	Lentil
cDNA	Marigold	**0,36**									
	Black oat	**0,17**	**0,17**								
	Hybrid ryegrass	**0,23**	0,15	0,12							
	Tall fescue	**0,32**	**0,29**	**0,30**	**0,19**						
	Oilseed radish E	**0,51**	**0,42**	**0,43**	**0,41**	**0,49**					
	Oilseed radish T	**0,49**	**0,36**	**0,39**	**0,36**	**0,47**	0,08				
	Borage	**0,44**	**0,29**	**0,35**	**0,29**	**0,25**	**0,46**	**0,42**			
	Phacelia	**0,41**	**0,30**	**0,35**	**0,27**	**0,25**	**0,41**	**0,38**	**0,17**		
	Lentil	**0,46**	**0,30**	**0,39**	**0,34**	**0,27**	**0,50**	**0,49**	**0,32**	**0,31**	
	Vetch	**0,43**	**0,23**	**0,35**	**0,27**	**0,22**	**0,49**	**0,47**	**0,20**	**0,22**	0,12

G **Protists - Pairwise PERMANOVA - R2**

		Fallow	Marigold	Black oat	Hybrid ryegrass	Tall fescue	Oilseed radish E	Oilseed radish T	Borage	Phacelia	Lentil
DNA	Marigold	**0,15**									
	Black oat	**0,16**	0,10								
	Hybrid ryegrass	**0,14**	0,11	0,07							
	Tall fescue	**0,22**	**0,24**	**0,22**	**0,21**						
	Oilseed radish E	**0,35**	**0,29**	**0,23**	**0,26**	**0,37**					
	Oilseed radish T	**0,36**	**0,30**	**0,23**	**0,25**	**0,39**	0,06				
	Borage	**0,23**	**0,18**	**0,20**	**0,21**	**0,20**	**0,33**	**0,34**			
	Phacelia	**0,26**	**0,23**	**0,22**	**0,24**	0,19	**0,34**	**0,36**	**0,16**		
	Lentil	**0,16**	**0,19**	**0,18**	**0,17**	0,13	**0,36**	**0,36**	**0,18**	**0,15**	
	Vetch	**0,18**	**0,20**	**0,17**	**0,17**	0,14	**0,34**	**0,35**	**0,17**	**0,16**	0,07

cDNA	Marigold	**0,20**									
	Black oat	**0,20**	**0,11**								
	Hybrid ryegrass	**0,15**	**0,16**	**0,10**							
	Tall fescue	**0,26**	**0,24**	**0,18**	**0,21**						
	Oilseed radish E	**0,40**	**0,30**	**0,28**	**0,31**	**0,39**					
	Oilseed radish T	**0,43**	**0,34**	**0,30**	**0,33**	**0,40**	0,06				
	Borage	**0,25**	**0,18**	**0,15**	**0,20**	0,14	**0,33**	**0,35**			
	Phacelia	**0,25**	**0,21**	**0,17**	**0,21**	0,14	**0,35**	**0,38**	0,11		
	Lentil	**0,21**	**0,18**	**0,14**	**0,17**	0,09	**0,34**	**0,36**	0,07	0,08	
	Vetch	**0,23**	**0,18**	**0,15**	**0,19**	0,12	**0,35**	**0,37**	0,08	0,07	0,05

H **Metazoa (DNA) - Pairwise PERMANOVA - R2**

	Fallow	Marigold	Black oat	Hybrid ryegrass	Tall fescue	Oilseed radish E	Oilseed radish T	Borage	Phacelia	Lentil
Marigold	0,14									
Black oat	0,14	**0,31**								
Hybrid ryegrass	0,07	0,16	0,04							
Tall fescue	0,12	0,30	0,04	0,05						
Oilseed radish E	**0,19**	**0,29**	**0,22**	**0,17**	**0,26**					
Oilseed radish T	**0,22**	**0,32**	**0,28**	**0,19**	**0,30**	0,04				
Borage	**0,27**	**0,33**	**0,24**	**0,21**	0,20	**0,32**	**0,33**			
Phacelia	0,15	0,22	0,13	0,11	0,12	**0,32**	**0,35**	0,16		
Lentil	**0,20**	**0,23**	0,14	0,10	0,14	**0,31**	**0,34**	**0,22**	**0,18**	
Vetch	**0,19**	0,22	0,12	0,10	0,13	**0,37**	**0,42**	**0,23**	0,13	0,09

Supplementary Table S2.7 | Alpha diversity results. Cover crop treatments and values of alpha diversity metrics: Observed, Shannon and Chao1, followed by significance groups (sign. groups) indicating significant differences among treatments. Highlighted in green the highest alpha diversity scores, in orange the lowest and in blue the scores for the fallow treatment.

A) Bacteria DNA

Observed	group value	sign. group	Shannon	group value	sign. group	Chao1	group value	sign. group
Vetch	65,00	a	Vetch	65,17	a	Vetch	65,00	a
Tall fescue	62,75	a	Tall fescue	63,75	ab	Tall fescue	62,75	a
Lentil	57,75	a	Lentil	56,25	abc	Lentil	58,00	a
Phacelia	56,75	a	Phacelia	53,75	abcd	Phacelia	56,75	a
Borage	48,67	ab	Borage	39,00	bcd	Borage	48,67	ab
Hybrid ryegrass	34,50	b	Hybrid ryegrass	37,43	cd	Hybrid ryegrass	34,43	b
Black oat	32,31	b	Fallow	35,13	d	Black oat	32,25	b
Marigold	31,50	b	Black oat	35,00	d	Marigold	31,50	b
Fallow	31,13	b	Marigold	32,67	d	Fallow	30,88	b
Oilseed radish-E	9,25	c	Oilseed radish-T	8,75	e	Oilseed radish-E	9,38	c
Oilseed radish-T	8,38	c	Oilseed radish-E	8,38	e	Oilseed radish-T	8,38	c

B) Bacteria cDNA

Observed	group value	sign. group	Shannon	group value	sign. group	Chao1	group value	sign. group
Tall fescue	70,13	a	Tall fescue	67,75	a	Tall fescue	70,25	a
Lentil	61,88	a	Phacelia	62,50	ab	Lentil	61,50	a
Phacelia	61,00	ab	Lentil	59,00	ab	Phacelia	60,75	a
Vetch	52,58	abc	Vetch	48,50	abc	Vetch	52,83	ab
Borage	51,33	abcd	Borage	40,50	abc	Borage	52,50	ab
Marigold	36,67	bcde	Marigold	40,50	abc	Marigold	36,50	bc
Hybrid ryegrass	32,25	cde	Hybrid ryegrass	35,75	bc	Hybrid ryegrass	31,75	c
Black oat	31,25	de	Black oat	35,38	bc	Black oat	31,63	c
Fallow	29,00	ef	Fallow	34,50	c	Fallow	28,63	cd
Oilseed radish-E	11,38	f	Oilseed radish-T	10,50	d	Oilseed radish-E	11,25	d
Oilseed radish-T	10,13	f	Oilseed radish-E	9,50	d	Oilseed radish-T	10,25	d

C) Fungi DNA

Observed	group value	sign. group	Shannon	group value	sign. group	Chao1	group value	sign. group
Vetch	65,33	a	Tall fescue	61,00	a	Vetch	66,25	a
Lentil	63,75	a	Vetch	55,50	a	Lentil	63,50	ab
Tall fescue	63,50	ab	Lentil	51,13	ab	Tall fescue	61,38	ab
Phacelia	50,50	abc	Phacelia	46,75	abc	Phacelia	47,50	abc
Borage	36,42	bcd	Hybrid ryegrass	45,50	abc	Borage	40,92	abcd
Marigold	34,75	bcd	Marigold	41,50	abc	Marigold	38,25	bcd
Fallow	32,81	cd	Black oat	32,50	abc	Fallow	31,88	cd
Hybrid ryegrass	28,56	cd	Oilseed radish-E	30,38	abc	Oilseed radish-E	27,81	cd
Oilseed radish-E	26,75	cd	Fallow	28,50	abc	Hybrid ryegrass	26,25	cd
Black oat	18,75	d	Oilseed radish-T	20,50	bc	Black oat	17,88	d
Oilseed radish-T	16,88	d	Borage	15,67	c	Oilseed radish-T	16,06	d

D) Fungi cDNA

Observed	group value	sign. group	Shannon	group value	sign. group	Chao1	group value	sign. group
Lentil	60,38	a	Phacelia	53,25	a	Lentil	60,75	a
Vetch	60,00	a	Lentil	52,13	a	Vetch	58,83	a
Phacelia	53,25	ab	Vetch	51,50	ab	Phacelia	53,50	a
Tall fescue	50,25	abc	Tall fescue	38,75	ab	Tall fescue	52,00	a
Borage	44,67	abc	Hybrid ryegrass	35,43	ab	Borage	51,00	a
Fallow	27,69	bcd	Oilseed radish-E	34,86	ab	Fallow	26,44	b
Oilseed radish-E	26,86	bcd	Fallow	32,38	ab	Oilseed radish-E	26,07	b
Hybrid ryegrass	25,71	cd	Oilseed radish-T	30,00	ab	Hybrid ryegrass	24,50	b
Marigold	25,00	cd	Borage	24,83	ab	Marigold	23,20	b
Oilseed radish-T	21,06	d	Marigold	24,00	ab	Oilseed radish-T	20,50	b
Black oat	11,00	d	Black oat	18,71	ab	Black oat	10,36	b

E) Protists DNA

Observed			Shannon			Chao1		
	group value	sign. group		group value	sign. group		group value	sign. group
Tall fescue	65,00	a	Tall fescue	68,00	a	Tall fescue	64,50	a
Phacelia	61,00	ab	Vetch	55,00	ab	Phacelia	62,75	a
Lentil	60,13	ab	Lentil	51,25	ab	Lentil	59,50	a
Vetch	57,17	ab	Fallow	43,13	ab	Vetch	55,83	a
Borage	46,58	abc	Borage	42,17	ab	Borage	48,83	ab
Fallow	44,00	bcd	Marigold	39,00	abc	Fallow	43,63	abc
Marigold	29,83	cde	Phacelia Hybrid	37,00	abcd	Marigold Hybrid	30,00	bcd
ryegrass	27,86	de	ryegrass	36,86	abcd	ryegrass	26,57	cd
Black oat	26,13	e	Black oat	34,88	bcd	Black oat	24,13	de
Oilseed radish-T	13,63	ef	Oilseed radish-T	12,50	cd	Oilseed radish-T	14,50	de
Oilseed radish-E	6,19	f	Oilseed radish-E	9,00	d	Oilseed radish-E	8,00	e

F) Protists cDNA

Observed			Shannon			Chao1		
	group value	sign. group		group value	sign. group		group value	sign. group
Tall fescue	65,50	a	Tall fescue	51,00	a	Tall fescue	65,00	a
Phacelia	60,88	a	Fallow	50,50	a	Phacelia	61,00	a
Vetch	60,83	a	Vetch	49,50	ab	Vetch	60,83	a
Lentil	60,31	a	Black oat	48,63	ab	Lentil	60,13	a
Borage	55,08	a	Phacelia	46,50	abc	Borage	56,67	a
Fallow	34,81	b	Lentil	44,38	abc	Fallow	34,00	b
Black oat Hybrid	33,44	b	Hybrid ryegrass	39,75	abc	Black oat Hybrid	32,88	b
ryegrass	29,00	b	Marigold	36,67	abc	ryegrass	29,25	b
Marigold	26,17	bc	Borage	26,00	abc	Marigold	26,17	bc
Oilseed radish-E	10,94	cd	Oilseed radish-E	15,75	abc	Oilseed radish-E	11,13	cd
Oilseed radish-T	8,63	d	Oilseed radish-T	15,00	ac	Oilseed radish-T	8,75	d

Metazoa DNA

Observed			Shannon			Chao1		
	group value	sign. group		group value	sign. group		group value	sign. group
Borage	59,83	a	Lentil Tall fescue	51,88	a	Borage	61,17	a
Vetch	50,75	a		46,25	a	Vetch	50,83	ab
Lentil	45,94	a	Phacelia	45,00	a	Phacelia	45,88	abc
Phacelia Tall fescue	45,50	ab	Vetch Hybrid ryegrass	44,00	a	Lentil Tall fescue	45,50	abc
Black oat Hybrid ryegrass	44,38	ab		42,43	a		42,88	abc
Marigold Oilseed radish-E	35,42	ab	Marigold	42,00	ab	Black oat Hybrid ryegrass	35,00	abc
Oilseed radish-T	32,50	ab	Black oat	40,83	ab		31,14	bc
Fallow	26,67	ab	Borage	34,50	ab	Marigold Oilseed radish-E	24,67	bc
	19,56	b	Fallow Oilseed radish-E	31,88	ab		22,38	c
	17,81	b		10,75	b	Oilseed radish-T	18,94	c
	17,06	b	Oilseed radish-T	10,75	b	Fallow	15,31	c

Supplementary tables from S2.8 to S2.13 are available online at https://doi.org/10.1016/j.soilbio.2023.109080

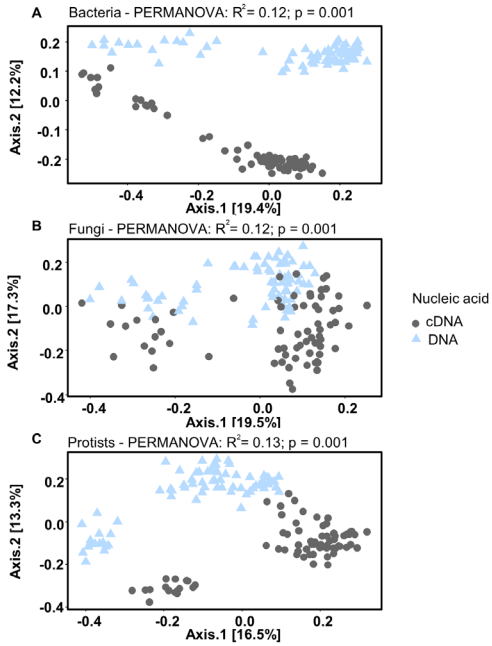
Supplementary Table S2.14 | Statistics of the network analyses. A) Summary of the network analysis parameters (by Cytoscape sw). Co-occurrence correlations were calculated with SparCC approach. Only significant (SparCC bootstrap = $p<0.05$) correlations higher than 0.6 or lower than -0.6 were used to build the network. B) Summary table of the interactions within and across organismal groups (multitrophic interactions).

A	Fallow	Marigold	Black Oat	Hybrid Ryegrass	Tall Fescue	Oilseed radish-E	Oilseed radish-T	Borage	Phacelia	Lentil	Vetch
nucleic acid	cDNA	cDNA	cDNA	cDNA	cDNA	cDNA	cDNA	cDNA	cDNA	cDNA	cDNA
#nodes	59	69	79	93	61	59	50	95	32	120	141
#edges	55	98	115	123	127	79	63	182	26	138	468
Average #neighbors	2,286	3,125	3,226	2,961	5,61	2,913	3,04	3,259	2,167	2,789	7,398
Network diameter	15	11	14	14	5	10	9	17	7	12	16
Network radius	8	6	8	7	3	6	5	9	4	6	8
Average path length	5,793	4,216	5,992	6,286	2,395	4,523	3,75	6,209	3,121	4,633	4,811
Clustering coefficient	0,245	0,243	0,293	0,281	0,235	0,204	0,403	0,272	0,167	0,308	0,397
Network density	0,067	0,101	0,053	0,039	0,14	0,065	0,127	0,061	0,197	0,075	0,061
Network heterogeneity	0,486	0,663	0,538	0,551	0,644	0,619	0,47	0,679	0,493	0,611	0,98
Network centralization	0,085	0,133	0,098	0,082	0,247	0,141	0,134	0,132	0,2	0,149	0,23
Connected components	10	10	7	8	9	4	7	4	8	19	6

* including interactions within the same organismal group and across groups

B

		Fallow	Marigold	Black oat	Hybrid ryegrass	Tall fescue	Oilseed radish - E	Oilseed radish - T	Borage	Phacelia	Lentil	Vetch
All con	overall	55	98	115	123	126	79	63	182	26	138	468
	positive	28	50	72	82	58	50	38	111	8	87	248
	negative	27	48	43	41	68	29	25	71	18	51	220
%	positive ratio	0,43	0,52	0,68	0,63	0,49	0,69	0,52	0,63	0,30	0,62	0,48
	negative ratio	0,57	0,48	0,32	0,37	0,51	0,31	0,48	0,38	0,70	0,38	0,52
bacteria-protists	total	22	30	31	35	64	15	17	57	7	43	80
	ratio on tot interactions	0,40	0,31	0,27	0,28	0,51	0,19	0,27	0,31	0,27	0,31	0,17
	positive	10	18	25	19	29	11	10	34	2	30	43
	negative	12	12	6	16	35	4	7	23	5	13	37
	ratio positive	0,45	0,60	0,81	0,54	0,45	0,73	0,59	0,60	0,29	0,70	0,54
	ratio negative	0,55	0,40	0,19	0,46	0,55	0,27	0,41	0,40	0,71	0,30	0,46
bacteria-fungi	total	9,00	31,00	16,00	21,00	14,00	14,00	10,00	23,00	3,00	33,00	123,00
	ratio on tot interactions	0,16	0,32	0,14	0,17	0,11	0,18	0,16	0,13	0,12	0,24	0,26
	positive	3,00	15,00	8,00	18,00	7,00	9,00	4,00	16,00	1,00	20,00	56,00
	negative	6,00	16,00	8,00	3,00	7,00	5,00	6,00	7,00	2,00	13,00	67,00
	ratio positive	0,33	0,48	0,50	0,86	0,50	0,64	0,40	0,70	0,33	0,61	0,46
	ratio negative	0,67	0,52	0,50	0,14	0,50	0,36	0,60	0,30	0,67	0,39	0,54
protists-fungi	total	4,00	14,00	3,00	9,00	7,00	7,00	4,00	16,00	0,00	13,00	44,00
	ratio on tot interactions	0,07	0,14	0,03	0,07	0,06	0,09	0,06	0,09		0,09	0,09
	positive	2,00	6,00	1,00	4,00	6,00	5,00	2,00	10,00	0,00	5,00	20,00
	negative	2,00	8,00	2,00	5,00	1,00	2,00	2,00	6,00	0,00	8,00	24,00
	ratio positive	0,50	0,43	0,33	0,44	0,86	0,71	0,50	0,63		0,38	0,45
	ratio negative	0,50	0,57	0,67	0,56	0,14	0,29	0,50	0,38		0,62	0,55



Supplementary Figure S2.1 | Principal Coordinate Analysis (PCoA) of the rhizosphere microbiome of cover crops and fallow. A) bacterial community, B) fungal community and C) protists communities. Grey circles represent the potentially active (cDNA) communities, blue triangles represent the resident (DNA) communities.

Supplementary Figure S2.2 | available online at https://doi.org/10.1016/j.soilbio.2023.109080

Chapter 3

On the legacy of cover crop-specific microbial footprints

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Abstract

Apart from improving the physical and chemical condition of arable soils, cover crops have the potential to boost and activate selected soil microbiota that could contribute to improved nutrient cycling and strengthened disease suppressiveness. However, a main crop can only benefit from cover crop-induced microbial shifts if these persist until the onset of the main growing season. Here, we map the persistence of microbiome changes by cover crops over time. We performed a field experiment on a sandy soil with ten different cover crop monocultures belonging to five plant families, one cover crop mixture and a fallow control. Cover crops were grown for 4.5 months under field conditions in 70-litre bottomless containers in a random block design with eight replications. We studied the total (DNA-based) and the potentially active (RNA-based) microbial fractions at the onset of the main growing season, and just after the harvest of the main crop, potato (respectively 3.5 and 10 months after cover crop termination), through MiSeq sequencing. All cover crops tested induced shifts in the soil microbiome that lasted at least until the onset of the main growing season. Cover crop treatments gave rise to species and even cultivar-specific microbial footprints, and - although roughly the same trends were observed - DNA-based microbial shifts were not necessarily paralleled by similar changes at RNA level. We conclude that cover crops have the potential to act as handles to steer the soil microbiome in a way that is supportive of sustainable crop production.

3.1 Introduction

Protection, restoration and promotion of sustainable use of terrestrial ecosystems belong to the targets of 'Life on Land', one of the United Nations Sustainable Development Goals (UN, 2015). Achieving this goal will require a wide range of adaptations of soil management practices to maintain productivity while supporting natural processes that promote and regenerate ecosystem services (Bommarco et al., 2013; Garibaldi et al., 2019; Kremen, 2020). The use of cover crops is considered a tool that could contribute to the realisation of some of these goals (Wittwer et al., 2017). Winter cover crops are non-cash crops that essentially are grown between cash crop growing seasons to protect against soil erosion and prevent nutrient leaching (Blanco-Canqui et al., 2015; Kaye and Quemada, 2017; Daryanto et al., 2018). At the end of their growing season, cover crops are mowed and incorporated into the topsoil and as such, they increase the organic matter content of soils (Blanco-Canqui et al., 2015). Furthermore, cover crops can amply affect soil microbial growth and activity (Vukicevich et al., 2016; Finney et al., 2017; Kim et al., 2020).

Rhizodeposition, the active and/or passive release of organic and inorganic components from living plant roots (Wichern et al., 2008), promotes a selection of the soil bacterial and fungal communities (Hartmann et al., 2009; Philippot et al., 2013). Plants invest heavily in the generation of a beneficial microbiome in the rhizosphere (Berendsen et al., 2012), which, in turn, supports the plant in nutrient acquisition and disease suppressiveness (Philippot et al., 2013). Together with plant exudation, predation by soil protists is the other main factor shaping the rhizosphere microbiome. By selective grazing on bacteria and fungi, protists contribute to enhancing nutrient turnover, which stimulates microbial activity (Gao et al., 2019; Xiong et al., 2020; Guo et al., 2022).

Soil microbial organisms should be metabolically active in order to have any effect on plants. In bulk soil typically 80% of the cells and 60% of the microbial taxa are dormant (Lennon and Jones, 2011). Although they can be resuscitated upon environmental changes, dormant microbes - as long as they are metabolically inactive - do not contribute to soil functioning. DNA-based community characterization will map both the active and the dormant fractions. To understand the effects of cover crops on the functioning of the soil microbiome it is informative to map the (RNA-based) potentially active microbial fraction in parallel (Jones and Lennon, 2010; Blagodatskaya and Kuzyakov, 2013). In some cases, ribosomal RNA (rRNA) was shown to be present in dormant bacterial cells (*e.g.,* Sukenik et al., 2012). For this reason, rRNA data are used here as an index for potential activity (Blazewicz et al., 2013), rather than for microbial activity *per se.* Contrasts between rDNA-based

and rRNA-based microbial communities in an agronomic setting have been mapped previously (*e.g.,* (Duineveld et al., 2001; Ofek et al., 2014; Bay et al., 2021).

Cover crops have the potential to be used for host-mediated engineering of microbiomes (French et al., 2021; Jing et al., 2022). Cover crops might be more suitable for this purpose than main crops, as cover crops comprise a wide range of plant species belonging to multiple plant families and functional groups, providing a broad palette of options. Regulation of the soil microbiome by cover crops occurs at two distinct stages: during plant growth through rhizodeposition, and during the decomposition of plant residues, after incorporation in the topsoil (Spedding et al., 2004). Leite et al. (2021) studied the impact of cover crops on soil microbial communities during plant growth. They demonstrated that cover crops significantly affected the indigenous bacterial community, such that the impact of the individual cover crops was detectable at a high taxonomic level (phylum level). These findings were corroborated by a recent study that compared the impact of ten different cover crop species on the rhizosphere microbiome (Cazzaniga et al., 2022). Cover crops were shown to produce species-specific microbial footprints, and Brassicaceous cover crops produced more pronounced microbial signatures than most other cover crops. In the second stage, the soil microbiome is affected by the incorporation of plant residues in the topsoil after cover crop termination. Decomposition of the cover crop residues by soil microbiota regulates the microbial community in a manner that deviates from the effect during cover crop growth. The effect of crop residues *per se* was pinpointed by Liu et al. (2021). By mixing soil with dried and milled cover crop material, they observed a promotion of root-associated microbial groups such as Proteobacteria, Bacillaceae and Mortierellomycetes. Similarly, Barel et al. (2019) studied the effect of residues of cover crops on the soil microbial community. Although significant changes in bacterial and fungal biomass were reported, no effect on the overall microbial community composition was detected.

A main crop can benefit from shifts in the microbial community that are induced by prior cover crops if such a shift would last- at least - until the onset of the main growing season. Such legacies have been described for maize. Ray et al. (2022) showed that the legacy of cover crops on the soil microbiome suppressed the spreading of the pathogen *Fusarium verticillioides*, whereas Murrell et al. (2020) demonstrated that mycorrhizal cover crops resulted in increased AMF colonisation of the main crop. Although these studies focused on very specific parts of the soil microbial community only, they demonstrate that main crops can benefit from shifts in the microbial community that are previously induced by cover crops.

To study the persistence of cover crop-induced changes in the soil microbiome over time, we performed a field experiment with ten winter cover crop monocultures, one mixture of eight cover crops and one fallow control. The cover crop monocultures belonged to five different families with different above and below-ground characteristics and were shown to induce distinct changes in the rhizobiome (Cazzaniga et al., 2022). To map the impact of winter cover crops on the soil microbiome, we compared the microbial community assembly in bulk soil just before the planting of the main crop (potato) and right after the harvest of the main crop with the initial cover crop-induced microbial shifts that were brought about in the rhizosphere (Cazzaniga et al., 2022). In this study, we mapped both the total microbial community and the potentially active fractions while focusing on three organismal groups: bacteria, fungi and protists.

We addressed three research questions all relating to the persistence of changes in the soil microbiome after cover crop termination: (1) Is the impact of cover crops that were incorporated in the topsoil in late autumn on the soil microbiome still detectable just before the planting of the main crop (early spring) (T2) and/or after the harvesting of the main crop (autumn) (T2)? (2) If the impact is still detectable at T2 and/or T2, do we see differences in the total (DNA-based) or in the potentially active (RNA-based) fractions of the microbial community among individual cover crop species? (3) Does the legacy of cover crops, either directly via residues or indirectly via persistent changes in the microbial community, affect plant performance and yield of the main crop, potato? A better understanding of the persistence of cover crops-induced changes in the soil microbiome over time will be instrumental in informed cover crop selection that - via its effect on microbiota – will contribute to a reduction of the use of pesticides and mineral fertilizers in agricultural practices.

3.2 Materials and Methods

3.2.1 Study site and sampling

The field experiment was located at the Wageningen University and Research experimental farm 'Vredepeel' in the southeast of the Netherlands (51°32'27''.5N 5°50"59''.4E). This field was characterized by sandy soil (1% clay, 7% silt, 88% sand) with an organic matter content of 4.5% (4.4-4.5%) and a pH of 5.7. For full details on the experimental layout see (Cazzaniga et al., 2023, **Chapter 2**). In brief, ten cover crop monocultures and one cover crop mixture (Table 3.1) were sown in 70 litres of bottomless containers (Æ 55 cm, height 47 cm) on the 1st of August 2019. Thus, eleven treatments with cover crops plus an unplanted control ('fallow') were represented in each of the

eight blocks. Treatments were randomized per block. In total the experiments comprised 96 containers, 14 were excluded from the experiment because of poor growth of the cover crop, and they were not concentrated on a particular treatment or replicate.

At the first sampling time point (3rd of October 2019, here referred to as T1), rhizosphere soils were collected for each of the cover crop species and processed as described in (Cazzaniga et al., 2022). Cover crop mixtures were not sampled at T1 because of the impossibility to collect representative samples from the highly intertwined root systems. On the 16th of December 2019, cover crops were cut and manually incorporated into the soil. About 3.5 months after cover crop incorporation (9th of April 2020), bulk soil was collected from each container. This constitutes the second sampling time point hereafter referred to as T2. An auger (diameter 15 mm, core length 20 cm) was used to collect three soil cores per container. After thorough mixing, subsamples (6-8 g) were snap-frozen in N_2 (l) and stored at -80 °C until further processing.

On the 4th of May 2020, three seed potatoes (var. Agria) were planted per container. Following standard practices, plants were sprayed with fungicides to prevent the spreading of potato late blight. On the 5th of June, plant performance (growth, health and colouration) was recorded by attributing scores on a 1 – 9 scale (from poor to optimal performance, following internally standardised crop assessment criteria developed and applied by Wageningen Research, Field Crops). On the 17th of October, the potato tubers were harvested, and the tuber weight was determined per container. Just after harvesting, bulk soil was sampled again (referred to as T2) by collecting soil cores as described above for T2 sampling. Soil samples were snap frozen and stored at −80 °C until further processing.

3.2.2 Nucleic acids extractions and sequencing library preparation

Nucleic acids (DNA and RNA) were co-isolated from subsamples of 2 g of soil following an in-house phenol-chloroform extraction protocol (Harkes et al., 2019). cDNA was synthesised from the extracted RNA using the Maxima First Strand cDNA Synthesis Kit for RT-PCR (Fermentas, Thermo Fisher Scientific Inc., USA) following the manufacturer's instructions. Metabarcoding of DNA and cDNA was carried out in two main PCR steps following the procedure described in Cazzaniga et al. (2023) (**Chapter 2**). In brief, the first organismal group-specific loci were amplified: V4 region of 16S rRNA of bacteria, and V9 and V7-V8 of 18S rRNA of protozoa and fungi, respectively (Supplementary Table S3.2). In the second PCR, sample-specific index combinations were added to the amplicons. The resulting products were used to generate three libraries

each covering 112 samples. Illumina MiSeq sequencing platform was used for paired-end sequencing (2 x 300 bp, V3 kit) at the Bioscience unit (Wageningen Research, Wageningen, The Netherlands). To guarantee sufficient sequencing depths, libraries from the three time points were sequenced in three separate MiSeq runs. Raw reads were demultiplexed by the sequencing service provider.

Table 3.1 | Cover crop common name, family, species and cultivars used in the field experiment, breeding companies providing the seeds

Common Name	Family	Species	Cultivar	Company
Vetch Lentil	Fabaceae	*Vicia sativa* *Lens culinaris*	Amelia Eston	Joordens Zaden (NL) Joordens Zaden (NL)
Oilseed Radish Oilseed Radish	Brassicaceae	*Raphanus sativus* var. *oleiformis*	Terranova E1039	Joordens Zaden (NL) Joordens Zaden (NL)
Black Oat Tall Fescue Hybrid Ryegrass	Poaceae	*Avena strigosa* *Festuca arundinacea* *Lolium hybridum*	Pratex Firecracker Daboya	PH Petersen (DE) Barenbrug (NL) Vandinter Semo (NL)
Phacelia Borage	Boraginaceae	*Phacelia tanacetifolia* *Borago officinalis*	Beehappy Wild Type	DSV-Zaden (NL) Nebelung (DE)
Marigold	Asteraceae	*Tagetes patula*	Ground Control	Takii Europe (NL)
Mix [a]	Multiple Families	*Trifolium resupinatum, Brassica carinata, Camelina sativa, Linum usitatissimum, Guizotia abyssinica, Avena strigosa, Raphanus sativus var. oleiferus, Vicia sativa*	Solarigol	DSV Zaden (Nl)

[a] : Mix was composed of: 6% Alexandrian Clover, 2% Ethiopian Mustard, 1% Camelina, 7% Flax, 15% Niger, 20% Black Oat, 14% Oilseed Radish, 35% Common Vetch

3.2.3 Pre-processing of raw sequencing data

Demultiplexed raw-sequencing data were processed following the procedure described in Cazzaniga et al. (2022). Reads were processed in QIIME2 and denoised with the DADA2 algorithm for paired-end sequences (Callahan et al., 2016). Amplicon Sequence Variants (ASVs) (Callahan et al., 2017) were assigned to taxa using the q2-feature-classifier plugin and *classify-sklearn* function (Pedregosa et al., 2011) with pre-trained reference databases for each primer-specific amplicon. Bacteria and fungi were assigned using two pre-

trained databases based on the non-redundant SILVA reference database (Glöckner et al., 2017) (*silva-138-ssu-nr99-seqs-derep-uniq*, version 138, 99% identity criterion). Protists were assigned based on the pre-trained pr2 reference database (Guillou et al., 2012). QIIME2 files were imported into Rstudio (v. 1.4.1106, R version 4.0.4) and processed following the same procedure and filtering cut-offs as used in Cazzaniga et al. (2022), utilizing the R packages phyloseq (v1.34.0) (McMurdie and Holmes, 2013), metagMisc (v0.0.4) (Mikryukov, 2017), microbiome (v.1.12.0) (Lahti, 2012-2019), and metagenomeSeq v. 1.32.0) (Paulson et al., 2013). To facilitate comparison with other studies using the same reference databases, the original names assigned to the ASVs from the reference databases were left unchanged. Unassigned ASVs, ASVs assigned to chloroplasts and mitochondria, ASVs from non-target organisms as well as ASVs with a low prevalence (<10 reads per sample) were filtered out. Samples with <5,000 bacterial, <1,000 fungal and <500 protist reads were excluded from the analyses.

After processing and filtering the three resulting datasets comprised 6,487,844, 6,779,998 and 7,401,812 reads, respectively. The T1 dataset had a median sequencing depth of 50,188 reads per sample (with a standard deviation (SD) of 16,566 reads). The T2 and T2 datasets had a mean sequencing depth of 85,128 ± 17,072 (SD) and 91,103 ± 18,668 (SD) reads per sample. 4,667,586 and 4,753,973 filtered reads belonged to the total and potentially active bacterial community and were assigned to 7,528 and 7,851 ASVs respectively. The fungal DNA and RNA datasets comprised 3,135,632 and 3,371,378 reads, assigned to 828 and 1,367 ASVs. 799,511 and 2,344,919 filtered reads belonged to the protist's DNA and RNA datasets and were assigned to 2,165 and 3,870 ASVs respectively. Further information on the sequencing depth can be found in Supplementary Table S3.3. In the next sections, we simplified and shortened the term 'potentially active fractions of the microbiome' to 'active fractions' solely to facilitate readability.

3.2.4 Statistical analyses of the soil microbial communities over time

The effect of the cover crop treatments on the active and total soil microbial communities was determined at each timepoint with the PERMANOVA test subtracting the random effect of the experimental blocks (blocks and cover crop treatment terms were added sequentially in the model). The R^2 values resulting from the PERMANOVA indicate the percentage of the variation in the microbiome explained by the cover crop treatments (effects with $p<0.05$ were considered to be statistically significant). Multiple comparisons among cover crop treatments and fallow were carried out with a Pairwise PERMANOVA test on Bray Curtis distances with Benjamini-Hochberg correction for multiple

testing and 999 permutations in RVAideMemoire R package (v.0.9-81-2) (Hervé and Hervé, 2020).

3.2.5 Cover crops' microbial footprints over time

To pinpoint changes in the microbiome over time, differential abundance analyses were performed using ANCOM-BC (Analysis of Compositions of Microbiomes with Bias Correction (Lin and Peddada (2020). This method was employed to pinpoint the effect of cover crops on the stimulation or repression of microbial taxa i) in the cover crop rhizosphere (T1), ii) after incorporation and partial digestion of cover crop remains (T2) and, iii) after the harvest of the main crop, potato (T2). For shifts in the microbial community, fallow soil from the same sampling time point was used as a reference.

We analysed each time point separately to remove possible variation due to the sequencing batches. In the ANCOM-BC model, cover crop treatments were used as covariates of interest and the fallow soil was the reference level for each time point, while the response variable was the individual microbial groups at the family level (bacteria and fungi) and order level (protists) (ASVs from the same type were agglomerated using *tax_glom* function in phyloseq). Taxa were considered differentially abundant when they had a p-value <0.05 after correction with the Holm–Bonferroni method for multiple testing. Furthermore, ANCOM-BC allowed us to distinguish between negative beta coefficients, pointing at the suppression of a given taxon, and positive beta coefficients, indicative of stimulation of a specific taxon.

To determine the legacy of cover crop treatments on the individual microbial taxa, we compared the differentially abundant taxa over time. To identify microbial footprints, microbial taxa differentially abundant in the cover crop rhizosphere (T1) and after the cover crop treatment throughout T2 and/or T2 were compared to the fallow at the same time point. We focused on three types of microbial footprints characterizing cover crop legacies over time: i) Type 1; a microbial taxon is significantly and consistently stimulated or repressed at T1 and T2 and T2; ii) Type 2: a microbial taxon is significantly stimulated or repressed at T1 and T2; iii) Type 3: a taxon is significantly affected with same directionality at T1 and T2 (not at T2). Patterns were represented as dot plots. The diameter of the dots represents the value of the beta coefficient, and the colour indicates stimulation (green) or repression (red) of a given microbial taxon.

3.2.6 Potato performance and yield

The effect of the preceding cover crop treatment on the potato plant performance and the tuber yield (kg) per container were analysed separately with mixed-models (lmerTest R package, Kuznetsova et al. (2017). In the mixed models, cover crops were the fixed effect, and blocks were treated as random effects. The pairwise comparisons were determined with Tukey HSD correction for multiple testing (emmeans R package, v. 1.7.2, Lenth, 2022).

3.3 Results

3.3.1 Legacy of cover crop-induced changes in native microbial communities.

The effects of cover crops on the bacterial, fungal and protist communities lasted over time in a cover crop treatment-dependent manner (Figure 3.1 A, B), but the effects gradually decreased over time both at DNA (explained variation T1 (**Chapter 2**): 35-43%, T2: 24-37% and T2: 16-19%, Figure 3.1 A) and at RNA level (T1, **Chapter 2**): 36-50%, T2: 25-34%, T2: 15-17%, Figure 3.1 B). Cover crop legacies were detected for all three organismal groups, and the percentages of variation explained was the highest for fungi (at T2 the variation explained by cover crops was 37% and 34% for fungi vs 24% and 27% for bacteria, and 32% and 25% for protists, at DNA and RNA level respectively, Figure 3.1 A, B).

The PCoA ordinations show that both oilseed radish cultivars, as well as the cover crop mixture (dominated by oilseed radish), had the greatest impact on both the total and the active microbial communities at all three time points (Figure 3.1 A, B). At T2, no separation between the cover crop treatments and the fallow control was observable for most treatments except for the oilseed radishes and the cover crop mixture (for all microbial groups at both DNA and RNA levels, Figure 3.1 A, B). Pairwise comparisons among treatments confirmed these observations (Table 3.2 and Supplementary Table S3.4). In all pairwise analyses, the impact of each cover crop treatment was determined by comparing it with the condition of the fallow control (black circles in Figure 3.1) at the corresponding time point (full pairwise comparisons presented in Supplementary Table S3.4). At T2, pairwise comparisons (Table 3.2 A, C, Supplementary Table S3.4 A, B) showed that oilseed radish and the mixture treatments significantly affected the bacterial, fungal and protist communities both at DNA and RNA levels.

Table 3.2 | R2 values from pairwise PERMANOVA between cover crop treatments and fallows at time points T2 and T2. Only significant treatments are shown ($p<0.05$). For a complete overview of pairwise comparisons see Supplementary Table S3.4.

A T2-DNA	Cover Crop Treatment	R^2	P	B T2-DNA	Cover Crop Treatment	R^2	P
Bacteria							
	Oilseed radish-E	0.28	0.004		Mix	0.13	0.017
	Mix	0.28	0.007		Oilseed radish-E	0.12	0.017
	Oilseed radish-T	0.22	0.004		Oilseed radish-T	0.11	0.017
Fungi							
	Oilseed radish-E	0.41	0.003		Oilseed radish-E	0.19	0.004
	Mix	0.39	0.003		Mix	0.18	0.004
	Oilseed radish-T	0.37	0.005		Oilseed radish-T	0.17	0.007
	Marigold	0.17	0.006				
	Hybrid ryegrass	0.12	0.020				
	Lentil	0.11	0.019				
	Mix	0.41	0.006				
Protists							
	Oilseed radish-E	0.41	0.005		Mix	0.18	0.006
	Oilseed radish-T	0.30	0.005		Oilseed radish-E	0.16	0.006
	Marigold	0.14	0.032		Oilseed radish-T	0.15	0.006
	Hybrid ryegrass	0.10	0.045				
C T2-RNA	**Cover Crop Treatment**	**R^2**	**P**	**D T2-RNA**	**Cover Crop Treatment**	**R^2**	**P**
Bacteria							
	Mix	0.30	0.003		Oilseed radish-E	0.11	0.008
	Oilseed radish-E	0.29	0.003		Mix	0.11	0.008
	Oilseed radish-T	0.24	0.006		Oilseed radish-T	0.10	0.022
	Marigold	0.11	0.041				
	Black Oat	0.10	0.003				
	Hybrid ryegrass	0.10	0.019				
Fungi							
	Mix	0.38	0.003		Oilseed radish-E	0.13	0.007
	Oilseed radish-E	0.37	0.003		Oilseed radish-T	0.12	0.007
	Oilseed radish-T	0.29	0.003		Mix	0.11	0.008
	Marigold	0.17	0.003				
	Hybrid ryegrass	0.13	0.008				
	Black Oat	0.12	0.003				
Protists							
	Mix	0.30	0.003		Mix	0.16	0.008
	Oilseed radish-E	0.26	0.003		Oilseed radish-E	0.14	0.008
	Oilseed radish-T	0.25	0.003		Oilseed radish-T	0.13	0.008
	Marigold	0.12	0.007				
	Vetch	0.12	0.015				
	Black Oat	0.12	0.005				
	Hybrid ryegrass	0.10	0.005				
	Lentil	0.10	0.017				

Also, marigold and hybrid ryegrass significantly affected the fungal and protist communities, although the corresponding R^2 values were considerably lower (Table 3.2, fungal community: 37-41% in oilseed radish and mixture vs 17 and 12% in marigold and hybrid ryegrass; in protist community 31-40% vs 14-11%). Lentil only had a significant effect on the fungal community at the DNA level, and on the protist community at RNA level. Black oat treatment only resulted in a significant effect on all organismal groups at RNA level, while tall fescue, borage and phacelia did not show a significant overall effect on the microbial communities.

At T2, just after harvesting the main crop (potato), only the impact of the two oilseed radish treatments and the cover crop mixture was still significant (Table 3.2 B, D, Supplementary Table S3.4 C, D). Focusing on the total communities, the cover crop treatment still explained 11-19% of the observed variation, whereas 11-16% of the variation was explained by the active microbial fractions (Table 3.2).

Hence, our data showed that both oilseed radish treatments and the cover crop mixture significantly affected each of the three organismal groups under investigation at both time points. It is noted that at T2, the onset of the main growing season, the impact of the cover crop treatments was more prominent at RNA than at DNA level.

3.3.2 Nature and legacy patterns of cover crop-induced microbial footprints

Differential abundance analysis (ANCOM-BC) was used to reveal the extent to which individual microbial taxa were contributing to the observed cover crop treatment effects on the different organismal groups over time. In total, we monitored 375 and 377 bacterial families at respectively DNA and RNA level, 130 and 144 fungal families, and 57 and 59 protist orders. To characterize the legacy of each of the cover crop treatments, the differentially abundant taxa at T2 (before the main growing season) and/or T2 (after the main growing season) were compared with the ones differentially abundant at T1. Based on this comparison, we defined a microbial footprint as a microbial family/order that is significantly promoted or repressed by a given cover crop treatment as compared to the corresponding fallow controls at - at least - two sampling times. We identified 232 cover crop-affected microbial taxa at DNA level, of which 170 belonged to bacteria, 46 to fungi and 16 to protists (Supplementary Figure S3.1, 2). At RNA level, we found 268 differentially affected microbial taxa, consisting of 206 bacterial, 49 fungal and 13 protist taxa (Supplementary Figure S3.3, 4).In our analyses, we focused on footprints displaying three distinct types of consistencies in the promotion or repression of a microbial

taxon over time, namely Type 1, Type 2 and Type 3, individually illustrated below. By focusing on shifts that were observed at - at least - two time points in the same direction we aimed to filter out stochastic effects.

Type 1 footprints refer to microbial taxa that are either consistently promoted or consistently repressed at all three time points for a given cover crop treatment. This type of footprint was relatively rare. It was observed only for 10 and 8 out of 232 and 268 microbial taxa at DNA and RNA level respectively. Among the ten cover crop monocultures, only six were shown to produce Type 1 microbial footprints, namely marigold, borage, phacelia, vetch and both oilseed radish cultivars (Figure 3.2 A, D). Oilseed radish treatments gave rise to the highest number of Type 1 microbial footprints. These patterns were observed for nine and seven microbial taxa at DNA and RNA level respectively (Figure 3.2 A and 3.2 D).

At the DNA level, eight bacterial families showed this footprint, five were consistently promoted and three were consistently repressed (Figure 3.2 A). Most notably, the bacterial families Kaistiaceae, Pseudomonadaceae, and Sanguibacteraceae were promoted by the oilseed radish treatments, whereas the Polyangiaceae were repressed. Cellulomonadaceae was increased upon borage treatment. The fungal family Plectosphaerellaceae was significantly promoted by both oilseed radish treatments. Among the protists, the order Physarales stood out as four cover crop treatments promoted the abundance of members of this order (Figure 3.2 A).

At the RNA level, four bacterial families were consistently promoted by one or more cover crop treatments, namely Alcaligenaceae, Pseudomonadaceae Rhodocyclaceae, and Sanguibacteraceae (Figure 3.2 D). Except for Alcaligenaceae, these families were also stimulated at DNA level. It is noted that members of the family Rhodocyclaceae were promoted by four distinct cover crop treatments. One fungal family, the Plectosphaerellaceae, was consistently promoted at DNA and RNA level upon the oilseed radish treatments (Figure 3.2 A, D). In contrast to our observations at DNA level, no significant effect of cover crops on the Physarales was detected at the RNA level (Figure 3.2 A, D).

Type 2 footprints refer to microbial taxa that are either significantly promoted or repressed at T1 and T2 (and not at T2). This type of footprint was found among all cover crop treatments. With 48 microbial taxa showing a Type 2 footprint at the DNA level (Figure 3.2 B), and 63 at the RNA level (Figure 3.2 E), this was the most widespread footprint type. The oilseed radish treatments gave rise to the highest number of microbial taxa with Type 2 footprints (48 and 63 at DNA and RNA level vs 2-9 in the other cover crops).

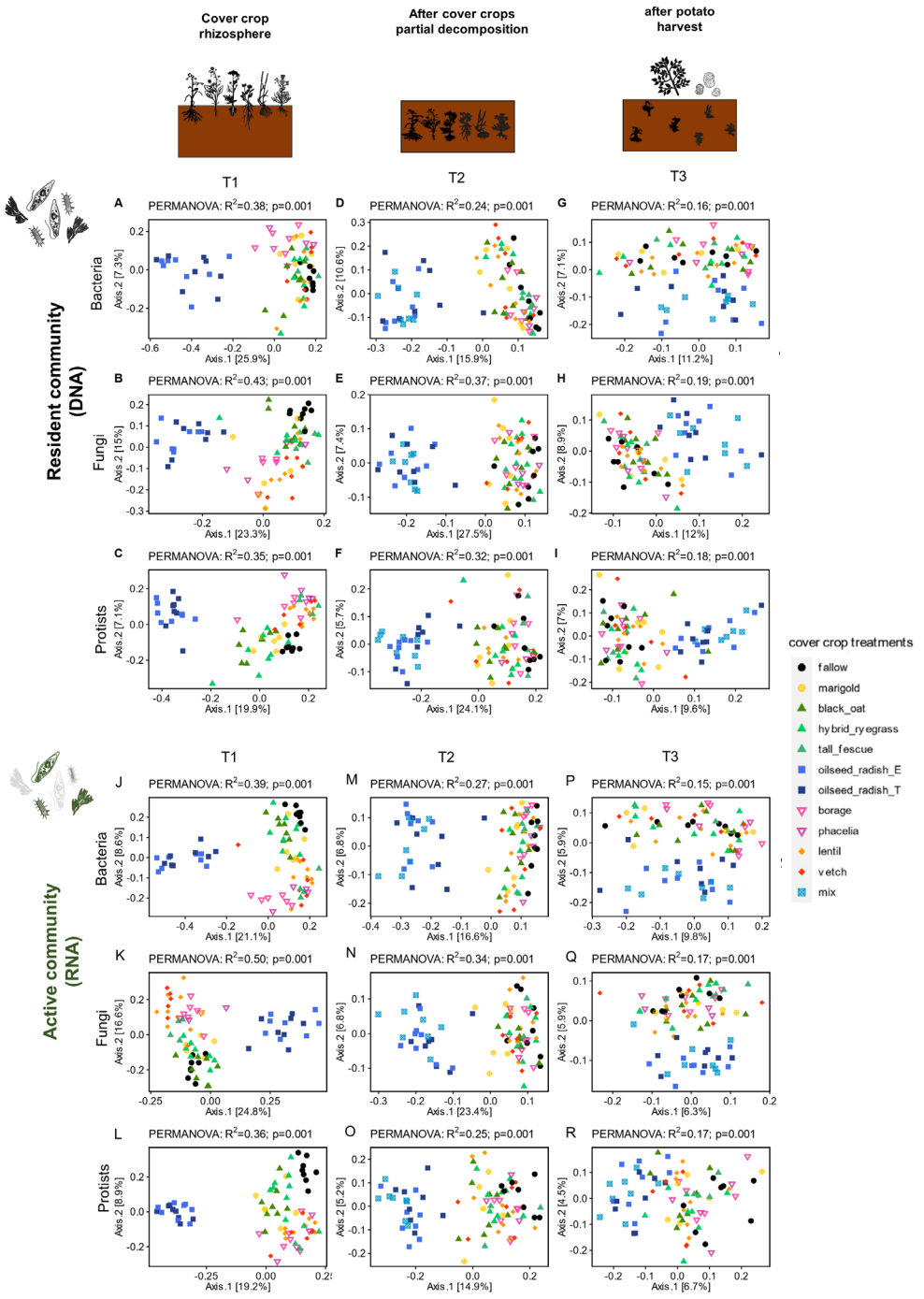

Figure 3.1 | Principal Coordinate Analysis (PCoA) of CSS normalised ASV data. The dissimilarity matrix was built on the Bray-Curtis metric and plotted to separate ASV based

on the cover crop treatment. Different colours are used to indicate different cover crop treatments and cover crops from the same plant family share the same shape. Microbial communities are represented at DNA (A) and RNA level (B) for bacteria, fungi and protists at the three sampling time points: in the rhizosphere during cover crop growth (T1), just before planting of the main crop (potato) (T2) and right after the harvest of the main crop (T2). The microbial communities collected from the rhizosphere were mutually more distinct than communities characterized in bulk soil (PERMANOVA, $p \leq 0.001$). For the cover crop mixture (light blue closed squares), the microbial community was determined in bulk soil only at T2 and T2. Both oilseed radish cultivars, as well as the cover crop mixture (dominated by oilseed radish), had the greatest impact on both the total and the active microbial communities at all three time points as shown by the clear separation of the blue squared dots representing the oilseed radish cultivars and the mixture from the other symbols.

It is worth noting that at the DNA level, the two oilseed radish treatments produced distinct microbial footprints.

Oilseed radish-E showed four times more repressed microbial taxa than cultivar Terranova (21 vs 5), but the same number of Type 2 promoted taxa. At RNA level, a less skewed Type 2 repression pattern was observed (19 vs 13 microbial taxa) between the two oilseed radish cultivars (Figure 3.2 B, E), but oilseed radish promoted two times more microbial taxa than the cultivar Terranova (13 vs 9).

Except for oilseed radish, a considerable number of bacterial taxa was promoted or repressed at the DNA level by a single cover crop treatment only. Examples are vetch promoting *C*ellulomonadaceae and repressing Planctomycetes, and phacelia repressing Babeliales and Thermomicrobiales. However, most of the taxa were significantly affected simultaneously by oilseed radish and a second cover crop treatment (*e.g.*, Dehalococcoidia repressed by oilseed radish-E and tall fescue, and Cellvibrionaceae promoted by oilseed radish-E and black oat (both at DNA level). Among the fungal families, five were stimulated at DNA level (Figure 3.2 B). Cladosporiaceae are exceptional as their presence was promoted by eight out of the ten cover crops tested (all but tall fescue and oilseed radish-T). Members of the Pleosporaceae were exclusively promoted upon marigold treatment. Repression Thelebolaceae was detected for three cover crop treatments (black oat, oilseed radish-E, and vetch). The protist order Physarales, showing a positive Type 1 footprint upon four different cover crop treatments (both oilseed radish, phacelia and vetch), gave an additional positive Type 2 footprint upon exposure to borage. As compared to the DNA level, more taxa were significantly promoted at the RNA level by multiple cover crops at the same time. This can be illustrated by the Armatimonadales, activated by seven cover crop treatments (not including phacelia and the two Fabaceae), and Kaistiaceae, which activity was promoted

by six cover crops (not including marigold, tall fescue and phacelia). Oxalobacteraceae and Spirochaetaceae are examples of bacterial taxa that were not significantly affected at the DNA level but were shown to be activated by two Poaceous cover crop species (hybrid ryegrass and tall fescue, respectively, Figure 3.2 E). The fungal family Cladosporiaceae, which presence was promoted by eight cover crops (Figure 3.2 B), was activated by four cover crop treatments only. On the contrary, the fungal family Pleosporaceae was promoted solely by marigold at the DNA level, whereas at the RNA level, it was shown to be activated by six distinct cover crop treatments (not including black oat, oilseed radish, and phacelia, Figure 3.2 B, E). Mortierellaceae and Pezizales were the only two fungal taxa which activities were repressed as compared to the fallow control. Notably, under the same treatment (oilseed radish-E) these taxa also showed a Type 2 footprint-like repression pattern at the DNA level (Figure 3.2 B, E). Among the protists, Sagenista was activated by hybrid ryegrass (Figure 3.2 B, E), while the activity of Bacillariophyta was repressed by the oilseed radish cultivar-T (Figure 3.2 E).

Type 3 footprints involve microbial taxa that are significantly promoted or repressed by cover crops at T1 (during cover crop growth) and T2 (just after the main growing season), while – as compared to the corresponding fallow control - no significant change was observed at T2. Both at DNA and RNA levels, 23 families showed a Type 3 footprint, consisting of 16 and 16 bacterial, four and three fungal, and three and four protist taxa (Figure 3.2 C, F).

At DNA level both the Chitinophagales and the Defluviicoccales were promoted by six and five different cover crop treatments. Repressive Type 3 footprints appeared to be more cover crop-specific; only the Halingiaceae were repressed by more than one cover crop species, namely marigold and oilseed radish (Figure 3.2 C). DNA-based analysis of the fungal community revealed a specific Type 3 promotion of Trichocomaceae only upon exposure to the two Fabaceae, lentil and vetch (Figure 3.2 C). Other fungal taxa were significantly repressed by tall fescue and oilseed radish (Gromochytriaceae, Hymenochaetales and Stachyobotryaceae). Concerning the protist community, we observed a positive Type 3 footprint for the Cryptomonadales order upon exposure to tall fescue.

RNA-based community analyses revealed positive Type 3 footprints induced on Reyranellaceae by all cover crop species except for tall fescue and oilseed radish-E. On the other hand, oilseed radish cultivar-E specifically gave rise to an activity reduction of the bacterial taxa Kapabacteriales, Sphingobacteriales KD3-93, Suttereliaceae and Vermiphilaceae (Figure 3.2 F). Lentil induced the highest number of positive Type 3 footprints (four out of five affected taxa). Notably, none of these taxa was significantly changed at the DNA level. RNA-based fungal community analyses showed an activation of the

Claroideoglomeraceae upon exposure to each of the two legumes, vetch and lentil as well as marigold (Figure 3.2 F).

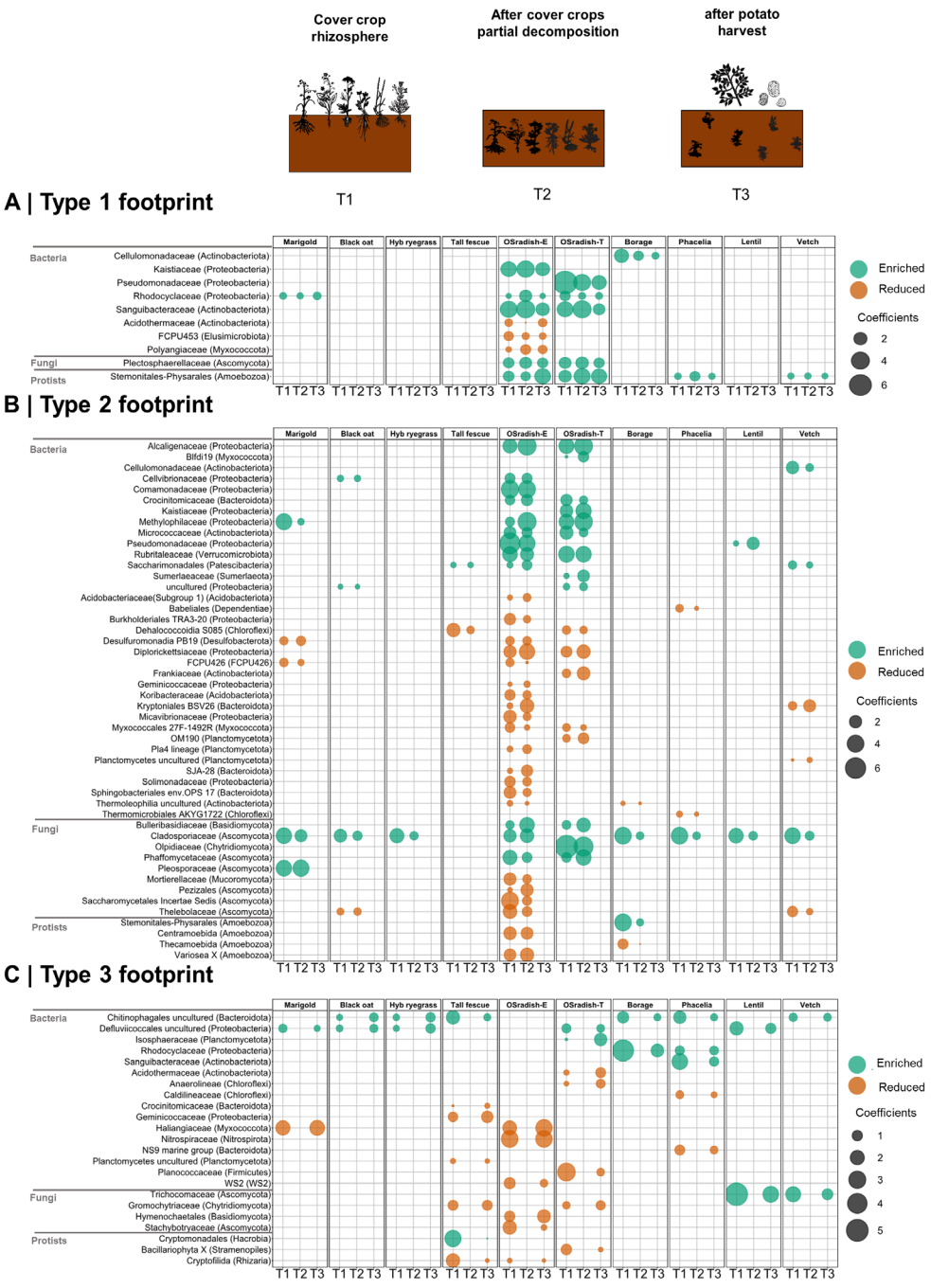

Figure 3.2, part 1, follows in the next page

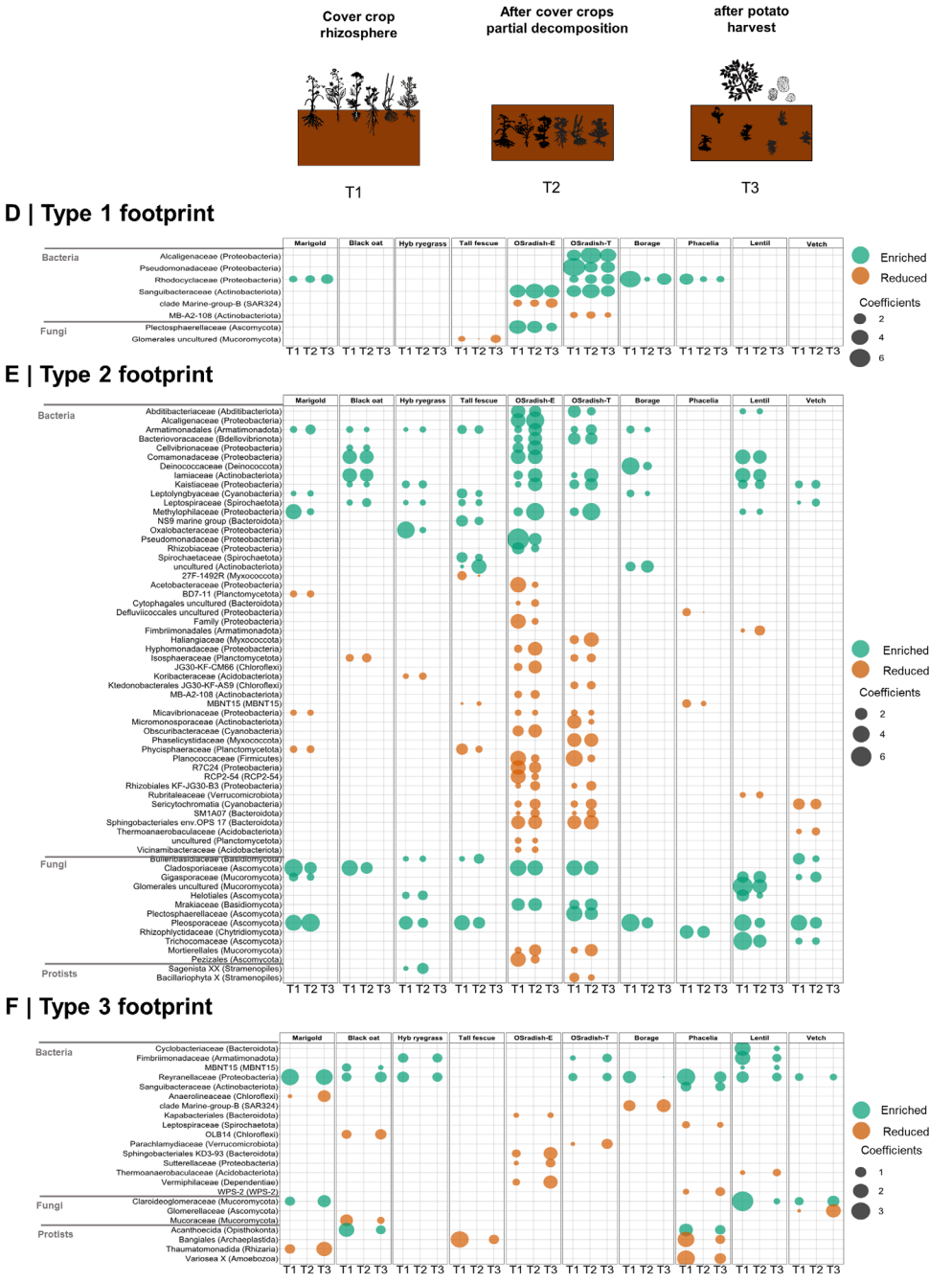

Figure 3.2 | Dot plots representing the cover crops' microbial footprints over time. The dots represent differentially abundant microbial features (significant at $p < 0.05$) and their size corresponds to the beta-coefficient value from the ANCOM-BC test. Differential abundances refer to microbial taxa (bacterial and fungal families and protist orders) showing a significantly different abundance after the cover crop treatment as compared

to the corresponding fallow control. T1 refers to the sampling of rhizosphere soil during cover crop growth, T2 refers to the bulk soil sampled right before the planting of the main crop (potato), and T2 refers to the bulk soil sampled right after the harvesting of the main crop. This figure is subdivided into A) taxa promoted (green) or repressed (orange) at all time points (Type 1 footprint), B) Taxa promoted or repressed at T1 and T2 and non-significant affected at T2 (Type 2), C) taxa promoted or repressed at T1 and T2, but non-significant affected at T2 (Type 3 footprint) at DNA level. C, D and E figures show Type 1, Type 2 and Type 3 footprints, respectively, at RNA level.

This shift was not observed at the DNA level. Concentrating on microbial activity levels, enhanced activity of members of the protist order Acanthoecida was observed upon the black oat and phacelia treatments (Figure 3.2 F).

Overall, our analyses of promotion or repression patterns revealed that many footprints are cover crop treatment-specific or shared between oilseed radish and another cover crop treatment. Patterns as we have seen at the DNA level for the fungal family Cladosporiaceae, and the bacterial family Reyranellaceae at the RNA level – in both cases promotion by eight of the ten cover crops – are rare. It is noted that the number of taxa showing Type 1, 2 and 3 microbial footprints is generally higher among members of the active community (at RNA level) than of the total community (at DNA level). All in all, our results show that changes at the level of individual members of the soil microbiome are mostly cover crop-specific, and often still present at the onset of the next main growing season (T2).

3.3.3 Effect of cover crop treatments on the main crop, potato.

The cover crop treatments significantly (adjusted $p<0.05$) affected tuber yield (kg/container). Pairwise comparisons showed that lentil and borage treatments significantly decreased potato yield in comparison to the fallow (Bonferroni adjusted p-value < 0.05) (Figure 3.3 A). For all other cover crop treatments, the tuber yield was not significantly different from the fallow. A non-significant trend in tuber yield was observed upon the oilseed radish and mixture treatments. This trend comprised an increase from 3.1 kg potato tuber/container in the fallow control, to 3.9 and 3.8 kg/container respectively (Figure 3.3 A). Potato plant performance was not significantly affected by the cover crop treatments (Figure 3.3 B).

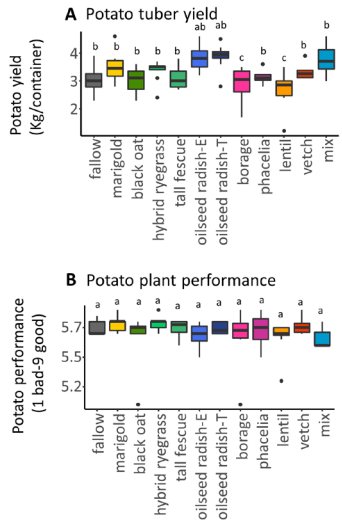

Figure 3.3 | Boxplots representing potato tuber yield in kg tuber per container (A) and potato plant performance (B). The potato plant performance was based on a scale between 1 and 9 which took into account plant size, growth, colouration and health. Different letters above the box indicate significant differences in tuber yield as calculated with a linear mixed model (with tuber yield and potato performance as response variables, block as a random effect and cover crop treatment as an explanatory variable) and post-hoc test with Bonferroni adjustment for multiple comparisons.

3.4 Discussion

3.4.1 Persistence of cover crop-induced changes in the soil microbiome

For all ten cover crop treatments, we found significant effects on microbial taxa approximately four months after the incorporation into the topsoil (T2) just before the planting of the main crop (potato). In the case of the two oilseed radish cultivars and in a cover crop mixture that was dominated by oilseed radish, soil-borne legacies were detectable for all three organismal groups, even after the harvest of the main crop, potato (T2). Borage, phacelia, vetch and lentil did not have a significant effect on the overall community composition, but significant effects on individual microbial taxa were observed. The persistence of cover crop effects over time is also biomass-dependent (Barel et al., 2018). In our field experiment, individual cover crops produced varying amounts of biomass. This factor may have contributed to the lack of overall community composition effects for these four cover crop species.

Previous studies by Walker et al. (2022) and Nevins et al. (2018) found persistent effects of cover crops on soil microbial communities under various agro-ecological conditions (*e.g.*, distinct growing periods and cover crop species). Upon exposure to brown mustard (*Brassica juncea*) and ryegrass (*Lolium multiflorum*) (Walker et al. (2022), and hairy vetch (*Vicia villosa*), cereal rye (*Secale cereale*), and a mixture of hairy vetch and cereal rye (Nevins et al., 2018), shifts in the microbial communities were still observable at the time the main crop was grown. Although the persistence of cover crop-induced shifts in the native soil microbiome at the onset of the main growing season had been demonstrated before, the effect of a prolonged crop-free post-incorporation period (as usual in the temperate climate zone) had not been assessed before. Here we demonstrate that after a crop-free period of about four months, distinct legacies could be detected for all ten cover crops tested.

3.4.2 Ecological understanding of observed microbial shifts.

Microbial footprints as defined here – microbial taxa that are consistently affected by a cover crop as compared to the corresponding control at, at least, two time points – were most frequently observed for bacteria. This does not come as a surprise as the number of bacterial ASVs manyfold exceeded the number of fungal and protist ASVs (see Results). Cover crops had the most persistent effect 9

Type 1 footprints comprise microbial taxa consistently affected in the same direction (increase or decrease) at all three time points and were relatively rare. It should be noted that taxa with a Type 1 footprint upon a given cover crop treatment, often produced Type 2 or Type 3 footprints upon other cover crop treatments. Illumina A noteworthy stimulation was observed for the bacterial family Pseudomonadaceae. A Type 1 stimulation was detected upon exposure to oilseed radish-T (both at DNA and RNA level), and a Type 2 footprint for oilseed radish-E (both at DNA and RNA level). *Pseudomonas* strains have been implicated as the main explanation for the decline of the take-all disease caused by *Gaeumannomyces graminis*. Members of the genus *Pseudomonas* have been involved in other types of disease suppressiveness, in particular towards fungal pathogens (*Fusarium oxysporum* and *Rhizoctonia solani*) and plant-parasitic nematodes (*Meloidogyne spp.*) (Kloepper et al., 1980; Mazurier et al., 2009; Adam et al., 2014). Sanguibacteraceae, a monotypic family that only comprises the genus *Sanguibacter,* showed a Type 1 stimulation for both oilseed radish cultivars, both at DNA and RNA level. *Sanguibacter* strains have been identified as potential lignocellulose degraders (de Lima Brossi et al., 2016). It is unknown, however, why this characteristic would be more boosted by oilseed radish cultivars at T2 and T2 than by other cover crop treatments.

Type 2 footprints comprise taxa that were consistently promoted or repressed during cover crop growth (T1) and just before the planting of the main crop (T2), whereas no significant change was observed after the main crop as compared to the corresponding fallow control (T2). T2 footprints were the most widespread type in our study. This was anticipated as the main crop also induced a specific shift in the soil microbiome. As a result, many cover crop induced-changes might have been nullified. The neutralisation of cover crop-induced shifts in the indigenous soil microbiome will depend on the identity of the main crop. This notion was nicely illustrated by Ulcuango et al. (2021)who showed that the differential effects of vetch, sweet clover and barley cover crops on the bacteria, fungal, AMF and archaeal communities depended on the identity of the main crop. In this case, vetch and sweet clover induced similar soil microbial responses in wheat and maize main crops, by promoting the total abundance of fungi, bacteria and archaea, while the effect of the barley cover crop was less pronounced and significantly distinct among the two main crops. Similarly, Manici et al. (2018) found that barley and hairy vetch cover crops had a more prominent effect on the bacterial community associated with the main crop tomato rather than zucchini. These studies indicate that the effect that cover crops have on the soil microbiome and its persistence co-depend on the nature of the cover crop-main crop combination.

Among the most notable taxa displaying a Type 2 footprint is the bacterial family Kaistiaceae that was shown to be activated by six cover crop treatments at RNA level. Interestingly, *Kaistia*, one of the two genera of this family, was shown to effectively suppress the fungal pathogen *F. oxysporum* (Fujiwara et al., 2016). Keeping in mind that this family was promoted at the RNA level might add to the relevance of this cover crop-specific stimulation. Also, the bacterial family Oxalobacteraceae showed a Type 2 footprint at RNA level upon exposure to hybrid ryegrass. This bacterial family has been associated with the suppressiveness of several fungal soil-borne pathogens, including *F. oxysporum, Verticillium dahliae and R. solani* (Gómez Expósito et al., 2017).

The fungal family Cladosporiaceae is exceptional as it produced Type 2 footprints at the DNA level in eight out of the ten cover crops tested (excluding tall fescue and oilseed radish-T). *Cladosporium,* the dominant and most widespread genus within this family (Bensch et al., 2015) may grow biotrophically on a wide range of plant species whereas other species can grow as a saprophyte on dead or senescing plant tissue (Bensch et al., 2012). Therefore, our results suggest that multiple and/or polyphagous representatives of the Cladosporiaceae used cover crops themselves and/or their residues as a substrate. Although not implicated in this study, it is worth mentioning that some *Cladosporium* species have been implicated in natural

soil suppressiveness against cyst (Song et al., 2016) and root-knot nematodes (Giné et al., 2016).

At the RNA level, six out of the ten cover crop treatments resulted in an activation of members of the fungal family Pleosporaceae. This family includes numerous genera among which *Alternaria* and *Pleospora* are the most speciose and ecologically diverse. *Alternaria* species are mostly saprobes, but a subset has evolved into plant parasites with broad host ranges (Thomma, 2003). *Pleospora* (anamorph *Stemphylium*) harbours a wide range of saprophytic and pathogenic species (see e.g. McDonald et al., 2022). Hence, we attribute the widespread activation by multiple cover crop treatments to saprobic and/or polyphagous parasitic members of this family.

Type 3 footprints are here defined as microbial taxa significantly promoted or repressed in the rhizosphere at T1, and after the harvesting of the main crop (T2), but showing no significant change just before the onset of the main growing season (T2). Hence, Type 3 footprints reflect cover crop treatment effects observed in the presence of living plants only. Striking examples of relatively widespread Type 3 footprints are the bacterial orders Chitinophagales and Defluvicoccales at DNA level, and Reyranellaceae at RNA level, which were stimulated by multiple cover crop treatments from different families. Chitinophagales and Defluvicoccales have high ecological diversity which hampers the interpretation of their ecological role. Our RNA data suggested that Reyranellaceae might have been activated under eight out of the ten cover crop treatments. Within this monogenetic family, representatives of the *Reyranella* genus have been characterized as dominant denitrifier populations in dry upland soils (Pessi et al., 2022). As such, increased nitrate (NO_3^-) availability during plant growth could have triggered the generalised activation of *Reyranella* spp upon most cover crop treatments.

Interestingly, a few Type 3 footprints were associated exclusively with cover crops from the same plant family. At DNA level, the fungal family Trichocomaceae was only promoted by the fabaceous cover crops lentil and vetch. Trichocomoceae constitute an ecologically diverse family of which some members are known as endophytes of legumes (Higginbotham et al., 2013). Whether the members of this family detected here acted as endophytes of lentil and vetch remains to be established. Another example is the arbuscular mycorrhizal fungal (AMF) family Claroideoglomeraceae, which was significantly stimulated at RNA level by both fabaceous cover crops and marigold. A study by Higo et al. (2019) showed that the cover crop identity determines the establishment of the AMF community in the subsequent crop, even more than the host (main crop) identity itself. Therefore, the choice of the cover crop may

also determine the success of the establishment of certain AMF taxa during the next main crop.

3.4.3 Added value of monitoring both resident and active fractions of the soil microbiome

We monitored changes over time in the resident (DNA-based) as well as the active (RNA-based) fractions of the soil microbial communities. Although similar general trends were observed for the individual cover crop treatments, DNA and RNA-based analyses revealed some remarkable contrasts. At the RNA level, we observed a higher number of significant contrasts as compared to the DNA level. This can be illustrated by the number of Type 2 footprints: 48 taxa at the DNA level, and 63 at the RNA level. This suggests that RNA-based community profiling may provide an augmented representation of how the soil microbial community composition is shaped by the cover crop treatments (Bay et al., 2021). It must be noted that the number of bacterial and fungal families and protist orders was comparable between DNA and RNA datasets. Therefore, differences in the number of families/orders *per se* are unlikely to be the cause of the contrasts between RNA and DNA-based microbial profiles.

We argue that the combined analysis of DNA and RNA community fractions represents a comprehensive approach to studying the highly dynamic soil microbial communities, especially when assessing community compositional changes over time. This approach allows for studying the active fraction of the microbiome at the time of the sampling (Blazewicz et al., 2013). In addition, the inclusion of DNA-based analyses allows us to take into account dormant and active microorganisms which can be activated in response to environmental changes or substrate input (Blagodatskaya and Kuzyakov, 2013).

The possibility to discriminate between active and dormant microbiota would be instrumental to assess the disease-suppressive as well as the plant-pathogenic potential of a given soil. The observed strong stimulation of, Pseudomonadaceae by both oilseed radish cultivars at DNA and RNA level could justify a more detailed study to investigate whether it could result in elevated levels of disease suppressiveness. On the other hand, stimulation of the pathogenic fungal family Olpidiaceae by the oilseed radish cultivar Terranova at the DNA level was not paralleled by activation at RNA level. This information could be taken along in the design of crop rotations.

3.4.4 Possible explanations for the limited effect of cover crop treatments on potato yield

In this study, cover crop treatments did not have a major effect on potato tuber yield. A reason for the limited effects of cover crop treatments on the potato yield could be related to the experimental field conditions. The experiment was

carried out on well-fertilized soil in the absence of major soil-borne pathogens, suggesting relatively optimal soil conditions for plant growth. A study by Porter and Sisson (1991) reported a significant tuber yield increase after red clover only when nitrogen was limiting potato growth. Similarly, Sincik et al. (2008) also found that tuber yield increases following cover crops were less pronounced with increased nitrogen fertilisation rates. Therefore, the beneficial effect of the cover crop-steered microbiomes on the succeeding main crop may have been obscured by the optimal nutritional status of the experimental field (Bokhari et al., 2019; Liu et al., 2020; Trivedi et al., 2022). Future work needs to reveal how the steering effect of cover crops should be understood in the context of soil fertility, pathogen pressure and other agronomically relevant factors.

3.5 Conclusions

Our data showed that each of the ten cover crop treatments tested here resulted in significant microbial changes that lasted at least until the onset of the main growing season (T2). The two oilseed radish cultivars and a cover crop mixture (dominated by oilseed radish) had the most persistent effect on the microbial community as shifts were even observable after the harvesting of the main crop (potato). Furthermore, each cover crop treatment resulted in qualitatively distinct microbial footprints. Most notably oilseed radish treatments significantly and consistently boosted the presence and potential activity of members of the families Sanguibacteraceae and Pseudomonadaceae. The observed stimulation of Pseudomonadaceae by both oilseed radish cultivars over time could justify a more detailed study to see whether it resulted in higher local levels of suppressiveness against plant-parasitic fungi or nematodes. In such a study, the direct effect of isothiocyanates released from Brassicaceous crops as well as the distinct sensitivities of fungal pathogens vis-à-vis pathogenic fungi should be taken along (Sarwar et al., 1998).

Other crop treatments had a significant effect on the potential activity of other individual microbial taxa, including Glomerales and Helotiales by lentil, Deinococcaceae by borage, Claroideoglomeraceae by vetch and marigold and

lentil. The data presented here demonstrate that cover crops induce species and even cultivar-specific changes in the local soil microbiome. Finally, by comparing rDNA and rRNA-based community profiles induced by cover crop treatments, we generally recognised similar trends. However, a considerable number of exceptions were observed in which the promotion or repression of a taxon at DNA level was not accompanied by similar changes at RNA level and *vice versa.* Our study demonstrates that cover crops can be used to regulate the soil microbiome in a cover crop-specific manner, and at least a part of these changes will persist in the topsoil until at least the onset of the main growing season in sandy soil. In a previous study, Harkes et al. (2019) reported that even within the same climatic zone, the soil microbiome differs among soil types, which limits the generalisation of our results beyond sandy soils with a comparable pH. For several microbial taxa, we could link induced changes to disease suppressiveness. Stimulation of microbiota that strengthen the antagonistic capacity of soil could contribute to more sustainable pest and pathogen management (Vukicevich et al., 2016). In follow-up studies, we will focus on the effects of cover crops-regulated microbiomes in the presence of soil-borne pathogens to further test the potential of cover crops in stimulating pathogen antagonists.

Data availability

All sequences have been submitted to the NCBI database. Data from T1 sampling (cover crop rhizosphere) are under BioProject ID PRJNA842568, Data from T2 and T2 sampling (bulk soil before and after potato) are under BioProject ID PRJNA926607.

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3.6 Supplementary material

Supplementary Table S3.1 | Number of cover crop treatments (containers) included in the experiment. 14 treatments were excluded because of poor growth due to extreme environmental conditions at germination (August 2019).

Cover crop Treatments	Sampled containers
Fallow	8
Black oat	8
Marigold	6
Tall fescue	4
Oilseed radish Terranova	8
Oilseed radish E1089	8
Phacelia	4
Borage	6
Vetch	6
Lentil	8
Hybrid ryegrass	8
Mixture	8
	Tot = 82 containers

Supplementary Table S3.2 | Primers used in the first PCR step for amplification of organismal group-specific 16S and 18S rRNA regions, with adaptor sequence (underlined) and locus-specific sequence (bold).

Organism	rDNA Target Region	Primer	Primer	Refs
Bacteria	V4	515 F	TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG **GTGCCAGCMGCCGCGGTAA**	(Caporaso et al., 2012)
	(16S)	806 R	GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAG **GGACTACHVGGGTWTCTAAT**	
Fungi	V7-8	FF 390.1	TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG **CGWTAACGAACGAGACCT**	(Verbruggen et al., 2012)
	(18S)	FR1	GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAG **AICCATTCAATCGGTAIT**	
Protists	V9	1391 F	TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG **GTACACACCGCCCGTC**	(Lane, 1991)
	(18S)	EukBr	GTCTCGTGGGCTCGG AGATGTGTATAAGAGACAG **TGATCCTTCTGCAGGTTCACCTAC**	(Medlin et al., 1988)

Supplementary Table S3.3 | Sequencing statistics of processed reads divided by organismal group, DNA and cDNA and sequencing time point

		time point	Min.	1stQu.	Median	Mean	3rdQu.	Max.	Tot	sd	Tot time point	n
bacteria	DNA	t1	9.088	15.118	17.509	22.012	28.480	51.885	1.606.890	10.292	4.753.973	73
bacteria	DNA	t2	10.234	15.604	19.648	19.449	22.279	32.873	1.575.348	4.916		81
bacteria	DNA	t3	12.467	16.126	18.359	19.168	21.097	35.157	1.571.735	4.295		82
bacteria	cDNA	t1	4.970	11.334	13.980	22.234	38.103	49.918	1.645.286	14.527	4.667.586	74
bacteria	cDNA	t2	9.378	15.357	18.399	17.946	20.348	27.549	1.471.555	4.146		81
bacteria	cDNA	t3	5.887	15.533	18.813	18.912	21.380	34.343	1.550.745	5.487		82
fungi	DNA	t1	1.044	4.055	5.626	7.340	8.957	27.999	543.147	4.914	3.135.632	74
fungi	DNA	t2	6.907	12.567	15.416	15.486	17.210	27.969	1.269.868	4.140		82
fungi	DNA	t3	9.334	14.327	16.729	16.129	17.947	23.658	1.322.617	2.741		82
fungi	cDNA	t1	589	1.434	2.218	4.434	6.070	33.496	328.091	5.066	3.371.378	74
fungi	cDNA	t2	5.161	16.448	18.383	18.836	21.262	32.702	1.506.847	4.478		80
fungi	cDNA	t3	6.099	16.024	18.312	18.737	21.053	31.581	1.536.440	4.161		82
protists	DNA	t1	1.149	2.393	3.641	5.851	9.009	24.486	427.092	5.137	649.990	73
protists	DNA	t2	646	981	1.328	1.434	1.655	4.153	116.191	605		81
protists	DNA	t3	761	1.081	1.226	1.301	1.464	3.177	106.707	372		82
protists	cDNA	t1	2.455	4.851	7.674	13.393	20.485	53.585	991.084	11.568	2.010.746	74
protists	cDNA	t2	1.955	3.782	4.936	5.292	6.604	11.140	433.971	1.981		82
protists	cDNA	t3	3.832	5.729	6.996	7.143	8.294	13.253	585.691	1.984		82

Supplementary Table S3.4 | R^2 values from the Pairwise PERMANOVA comparisons with BH correction for multiple testing. R^2 values in bold were supported by significant p value (<0.05) and indicate significant differences in the microbiome assembly between the treatments.

Bacteria T1 -DNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil
marigold	**0,14**									
black oat	**0,13**	**0,11**								
hybrid ry.	**0,12**	**0,13**	0,08							
tall fescue	**0,21**	**0,19**	**0,14**	**0,14**						
osr-E	**0,44**	**0,38**	**0,34**	**0,36**	**0,41**					
osr-t	**0,42**	**0,35**	**0,32**	**0,34**	**0,39**	0,07				
borage	**0,27**	**0,20**	**0,20**	**0,22**	**0,26**	**0,36**	**0,34**			
phacelia	**0,20**	0,13	**0,15**	**0,16**	0,19	**0,35**	**0,33**	**0,19**		
lentil	**0,16**	**0,16**	**0,13**	0,10	0,11	**0,39**	**0,37**	**0,21**	**0,15**	
vetch	**0,20**	**0,17**	**0,15**	**0,14**	0,12	**0,41**	**0,39**	**0,21**	0,14	0,07

Bacteria T1-RNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil
marigold	**0,16**									
black oat	**0,16**	**0,12**								
hybrid ry.	**0,13**	**0,12**	**0,10**							
tall fescue	**0,29**	**0,25**	**0,21**	**0,19**						
osr-E	**0,41**	**0,37**	**0,33**	**0,33**	**0,43**					
osr-t	**0,37**	**0,32**	**0,29**	**0,29**	**0,39**	**0,06**				
borage	**0,34**	**0,25**	**0,28**	**0,26**	**0,33**	**0,38**	**0,35**			
phacelia	**0,27**	**0,19**	**0,22**	**0,20**	**0,21**	**0,38**	**0,34**	**0,23**		
lentil	**0,24**	**0,21**	**0,21**	**0,17**	**0,14**	**0,41**	**0,38**	**0,27**	**0,18**	
vetch	**0,21**	**0,16**	**0,17**	**0,14**	0,14	**0,35**	**0,31**	**0,20**	0,14	0,08

Bacteria T2-DNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	0,09										
black oat	0,08	0,07									
hybrid ry.	0,07	0,07	0,06								
tall fescue	0,10	0,12	0,09	0,10							
osr-E	**0,28**	**0,22**	**0,22**	**0,24**	**0,27**						
osr-t	**0,22**	**0,17**	**0,17**	**0,18**	**0,21**	0,06					
borage	0,07	0,10	0,08	0,08	0,11	**0,26**	**0,20**				

Bacteria T2-RNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	**0,11**										
black oat	**0,10**	0,09									
hybrid ry.	**0,10**	0,10	0,07								
tall fescue	0,10	0,12	0,10	0,09							
osr-E	**0,29**	**0,22**	**0,23**	**0,24**	**0,26**						
osr-t	**0,24**	**0,19**	**0,19**	**0,20**	**0,22**	0,07					
borage	0,08	0,12	**0,11**	**0,11**	0,12	**0,27**	**0,23**				

phacelia	0,09	0,14	0,11	0,10	0,16	**0,27**	**0,22**	0,09			
lentil	0,07	0,07	0,06	0,06	0,10	**0,23**	**0,17**	0,08	0,12		
vetch	0,09	0,08	0,07	0,07	0,11	**0,23**	**0,17**	0,10	0,13	0,07	
mix	**0,28**	**0,22**	**0,22**	**0,24**	**0,27**	0,06	0,07	**0,25**	**0,27**	**0,22**	**0,23**

phacelia	0,09	0,12	0,10	0,09	0,14	**0,27**	**0,22**	0,11			
lentil	0,09	0,09	0,08	0,08	0,11	**0,24**	**0,20**	0,10	0,10		
vetch	0,10	0,09	0,08	0,08	0,10	**0,23**	**0,19**	0,11	0,10	0,07	
mix	**0,30**	**0,24**	**0,24**	**0,25**	**0,26**	0,07	0,08	**0,28**	**0,27**	**0,25**	**0,23**

Bacteria T3-DNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	0,06										
black oat	0,07	0,06									
hybrid ry.	0,06	0,06	0,06								
tall fescue	0,07	0,10	0,08	0,08							
osr-E	**0,12**	**0,12**	**0,11**	**0,11**	**0,14**						
osr-t	**0,11**	0,11	0,10	0,09	**0,13**	0,05					
borage	0,09	0,09	0,08	0,08	0,12	**0,14**	**0,14**				
phacelia	0,08	0,08	0,08	0,08	0,12	**0,13**	0,12	0,11			
lentil	0,06	0,07	0,06	0,05	0,08	**0,10**	0,09	0,09	0,08		
vetch	0,07	0,06	0,06	0,06	0,09	**0,13**	0,11	0,09	0,09	0,07	
mix	**0,13**	**0,12**	**0,12**	**0,12**	**0,15**	0,05	0,05	**0,15**	**0,13**	**0,11**	**0,13**

Bacteria T3-RNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	0,07										
black oat	0,06	0,07									
hybrid ry.	0,06	0,06	0,06								
tall fescue	0,08	0,10	0,08	0,08							
osr-E	**0,11**	0,11	**0,10**	**0,10**	**0,14**						
osr-t	**0,10**	0,10	0,09	0,09	0,12	0,06					
borage	0,07	0,09	0,08	0,08	0,10	**0,14**	**0,12**				
phacelia	0,08	0,09	0,09	0,07	0,12	**0,13**	0,11	0,10			
lentil	0,07	0,07	0,06	0,06	0,08	**0,11**	0,08	0,08	0,08		
vetch	0,06	0,06	0,06	0,06	0,09	**0,11**	0,09	0,08	0,09	0,07	
mix	**0,11**	0,11	**0,10**	**0,11**	**0,14**	0,05	0,06	**0,14**	**0,13**	**0,10**	**0,11**

Fungi T1-DNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil
marigold	**0,31**									
black oat	**0,12**	**0,15**								
hybrid ry.	**0,18**	**0,14**	0,08							
tall fescue	**0,25**	**0,22**	**0,21**	**0,18**						
osr-E	**0,52**	**0,35**	**0,43**	**0,40**	**0,53**					
osr-t	**0,42**	**0,28**	**0,33**	**0,30**	**0,43**	0,07				
borage	**0,35**	**0,15**	**0,23**	**0,23**	**0,32**	**0,44**	**0,32**			
phacelia	**0,29**	0,13	**0,19**	**0,18**	**0,28**	**0,36**	**0,26**	0,14		
lentil	**0,35**	**0,19**	**0,28**	**0,24**	**0,25**	**0,45**	**0,37**	**0,25**	**0,21**	
vetch	**0,36**	0,16	**0,28**	**0,25**	**0,27**	**0,49**	**0,41**	**0,23**	**0,19**	0,13

Fungi T1-RNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil
marigold	**0,38**									
black oat	**0,19**	**0,17**								
hybrid ry.	**0,22**	**0,15**	0,11							
tall fescue	**0,32**	**0,31**	**0,30**	**0,16**						
osr-E	**0,52**	**0,42**	**0,44**	**0,40**	**0,48**					
osr-t	**0,49**	**0,36**	**0,40**	**0,36**	**0,47**	0,09				
borage	**0,44**	**0,31**	**0,35**	**0,27**	**0,25**	**0,46**	**0,42**			
phacelia	**0,41**	**0,31**	**0,34**	**0,24**	**0,25**	**0,40**	**0,38**	**0,17**		
lentil	**0,46**	**0,31**	**0,40**	**0,33**	**0,27**	**0,51**	**0,49**	**0,32**	**0,31**	
vetch	**0,43**	**0,24**	**0,36**	**0,26**	**0,22**	**0,49**	**0,47**	**0,20**	**0,22**	0,12

Fungi T2-DNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	**0,17**										
black oat	0,08	**0,14**									
hybrid ry.	**0,12**	**0,17**	0,07								
tall fescue	0,09	0,15	0,08	0,07							
osr-E	**0,41**	**0,31**	**0,35**	**0,37**	**0,35**						
osr-t	**0,37**	**0,28**	**0,30**	**0,34**	**0,31**	0,07					
borage	0,08	0,14	0,10	**0,12**	0,12	**0,37**	**0,33**				
phacelia	0,09	0,15	0,10	0,11	0,16	**0,37**	**0,33**	0,10			
lentil	**0,11**	0,11	**0,11**	**0,14**	0,14	**0,37**	**0,33**	**0,12**	0,11		
vetch	0,09	0,12	0,06	0,11	0,12	**0,36**	**0,31**	0,11	0,10	0,08	
mix	**0,39**	**0,30**	**0,33**	**0,35**	**0,33**	0,07	0,07	**0,36**	**0,36**	**0,36**	**0,33**

Fungi T2-RNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	**0,17**										
black oat	**0,12**	**0,15**									
hybrid ry.	**0,13**	**0,15**	0,09								
tall fescue	0,09	0,17	0,11	0,08							
osr-E	**0,37**	**0,25**	**0,32**	**0,33**	**0,33**						
osr-t	**0,29**	**0,21**	**0,26**	**0,28**	**0,25**	0,08					
borage	0,07	**0,14**	0,09	**0,11**	0,10	**0,34**	**0,27**				
phacelia	0,08	0,15	0,09	0,09	0,13	**0,30**	**0,23**	0,08			
lentil	0,10	**0,13**	**0,12**	**0,12**	0,12	**0,34**	**0,28**	0,11	0,11		
vetch	0,10	0,11	0,09	0,11	0,12	**0,30**	**0,25**	0,10	0,10	0,09	
mix	**0,38**	**0,26**	**0,34**	**0,33**	**0,31**	0,11	0,08	**0,34**	**0,31**	**0,34**	**0,32**

Fungi T3-DNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	0,06										
black oat	0,07	0,07									
hybrid ry.	0,08	0,07	0,07								
tall fescue	0,05	0,05	0,05	0,08							
osr-E	**0,19**	**0,16**	**0,14**	**0,17**	**0,17**						
osr-t	**0,17**	**0,14**	**0,13**	**0,15**	**0,14**	0,05					
borage	0,06	0,09	0,08	0,09	0,07	**0,18**	**0,15**				
phacelia	0,07	0,08	0,08	0,08	0,08	**0,16**	**0,16**	0,10			
lentil	0,06	0,06	0,07	0,07	0,05	**0,15**	**0,14**	0,09	0,06		
vetch	0,08	0,07	0,08	0,09	0,07	**0,17**	**0,16**	0,08	0,09	0,06	
mix	**0,18**	**0,15**	**0,13**	**0,17**	**0,16**	0,06	0,06	**0,19**	**0,16**	**0,14**	**0,17**

Fungi T3-RNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	0,07										
black oat	0,07	0,09									
hybrid ry.	0,07	0,08	0,08								
tall fescue	0,11	0,13	**0,12**	0,12							
osr-E	**0,13**	**0,12**	**0,13**	**0,13**	**0,18**						
osr-t	**0,12**	**0,12**	**0,10**	**0,12**	**0,16**	0,06					
borage	0,07	0,09	0,09	0,08	0,13	**0,13**	**0,12**				
phacelia	0,09	0,11	0,11	0,08	0,17	**0,14**	**0,14**	0,12			
lentil	0,07	0,08	**0,09**	0,07	**0,13**	**0,12**	**0,11**	0,10	0,09		
vetch	0,07	0,08	0,07	0,08	0,10	**0,13**	**0,12**	0,06	0,11	0,08	
mix	**0,11**	**0,12**	**0,10**	**0,11**	**0,16**	0,06	0,06	**0,12**	0,13	**0,10**	**0,11**

Protists T1-DNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil
marigold	**0,16**									
black oat	**0,15**	0,10								
hybrid ry.	**0,13**	**0,11**	0,07							
tall fescue	**0,22**	**0,25**	**0,20**	**0,20**						
osr-E	**0,35**	**0,29**	**0,22**	**0,24**	**0,37**					
osr-t	**0,36**	**0,29**	**0,22**	**0,24**	**0,38**	0,05				
borage	**0,21**	**0,17**	**0,17**	**0,19**	**0,20**	**0,33**	**0,33**			
phacelia	**0,26**	**0,23**	**0,21**	**0,23**	0,19	**0,35**	**0,36**	**0,17**		
lentil	**0,16**	**0,19**	**0,17**	**0,17**	0,12	**0,35**	**0,36**	**0,16**	**0,15**	
vetch	**0,17**	**0,20**	**0,15**	**0,16**	0,14	**0,34**	**0,34**	**0,16**	**0,16**	0,07

Protists T1-RNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil
marigold	**0,21**									
black oat	**0,19**	**0,11**								
hybrid ry.	**0,14**	**0,16**	0,10							
tall fescue	**0,26**	**0,24**	**0,18**	**0,20**						
osr-E	**0,40**	**0,29**	**0,27**	**0,31**	**0,39**					
osr-t	**0,42**	**0,32**	**0,29**	**0,33**	**0,40**	0,06				
borage	**0,25**	**0,17**	**0,14**	**0,18**	0,14	**0,32**	**0,34**			
phacelia	**0,24**	**0,20**	**0,16**	**0,19**	0,14	**0,35**	**0,37**	0,10		
lentil	**0,21**	**0,18**	**0,14**	**0,16**	0,09	**0,34**	**0,35**	0,07	0,08	
vetch	**0,23**	**0,18**	**0,14**	**0,17**	0,11	**0,35**	**0,36**	0,08	0,07	0,05

Protists T2-DNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	**0,14**										
black oat	0,10	0,10									
hybrid ry.	**0,10**	0,11	**0,10**								
tall fescue	0,07	0,12	0,11	0,13							
osr-E	**0,40**	**0,29**	**0,31**	**0,31**	**0,40**						
osr-t	**0,30**	**0,21**	**0,21**	**0,23**	**0,29**	0,08					
borage	0,06	0,13	**0,11**	0,11	0,08	**0,39**	**0,28**				
phacelia	0,10	0,12	0,11	0,12	0,14	**0,35**	**0,26**	0,10			
lentil	0,10	0,08	0,08	0,09	0,13	**0,31**	**0,22**	0,10	0,13		
vetch	0,08	0,05	0,06	0,05	0,10	**0,27**	**0,20**	0,09	0,08	0,07	
mix	**0,41**	**0,31**	**0,31**	**0,33**	**0,39**	0,08	0,07	**0,39**	**0,36**	**0,33**	**0,30**

Protists T2-RNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	**0,12**										
black oat	**0,12**	0,08									
hybrid ry.	**0,10**	0,08	0,07								
tall fescue	0,11	0,11	0,10	0,10							
osr-E	**0,26**	**0,17**	**0,18**	**0,21**	**0,23**						
osr-t	**0,25**	**0,18**	**0,15**	**0,20**	**0,23**	0,07					
borage	0,09	0,11	0,10	**0,10**	0,12	**0,22**	**0,22**				
phacelia	0,09	0,12	0,11	0,09	0,14	**0,21**	**0,22**	0,10			
lentil	**0,10**	0,08	0,06	0,07	0,10	**0,21**	**0,19**	0,09	0,10		
vetch	**0,12**	0,08	0,06	0,09	0,10	**0,20**	**0,18**	0,10	0,12	0,07	
mix	**0,30**	**0,23**	**0,21**	**0,25**	**0,27**	0,06	0,08	**0,27**	**0,26**	**0,25**	**0,23**

Protists T3-DNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	0,09										
black oat	0,08	0,08									
hybrid ry.	0,08	0,09	0,08								
tall fescue	0,07	0,09	0,08	0,07							
osr-E	**0,16**	**0,12**	**0,12**	**0,14**	0,12						
osr-t	**0,15**	**0,15**	**0,12**	**0,14**	**0,13**	0,05					
borage	0,08	0,12	0,08	0,09	0,08	**0,15**	**0,15**				
phacelia	0,09	0,08	0,09	0,09	0,11	0,13	**0,15**	0,08			
lentil	0,07	0,09	0,05	0,06	0,06	**0,11**	**0,13**	0,08	0,08		
vetch	0,09	0,10	0,08	0,09	0,07	**0,13**	0,11	0,10	0,09	0,07	
mix	**0,18**	**0,14**	**0,13**	**0,16**	**0,17**	0,04	0,05	**0,17**	**0,15**	**0,14**	**0,13**

Protists T3-RNA

	fallow	marigold	black oat	hybrid ry.	tall fescue	osr-E	osr-T	borage	phacelia	lentil	vetch
marigold	0,08										
black oat	0,09	0,07									
hybrid ry.	0,08	0,08	0,06								
tall fescue	0,10	0,11	0,10	0,10							
osr-E	**0,14**	**0,11**	0,08	**0,11**	**0,14**						
osr-t	**0,13**	**0,11**	0,08	**0,10**	**0,13**	0,06					
borage	0,07	0,07	0,07	0,08	0,10	**0,13**	**0,11**				
phacelia	0,10	0,11	**0,12**	0,11	0,16	**0,15**	**0,14**	0,11			
lentil	0,07	0,07	0,06	0,07	0,09	**0,09**	0,08	0,08	0,09		
vetch	0,10	0,08	0,07	0,08	0,12	0,10	0,09	0,08	0,12	0,07	
mix	**0,16**	**0,13**	**0,10**	**0,12**	**0,16**	0,06	0,06	**0,15**	**0,16**	**0,10**	**0,12**

Supplementary Table S3.5 | Potato tubers yield and potato plant assessment

Cover crop treatment	**average tuber yield per cover crop treatment (kg/bucket)**	average crop performance (between poor = 1 and good = 9)
Fallow	3,1	7,4
Black oat	3,0	6,7
Marigold	3,5	7,9
Tall fescue	3,0	7,3
OSR-T	3,9	7,4
OSR-E	3,9	6,9
Phacelia	3,1	7,4
Borage	2,9	6,8
Vetch	3,1	7,6
Lentil	2,7	6,6
Hybrid ryegrass	3,3	7,9
Mix	3,8	6,6

Supplementary Figures S3.1-4 | Available online at https://doi.org/10.1016/j.soilbio.2023.109080

Chapter 4

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

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Abstract

Agricultural intensification has resulted in a decline in soil biodiversity, and concerns about the deterioration of the biological condition of soils prompted the development of measures to restore soil life. Due to the overwhelming biodiversity of soils, evaluation of such measures is not straight-forward, 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 indica-tors. 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 the species level, including representatives of all trophic groups. As a validation, all samples were analysed microscopically for *M. chitwoodi*, and a comparison of count and DNA read data revealed highly similar results. Treatments did not only affect plant-parasitic nematodes but also free-living nematodes in a cover crop-specific manner. Free-living nematodes from the same trophic group, and even congeneric species, responded differentially to plant-mediated manipulations of the soil microbiome. Hence, nanopore-based SSU rDNA sequencing could facilitate a substantial refinement of the use of nematodes as indicators for soil health.

4.1 Introduction

Soils belong to the most densely inhabited and biodiverse habitats on Earth. Microbiota in terrestrial soils are pivotal to major ecosystem functions such as carbon, nitrogen and phosphorous cycling, the generation of plant-available forms of macro and micronutrients, and soil aggregate formation (Bahram et al., 2018). Current agricultural intensification practices have been shown to result in a decline in soil biodiversity (Tsiafouli et al., 2014), and this may threaten the ecological functioning of soils. Currently, there is an urgent need for management practices that could contribute to the restoration of these ecosystem services. 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 et al. (2021).

Mapping and monitoring the effectiveness of sustainable soil management measures is non-trivial as soils harbour overwhelming biodiversity. In terms of biomass and biodiversity, bacteria and fungi are the dominant organismal groups in terrestrial ecosystems. 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 condition but also reflect the condition of their main food source(s) (Geisen et al., 2018, Cortet et al., 2002, Ewald et al., 2022).

Soil food webs are a schematic way to map and analyse 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, 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. Therefore, they are considered an informative group for soil health assessment (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 relatively uniform shape, their specific gravity, and their mobility, nematode extraction from soil samples in the range of hundreds of grams is relatively straightforward (Verschoor and de Goede, 2000). Although nematodes meet some major requirements to serve as a proxy for the soil's biological condition, their routine use is hampered by their morphological uniformity.

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

DNA-based characterization of nematode communities requires a versatile molecular framework. Various molecular markers have been proposed for such a framework, and the small subunit of the ribosomal DNA (SSU rDNA, also referred to as 18S rDNA) is currently, by far, the most used molecular marker for nematodes. NCBI (https://www.ncbi.nlm.nih.gov), for example, harbours about 30,000 partial or complete nematode SSU rDNA sequences. SSU rDNA is known as a conserved gene, and probably because of the ancient nature of the phylum Nematoda, this gene (≈ 1,700 bp) offers a remarkably good taxonomic resolution (e.g., Holterman et al., 2006; Meldal et al., 2007). Short-read metabarcoding to characterize (artificial) nematode communities was first used by Porazinska et al. (2009). Later on, Illumina MiSeq sequencing of the V4 or the V5-V7 region of SSU rDNA was applied to map nematode communities (Du et al., 2020; Harkes et al., 2020; Kitagami and 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 an experimental field setting, the effect of ten cover crop treatments that are known to differentially affect the soil microbial community (Cazzaniga et al., 2023), as well as a fallow control on nematode communities, was tested. It should be noted that one trophic group, the plant-parasitic nematodes, is directly impacted by cover crops. At the onset of this research, the experimental field was found to be infected with a low density of the Columbia root-knot nematode *Meloidogyne chitwoodi.* This allowed us to investigate – next to the cover crop effects – the impact of elevated *M. chitwoodi* densities on other plant-parasitic nematodes as well as on the free-living fraction of the nematode community. First, a full overview of the nematode communities present in the field was generated employing nanopore sequencing. This was followed by a validation step in which SSU rDNA sequence reads were compared with count data from microscopic sample analysis. This was done for *M. chitwoodi*, as this plant-parasitic nematode species can routinely be detected and quantified based on its morphological characteristics. In the next step, the following soil ecological questions were addressed: 1) Does a strongly increased density of the plant-parasitic nematode *M. chitwoodi* impact other plant-parasitic and/or free-living nematodes? 2) How do cover crop treatments affect free-living and plant-parasitic representatives of nematode communities? 3) Does the high-resolution characterization of nematode

communities (until genus and/or species level) have an ecological or agronomical added value?

4.2 Materials And Methods

4.2.1 Experimental field set-up

The field experiment was set up at the Vredepeel experimental field station of Wageningen University and Research, Field Crops (WUR-FC). The Vredepeel farm is located in the southeastern part of the Netherlands (700 – 800 mm precipitation $year^{-1}$, mean temperatures of 11°C) on sandy soil (93.3% sand, 4.5% silt, 2.2% clay) (Quist et al., 2016). The current experiment was embedded in a larger trial by WUR-FC aimed at assessing the host plant status of a selection of arable and cover crops in a field with a low density of the root-knot nematode (RKN) *M. chitwoodi*. The field experiment comprised six rectangular strips (each 6 x 42 m) organized in three blocks (Supplementary S4.1). In half of the strips, the initial RKN concentration was raised by growing an excellent host, black oat (*Avena strigosa*, cultivar Pratex). On the other half of the strips, perennial ryegrass (*Lolium perenne*, cultivar Mercedes), a poor host of *M. chitwoodi,* was grown. For the pre-crop treatments, no fallow controls were included. Both poaceous crops were grown in the field between August 2018 and July 2019 and are referred to as "pre-crops". Perpendicular to the longitudinal direction of these strips, 11 plots (each 6x3 m) were defined, and after pre-crop treatment, plots were exposed to ten cover crop treatments, whereas the 11^{th} plot remained unplanted (fallow control). Hence, corresponding to the two pre-crop treatments, two types of fallow control are included. Cover crop treatments included six monocultures and four mixtures (Table 4.1). Cover crops were sown on August 7th 2019, and grown for five months. On December 2nd 2019, cover crops were mechanically terminated using a rotary tiller and residues were incorporated into the topsoil. In spring 2020, soil was tilled, and on April 30th, the main crop potato (*Solanum tuberosum,* cultivar 'Hansa') was planted. Potato was harvested on October 14th 2020.

4.2.2 Nematode extraction and microscopic *M. chitwoodi* quantification

To assess the nematode soil community, bulk soil samples were collected at two time points: i) at cover crop termination (December 2nd, 2019, hereafter referred to as T1) and ii) after potato harvest (October 15th 2020, hereafter referred to as T2). In both samplings, 1.5 l of topsoil was collected from the central area (1.5 × 2.7 m) of each plot with an auger (sampling depth 25 cm,

diameter 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 fractions were used in subsequent steps.

4.2.3 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 fibre filtration plates. Purified nematode community DNA was eluted and immediately stored at −20°C. The DNA concentration of the combined purified lysates was quantified using a Qubit Fluorometer and subsequently diluted to an end concentration of 0.1 ng/µl. Primers 988F (5’-ctcaaagattaagccatgc-3') and 2646R (5’-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 sample multiplexing. 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 Supplementary Table S4.1. As the samples primarily consisted of nematode DNA, a reversed touchdown-PCR could be used that allows for SSU rDNA amplification even if, for some taxa, the flanking region does not perfectly match the PCR primers. After DNA amplification, 4 µl of PCR product was loaded on a 1.5% agarose gel to verify the amplification and the concentration of all PCR products was measured using a Qubit 4 Fluorometer.

Table 4.1 | Details of the cover crop species and cultivars used in this study, including the origin of seeds, sowing density and host status for *Meloidogyne chitwoodi.*

Treatment	Cover crop	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 Radical	multiple	Pratex + Radical	40+15	Good + moderate
BLO_OSR_T	Black oat + Oilseed radish Terranova	multiple	Pratex + Terranova	40+15	Good + non-host
PHA_OSR_T	Phacelia + Oilseed radish Terranova	multiple	BeeHappy + Terranova	7+15	Poor + non-host
VET_OSR_T	Vetch + Oilseed radish Terranova	multiple	Ameli + Terranova	70+15	Poor + non-host
FW	Fallow	-	-	-	-

4.2.4 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 (< 1,000 bp), 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.

4.2.5 Data processing

Basecalling of raw reads was performed using Guppy (v6.2.1, Oxford Nanopore Technologies Plc., UK) in super-accuracy mode. Guppy was then used to demultiplex samples and remove adapters and barcodes. For a single sample, <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 into Decona was used for taxon delineation against an in-house curated nematode SSU rRNA reference database, and the top hit was selected. This in-house reference database consists of >5,000 nearly full SSU nematode sequences (nearly all are available on GenBank; see M. Holterman et al., 2017 and M. Holterman, Schratzberger, & Helder, 2019). Decona output files were merged into an OTU table using a custom Python script, and identifications with an ID percentage below 97% were excluded. Before statistical analyses, nematode taxa that were detected only once were excluded. The OTU table and metadata were subsequently processed using phyloseq (v. 1.42.0) (McMurdie & Holmes, 2013) in R Software (v. 4.2.2) (R Core Team, 2021). An overview of the workflow is presented in Figure 4.1.

4.2.6 Statistical analysis

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 4.1. The sequencing data were 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 the response variable in a generalized linear mixed model with a negative binomial distribution (GLMM-NB), with cover crops, pre-crops and time point as fixed factors and block as a random factor. Zero-Inflated Negative Binomial mixed models (GLMM-ZINB) (Zhang & Yi, 2020) were used in excess of zeros (zero-inflation tested with performance R package). Microscopic *M. chitwoodi* counts were used in a GLMM-NB model with

interaction between cover crops and pre-crops and time as fixed factors and block as a random factor. Box plots with log-transformed reads or counts were generated in ggploT2 (v. 3.4.1) (Wickham, 2016) and statistical significance was indicated based on the output of the mixed models using the R package glmmTMB (v. 1.1.6, Brooks et al., 2017).

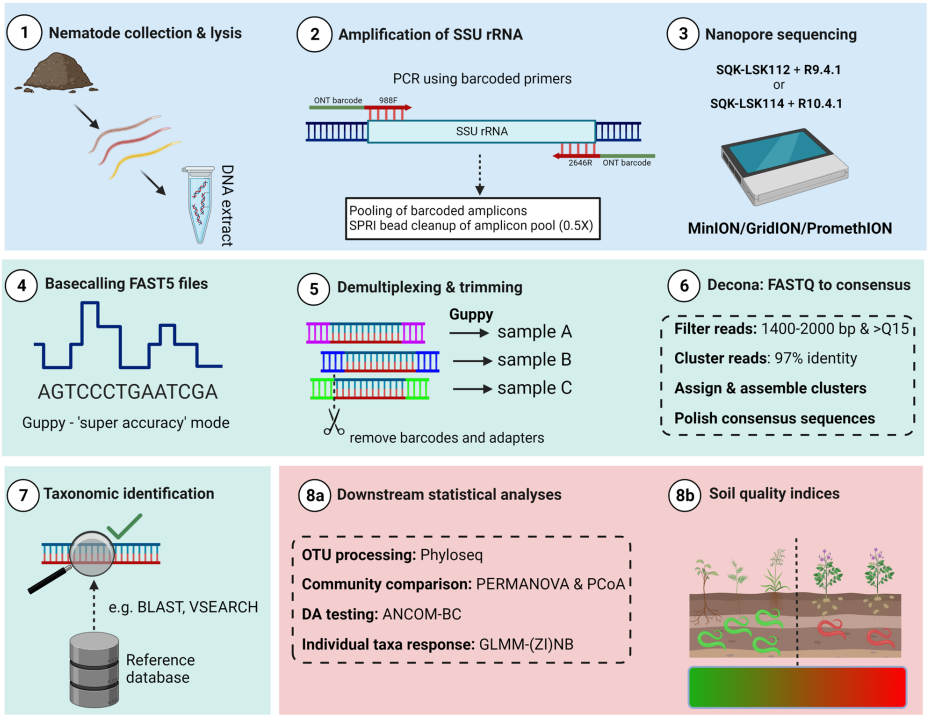

Figure 4.1 | Workflow for nanopore sequencing-based nematode community analysis. 1. Nematodes are separated from the soil matrix, concentrated and lysed. 2. Amplicons spanning the complete SSU rDNA gene are generated, and (3) resulting libraries ran on a nanopore sequencing device. After high accuracy base-calling (4), demultiplexing and trimming (5), polished consensus sequences are generated (6). A curated reference database is used for nematode taxon identification (7) and resulting community composition data are statistically analyzed (8a) and, for example, used for nematode-based soil quality indices (8b).

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

Sequencing counts were normalized 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, the block was always added as the first term to remove the variability attributed to a block effect from the variability attributable to the following factors. ANCOM-BC (v1.4.0, default parameters) (Lin & Peddada, 2020) was used to investigate the overall impact of pre-crop and cover crop treatments on the nematode community. Non-transformed reads were used to characterize the impact of M. chitwoodi stimulation by black oat as pre-crop on nematode communities, as compared to the impact of perennial ryegrass as a non-host.

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

Effect of cover crop treatments on four *Pratylenchus* species

Root lesion nematodes (*Pratylenchus spp*.) are known as a stenomorphic genus. While members of this genus are easily recognizable, species are difficult to separate. Four *Pratylenchus* species were present in the experimental field, and we analyzed whether individual *Pratylenchus* species showed distinct responses upon exposure to a range of cover crop treatments (T1) and to the potato cultivar Hansa (T2). *Pratylenchus* counts were selected from the rarefied dataset (see 2.6.1) and fitted in GLMM-NB models with cover crop treatment, time point and pre-crop treatment as fixed factors and block as a random factor with the glmmTMB (v. 1.1.6). Estimated marginal means per group and statistical differences between groups were calculated with emmeans (v. 18.5, Lenth, 2022) and cld (multcomp, v.1.4-23, Hothorn et al., 2008) with Benjamini-Hochberg correction for multiple testing.

4.3 Results

4.3.1 Nematode community characterization by long-read amplicon sequencing

In an experimental field setting, we aimed to map the effects of local manipulation of the density of the plant-parasitic nematode *Meloidogyne chitwoodi* at the nematode community level using MinION-based full-length SSU rDNA sequencing. For this, a total of 132 soil samples were collected at two times points, in late Autumn, just after cover crop termination ('T1') (66 samples) and 10.5 months later, just after the harvest of the main crop, potato ('T2') (66 samples).

Amplicons covering almost the complete SSU rDNA (≈ 1,7 kb) were generated for all but one of the samples, and MinION sequencing resulted in the generation of 3,013,020 filtered reads for T1 and 5,165,791 filtered reads for T2. For T1 and T2, the median number of read counts per sample was respectively 47,378 and 81,005, with a median number of OTUs of 19.50 and 20.00. Blasting OTUs against a curated nematode SSU rDNA database resulted in the identification of 86 nematode taxa 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 4.3). Next to 13 plant-parasitic nematode species, nematode communities harboured bacterivores (27 taxa), fungivores (9 taxa), omnivores (11 taxa), predators (4 taxa) and one insect parasitic taxon. Notably, we found one widespread bacterivorous taxon referred to as Rhabditidae_fam (in 97% of the samples). Full-length SSU rDNA sequences demonstrated this taxon belonged to the family Rhabditidae, but the BLAST identity was too low to assign it to a Rhabditidae genus (sequences were similar to the Rabditidae genera *Cephaloboides* and *Pellioditis* with respectively 96% and 95% identity).

As expected, *M. chitwoodi* was present in most samples (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 against our database showed an average overall identity of 97.2% with *M. exigua*. The associated consensus sequence was subsequently also identified using BLAST against the complete NCBI database, which yielded a < 97% ID with a *Meloidogyne* species. We conclude this presumably is an RKN species related but not identical to *M. exigua.* For this reason, it is referred to as *Meloidogyne cf. exigua* (Table 4.3).

Among the bacterivores, the broad distribution of members of the family Cephalobidae (*Acrobeles* sp., *Acrobeloides* sp., *Chiloplacus* sp., *Eucephalobus* sp., present in > 75% of the samples) is noteworthy. In contrast, the distribution of fungivores was patchier; the most widespread genera, *Aphelenchus* and *Aphelenchoides,* were detected in around 20% of the samples. The entomopathogenic nematode *Steinernema affine* known to be native to The Netherlands (Spiridonov et al., 2004), was present in 3% of the samples. Among the omnivores, *Aporcelaimellus obtusicaudatus* stood out as it was present in about 70% of the samples. Predatory nematodes showed a patchy distribution in the experimental field, with *Mononchoides* being the most widespread (present in 24% of the samples). In the taxon overview (Table 4.3), 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 seven bacterivores and two plant parasites.

4.3.2 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 4.2 A, B, $p \leq 0.01$ and $p \leq 0.001$ respectively). Irrespective of the detection method and pre-crop identity, cultivation of the susceptible potato cultivar Hansa resulted in a further increase in *M. chitwoodi* levels (orange box plots and black horizontal bars in Figure 4.2 A, B, $p \leq 0.001$ for all four combinations). 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 4.2 A, B). So, although read counts cannot easily be translated into numbers of individuals for *M. chitwoodi*, the effects of treatments on *M. chitwoodi* densities in an experimental field setting look highly similar, irrespective of whether communities were analysed microscopically or by a MinION-based DNA sequencing approach.

4.3.3 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 4.4). The strongest effect was observed for cover crops (explaining 21% of the variation, Supplementary Figure S4.2 A), 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 the harvest of potatoes, the composition of the nematode community was characterized again. As can be seen in Table 4.4, 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. PCoA graphs of the two time points based on Bray-Curtis dissimilarity (Supplementary Figures S4.2 A, B) clearly show the pre-crop effects. The presence of a cover crop effect at T1 is exemplified by highlighting the effect of vetch in Supplementary Figures S2 A and B.

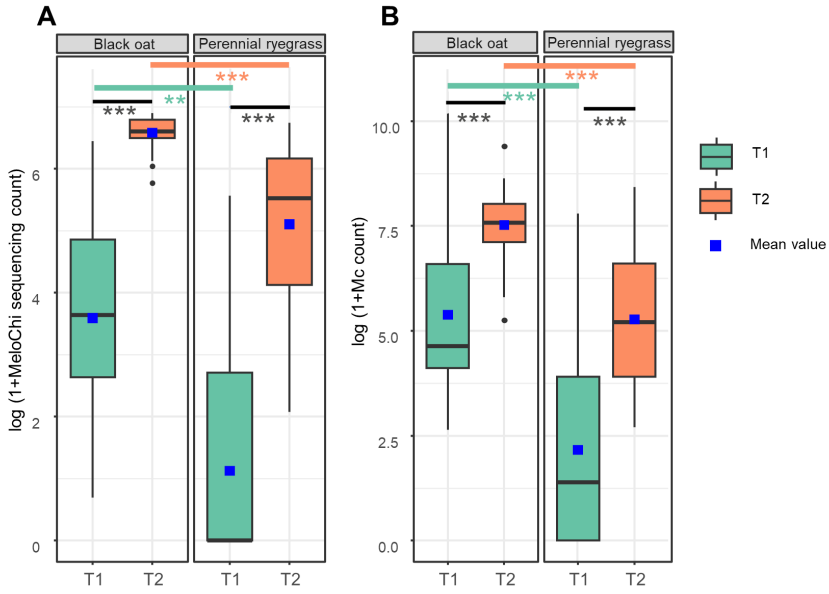

Figure 4.2 | Comparison of two methods to determine *Meloidogyne chitwoodi* densities at T1 (after pre-crop and cover crop treatments) and T2 (after potato): (A) Nanopore sequencings reads (rarefied to minimum library size, 5932; (B) microscopic counts. Counts were compared at T1 and T2 on the basis of the pre-crop used to create the initial two M. chitwoodi population densities: black oat = good host gave highest M. chitwoodi densities, perennial ryegrass = poor host, gave initial lowest M. chitwoodi densities. After pre-crop and cover crops, potato (a good host for *M. chitwoodi*) was grown, and soil samples were collected just after harvest. *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$. Supplementary Figure 4.2 B suggests that a significant effect of vetch could still be present at T2, but this is largely attributable to the substantial block effect (Table 4.4).

Table 4.3 | Nematode biodiversity in experimental fields at the Vredepeel field station (The Netherlands). Nematodes are identified on the basis nearly full length SSU rDNA sequences (» 1.700 bp). Taxa are clustered according to their trophic preferences. Taxa are included only if they were detected in at least two soil samples. The percentage of samples in which in dividual taxa were detected at T1 (after cove crop) and T2 (after potato) is provided in separate columns. Bold: taxa on average (T1 +T2) present in > 80% of the samples.

Bacterivores	T1 (%)	T2 (%)	**Fungivores**	T1 (%)	T2 (%)	**Predators**	T1 (%)	T2 (%)
Achromadora ruricola	0	6	*Anomyctus xenurus*	3	3	*Clarkus papillatus*	20	18
Acrobeles ciliatus	36	57	*Aphelenchoides bicaudatus*	5	0	*Clarkus sp.*	9	8
Acrobeles complexus	**91**	**72**	*Aphelenchoides blastophthorus*	0	5	*Mononchoides sp.* (and bacterivore)	23	25
Acrobeles sp.	**95**	**94**	*Aphelenchoides sp.*	15	25	*Mylonchulus hawaiiensis*	0	3
Acrobeloides apiculatus	**95**	**91**	*Aphelenchus avenae*	0	6			
Acrobeloides maximus	3	0	*Aphelenchus sp.*	2	42	**Plant parasites**		
Acrobeloides varius	**95**	**88**	*Filenchus misellus* (and plant parasite)	18	23	*Ditylenchus destructor*	33	45
Alaimus sp.	14	32	*Filenchus vulgaris* (and plant parasite)	9	14	*Ditylenchus sp.*	11	28
Anaplectus porosus	36	18	*Tylenchidae* (and plant parasite)	3	3	***Meloidogyne chitwoodi***	**68**	**100**
Chiloplacus propinquus	**100**	**98**				*Meloidogyne* cf. *exigua*	2	26
(Chilo)Plectus andrássyi	17	12	**Insect parasites**			*Meloidogyne naasi*	5	0
Cruznema sp.	45	17	*Steinernema affine*	2	5	*Paratrichodorus pachydermus*	2	3
Diploscapter sp.	33	34				*Paratrichodorus teres*	6	15
Eucephalobus oxyuroides	82	68	**Omnivores**			*Pratylenchus crenatus*	18	54
Eucephalobus striatus	**98**	**97**	*Aporcelaimellus obtusicaudatus*	65	74	*Pratylenchus fallax*	32	29
Mesorhabditis sp.	53	20	*Aporcelaimellus paraobtusicaudatus*	24	11	*Pratylenchus neglectus*	29	54
Oscheius tipulae	0	3	*Aporcelaimellus sp.*	3	23	*Pratylenchus scribneri*	21	17
Panagrolaimus sp.	2	5	*Calcaridorylaimus sp.*	14	0	*Trichodorus viruliferus*	3	8
Pelodera cylindrica	3	0	*Dorylaimoides sp.*	2	11	***Tylenchorhynchus dubius***	**91**	**100**
Pelodera teres	80	65	*Ecumenicus sp.*	8	9			
Plectus sp.	2	5	*Microdorylaimus miser*	32	58			

Rhabditidae_fam	**95**	**98**	*Microdorylaimus modestus*	3	2
Rhabditis sp.	76	35	*Thonus circulifer*	17	17
Rhabditis terricola	39	26	Tylencholaimellidae	36	23
Rhabditophanes sp.	86	51	*Tylencholaimus sp.*	6	6
Zeldia sp.	0	3			
Pristionchus uniformis	5	2			

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

Differential abundance testing (ANCOM-BC) was used to characterize the impact of the pre-crop black oat, known as a good host for *M. chitwoodi*, as compared to the effect of perennial ryegrass, known as a poor host for this root-knot nematode, over all cover crop treatments (Figure 4.3). 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 4.3). Remarkably, other widespread and closely related relatives, such as *Acrobeles ciliatus* and *Eucephalobus oxyuroides* (see Table 4.3) 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.

Table 4.4 | PERMANOVA analysis with Bray-Curtis dissimilarity metric to assess the variation explained by block, pre-crop, cover crop, and interaction effect between pre-crop and cover crop upon CSS normalization of data A) after cover crop termination and incorporation in topsoil (T1) and B) just after the harvest of the main crop, potato (T2).

	A) T1			**B)** T2		
	Df	R^2	p-value (F)	Df	R^2	p-value (F)
Block	3	0.16	0.001 (***)	3	0.15	0.001 (***)
Pre-crop	1	0.06	0.001 (***)	1	0.18	0.001 (***)
Cover crop	10	0.21	0.002 (**)	10	0.13	0.141
pre.crop:cover.crop	10	0.11	0.475	10	0.13	0.125

4.3.5 Effects of cover crop treatments at the nematode community level

For each of the two pre-crops, perennial ryegrass and black oat, the impact of individual cover crop treatments upon manipulation of the *M. chitwoodi* density at T1 was analyzed taking only into consideration taxa with an estimated coefficient (from MaAsLin2) lower than -2, or above 2. When perennial ryegrass was used as pre-crop, as shown in Figure 4.4 A, 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 4.4 B). Mixtures with oilseed radish cv. Terranova (OSR_T in Figure 4.3) all had a strong negative impact on the omnivore *Aporcelaimellus paraobtusicaudatus* (Figure 4.4 B). Moreover, two specific treatments that included oilseed radish cv. Radical negatively affected the plant parasite *T. dubius.* Only two treatments that both included black oat (black oat and oilseed radish Terranova, and black oat) resulted in a stimulation of a community member, namely non-identified member(s) of the bacterivorous family Rhabditidae.

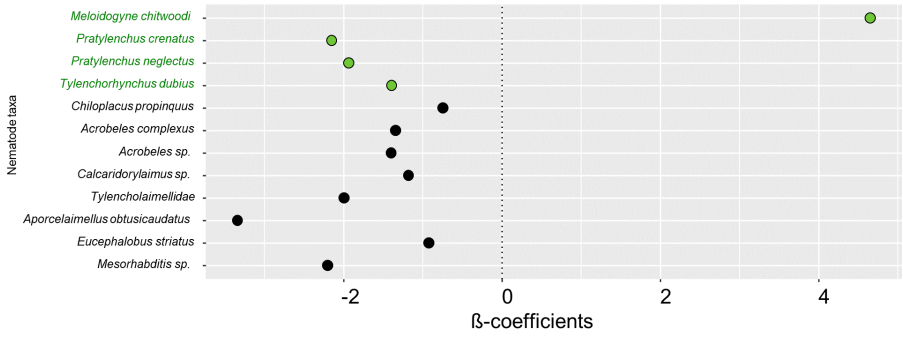

Figure 4.3 | Differential abundance testing (ANCOM-BC) was used to characterize the impact of the pre-crop black oat, a good host for M. chitwoodi, as compared to the effects of perennial ryegrass, a poor host for this root-knot nematode, on nematode communities over all cover crop treatments at T1. The displayed taxa were differentially abundant in black oat compared to perennial ryegrass, according to the test. The ANCOM-BC beta coefficient is a measure to show the extent by which individual nematode taxa are affected by one pre-crop compared to the other.

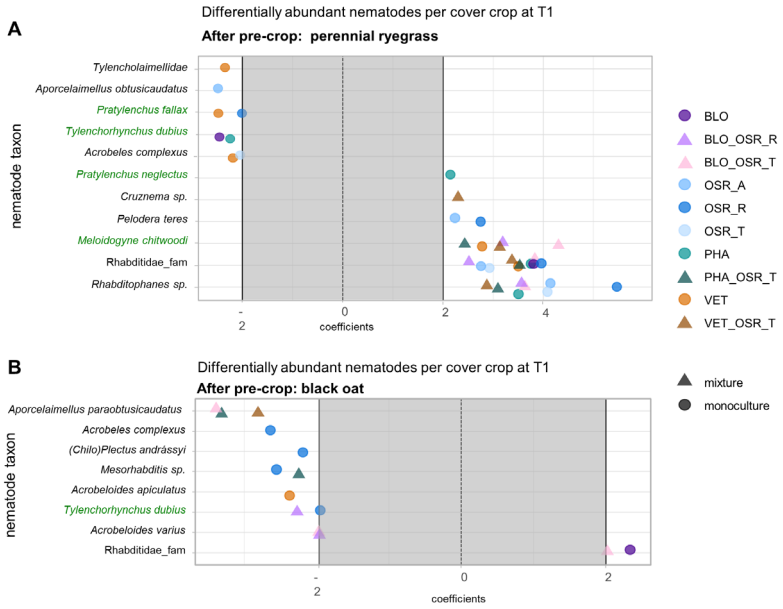

Figure 4.4 | Differential abundance testing (MaAsLin2) was used to characterize the impact of cover crop treatments on nematode communities at T1 in plots that were initially exposed to the pre-crop perennial rye (A) or to black oat (B). Only nematode taxa with regression coefficients lower than -2, or above 2 are shown.

4.3.6 Effect of cover crop treatments on four *Pratylenchus* species

Four *Pratylenchus* species were present in the experimental field, and in Table 4.5, 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). *P. crenatus* was the only root lesion nematode species that was negatively affected by black oat. In contrast to the other three *Pratylenchus* species, neither the type of cover crop nor the pre-crop treatments affected *P. fallax*. *P. neglectus* was negatively impacted by all three oilseed radish monocultures and by three out of the four cover crop mixes that included oilseed radish. *P. scribneri* was not stimulated nor repressed by any cover crop. Except for *P. fallax,* all root lesion nematode species were negatively affected by the pre-crop black oat as compared to perennial rye. From this analysis, we conclude that despite their morphological resemblance, individual root lesion nematode species respond in species-specific ways upon exposure to both cover and main crops.

Table 4.5 | Effect of monocultures and simple mixtures of cover crops and the subsequent cultivation of potato of four lesion nematode species, *Pratylenchus crenatus*, *P. fallax, P. neglectus* and *P. scribneri*. Pratylenchus reads were rarefied and fitted in negative binomial mixed models (GLMM-NB) with cover crop treatment, time point and pre-crop treatment as fixed and block as a random factor. In the table, estimated adjusted means of nematode reads per group (emm), the corresponding standard error (se.emm) and significant differences between groups (group) are indicated. Table follows in the next page.

	Pratylenchus crenatus			*Pratylenchus fallax*			*Pratylenchus neglectus*			*Pratylenchus scribneri*		
Cover crop (T1)	emm	se.emm	group	emm	se.emm	group	emm	se.emm	group	emm	se.emm	group
FW	1.91	1.51	bc	2.46	2.11	abc	11.42	7.12	c	0.73	0.85	a
BLO	0.06	0.06	a	1.31	1.29	abc	6.24	3.54	bc	2.18	3.79	a
OSR-R	1.85	1.53	bc	1.45	1.25	abc	0.15	0.12	a	0.00	0.00	a
OSR-A	0.69	0.61	ab	4.99	4.71	abc	0.24	0.18	a	3.54	4.30	a
OSR-T	1.01	0.85	bc	0.25	0.27	ab	0.22	0.16	a	0.19	0.28	a
PHA	12.72	11.38	c	8.90	7.49	bc	26.18	14.17	c	6.17	6.88	a
VET	0.23	0.21	ab	5.37	4.83	abc	1.14	0.73	ab	4.17	5.00	a
BLO.OSR-R	0.75	0.67	ab	0.95	0.83	abc	1.32	0.81	ab	0.45	0.54	a
BLO.OSR-T	0.23	0.21	ab	0.19	0.20	a	0.52	0.35	a	0.29	0.36	a
PHA.OSR-T	1.82	1.45	bc	26.23	22.88	c	22.56	13.52	c	3.93	4.20	a
VET.OSR-T	1.40	1.12	bc	0.23	0.23	ab	0.34	0.24	a	0.73	0.84	a
Time point												
At T1	0.24	0.16	a	1.34	0.63	a	0.64	0.21	a	0.18	129	a
At T2	3.04	1.80	b	2.11	0.99	a	3.50	0.97	b	0.19	140	a
Pre-crop												
bo	0.18	0.12	a	1.03	0.54	a	0.36	0.14	a	0.01	89.7	a
er	4.1	2.5	b	2.75	1.34	a	6.24	1.65	b	0.05	813.4	b

4.4 Discussion

4.4.1 A Nanopore sequencing approach for nematode community analyses

Being abundant in virtually any soil, trophically diverse, and ecologically relatively well-characterized, nematode communities have the potential to be used as a proxy for the soil's biological condition (Ferris et al., 1999). However, microscopy-based methods for community analysis require extensive taxonomical expertise, are labour-intensive, and, most often, juvenile life stages are not taken into consideration due to a lack of informative morphological characteristics. In essence, DNA sequencing-based approaches can overcome these hurdles, but most high throughput sequencing methods produce relatively short reads that intrinsically limits the taxonomic resolution. Here we show that Nanopore sequencing allows for the routine sequencing of full-length SSU rDNA (≈ 1,700 bp), by far the most popular barcoding gene for nematodes, and results in complete overviews of nematode communities either until genus or (most often) to species level. Nanopore sequencing has been used before by Knot et al. (2020) to identify nematodes within an artificial community of four nematode species. Here, we present a nanopore sequencing-based workflow that allows for routine analyses of nematode communities with a high taxonomic resolution and present data that demonstrate the ecological and agronomic relevance of high-resolution community analyses.

4.4.2 Nematode community composition

In our experimental field, we detected 65 nematode taxa with representatives from all trophic groups. This nematode diversity lies in the same order of magnitude as the diversity of other agricultural fields in the Netherlands (Mulder et al., 2005) or Sweden (Sohlenius et al., 1987). In the current community composition overview, members of the bacterivorous family Cephalobidae including the genera *Acrobeles, Acrobeloides, Chiloplacus* and *Eucephalobus*, are amply represented. In many studies, the abundance of the family of bacterivores has been reported in both agricultural (Sohlenius et al., 1987) and natural habitats (Porazinska et al., 2012). A striking characteristic of Cephalobidae is the diversity in elaborations of the body wall cuticle surrounding the mouth and lips ('probolae'). *Acrobeles* is characterized by extensive, deeply bifurcated probolae, whereas *Eucephalobus* members are equipped with particularly short probolae. These elaborations are thought to play a role in feeding (De Ley 1992). If this assumption is correct, it would imply that members of Cephalobidae differ in their feeding strategy, and

apparently, this diversification contributed to their evolutionary success. From a soil ecological perspective, it would therefore be preferable not to lump these members into a single category, as the presence of the individual taxa might reflect the condition of distinct categories of soil bacteria.

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

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

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

4.4.3 Quantification of nematode community data – sequence reads *versus* microscopic counts

DNA read counts cannot easily be translated into numbers of nematode individuals. Nevertheless, we made the comparison between morphology- and DNA-based analysis, and the contrasts were remarkably similar both in directionality and statistical robustness. It should be noted, however that for *M. chitwoodi* (like for all RKNs), this comparison might be more straightforward than for most other nematode species. *M. chitwoodi* has mainly one mobile life

stage in soil, the pre-parasitic second-stage juveniles. Males are the other mobile life stage, but males are only formed under stress conditions for this facultative meiotic parthenogenetic nematode species (Castagnone-Sereno et al., 2013). It is expected that the DNA content of individual pre-parasitic juveniles is more or less a constant, and this would suggest a linear relationship between numbers *M. chitwoodi* and the *M. chitwoodi*-derived DNA concentration in the community lysates. So, it should be noted that for most other nematode species, the relationship between counts and sequence reads could be less comparable. The one example that is comparable to *M. chitwoodi* in Table 4.1 is the entomopathogenic nematode *Steinernema affine.* Also, for this nematode, only a single mobile life stage, the Dauerlarva, is found in soil. All other life stages can be found inside their host, insect larvae. For most other nematode species mentioned in Table 4.1, probably multiple life stages were present in the samples under investigation.

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

Stimulation of *M. chitwoodi* by growing the good host black oat as a pre-crop also resulted in the repression of multiple other nematode taxa. This repression could be caused (in)directly by the plants as they can alter the soil microbiome locally (Koprivova and 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, stimulation of the sedentary endoparasite *M. chitwoodi* had a negative effect on two migratory endoparasites, *P. crenatus* and *P. neglectus*. Competition between (*Meloidogyne)* and a (*Pratylenchus*) has been investigated before. Co-inoculation of barley with *M. chitwoodi* and *P. neglectus* revealed that the species that parasitized the roots first lowered the parasitic success of the other (Umesh et al., 1994). In this system, the lesion nematode outcompeted *M. chitwoodi.* Hence, our findings might be the result of competition between lesion and root-knot nematodes, and - if correct - the nature of the interaction appears to be context-dependent. Alternatively, differences in host plant status might have contributed to the observed suppression of some root lesion nematode species (host plant status of cover crops for *Pratylenchus* species is largely unknown). Also, the ectoparasite *T. dubius* was negatively affected by the stimulation of *M. chitwoodi. T. dubius* belongs to the nematode family Telotylenchidae. Under field conditions in a vegetable cropping system (all vegetables were susceptible to RKNs) (Mateille et al., 2020) also observed a competition between Telotylenchidae and RKNs.

As such, we can conclude that the difference in feeding strategy does not rule out competition between obligatory plant-parasitic nematodes.

The negative impact of *M. chitwoodi* stimulation on the omnivore *Aporcelaimellus obtusicaudatus*, a very common predaceous nematode that feeds on microdrile oligochaete 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.

4.4.5 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 (Figure 4.4 A, B). In the context of an initially low *M. chitwoodi* density, six nematode taxa, including two plant parasites, were strongly stimulated. In the case of *M. chitwoodi,* this was associated 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., 2023b, **Chapter 3**). Therefore, it is tempting to suggest that *Rhabditophanes* sp. benefitted from the local increase in a potential food source, active Gammaproteobacteria.

After growing black oat as a pre-crop, most nematode taxa were significantly repressed. This is especially true for *Aporcelaimellus paraobtusicaudatus,* an omnivorous nematode that was repressed in three cover crop mixtures that all included oilseed radish. Although rDNA sequences support the distinction between *A. paraobtusicaudatus* (Figure 4.4 B) and *A. obtusicaudatus* (Figure 4.4 A) (Holterman et al., 2008), it is uncertain whether or not these should be considered two species (Álvarez-Ortega and Peña-Santiago, 2013). Assuming that microdrile Oligochaeta and other nematodes are also the main food source of *A. paraobtusicaudatus*, we hypothesize that these food sources were repressed or repelled by oilseed radish. 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 conditions and respond rapidly to local bacterial bloom, which probably has happened upon the incorporation of terminated cover crop material into the topsoil.

4.4.6 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 until the 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 of the stimulation of a single plant-parasitic nematode on the nematode community as a whole, as well as the effect of individual cover crop treatments on nematode communities. Moreover, we showed that this long-read approach was able to distinguish species within the stenomorphic plant-parasitic genus *Pratylenchus*, and our analyses also showed that this resolution matters, also from an agronomic perspective.

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

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 to the preparation of the sequencing library.

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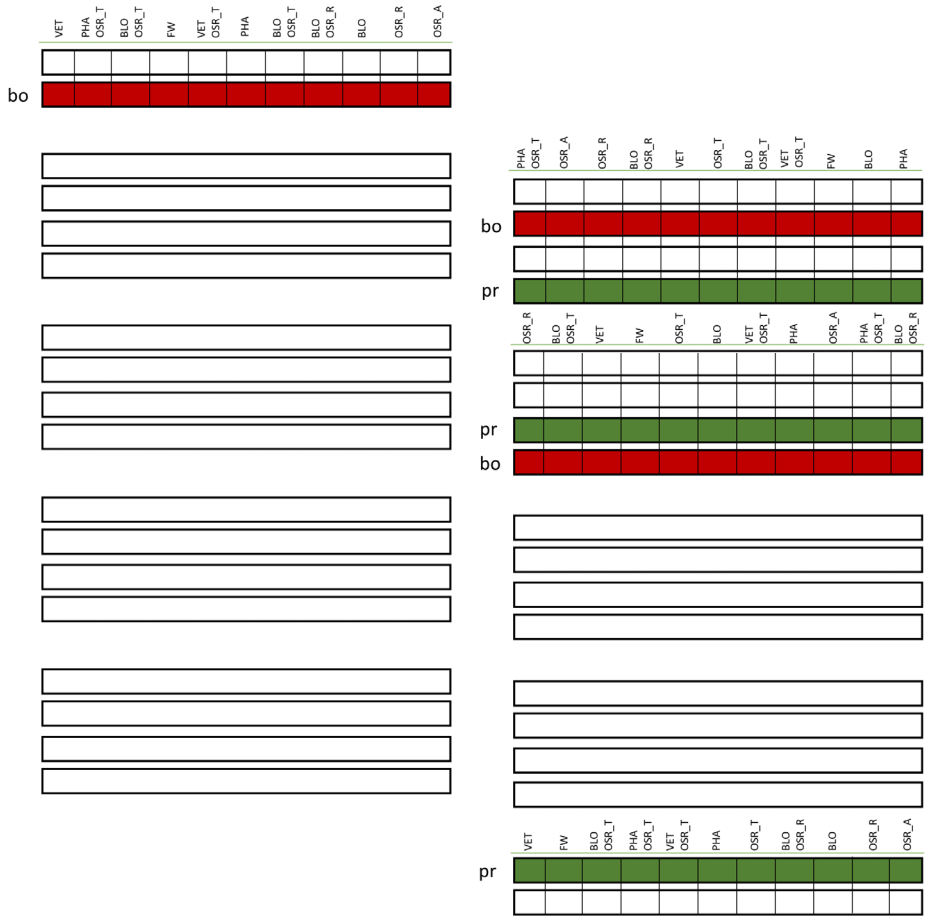
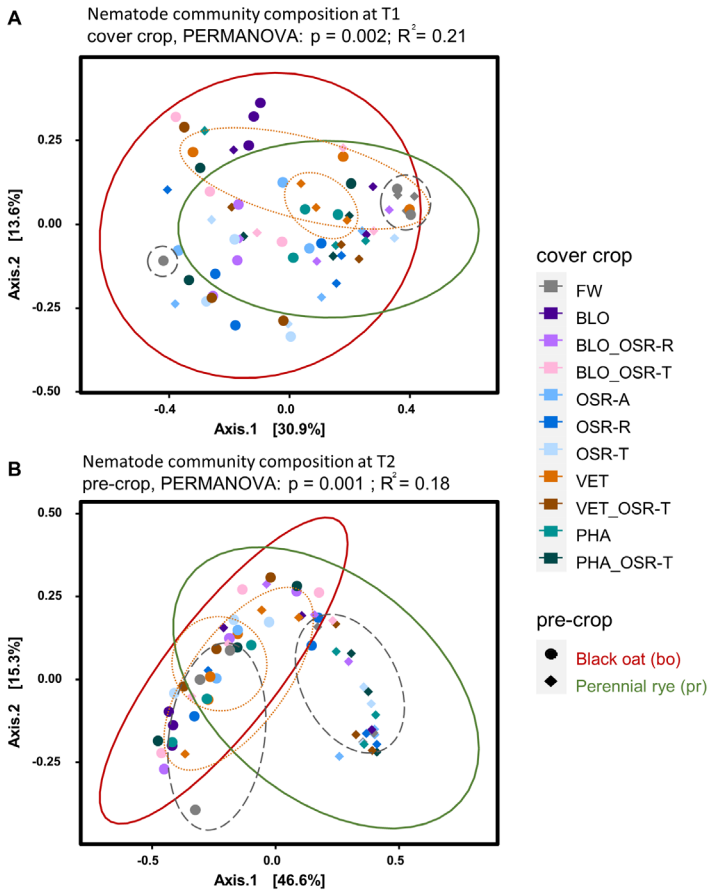
4.5 Supplementary Materials

Supplementary Table S4.1 | Temperature profile for PCR amplification of nearly full length nematode SSU

	Temperature	*Time*	*# cycles*		*Temperature*	*Time*	*# cycles*
Initiation	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	2 min 30 s	
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	3min			65°C	2 min	
					65°C	5 min	1X
					12°C	Continuous	1X



Supplementary Figure S4.1 | Layout of field experiment at Vredepeel. 'bo' refers to black oat as a pre-crop treatment, 'pr' to perennial rye. Perpendicular to the longitudinal direction of the pre-crop strips, 11 plots (each 6x3 m) were defined, where ten cover crop treatments were grown, and one was left fallow (unplanted control). VET= vetch, BLO= black oat, PHA= phacelia, BLO.OSR_T= black oat – oilseed radish Terranova, PHA.OSR_T= phacelia – oilseed radish Terranova, VET.OSR_T= vetch - oilseed radish Terranova, BLO.OSR_R= black oat – oilseed radish Radical, OSR_T= oilseed radish Terranova, OSR_R= oilseed radish Radical, OSR_A= oilseed radish Adios.



Supplementary Figure S4.2 | Effect of cover crop treatments (colours) and pre-crops (shapes) on nematode communities at T1 (A) and after the main crop (potato) at T2 (B). Shown is the principal Coordinate Analysis (PCoA) of CSS normalised OTU data with distance based on Bray-Curtis metric. Fallow control after each pre-crop is highlighted with solid grey ellipses, vetch, as an example, is highlighted with dashed orange ellipses. The factor explaining most of the nematode community variation per time point is indicated above the figure.

Chapter 5

Impact of cover crops on native nematode antagonists in arable soil

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In preparation

Abstract

Plant-parasitic nematodes are among the most harmful pests of cultivated crops causing important economic losses. Most root-knot nematodes (*Meloidogyne* spp.) are highly polyphagous endoparasites and are notoriously hard to control. While the introduction of nematode antagonists has been considered a potential strategy to curb root-knot nematode proliferation, past investigations have underscored the challenges linked to the competitive attributes of the soil's microbiome when attempting to inoculate antagonists. Stimulation of native nematode antagonists could be a viable alternative to stimulate the suppressive potential of soils. We performed a field experiment with three different densities of the Columbia root-knot nematode *Meloidogyne chitwoodi*. We mapped the effects of 10 cover crop treatments against the background of distinct *M. chitwoodi* levels on both the resident (DNA) and the active (RNA) fraction of the bacterial and fungal communities, with a specific focus on those referred to as nematode antagonists in literature. Among the eight bacterial and 26 fungal genera known to harbour nematode antagonists, respectively five and thirteen were detected in the rhizosphere of cover crops. Generally, cover crops had a stronger impact on genera harbouring nematode antagonists than *M. chitwoodi* density. Furthermore, cover crop treatments stimulated genera of nematode antagonists in a genus-specific manner, but increased abundances of nematode antagonists by cover crops were usually not paralleled by increased activity levels. This study reveals a rich representation of microbial genera associated with nematode antagonism in a conventionally managed arable field. The richness in putative nematode antagonists did not translate in *M. chitwoodi* suppression probably because most antagonists have a facultative nematophagous lifestyle and will only predate nematode under poorer nutritional conditions.

5.1 Introduction

Global crop yield is severely limited by diseases as a recent study reported that crop pests and pathogens are the direct cause of up to 40% losses on a wide range of economically important crops worldwide (Savary et al., 2019). Plant-parasitic nematodes alone account for an estimated loss of US$ 173 billion every year (Elling, 2013), which makes the implementation of disease control measures a necessity in agriculture. Among the plant-parasitic nematodes, root-knot nematodes (RKN, *Meloidogyne* spp.) are the most impactful worldwide (Jones et al., 2013).

Broadly speaking three types of measures have been developed to control plant-parasitic nematodes. First, crop rotation is the practice by which the growing of susceptible plant species is alternated with non-host plants over time. The discontinuous presence of hosts has been shown to effectively reduce the densities of several plant-parasitic nematode species (Azlay et al., 2023). This practise is effective for nematodes feeding on a limited range of plant species only. The applicability of crop rotation is problematic in the case of nematodes with a broad host range, especially because our agricultural crop range is narrowed to a few economically important crops (Maleita et al., 2012). Crop rotation is barely effective against RKN as all agronomically relevant RKN species are highly polyphagous. Second, breeding for host plant resistance has been very effective in the case of several plant-parasitic nematodes. For over 60 years the dominant resistance Mi1.2 gene has been effective in reducing the damage caused by three 'tropical' root-knot nematode species in tomato (Milligan et al., 1998). However, the transferability of this R gene was shown to be limited to close relatives of tomato (Goggin et al., 2006). The scarceness of effective R genes and their limited transferability to other crops has limited the broadening of the application of this type of control. Third, a range of synthetic nematicides has been introduced to control plant-parasitic nematodes. General soil fumigants and inhibitors of the neurotransmitter acetylcholinesterase have been widely applied to control plant-parasitic nematodes for decades. Mainly due to their strong negative side effects on non-target organisms as well as because of serious risks to human health (Heckel, 2012; Gill and Garg, 2014; Prashar and Shah, 2016), many of these nematicides have been phased out lately. Hence, due to the limited applicability of crop rotation, the limited availability of effective host plant resistance genes, and the widely supported endeavour to reduce the application of synthetic nematicides, there is an urgent need for the development of alternative sustainable nematode management practices.

In soil, a diversity of nematode antagonists has been identified. Among several taxa of bacteria and fungi, mechanisms have evolved to capture nematodes as

a food source. The bacterial genus *Pasteuria* has diversified in a number of species that all parasitize on (mainly) plant-parasitic nematodes, with species-specific food preferences (Ciancio, 2018).

Among the fungal nematode antagonists, *Arthrobotrys oligospora* can produce adhesive trapping nets in the presence of nematodes (Nordbring-Hertz and Mattiasson, 1979) and a closely related species *A. dactyloides* forms three-celled constricting rings to catch plant-parasitic nematodes (Hlggins and Pramer, 1967). Moreover, *Pochonia chlamydosporia* is a fungal egg parasite that can also trigger defence responses in the plant root (Gouveia et al., 2023). Overviews of terrestrial nematode antagonists have been presented by, *e.g.,* Li et al. (2015b) and Topalovic et al. (2020).

Numerous attempts to introduce mass-produced nematode antagonists in soil have not resulted in practical outcomes. Generally speaking, the chances of successfully introducing antagonists into a highly competitive environment such as soil are low (Giuma and Cooke, 1974; Jaffee et al., 1996; Stirling, 2011). As an alternative, it has been proposed to identify factors in cropping systems that could be used to support native nematode antagonists (Stirling, 2011). One of these factors could be the addition of specific cover crops that support the growth and the activity of nematode antagonists.

Here, we focused on the root-knot nematode *Meloidogyne chitwoodi*, a highly polyphagous obligatory plant parasite that poses a significant threat to crops globally in both temperate and tropical regions (O'Bannon et al., 1982; Azlay et al., 2023). Because of its broad host range, *M. chitwoodi* cannot be effectively controlled by crop rotation, and although host plant resistances have been identified (Mojtahedi et al., 1995), they have not been introduced yet in commercial varieties of any main crop (Teklu et al., 2023). Hence, we investigated whether it would be possible to stimulate native nematode antagonists to control *M. chitwoodi* in an experimental field setting using cover crops.

Cover crops are non-economic crops grown in between main crops to minimise nutrient leaching and soil erosion, and to increase the soil organic matter content (Blanco‐Canqui and Ruis, 2020). A substantial number of plant species can be grown as a cover crop provided they grow well outside the main growing season and as long as they can be terminated easily. Recent evidence has shown that different cover crop species can differentially select and simulate distinct fractions of the soil microbial communities (Cazzaniga et al., 2023a). Such manipulation of the soil microbial community could be instrumental in the defence against pathogens (Berendsen et al., 2012; Philippot et al., 2013). Because cover crops steer the soil microbiome in a

species-specific manner (Cazzaniga et al., 2023a), we hypothesised that cover crops can be applied to boost the antagonistic potential of the microbial community in soils.

In this study, we performed an experiment in a field with known presence of *M. chitwoodi*. We began by experimentally generating four *M. chitwoodi* densities by growing four different grass species with different host statuses for this pathogen. Subsequently, we grew a range of cover crop monocultures and cover crop mixtures in plots with distinct nematode densities. With this setup, we aimed to assess the impact of two main variables, cover crop identity and *M. chitwoodi* density, on the resident (DNA-based) and active (RNA-based) fractions of the bacterial and fungal communities. We focused on changes in genera known to comprise putative nematode antagonists. With this set up we aimed to address the following questions: 1) Do cover crops differentially affect fractions of the soil microbiome associated with nematode antagonisms? 2) Does the generation of distinct *M. chitwoodi* levels affect the native nematode antagonist community? 3) Can cover crops be used to stimulate the abundance and/or the activity genera harbouring nematode antagonists? The overall aim would be to find affordable and implementable management tools that could be used to strengthen the native nematode-suppressive potential of soils.

5.2 Materials and Methods

5.2.1 Experimental field set-up

The experimental field was located in Vredepeel (North Limburg, the Netherlands), an experimental field station of the Field Crops unit (WUR-FC)of Wageningen University and Research. This field was characterized by sandy soil (1% clay, 8% silt and 87% sand) with an organic matter content of ≈ 4% (4.1 - 4.4%) and a pH of around 6 (5.4 - 6.1). Our experiment was embedded in a larger experiment by WUR-FC aimed at assessing the host plant status of a selection of arable crops and cover crops in an arable field naturally infested with *M. chitwoodi* (Visser et al., 2022). To generate four different initial population densities of *M. chitwoodi*, four pre-crops belonging to the Poaceae family but with distinct host statuses for this plant-parasitic nematode were selected (Table 5.1). Pre-crops were sown in August in essentially three blocks each subdivided into four rectangular strips (6 x 42 m) (Supplementary Figure S5.1). Two blocks (blocks 2 and 3) were placed next to one another (24 x 42 m), while block 1 was separated into two sub-blocks (each 12 x 42 m) (Supplementary Figure S5.1). In mid-March 2019, pre-crops were chemically terminated and incorporated into the soil. To further boost the contrasts between the four *M. chitwoodi* densities (RKN densities), pre-crops (Table 5.1)

were re-sown on May 7th and mowed on July 16th. Right before the sowing of the cover crops, pre-crop stubble was milled and incorporated into the topsoil. To measure the soil nutritional status after pre-crops, 500 g of bulk soil was collected per strip and then pooled in four composite samples, one per pre-crop. Analyses by Eurofins Agro (Wageningen, NL) gave the following results: C/N ratio = 18 - 21, total nitrogen (kg N/ha) = 3750 - 4280, total phosphorus (kg P/ha) = 1125 – 1240, of which plant available (kg P/ha) = 20.7 - 29.1; total potassium (kg K/ha) = 110 – 270, of which plant available (kg K/ha) = 110 – 155.

Table 5.1 | Pre-crops sowed in the field between August 2018 and July 2019 to generate four different population densities of *Meloidogyne chitwoodi*.

Code	Treatment	Species	Cultivars	Plant host status for ***M. chitwoodi****
pr	perennial ryegrass	*Lolium perenne*	Mercedes	Poor
ar	annual ryegrass	*Lolium multiflorum*	Barprisma	Moderate to good
ry	rye	*Secale cereale*	Ducato	Very good
bo	black oat	*Avena strigosa*	Pratex	Good

*according to best4soil.eu (https://nematodes.soilhealthtool.eu/en-gb/Nematode-scheme)

5.2.2 Selection of cover crop species

Cover crops were sown on August 7th, 2019. Per strip (6 x 42 m), 11 plots (6 x 3 m, ≈ 0.9 m spacing between plots) were defined to accommodate six cover crop monocultures, four cover crop mixtures and one unplanted control (fallow) (Table 5.2). Every cover crop treatment and the fallow control were represented in each strip. Cover crop treatments were sown in the same order per block and in randomized order between blocks (Supplementary Figure S5.1). Cover crops were mowed on December 2nd, and the plant residues were incorporated into the topsoil with a rotary tiller.

Table 5.2 | Details of the cover crop species and cultivars used in this study, including the origin of seeds, sowing density and expected host status for *Meloidogyne chitwoodi*.

Code	Treatment	Species	Cultivar	Sowing density (kg/ha)	Plant host status for *M. chitwoodi*
BO	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
BO-OSR-R	Black oat + Oilseed radish-R	multiple	Pratex + Radical	40+15	Good + moderate
BO-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	none	none	none	Natural decrease

5.2.3 Soil sampling for determination of *M. chitwoodi* densities

To determine the effect of cover crops on *M. chitwoodi* densities, bulk soil samples were collected just before sowing the cover crops (August 5th, 2019; $T0_{nem}$) and on the day of cover crop termination (December 2nd, 2019; $T1_{nem}$) (Figure 5.1). One litre of topsoil soil was collected with augers (∅ 12 mm, core length 25 cm) from the central area (1.5 × 2.7 m) of each plot. The soil sampled from each plot was carefully mixed, after which a subsample of 100 mL (120 g) was taken to determine the nematode densities as reported in van Himbeeck et al. (2023)(**Chapter 4**). Soil subsamples were rinsed through 180 µm sieves. The organic material (> 180 µm) remaining on the sieve was incubated for four weeks at 20°C to allow the eggs present in the sample to mature and hatch (= 'incubation fraction'). The soil suspension that passed the sieves (= 'mineral fraction') was extracted with an Oosterbrink funnel, and concentrated on three stacked 45 µm sieves. The material collected on these sieves was incubated on a filter for three days at 20 °C. The resulting nematode suspension was concentrated in 100 mL of tap water. *M. chitwoodi* individuals were counted under a microscope (Leica DMi8, 40x or 400x magnification) for

two 10 mL subsamples from both the mineral and incubation fractions. When < 100 *M. chitwoodi* juveniles were found in a 10 ml subsample, the number of *M. chitwoodi* nematodes in the residual suspensions was counted as well. Soil sampling and nematode counting were conducted at the facilities of WUR-FC in Lelystad (NL).

5.2.4 Soil sampling for microbiome analyses

The first soil sampling ('T0') (Figure 5.1) was conducted on June 26th (2019), during pre-crops growth. For each strip, bulk soil and rhizosphere soil were collected. Bulk soil was sampled by taking soil cores in between plants with an auger (diameter 12 mm, core length 20 cm). Each strip was randomly sampled by collecting 20 cores while avoiding the strip edges. Cores were thoroughly mixed and subsequently sieved (using a 2 mm mesh sieve). In total 12 bulk soil samples (4 pre-crops treatments x 3 replicates) were collected. Next to this, four randomly selected pre-crop plants were carefully extracted from each strip and taken to a nearby WUR-FC lab facility. Roots were mildly shaken to remove non-rhizosphere soil. Subsequently, rhizosphere soil was collected by gently brushing off soil adhering to the roots. Rhizosphere samples from four plants from the same strip were lumped and mixed. Hence, in total 12 rhizosphere samples (4 kinds of pre-crops x 3 replicates) were collected. Rhizosphere soil was collected from the roots of four plants per plot. In the case of cover crop mixtures, the same amount of rhizosphere soil was collected from four couples of allospecific neighbouring plants. Three soil cores were collected from each of the fallow plots with an auger (diameter 12 mm, core length 20 cm). Soil and plants were collected in the central part of the plots (1.5 x 3 m) to avoid a border effect. Hence, per strip 11 samples were collected at T1, and this should have resulted in 132 rhizosphere soil samples. However, two samples were lost during the sampling process, so effectively 130 samples were analysed. For all rhizosphere and bulk soil samples, subsamples of 10 g were taken, snap-frozen in N_2 (l) and transported on dry ice to the Laboratory of Nematology (WUR).

5.2.5 Illumina NovaSeq sequencing of 16s and 18S rDNA and rRNA

For all 10 g subsamples, an aliquot of 2 g was used to isolate total DNA and RNA using an in-house developed, phenol-chloroform-based extraction protocol (Harkes et al., 2019). cDNA was synthesized from the isolated RNA using the Maxima First Strand cDNA Synthesis Kit for RT-PCR (Fermentas, Thermo Fisher Scientific Inc., USA) following the manufacturer's instructions.

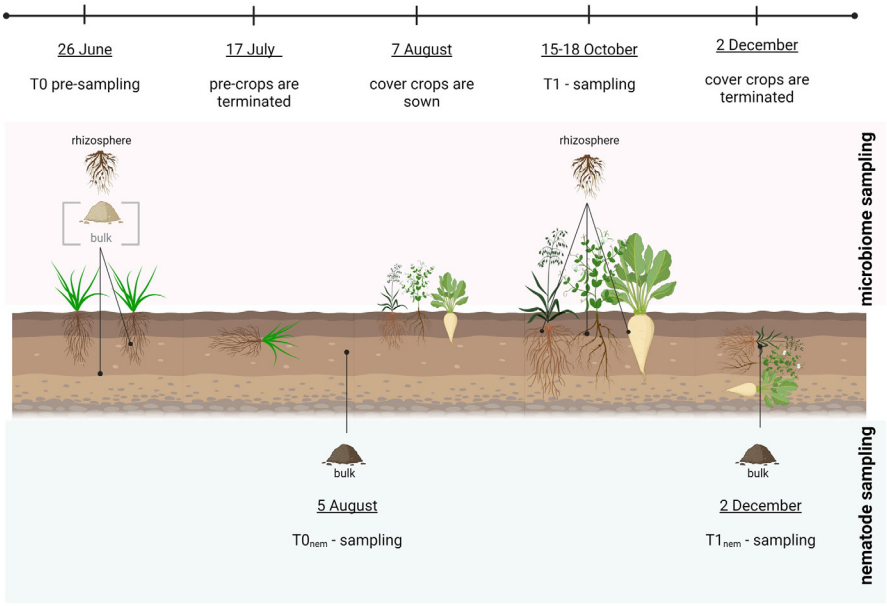

Figure 5.1 | Timeline of the experimental field. Pre-crops were sown in May 2019 and bulk and rhizosphere soil were sampled on June 26th during the pre-crops vegetative stage to study the initial composition of the soil microbiome. At pre-crop termination, on July 17th, bulk soil was collected to measure the initial *M. chitwoodi* densities in the plots ($T0_{nem}$). Cover crops were sown one month after mowing pre-crops on August 16th. Rhizosphere soil was sampled from cover crops during the vegetative stage of cover crops (on October 15-18th) to study the rhizosphere microbiome composition of each cover crop treatment. Cover crops were terminated on December 2nd, and on the same day soil was collected to assess the final *M. chitwoodi* densities in the plots after cover crops ($T1_{nem}$).

Library amplification was performed on DNA and cDNA extracts with the following primer combinations: 515F/806R (Caporaso et al., 2012) targeting the V4 region of 16S (bacteria) and gITS7/ITS4ngs (Tedersoo et al., 2018) targeting the ITS2 region of fungi. DNA and cDNA samples were diluted to 1 ng μl^{-1} and 0.1 ng μl^{-1} respectively. 3 µl of the diluted samples were mixed with 10 µl of IQ Supermix (Bio-Rad Laboratories, Inc.), 5 µl of Milli-Q and 1 µl of 5µM primers. For the first PCR reaction, the following temperature profile was used: 3 min. at 95°C for initial denaturation, followed by 35 cycles of 10s at 95°C, 30s at 56°C, 30s at 72°C and a final extension time of 5 min. at 72°C. After the first amplification, PCR amplicons were diluted 40-fold with Milli-Q water. Two µl of the diluted product was combined with 5 µl of Phire Hot Start II PCR Master Mix (ThermoFisher Scientific), 2 µl of Milli-Q water and 0.5 µl of forward and reverse primers (5µM). These primers included the Illumina sequencing adaptors and index sequences for sample multiplexing. For the

second PCR reaction, the following temperature profile was used: 3 min at 98°C for initial denaturation, followed by 15 cycles of 10 s at 98°C, 30 s at 60°C, 30 s at 72°C and a final extension time of 5 min at 72°C. Control samples including Milli-Q water only were taken along in the library preparation. Gel electrophoresis was used to check for correct amplicon size and purity for a random selection of PCRs products. Amplicons were pooled in one library and size selection and clean-up were carried out using AMPure XP Reagents (Beckman Coulter, Inc.). Finally, the library was sequenced using a standard Illumina NovaSeq SP2 (2×250bp) protocol (Illumina, San Diego, CA) and demultiplexed at Useq (Utrecht, The Netherlands). Sequences are available online in the NCBI SRA (Sequence Read Archive) database under BioProject number PRJNA973547.

5.2.6 Pre-processing of raw sequence data

Demultiplexed reads were sorted into the two organismal groups based on their locus-specific primer sequences. Forward and reverse reads were paired (merged on overlapping sequences) and clustered into amplicon sequence variants (ASVs) using the DADA2 pipeline (Callahan et al., 2016). For the ITS dataset, we applied the following filtering parameters: maximum expected error (maxEE) of 2 for both forward and reverse reads, truncation quality (truncQ) of 2 and no truncation (truncLen) of the amplicons, as advised for ITS reads. Taxonomic assignment for the ITS dataset was carried out using the IdTaxa method from DECIPHER (Wright, 2016), and the UNITE_v2021 database. For the bacterial dataset, we employed a maxEE of 2 for both forward and reverse reads, a truncQ of 2, and a truncation length (truncLen) of 230 for both forward and reverse reads. Taxonomic assignment for the bacterial dataset was performed using the default dada2 method, with the SILVA_SSU_v138 database being utilized. As the quality assumption of the base call is different for NovaSeq sequencing data as compared to MiSeq, we applied a non-default method to estimate the error rate by using the DADA2 *errorEstimationFunction* parameter in the *learnErrors* function (https://gist.github.com/Jorisvansteenbrugge/a4f26030a047af6197b37f410f189fd4). Phyloseq objects (McMurdie and Holmes, 2013) were created for the bacterial and the fungal ITS datasets by merging the ASV tables, taxonomy tables and phylogenetic trees resulting from the DADA2 pipeline and the metadata tables.

The decontam package (Davis et al., 2018) with default settings was used to remove potential bacterial contaminants based on the ASV composition of the control samples (MilliQ water without DNA or cDNA from the soil samples) (no contaminants were identified in the fungal ITS dataset). We discarded ASVs that were not annotated at the highest taxonomic level (phylum), singletons

(accounting for 1.8% and 1% of the total bacterial and fungal ASVs, respectively) and ASVs belonging to non-target organismal groups. After this step, only annotated bacterial ASVs were included in the bacterial phyloseq and fungal ASVs in the fungal-ITS dataset. Furthermore, we filtered out rare ASVs (present in one sample only) and ASVs represented by less than 10 reads. Samples with < 10,000 bacterial and < 1,000 fungal reads were discarded (true for 1 DNA and 1 RNA sample).

For bacteria, 29,890,835 reads were generated at the DNA level which clustered into 15,489 ASVs. The median number of reads and ASV per sample were 193,687 and 1,675 in 153 samples (one DNA sample had < 10,000 reads). At the RNA level, 28,439,842 reads and 17,302 ASVs were produced from 154 samples, with a median read number of 184,569, and a median ASV number of 1,546.

From the fungal dataset, based on ITS sequencing, 22,013,785 DNA reads were retained after pre-processing, which clustered into 2,850 ASVs. The median number of reads and ASVs per sample were respectively 127,284 and 201 (154 samples). At the RNA level, 15,087,455 reads were employed that clustered into 2,011 ASVs. The median number of reads per sample was 93,148, while the median number of ASVs was 177 for 153 samples (one RNA sample had < 1,000 reads). The filtered bacterial and fungal phyloseq objects were used as input for statistical analyses.

5.2.7 Selection of putative nematode antagonists towards *M. chitwoodi*

Based on the reviews by (Li et al., 2015a) and Topalovic et al. (2020), we made an overview of bacterial and fungal genera harbouring at least one species that has been described as an antagonist of plant-parasitic nematodes (Supplementary Table S5.1). We used this overview to identify genera harbouring nematode antagonists in our datasets. We refer to the shortlisted genera as putative nematode antagonists throughout the study for simplicity.

5.2.8 Statistical analyses

All statistical analyses were performed using R v.4.2.2 (R Core Team, 2021) with the aid of relevant packages, including phyloseq v.1.42.0 (McMurdie and Holmes, 2013) and vegan v.2.6-4 (Oksanen et al., 2013).

Effects pre-crops on RKN densities. Generalised linear mixed models with negative binomial error distributions (glmm-nb) were used to assess the effects of pre-crops (at $T0_{nem}$) and cover crop treatments (at $T1_{nem}$) on *M. chitwoodi*

densities. Raw nematode counts were used as response variables in the models, with block as a random factor to account for the positional effect of the field. Analysing the effect of pre-crops on the $T0_{nem}$ counts, we determined the initial population density levels of *M. chitwoodi*. These levels will be referred to as 'RKN densities'. Analysing the effect of cover crop treatments and RKN densities on the $T1_{nem,}$ an interaction factor (cover crop treatments * RKN density) was added to the model.

Effects pre-crops, cover crop, and RKN densities on soil microbiome. The effects of pre-crops, cover crop treatments and the distinct *M. chitwoodi* levels on the total and active bacterial and fungal community structure were assessed by Permutational analysis of variance (PERMANOVA – *adonis2* function in vegan) and Principal Coordinate Analysis (PCoA – *plot_ordination* function in phyloseq). For both tests, ASVs were normalised through Cumulative Sum Scaling (CSS) transformation (Paulson et al., 2013) and the distance matrix was calculated using the Bray-Curtis dissimilarity index. As PERMANOVA tests each term in sequential order, the positional effect of blocks was accounted for by introducing the term 'block' as the first term in the PERMANOVA. The same tests, PERMANOVA and PCoA, were used to assess the community structure of the selection of putative nematode-antagonistic microbial genera. Cover crop treatments were compared with pairwise PERMANOVAs (*pairwise.perm.manova* function, RVAideMemoire v.0.9 (Hervé and Hervé, 2020) carried out based on Bray-Curtis multivariate distances with Benjamini-Hochberg correction for multiple testing and 999 permutations.

Effects of cover crop treatments on the diversity of putative nematode antagonists. Alpha diversity indices describing richness (Observed index) and diversity (Shannon index) were calculated on the selection of putative nematode antagonists from the rarefied bacterial and fungal ASVs table using the phyloseq package. Rarefying was conducted to the minimum library size without replacement. The smallest library size between DNA and RNA datasets was used for bacteria and fungi (RNA had in both cases the smallest library size). Differences in alpha diversity scores among pre-crops were tested with linear models, using the logarithm of the Observed and Shannon index as the response variables. The effects of cover crop treatments were tested with the Kruskal-Wallis rank sum test (KW), after rejecting the normality of data and residuals from linear models implemented with raw or log-transformed data. As KW can only test one factor at a time, the effect of pre-crops and the positional effect (= block effect) on the T1 samples were also tested separately and reported if significant. When the KW statistical test was significant, post-hoc tests were conducted with Dunn's test and Holm p-value adjustment for multiple testing with ggstatsplot v 0.10.0 (Patil, 2021) which was also used to generate the plots. Rarefied ASV tables were also used to calculate the relative

abundance of putative nematode-antagonistic genera per pre-crop and cover crop treatment at DNA and RNA levels which were potted in barplots.

Effects of cover crops and nematode densities on the abundance putative nematode antagonists. Glmm-nb models were also used asses if the RKN densities and cover crops related to the abundance of putative bacterial and fungal nematode antagonists (Zhang and Yi, 2020). ASVs of putative nematode antagonists were aggregated to genus level, and raw DNA and RNA read counts of each genus were used as a response variable in the mixed models. Only antagonists with a prevalence of > 25% (present in > 3 and > 33 samples at T0 and T1, respectively) were tested with models. To account for differences in the sequencing depth, the logarithm of the total read counts per sample was added as an offset term. In addition, the block term was introduced as a random effect in the models to account for the positional effect. When assessing the effect of cover crops and RKN densities on the read counts of the antagonists, these two terms were first added as an interaction effect (cover crop treatments * RKN densities). In the absence of a significant interaction term, the individual terms were reciprocally included as both fixed and random effects in the two models. In these models, the fallow samples (bulk soil) were excluded and only rhizospheric samples from cover crops were tested. Glmm-nb were implemented in glmmTMB v. 1.1.6 (Brooks et al., 2017) and the fit of each model and zero inflation were tested with DHARMa v. 0.4.6 (Hartig, 2020). Zero-inflated negative binomial models were used when zero inflation was significant. Pairwise comparisons of estimated marginal means were assessed with emmeans (v. 1.8.5 (Lenth, 2022) and multcomp v.1.4-23 (Hothorn et al., 2008) and p-values were corrected with the Benjamini-Hochberg procedure. To present the results of the models and show significant comparisons, box plots were built on the non-rarefied ASVs for those genera tested in the models (provided as supplementary material). All tests were considered statistically significant at $P < 0.05$.

5.3 Results

5.3.1 Effect of pre-crop and cover crop treatments on *M. chitwoodi* population density.

Growing four grass species with distinct host status toward the root-knot nematode *M. chitwoodi* significantly affected the densities of this plant parasite ($X^2_{3,132} = 156.57$, $P < 0.001$; $R^2 = 63.47\%$, Figure 5.2 A) at $T0_{nem}$. Black oat, an excellent host for *M. chitwoodi*, resulted in the highest nematode densities ($estimate_{bo}$ = 4,730 individuals 100g $soil^{-1}$). Rye (ry) and annual ryegrass (ar) gave rise to intermediate *M. chitwoodi* densities with estimates of respectively

1,104 and 772 individuals 100 g soil^{-1}. Exposure to perennial ryegrass (pr) resulted in the establishment of the lowest *M. chitwoodi* levels (estimate$_{pr}$ = 180 individuals 100 g soil^{-1}). After correction for block effect (Figure S5.2), the four poaceous pre-crop treatments resulted in the generation of three distinct RKN levels ('high', 'medium' and 'low RKN' in Figure 5.2 B). Hence, no significant difference was observed between the rye and annual ryegrass pre-crop treatments.

At the time of cover crop termination ($T1_{nem}$), a strong interaction was observed between the pre-crop treatments and the subsequent impact of the cover crops ($X^2_{30,132}$ = 65.01, P < 0.001, R^2 = 80.33%). Therefore, the effect of each cover crop treatment on *M. chitwoodi* was assessed separately for each of the four pre-crops (Figure 5.2 B). Among all pre-crop treatments, cover crop treatments including black oat (the monoculture BO and the mixtures BO-OSR-T, BO-OSR-R, detailed in Table 5.2) showed the highest *M. chitwoodi* densities (estimate$_{treatment\ with\ BO}$= 1,1906 (lowest) - 105,384 (highest) individuals 100g soil^{-1}, Figure 5.2 B). Plots exposed to fallow (FW), oilseed radish monocultures (OSR-T, -R and -A), and phacelia (PHA) typically showed the lowest *M. chitwoodi* densities (Figure 5.2 B). This is illustrated by the effect of oilseed radish cv. Terranova, resulting in estimates between 1 (lowest) and 81 (highest) *M. chitwoodi* individuals 100 g soil^{-1}.

5.3.2 Effects of pre-crops on soil bacterial and fungal communities

NovaSeq sequencing of the V4 region of the ribosomal DNA for bacteria, and the ITS2 region for fungi resulted in the generation of 15-30 million reads for each of the two barcoding regions, and both the DNA and the RNA derived amplicons (for further details see M&M 5.2.6).

At T0, the four grass species used as pre-crops (Figure 5.1, Table 5.1) had uniform bacterial and fungal community structures at DNA and RNA level irrespective of whether rhizosphere or bulk soil was sampled (PERMANOVA after removing the variation due to block effect, P > 0.05, Supplementary Table S5.2 A, Supplementary Figure S5.2). As the four pre-crops did not result in significant shifts in the bacterial and fungal communities, plots were pooled on the basis of their RKN densities only. Hence, for further analyses, we compared three categories, namely plots with low, intermediate and high RKN densities (Figure 5.2 A). After pooling plots exposed to four pre-crop treatments into three categories based on the resulting RKN levels, the microbial community composition was re-analysed. A PERMANOVA test showed no significant effects of RKN densities on bacterial and fungal communities in the rhizosphere, neither at the DNA nor at the RNA level (Supplementary Table S5.2 A).

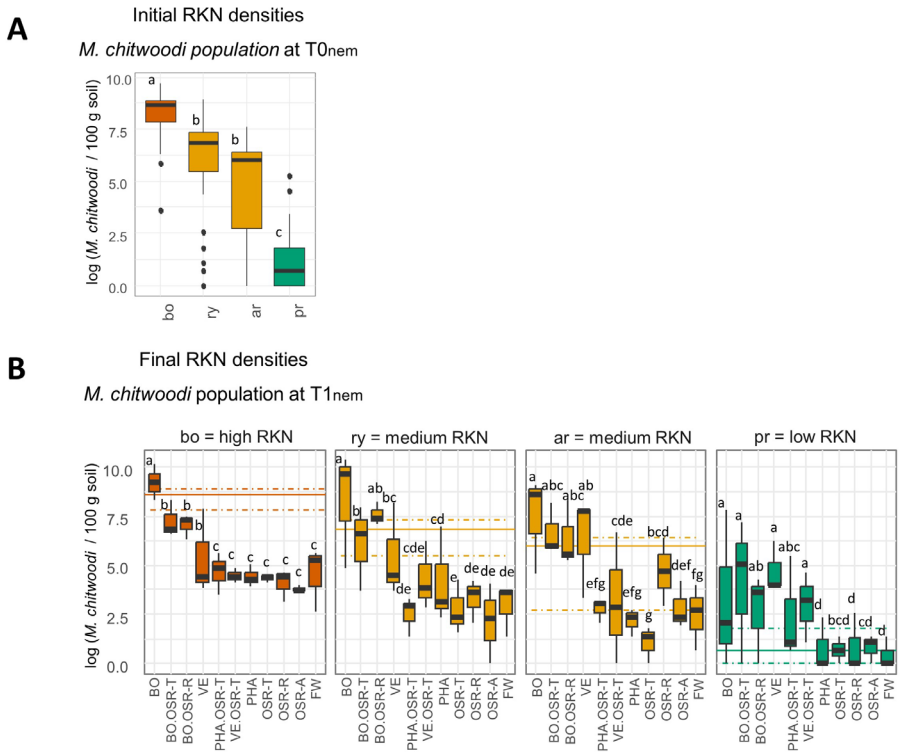

Figure 5.2 | *M. chitwoodi* population density after pre-crops ($T0_{nem}$) (A) and after the cover crop treatments ($T1_{nem}$) (B). A. Four pre-crops were used: black oat (bo), rye (ry), annual ryegrass (ar) and perennial ryegrass (pr). Different letters above box plots indicate statistically significant differences among the pre-crop treatments. B) To visualize the impact of cover crops in plots with distinct RKN levels, initial RKN levels are provided in each of the four graphs. Solid lines and dotted lines indicate the median and respectively the 0.25 and 0.75 quantiles of initial *M. chitwoodi* density counts for each pre-crop ($T0_{nem}$). Cover crop treatments are abbreviated, and further explained in Table 5.2. Box plots show the impact of cover crops on *M. chitwoodi* for each of the pre-crop treatments.

5.3.3 Effect of cover crop treatments on the overall microbiome community compositions

At T1, rhizosphere soil from all ten cover crop treatments and bulk soil from the fallow plots were collected, and microbiome analyses revealed that all cover crop treatments significantly affected the resident (DNA) and the active (RNA) fractions of the bacterial and fungal communities (Figure 5.3) (PERMANOVA, $R^2_{bact\text{-}DNA}$ = 22%, $R^2_{bact\text{-}RNA}$ = 25%; $R^2_{fun\text{-}DNA}$ = 27%, and $R^2_{fun\text{-}RNA}$ = 21%, $P < 0.001$, Figure 5.3). Initial RKN densities had no significant effect on the

bacterial DNA and RNA communities, and only explained 2% of variation for the fungal communities (both at DNA and RNA level) ($P < 0.01$) (Supplementary Table S5.2 B). The interaction effect of cover crop treatments and RKN densities was not significant (PERMANOVA, $P > 0.05$) (Supplementary Table S5.2 B).

Pairwise comparisons of cover crop treatments (Supplementary Table S5.2 C) revealed significant differences between all rhizosphere communities and the bulk communities in the fallow plots ($P < 0.01$). Hence, as compared to fallow bulk soil, all cover crop treatments resulted in a shift in the rhizosphere microbiome. Furthermore, no differences were observed in rhizosphere microbiome composition between the oilseed radish cultivars Adios, Radical and Terranova ($P > 0.05$). In most cases, the microbiome of cover crop mixtures that included oilseed radish Terranova did not significantly differ from the Terranova monoculture ($P > 0.05$). On the other hand, the microbiome composition of cover crop mixtures such as BO-OSR-T, PHA-OSR-T and VET-OSR-T deviated from the microbiomes in the rhizospheres of monocultures of black oat, phacelia and vetch.

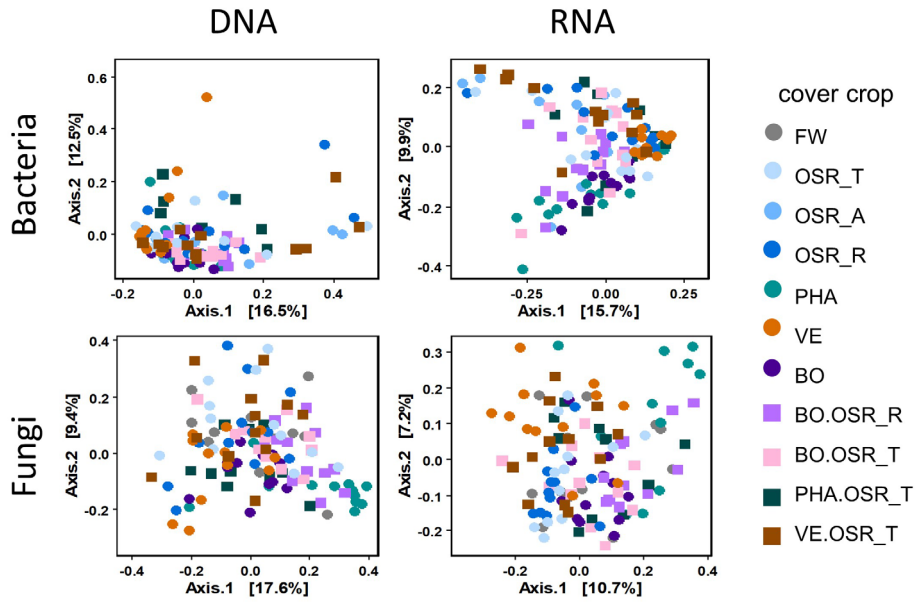

Figure 5.3 | Community composition of the rhizospheres of six cover crop monocultures, four mixtures and fallow bulk soil. Colours indicate cover crop treatments; shapes are used to discriminate between monocultures and mixtures. Principal Coordinate Analysis (PCoA) of CSS-normalised ASVs based on Bray-Curtis-based dissimilarity matrices show that microbial communities are separated along the axes as cover crop treatment accounted for 10.7-17.6% of variation along the principal PCoA axis (PERMANOVA, $p \leq .001$).

These analyses revealed that in the case of cover crop mixtures oilseed radish cv. Terranova had a dominant effect on the composition of the rhizosphere microbiome ($P < 0.01$). This dominance was not characteristic for all oilseed radish cultivars; the microbiome of the black oat - oilseed radish cv. Radical mixture (BO.OSR-R) deviated significantly from the microbiomes associated with each of the constituents ($P < 0.01$).

5.3.4 Diversity of native nematode antagonists detected in the experimental field

Eight bacterial and 28 fungal genera have been described in literature to comprise nematode antagonists *sensu lato* (reviewed by Li et al., 2015 and Topalovic et al., 2020) (Supplementary Table S5.1). Among these, fungal genera comprising endophytes known to strengthen plant defence responses against plant-parasitic nematodes such as *Acremonium* and *Fusarium* were also included. Especially for fungi, the afore-mentioned number is an approximation as the systematics of some of the genera is still volatile. Within the 130 samples analysed from the Vredepeel experimental field, five bacterial and thirteen fungal genera of putative nematode antagonists were detected, covering 63% and 50%, respectively, of the overall diversity of bacterial and fungal genera harbouring plant-parasitic nematode antagonists (as listed in Li et al. 2015 and Topalovic et al. 2020, Supplementary Table S5.1). It is noted that some genera, such as *Arthrobotrys* exclusively comprise nematode antagonists (Zhang et al., 2022), while, for example, within the speciose genus *Fusarium,* nematode antagonists constitute only a small minority (Benitez-Malvido et al., 2021).

Five out of the eight bacterial genera of nematode putative antagonists were represented in almost all samples (98-100% at the DNA level) (Table 5.3) and shown to be metabolically active (97-99% detected at the RNA level). Within four out of the five genera of putative nematode antagonists, species described in literature as being antagonists of plant-parasitic nematodes were found in the experimental field (*e.g. Pseudomonas putida*, Table 5.3).

Among the thirteen fungal genera of putative nematode antagonists, large differences in distribution over the experimental field were observed (Table 5.3). Whereas members of the genus *Arthrobotrys* were present and active in around 90% of the samples, *Nematoctonus* was shown to be present in 27% of the samples while they were active in 5% of the samples only (Table 5.3). For several genera of putative nematode antagonists (*e.g. Hirsutella*), we detected a species that is indeed known as a nematode antagonist (*e.g. Hirsutella vermicola,* Xiang et al., 2006). However, some fungal species identified within our dataset, such as *Coprinus heptemerus,* could not be linked to nematode suppression. This species was actually described as an inhibitor

of spore germination in the pathogenic fungus *Magnaporthe grisae* (Valdivia et al. 2005).

Table 5.3 | Overview of the putative nematode antagonists found in the Vredepeel experimental field at T1. Putative bacterial and fungal nematode antagonists detected, as well as their prevalence in rhizosphere soil samples at the genus level. Also shown are the species found within each genus. Note that for many species it is unknown whether they interfere in plant-parasitic nematode – plant interactions (ex. *Coprinus heptemerus*). Underlined species are representatives for which a documented example of nematode antagonism is included. Prevalence at T1 (= % of the 118 rhizosphere samples harbouring a given genus) is determined at DNA (resident community) and RNA level (active community). Table follows in the next page.

Bacterial genera	Species detected in sequencing data	Documented example of nematode antagonism	Documented antagonistic mechanism	Prevalence (% DNA samples)	Prevalence (% RNA samples)
Bacillus	*B. asahii* *B. licheniformis* *B. longiquaesitum* *B. murimartini* *B. oleronius* *B. plakortidis* *B. thermoamylovorans* *B. smithii*	*B. licheniformis* JF-22 (Du et al., 2022)	Strengthening host defences / producing toxic substances	98	95
Lysobacter	*L. concretionis* *L. dokdonensis* *L. ginsengisoli* *L. niastensis* *L. soli*	*L. enzymogenes* B25 (Martínez-Servat et al., 2023)	Strengthening host defences / producing toxic substances	98	97
Pseudomonas	*P. putida* *P. stutzeri* *P. alcaligenes* *P. caeni*	*P. putida* (Siddiqui et al., 2007)	Strengthening host defences / producing toxic substances	100	99
Rhizobium	*R. arenae* *R. azooxidifex* *R. giardinii* *R. leguminosarum* *R. mongolense* *R. soli* *R. yantingense*	*R. leguminosarum* (Siddiqui et al., 2007)	Strengthening host defences / producing toxic substances	100	99
Variovorax	*V. paradoxus* *V. soli*	*V. paradoxus* (Wolfgang et al., 2019)	Strengthening host defences / producing toxic substances	100	98
Fungal genera	Species detected in sequencing data	Documented example of nematode antagonism	Documented type of antagonism	Prevalence (% DNA samples)	Prevalence (% RNA samples)

Acremonium	*A. biseptum* *A. furcatum* *A. rutilum* *A. verruculosum*	*A. strictum* (Goswami et al., 2008)	Strengthening host defences, (endophytes)	44	11
Arthrobotrys	*A. brochopaga* *A. dactyloides* *A. iridis* *A. oligospora* *A. oudemansii* *A. reticulata*	*A. oligospora* (Liang et al., 2016)	Facultative parasite (nematode-trapping fungus)	97	89
Coprinus Coprinellus	*C. heptemerus*	*C. comatus* (Luo et al., 2007)	Facultative parasite (nematode-trapping fugus)	13	3
Dactylellina, Dactylella Monacrosporium	*D. tenuis* *M. drechsleri*	*D. oviparasitica* (Verdejo-Lucas et al., 2002) *M. drechsleri* (also known as *Dactylella drechsleri*) (Meyer et al., 2005)	Facultative parasite (nematode-trapping fugus)	16	12
Fusarium	*F. poae*	*F. oxysporum* strain Fo162 (Martinuz et al., 2013)	Strengthening host defences / producing toxic substances (endophytes)	100	83
Haptocillium	*H. balanoides (*also known as *Verticillium balanoides)* *H. sinense*	*H. balanoides* (Glockling and Holbrook, 2005)	Obligate endoparasite of nematodes and rotifers	23	1
Hirsutella	*H. vermicola*	*H. vermicola* (Xiang et al., 2006)	Obligate endoparasite	37	3
Mortierella	*M. elongatula* *M. fatshederae* *M. globalpina*	*M. globalpina* (Dilegge et al., 2019)	Facultative parasite	88	70
Nematoctonus	*N. leiosporus*	*N. leiosporus* (Jaffee et al., 1998)	Facultative parasite (nematode-trapping fungus)	27	5
Orbilia	*O. rectispora*	*O. querci* (Liu et al., 2005)	Facultative parasite (nematode-trapping fungus)	3	2
Penicillium	No resolution beyond genus level	*P. digitatum* (Eapen et al., 2005)	Facultative parasite (eggs or females), endophytes	100	34
Stropharia	No resolution beyond genus level	*S. rugosoannulata* (Luo et al., 2006)	Facultative parasite	0	1
Trichoderma	*T. albolutescens*	*T. harzianum* (Eapen et al., 2005)	Facultative parasite (eggs or females)	95	66

5.3.5 Initial composition of the community of putative nematode antagonists under distinct RKN densities

At T0, we investigated whether the pre-crop treatments that gave rise to three distinct RKN densities had resulted in differences in putative nematode antagonist communities. No such effect was detected as both richness (Observed) and diversity (Shannon) did not differ among the three RKN densities ($P > 0.05$ for all combinations) (Supplementary Figure S5.3 B, D). Overall, the putative nematode antagonists' community, as presented in Table 5.3 (and Supplementary Table S5.1) in the rhizosphere was uniformly distributed among the three RKN densities at T0 (PERMANOVA, $P > 0.05$ at DNA and RNA level for bacteria and fungi, Supplementary Figure S5.3 A, C, Supplementary Table S5.3).

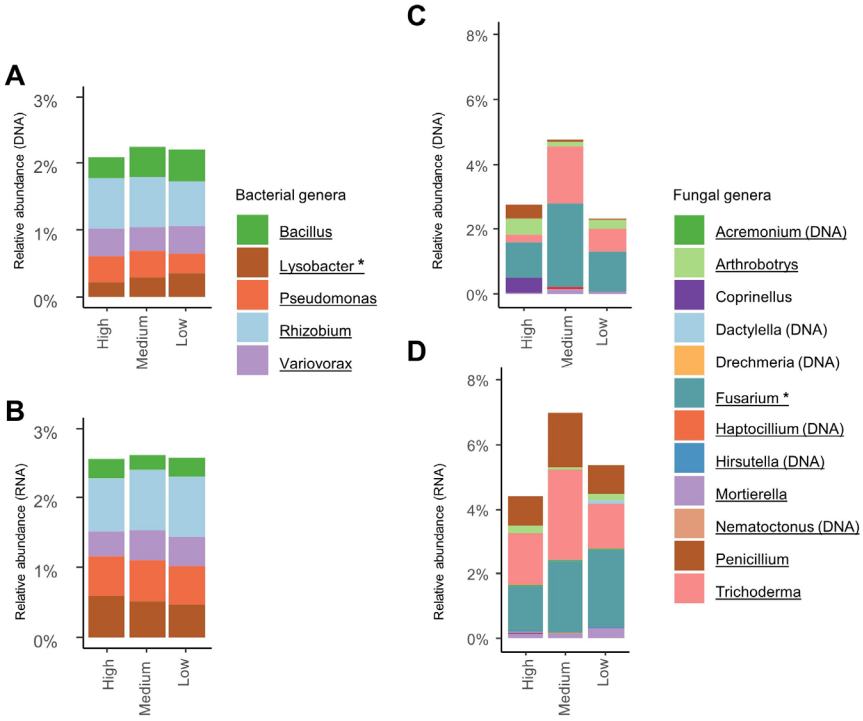

Figure 5.4 | Relative abundances of putative nematode antagonists in the rhizosphere of pre-crops at T0. Relative abundances were calculated for the three RKN densities individually as [read counts for putative antagonists per sample / total read count per sample] * 100%. **A, B.** Resident (DNA) and active (RNA) bacterial genera of putative nematode antagonists. **C, D.** Resident (DNA) and active (RNA) fungal genera of putative nematode antagonists. In case genera were detected at DNA level only, this is specified behind the genus name.

5.3.6 Impact of cover crop treatments and RKN densities on the community of putative nematode antagonists

Bacterial putative nematode antagonists. At T1, cover crop treatments had a significant effect on the community structure of putative bacterial antagonists (PERMANOVA, $P < 0.05$, Supplementary Figure S5.4, Supplementary Table S5.3 B) accounting for 31% to 19% of the microbiome variation at respectively DNA and RNA level (Supplementary Table S5.3 A). On the other hand, RKN densities did not affect the community structure of putative bacterial antagonists (PERMANOVA for RKN densities, $P > 0.05$). The diversity and richness of putative bacterial antagonists were significantly affected by cover crops both at DNA and RNA levels (Table 5.4). In most cases, rhizospheric communities of putative nematode antagonists displayed a higher diversity and richness as compared to the bulk soil antagonist communities of the fallow (Supplementary Figure S5.5 A, B).

At T1, the relative abundance of putative nematode antagonists (Table 5.3) in the rhizosphere of cover crops ranged from 2.7% in vetch to 6.1% in oilseed radish cv. Adios at the DNA level (Figure 5.5 A), and from 1.6% in black oat monoculture and mixtures to 2.5% in vetch at the RNA level (Figure 5.5 B). In fallow plots, putative bacterial antagonists accounted for 0.9% and 1.7% of the total bacterial community in bulk soil at DNA and RNA level respectively.

Model-based statistical analyses revealed that putative bacterial antagonists were stimulated by cover crops in a genus-specific manner (Table 5.5). *Bacillus* spp. were activated in the present of a cover crop mixture of vetch and oilseed radish Terranova. The abundance of *Pseudomonas* was influenced by the interaction between cover crop treatments and RKN densities. The vetch rhizosphere was characterized by the lowest *Pseudomonas* abundance (at DNA level) in combination with the highest *Pseudomonas* activity (at RNA level) (Table 5.5, Supplementary Figure S5.6). Monocultures of the oilseed radish cultivar Adios most strongly promoted the resident *Pseudomonas* community (Table 5.5).

Fungal putative nematode antagonists. Cover crop treatments accounted for ≈ 14% and 9% of the variation among the putative fungal antagonist community at respectively DNA and RNA levels ($P < 0.05$, Supplementary Table S5.3 B, Supplementary Figure S5.4). A small but significant effect of RKN densities on the putative nematode-antagonistic community was observed at DNA level ($R^2 = 2.8\%$, $P < 0.05$), while no effect on the active community was detected. The interaction between cover crop treatments and RKN densities was nonsignificant ($P > 0.05$, Supplementary Table S5.3 B).

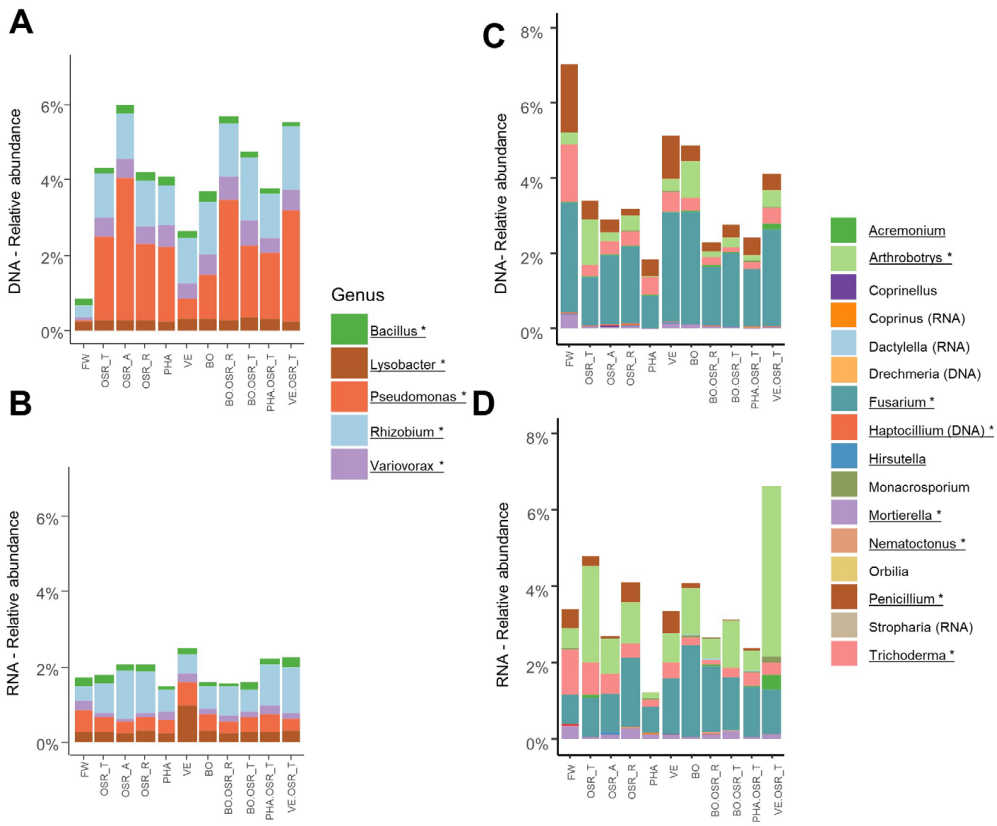

Figure 5.5 | Relative abundance of putative nematode antagonists in the rhizosphere of cover crops and the fallow control (calculated for each sample as [read counts of putative nematode antagonist per sample / total read count per sample] * 100%) at T1. Cover crop treatments are abbreviated, and further explained in Table 5.2. **A, B.** Resident (DNA) and active (RNA) bacterial genera comprising putative nematode antagonists. **C, D.** Resident (DNA) and active (RNA) fungal genera comprising putative nematode antagonists. In case genera were detected at DNA or RNA level only, this is specified behind the genus name. The most abundant and most prevalent genera (underlined) are used to test for differences among treatments with glmm-nb models. Genera significantly responding to cover crop treatments and/or RKN densities are marked with asterisks.

Cover crop treatments had a significant impact on the diversity and richness (at the level of ASVs) of putative nematode antagonists solely at the DNA level, as most cover crops' rhizosphere displayed higher richness and diversity of antagonists as compared to fallow (Table 5.4, Supplementary Figure S5.5 C), but no significant effects were observed at RNA level.

Fungal putative nematode antagonists constituted between 2.2% and 6.1% of the fungal community in phacelia and vetch at DNA level, respectively (Figure 5.5 C). At the RNA level, putative nematode antagonists' abundance ranged from 1.5% to 7.4% in phacelia and vetch-oilseed radish cv. Terranova mixture,

respectively (Figure 5.5 D). In fallow soil, putative nematode antagonists represented 8.4% at the DNA level and 4.1% at the RNA level of the total community in bulk soil.

Model-based statistical analyses revealed that *Arthrobotrys* to be significantly influenced by the interaction of cover crops and RKN densities (interaction: $X^2_{20,130}$ = 50.10, P < 0.001, Supplementary Figure S5.6). In plots with the highest RKN densities, this genus was least abundant in phacelia and most abundant in oilseed radish cv. Terranova monoculture and its mixtures with black oat and vetch (at both DNA and RNA levels, Table 5.5, Supplementary Figure S5.6). The genus *Nematoctonus* (only tested at the DNA level, Table 5.5) responded significantly to cover crops ($X^2_{10,130}$ = 39.04, P < 0.001) and was most abundant in oilseed radish cv. Adios and least abundant in plots with black oat. The fungal genera of obligate nematode parasites, *Hirsutella* and *Haptocillium* (only tested at the DNA level) were not affected by cover crops (P > 0.05), but *Haptocillium* abundance was affected by RKN densities ($X^2_{10,130}$ = 22.72, P < 0.001), with the highest read counts associated with the highest RKN density (Table 5.5). The abundances of *Trichoderma* spp. and *Fusarium* spp. were influenced by cover crops only in high and medium RKN densities, while no difference in read count was assessed at the low RKN density (Table 5.5 and Supplementary Table S5.4). In high RKN densities, both genera were enriched in oilseed radish cultivars, and *Trichoderma,* especially in oilseed radish cv. Adios. The abundance of the genus *Mortierella* did not vary among cover crops, but it was significantly more active upon exposure to medium and high RKN densities (RNA level) (Table 5.5, Supplementary Figure S5.6).

It is concluded that both the abundance and activity of putative nematode-antagonistic genera can be stimulated by cover crops and in particular by oilseed radish and, to a lower extent, its mixtures. It is noted that treatments – cover crop and RKN density – that stimulate the presence of a given putative nematophagous genus, did not necessarily have an effect on the activity of this genus, and *vice versa.*

Table 5.4 | Effects of cover crop treatments on the diversity and richness of putative nematode-antagonistic genera as presented in Table 5.3. Diversity and richness indexes were calculated at the ASV level. P-values were generated with the Kruskal-Wallis rank sum test for non-normally distributed data.

	Diversity (Shannon)	**Richness (Observed)**		**Diversity (Shannon)**	**Richness (Observed)**
Bacteria DNA	$X^2_{10,129}$ = 50.59, P < 0.001	$X^2_{10,129}$ = 59.31, P < 0.001	**Fungi DNA**	$X^2_{10,130}$ = 36.36, P < 0.001	$X^2_{10,130}$ = 52.18, P < 0.001
Bacteria RNA	$X^2_{10,129}$ = 35.22, P < 0.001	$X^2_{10,129}$ = 26.95, P < 0.001	**Fungi RNA**	$X^2_{10,130}$ = 9.17, P = 0.516	$X^2_{10,130}$ = 10.51, P = 0.3968

5.4 Discussion

We investigated the impact of ten cover crop treatments on fungal and bacterial communities against a background of three density levels of the root-knot nematode (RKN) *M. chitwoodi* with a focus on microbial genera with nematode-antagonistic activity. As microbial abundance and activity are equally relevant in this context, both the resident and the active fractions were mapped. Bacterial and fungal taxa interacting with plant-parasitic nematodes have been extensively studied in the past, and this pre-existing knowledge allowed us to identify a remarkable diversity of genera that are known to comprise putative nematode antagonists. In a conventionally managed experimental field, five bacterial and thirteen fungal genera were identified that comprise putative nematode antagonists. This represents 63% and 50% of the overall genera that are described in the literature to harbour antagonists of plant-parasitic nematodes. We showed that both cover crop treatments and RKN density impacted the abundance and activity of the identified putative nematode-antagonistic genera. However, the selection of the putative nematode-antagonistic genera responded distinctively to these treatments, and stimulation of the abundance of a taxon (at the DNA level) was not necessarily paralleled by an increase in the activity of the same taxon (at the RNA level) and *vice versa.* Overall, we observed a remarkably rich representation of bacterial and fungal genera associated with nematode antagonism in a common arable field, and these genera were shown to be more readily manipulatable by cover crops than by *M. chitwoodi* density.

5.4.1 Nematode antagonists are abundant and diverse in a conventionally managed experimental field.

Nematodes evolved in soils in the presence of bacterial and fungal communities for millions of years, and it is no surprise that nematode antagonists arose and diversified in both organismal groups. Among fungi, active nematophagous lifestyles were shown to diverge from 246 Mya onwards (Yang et al., 2012). Based on two recent overviews by (Li et al., 2015a) and (Topalovic et al., 2020) we estimated that mechanisms to control or parasitise plant-parasitic nematodes evolved in eight bacterial and 28 fungal genera.

Table 5.5 | Effects of cover crops (rhizosphere samples) and *M. chitwoodi* levels (RKN densities) on the abundance (number of reads) of genera of putative nematode antagonists at T1. Results of the negative binomial generalized linear mixed models are presented: estimated read number of putative genera per cover crop treatment and/or RKN density, and statistical significance between cover crop treatments or RKN densities assigned with post-hoc tests. Background grey pinpoints significantly higher read counts. This table is an excerpt of the complete Supplementary Table S5.4. Table is in the next page.

	Bacillus		***Pseudomonas***		***Arthrobotrys***				***Nematoctonus***		***Trichoderma***				***Haptocillium***		***Mortierella***	
	active		resident		active		resident		active		resident		active		resident		active	
Cover crop treatment	All RKN		RKN High[1]		RKN High[1]		RKN High[1]		RKN High[1]		all RKN		all RKN		RKN High[1]		RKN densities[3]	
OSR-T	436	cd	5,797	bcde	745	bc	3,517	c	5,820	b	10	a	388	ab	525	ab	**Low**	10 a 104 a
OSR-A	268	abc	16,687	e	273	a	220	b	65	a	71	c	282	ab	1,215	b	**Medium**	25 a 167 b
OSR-R	350	bcd	2,247	bc	794	bc	416	b	984	ab	36	bc	363	ab	591	ab	**High**	132 b 213 b
PHA	186	ab	5,090	bcd	713	bc	29	a	46	a	27	abc	338	ab	87	a		
VE	326	abcd	454	a	1,160	c	571	bc	729	ab	12	ab	666	b	306	ab		
BO	205	ab	1,868	b	804	bc	380	b	1,192	ab	9	a	353	ab	137	ab		
BO.OSR-R	171	a	3,142	bc	733	bc	207	b	556	ab	19	ab	195	a	0	ab		
BO.OSR-T	292	abcd	6,390	cde	694	bc	848	bc	2,608	b	15	ab	214	a	187	ab		
PHA.OSR-T	295	abcd	6,643	cde	816	bc	347	b	559	ab	19	ab	245	ab	329	ab		
VE.OSR-T	513	d	10,194	de	512	b	1,088	bc	12,101	b	17	ab	373	ab	195	ab		

[1] When the interaction between cover crop treatment and RKN density was significant, the effect of cover crop treatments was assessed within each RKN density: Low, Medium and High. In several instances, only the contrasts within the high RKN are included. For all levels see Supplementary Table S5.4. When the interaction was not significant, the effect of cover crop treatments was tested over all RKN densities ('All RKN'). [2] different lowercase letters indicate significant differences ($p < 0.05$) in the read count of putative nematode-antagonistic genera among cover crop treatments or RKN densities (calculated with post-hoc tests with Benjamini-Hochberg adjustment for multiple comparisons).[3] When only the effect of RKN density was significant, contrasts are reported between the three RKN densities: Low, Medium and High

These are estimations as the taxonomy of especially some of the fungal groups is volatile (e.g., Scholler et al., 1999). Assuming that these numbers are in the correct order of magnitude, the ample representation and the diversity found in a conventionally managed experimental field in the south-east of The Netherlands are remarkable. Some nematophagous fungi such as *Arthrobotrys oligospora* have been recorded from various continents and a wide range of habitats (Niu and Zhang 2011) and were expected to be represented in our survey. Essentially the same holds for *Nematoctonus leiosporus,* the most frequently isolated endoparasite among the Basidiomycota (Gray, 1983). On the other hand, the nematode antagonist *Mortierella globalpina* has been isolated and characterized only recently (Dilegge et al 2019), and virtually nothing is known about its distribution. This also holds for *Haptocillium balanoides* (also known as *Verticillium balanoides)* a species that was reported from a coastal reserve in northern California (Farrell et al., 2006). Hence, the putative nematode antagonist community detected in the Vredepeel experimental field comprised antagonists whose presence could be anticipated, and it was supplemented with a range of taxa that we did not foresee to be present in this experimental field. An exception is the fungus *Pochonia chlamydosporia* which was not found in our experimental field, although being the most abundant and widespread naturally occurring egg-parasite of *Meloidogyne* in organic and integrated vegetable production systems (Giné et al., 2013; Ghahremani et al., 2022).

5.4.2 Limitations regarding the identification of putative nematode antagonists

The diversity of putative nematode antagonists found in this study is likely an underestimation of the overall diversity of antagonists in the field. This is because of the limits of the amplicon size that can be sequenced in a high throughput scale (here Illumina NovaSeq 2x 250 bp). By sequencing ≈ 500 bp per microbial genotype, only a fraction of the reads could be accurately assigned to the genus level (17% and 19% for bacteria and fungi, respectively), while an even smaller fraction could be classified until species level (~5% for both bacteria and fungi). It is noted that due to the massive output of NovaSeq sequencing (around 1 billion reads per SP flow cell), these are still considerable numbers. Hence, especially at the species level, we might have missed antagonists. As a consequence, the richness in putative nematode antagonists present in the arable field under investigation could be higher than currently reported. With the advent of long-read technologies such as Oxford Nanopore sequencing as presented in **Chapter 4** of this thesis, surveys for native nematode antagonists come within reach.

5.4.3 Soil nutritional condition determines the trophic lifestyle of most nematode antagonists.

Although some antagonists have an obligate nematophagous lifestyle such as *Pasteuria penetrans* (Cetintas and Dickson, 2004) and *Drechmeria coniospora* (Wan et al., 2021), most nematophagous microbes are facultative nematode antagonists. The switch from a saprophytic to a nematophagous lifestyle is triggered by the environment. Traps required to capture nematodes are only formed in the presence of preys and under limited nutritional conditions (Nordbring - Hertz, 1968). Physical contact between *Arthrobotrys hyphae* and a nematode constitutes a strong trigger for trap formation (Tunlid et al., 1992), but the actual response is co-determined by N and C availability. On substrates without nitrogen, traps were formed irrespective of the C availability, and limiting the carbon availability in the presence of nitrogen resulted in a stronger nematophagous response of *Arthrobotrys oligospora* (Scholler and Rubner, 1994). Although this nutritional response is mainly characterized for *A. oligospora*, we hypothesize that most facultative nematode antagonists will respond to the environment in a comparable way. We characterized the soil nutritional status of the Vredepeel experimental field (Materials and Methods) and with a C/N ratio = 18 – 21, and N content of ≈ 4,000 kg N ha^{-1} this soil had nutrient status that is unlikely to trigger a switch from a saprophytic to a nematophagous lifestyle among the facultative nematode antagonists present.

5.4.4 Impact of cover crops on the native nematode antagonist' community

The growing of cover crops caused significant shifts in the community structure, diversity and abundance of putative nematode antagonists. Oilseed radish for example strongly promoted the resident *Pseudomonas* community (Table 5.5), and whereas the oilseed radish cultivar Terranova induced an increase of *Arthrobotrys* spp at DNA level, in combination with vetch it had the highest impact on the activity of representatives of this genus (Table 5.5). From our results, it can be concluded that cover crops have distinct effects on microbial genera harbouring nematode antagonists, and - in addition - cover crop treatments that promote the presence of a given genus, do not necessarily have a positive effect on the activity of the members of the same genus.

Another striking observation is the intraspecific variation with regard to stimulation of nematode antagonists that was observed for oilseed radish. Comparison of the impacts of the cultivars Adios, Radical, and Terranova on

Arthrobotrys spp. revealed that Terranova had a significantly stronger stimulating effect on both the density and the activity of members of this genus as compared to the cultivars Adios and Radical. This implies the presence of genotypic variation in oilseed radish with regard to an agronomically relevant threat. This observation confirms earlier results that were generated in a distinct experimental set-up while using a comparable technical approach (Cazzaniga et al., 2023a; Cazzaniga et al., 2023b). Genotypic variation regarding microbiome selection has been shown before for various plant species. In the case of tomato, genotypic variation accounted for 10% of the variation in root microbiota (French et al., 2020) In another study, the effect of domesticated and wild barley varieties on the soil microbiome was tested, and elite varieties exerted a stronger effect on the rhizosphere microbiome than the non-domesticated genotypes (Alegria Terrazas et al., 2020). It is concluded that cover crops can be used to stimulate microbial genera of putative nematode antagonists, but they do so in a species- and even genotype-specific manner.

5.4.5 Impact of *M. chitwoodi* density on the native antagonist community

As shown in Table 5.5, not all putative nematode-antagonistic genera responded to differences in *M. chitwoodi* level. Whereas *Bacillus* and *Nematoctonus* were unaffected by the densities of this plant-parasitic nematode, other genera such as *Trichoderma and Fusarium* responded to cover crop treatments only in plots with the highest *M. chitwoodi* densities. The genus *Haptocillium* constituted the clearest example of a genus harbouring nematode antagonists showing higher abundances in plots with elevated *M. chitwoodi* irrespective of the cover crop treatment (Table 5.5). This can – at least in part - be explained by its "low saprophytic ability", which was reported for one of the detected species, *H. balanoides* (Zare and Games, 2003). No similar ecological information could be found about the second species, *H. sinense.* Hence, the increased presence of the (semi-) obligate nematophagous fungal genus *Haptocillium* can probably best be explained by its trophic ecology. Another significant effect of *M. chitwoodi* density was observed for the fungal genus *Mortierella,* represented in the Vredepeel experimental field with three species. *M. globalpina* was recently identified as a potential biocontrol agent against *M. chitwoodi* (Dilegge et al., 2019). On the other hand, virtually no ecological information is available for *M. elongatula* and *M. fatshederae.* It is concluded that *M. chitwoodi* density stimulated some native putative nematode antagonists, but the effect of nematode density was less pronounced than the effect of cover crop treatments.

5.5 Conclusion and future outlook

Antagonists of plant-parasitic nematodes have been studied for decades, and numerous examples of nematode antagonists have been documented (Li et al., 2015a; Topalovic et al., 2020). These efforts have provided important insights into the diverse and often elegant mechanisms by which microbial antagonists predate nematodes. However, these insights could seldom be translated into effective biocontrol measures as the introduction of biocontrol agents in soil – a highly competitive environment – appeared often problematic. In this study, we focused on ways to stimulate the native nematode-antagonistic community in arable soil. We found a striking richness of putative nematode antagonists in this system. We demonstrated that individual antagonists can be promoted by cover crops in an antagonist-specific manner. We also showed that some nematode antagonists were stimulated by cover crops in the presence of high *M. chitwoodi* densities. If common arable soils indeed generally harbour a more diverse nematode-antagonistic community than previously anticipated, future research could focus on understanding the cues that promote the switch from a saprophytic to a nematophagous lifestyle for a relevant fraction of the native nematode antagonist community.

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5.6 Supplementary Materials

Supplementary Table S5.1 | Table of genera harbouring putative nematode antagonists considered in this study based on literature research. Genera found in the Vredepeel experimental field are presented including their prevalence in soil samples collected at T0 and T1. Sources are A for (Topalovic et al., 2020) or B for (Li et al., 2015)

	Bacterial genera	Source	species found in the dataset	DNA-T0 Prevalence (12 samples)	RNA-T0 Prevalence (12 samples)	DNA-T1 Prevalence (129 samples)	RNA-T1 Prevalence (130 samples)
1	*Arthrobacter*	A					
2	*Bacillus*	A, B	*B.asahii, B. licheniformis, B.longiquaesitum, B.murimartini, B.oleronius, B.plakortidis, B. thermoamylovorans, B. smithii*	12	11	125	124
3	*Lysobacter*	A	*L.concretionis, L.dokdonensis, L.ginsengisoli, L.niastensis, L.soli*	12	12	129	129
4	*Pasteuria*	A, B					
5	*Pseudomonas*	A, B	*P.putida, P.stutzeri, P.alcaligenes, P.caeni*	12	12	127	128
6	*Rhizobium*	A	*R.arenae, R.azooxidifex, R.giardinii, R.leguminosarum, R.mongolense, R.soli, R.yantingense*	12	12	124	129
7	*Streptomyces*	A					
8	*Variovorax*	A, B	*V.paradoxus, V.soli*	12	12	127	127

	Fungal genera	Source	species found in the dataset	DNA-T0 Prevalence (12 samples)	RNA-T0 Prevalence (12 samples)	DNA-T1 Prevalence (130 samples)	RNA-T1 Prevalence (129 samples)
1	*Acremonium*	B	*A.biseptum, A.furcatum, A.rutilum, A.verruculosum*	8		55	13
2	*Arthrobotrys /Orbilia*	A, B	*A.brochopaga, A.dactyloides, A.iridis, A.oligospora, A.oudemansii, A.reticulata*	12	7	126	112
3	*Catenaria*	A, B					
4	*Coprinus + Coprinellus*	B	*C.heptemerus*	2	1	17	4
5	*Cystopage*	B					

6	*Dactylella*	A, B	*D.tenuis, O.rectispora* (teleomorph)	1		4	5
7	*Dactylellina / Monacrosporium / Orbilia*	A, B	*M.drechsleri*	1		18	11
8	*Drechmeria*	B	*D.coniospora*	1		1	
9	*Drechslerella / Orbilia*	A, B					
10	*Fusarium*	B	*F.poae*	12	6	130	106
11	*Glomus*	B					1
12	*Haptocillium / Cordyceps*	A, B	*H.balanoides, H.sinense*	5		30	1
13	*Harposporium/ Podocrella*	B					
14	*Hirsutella*	A, B	*H.vermicola*	5		46	3
15	*Lecanicillium / Cordyceps*	B		1			
16	*Mortierella*	A	*M.elongatula M.fatshederae M.globalpina*	12	9	116	93
17	*Nematoctonus / Hohenbuehelia*	B	*N.leiosporus*	8		34	6
18	*Nematophthora*	A					
19	*Neotyphodium*	B					
20	*Penicillium*	B		12	6	130	44
21	*Pleurotus*	B					
22	*Pochonia / Metacordyceps*	A, B					
23	*Purpureocillium*	A					
24	*Stropharia*	B				1	
25	*Stylopage*	B					
26	*Trichoderma*	A, B	*T.albolutescens*	12	10	124	85

Supplementary Table S5.2 | PERMANOVA analyses on the bacterial and fungal communities at T0 (A), T1 (B) and pairwise comparisons at T1 (C)

A PERMANOVA analysis of the bacterial and fungal community composition at T0 - during pre-crops

	Bacteria DNA	Bacteria RNA	Fungi DNA	Fungi RNA
block	R^2 = 22.4%, P=0.001	R^2=17.3%, P=0.002	R^2=38.1%, P=0.001	R^2 = 15.4%, P=0.032,
sample type	R^2=18.7%, P=0.001	R^2=20.6%, P=0.001	R^2=14.0%, P=0.001	R^2=18.0%, P=0.001,
pre crop	ns[a]	ns	ns	ns
RKN density	ns	ns	ns	ns

B PERMANOVA analysis of the bacterial and fungal community composition at T1 - cover crop rhizosphere + fallow

	Bacteria DNA	Bacteria RNA	Fungi DNA	Fungi RNA
block	R^2 = 5.9%, P=0.001	R^2=4.7%, P=0.001	R^2=6.1%, P=0.001	R^2=3.3%, P=0.001
RKN density	ns	ns	R^2=2.3%, P=0.005	R^2=2.2%, P=0.002

cover crop	R^2=22.4%, P=0.001	R^2=25.5%, P=0.001	R^2=27.1%, P=0.001	R^2=21.0%, P=0.001
RKN density * cover crop	ns	ns	ns	ns

[a]ns: not statically significant (P > 0.005)

C Pairwise comparisons of the microbiomes associated with cover crops rhizosphere and fallow at T1. P-value adjusted with the Bonferroni-Holmes method. Highlighted in grey are significant differences (P < 0.05).

Bacteria - DNA

	FW	OSR_T	OSR_A	OSR_R	PHA	VE	BO	PHA. OSR_T	VE. OSR_T	BO. OSR_T
OSR_T	0.0016	-	-	-	-	-	-	-	-	-
OSR_A	0.0016	0.567	-	-	-	-	-	-	-	-
OSR_R	0.0016	0.3269	0.5184	-	-	-	-	-	-	-
PHA	0.0016	0.0016	0.0016	0.0016	-	-	-	-	-	-
VE	0.0138	0.003	0.0016	0.0056	0.0016	-	-	-	-	-
BO	0.0016	0.0016	0.0016	0.0016	0.0016	0.0016	-	-	-	-
PHA.OSR_T	0.0016	0.0928	0.1111	0.0854	0.0056	0.021	0.0016	-	-	-
VE.OSR_T	0.0016	0.1745	0.1531	0.1066	0.0016	0.0016	0.0016	0.0682	-	-
BO.OSR_T	0.0016	0.023	0.0263	0.0161	0.0016	0.0016	0.0016	0.003	0.033	-
BO.OSR_R	0.0016	0.0016	0.003	0.0016	0.0016	0.0016	0.0016	0.0016	0.0016	0.0016

Bacteria - RNA

	FW	OSR_T	OSR_A	OSR_R	PHA	VE	BO	PHA. OSR_T	VE. OSR_T	BO. OSR_T
OSR_T	0.0016	-	-	-	-	-	-	-	-	-
OSR_A	0.0016	0.345	-	-	-	-	-	-	-	-
OSR_R	0.0016	0.2455	0.1523	-	-	-	-	-	-	-
PHA	0.0016	0.0016	0.003	0.0016	-	-	-	-	-	-
VE	0.0016	0.0016	0.0016	0.0016	0.0016	-	-	-	-	-
BO	0.0016	0.0016	0.0016	0.0016	0.0016	0.0016	-	-	-	-
PHA.OSR_T	0.0016	0.1806	0.0985	0.0425	0.003	0.0016	0.0016	-	-	-
VE.OSR_T	0.0016	0.1122	0.1154	0.0477	0.0016	0.0016	0.0016	0.0971	-	-
BO.OSR_T	0.0016	0.1033	0.0183	0.0183	0.0016	0.0016	0.0016	0.0789	0.0183	-
BO.OSR_R	0.0016	0.013	0.0183	0.0016	0.0016	0.0016	0.0016	0.0016	0.0016	0.0217

Fungi – DNA

	FW	OSR_T	OSR_A	OSR_R	PHA	VE	BO	PHA.OSR_T	VE.OSR_T	BO.OSR_T
OSR_T	0.0016	-	-	-	-	-	-	-	-	-
OSR_A	0.0016	0.5113	-	-	-	-	-	-	-	-
OSR_R	0.0016	0.3196	0.689	-	-	-	-	-	-	-
PHA	0.0016	0.0016	0.0016	0.0016	-	-	-	-	-	-
VE	0.0016	0.0016	0.0016	0.0016	0.0016	-	-	-	-	-
BO	0.0016	0.0016	0.0016	0.0016	0.0016	0.0016	-	-	-	-
PHA.OSR_T	0.0016	0.0358	0.2729	0.1914	0.0016	0.0016	0.0016	-	-	-
VE.OSR_T	0.0016	0.248	0.1897	0.165	0.0016	0.0043	0.0016	0.0375	-	-
BO.OSR_T	0.0016	0.0587	0.1369	0.0069	0.0016	0.0016	0.0016	0.0157	0.0861	-
BO.OSR_R	0.0016	0.003	0.0094	0.0016	0.003	0.0016	0.0016	0.0016	0.0016	0.0069

Fungi - RNA

	FW	OSR_T	OSR_A	OSR_R	PHA	VE	BO	PHA.OSR_T	VE.OSR_T	BO.OSR_T
OSR_T	0.0018	-	-	-	-	-	-	-	-	-
OSR_A	0.0018	0.809	-	-	-	-	-	-	-	-
OSR_R	0.0018	0.4717	0.4325	-	-	-	-	-	-	-
PHA	0.0018	0.0018	0.0031	0.0018	-	-	-	-	-	-
VE	0.0018	0.0018	0.0046	0.0018	0.0018	-	-	-	-	-
BO	0.0018	0.0018	0.0018	0.0018	0.0018	0.0018	-	-	-	-
PHA.OSR_T	0.0018	0.0859	0.5583	0.0217	0.0031	0.0031	0.0018	-	-	-
VE.OSR_T	0.0018	0.1351	0.1785	0.1162	0.0018	0.0018	0.0018	0.089	-	-
BO.OSR_T	0.0018	0.0985	0.662	0.0591	0.0018	0.0018	0.0018	0.1271	0.2849	-
BO.OSR_R	0.0018	0.0018	0.1381	0.0018	0.0059	0.0018	0.0018	0.1581	0.0031	0.0381

Supplementary Table S5.3 | PERMANOVA analyses on the community of bacterial and fungal antagonists at T0 (A), T1 (B) and pairwise comparisons at T1 (C)

A PERMANOVA analysis of the antagonist's community composition at T0 - during pre-crops

	Bacteria DNA	Bacteria RNA	Fungi DNA	Fungi RNA
block	ns	R^2=15,6%, P=0.035	R^2=20.0%, =0.005	R^2=20.5%, P=0.006
sample type	R^2=49%, P=0.001	R^2=25.8%, P=0.001	R^2=12.4%, =0.001	R^2=9.7%, P=0.005
pre crop	ns	ns	ns	ns
RKN density	ns[a]	ns	ns	ns

B PERMANOVA analysis of the antagonist's community composition at T1 - in cover crop rhizosphere

	Bacteria DNA	Bacteria RNA	Fungi DNA	Fungi RNA
block	R^2=3.1%, P=0.003	R^2 =4.6%, P=0.001	R^2=3.7%, P=0.023	ns
RKN density	ns	ns	R^2=2.8%, P=0.027	ns
cover crop	R^2=30.7%, P=0.001	R^2=19.3%, P=0.001	R^2=14.2%, P=0.001	R^2=9.5%, P=0.027
RKN density * cover crop	ns[a]	ns	ns	ns

[a]ns: not statically significant (P > 0.005)

C Pairwise comparisons of the communities of antagonists associated with cover crops rhizosphere and fallow at T1. P-value adjusted with Bonferroni -Holmes method. Highlighterd in grey significant results (P < 0.05).

Bacteria _DNA

	FW	OSR_T	OSR_A	OSR_R	PHA	VE	BO	BO.OSR_R	BO.OSR_T	PHA.OSR_T
OSR_T	0.002	-								
OSR_A	0.002	0.392	-							
OSR_R	0.002	0.265	0.376	-						
PHA	0.002	0.002	0.002	0.002	-					
VE	0.002	0.003	0.002	0.002	0.002	-				
BO	0.002	0.002	0.002	0.002	0.002	0.002	-			
BO.OSR_R	0.002	0.052	0.219	0.013	0.003	0.002	0.002	-		
BO.OSR_T	0.002	0.397	0.136	0.204	0.002	0.002	0.002	0.006	-	
PHA.OSR_T	0.002	0.370	0.332	0.906	0.003	0.003	0.002	0.009	0.280	-
VE.OSR_T	0.002	0.392	0.025	0.013	0.002	0.002	0.002	0.003	0.081	0.043

Bacteria - RNA

	FW	OSR_T	OSR_A	OSR_R	PHA	VE	BO	BO.OSR_R	BO.OSR_T	PHA.OSR_T
OSR_T	0.003	-								
OSR_A	0.003	0.721	-							
OSR_R	0.003	0.165	0.370	-						
PHA	0.003	0.030	0.121	0.003	-					
VE	0.003	0.003	0.003	0.003	0.003	-				
BO	0.003	0.033	0.013	0.005	0.013	0.003	-			
BO.OSR_R	0.003	0.357	0.185	0.003	0.195	0.003	0.020	-		
BO.OSR_T	0.003	0.467	0.152	0.299	0.036	0.003	0.343	0.042	-	
PHA.OSR_T	0.005	0.259	0.661	0.357	0.399	0.003	0.078	0.034	0.394	-
VE.OSR_T	0.003	0.440	0.357	0.152	0.015	0.003	0.005	0.024	0.259	0.399

Fungi - DNA

	FW	OSR_T	OSR_A	OSR_R	PHA	VE	BO	BO.OSR_R	BO.OSR_T	PHA.OSR_T
OSR_T	0.037	-								
OSR_A	0.028	0.401	-							
OSR_R	0.087	0.168	0.401	-						
PHA	0.067	0.179	0.401	0.168	-					
VE	0.168	0.345	0.399	0.179	0.321	-				
BO	0.028	0.110	0.241	0.168	0.391	0.082	-			
BO.OSR_R	0.033	0.231	0.932	0.401	0.389	0.287	0.343	-		
BO.OSR_T	0.033	0.062	0.343	0.168	0.343	0.039	0.766	0.401	-	

PHA.OSR_T	0.168	0.406	0.401	0.343	0.458	0.814	0.102	0.310	0.085	-
VE.OSR_T	0.033	0.168	0.440	0.310	0.736	0.168	0.401	0.401	0.453	0.310

Fungi - RNA

	FW	OSR_T	OSR_A	OSR_R	PHA	VE	BO	BO.OSR_R	BO.OSR_T	PHA.OSR_T
OSR_T	0.780	-	-	-	-	-	-	-	-	-
OSR_A	0.700	0.730	-	-	-	-	-	-	-	-
OSR_R	0.560	0.460	0.520	-	-	-	-	-	-	-
PHA	0.460	0.520	0.900	0.670	-	-	-	-	-	-
VE	0.640	0.520	0.770	0.320	0.460	-	-	-	-	-
BO	0.210	0.210	0.720	0.210	0.500	0.520	-	-	-	-
BO.OSR_R	0.180	0.210	0.600	0.180	0.460	0.460	0.520	-	-	-
BO.OSR_T	0.460	0.400	0.940	0.730	0.730	0.520	0.720	0.600	-	-
PHA.OSR_T	0.600	0.800	0.980	0.700	0.980	0.860	0.720	0.730	0.940	-
VE.OSR_T	0.180	0.210	0.770	0.460	0.900	0.460	0.460	0.210	0.560	0.770

Supplementary Table S5.4 | Results of the generalised negative binomial mixed models on the counts of putative nematode antagonists. Post-hoc tests were conducted with emmeans and BH procedure for multiple comparisons. N.s. stands for non-significant (P > 0.05).

Fungi

Acremonium

DNA				RNA
All RKN				All RKN
cover crop	response	SE	group	no model, prevalence too low
OSR_T	45.8	15.1	a	
OSR_A	65.5	23.1	a	
OSR_R	221	133.6	ab	
PHA	44.7	17.2	a	
VE	59.4	25.7	a	
BO	53.6	14.6	a	
BO.OSR_R	85.1	27.7	a	
BO.OSR_T	62.3	26.8	a	
PHA.OSR_T	69	41	a	
VE.OSR_T	676.1	257.6	b	

Arthrobotrys

DNA				RNA			
RKN: Low				RKN: Low			
cover crop	response	SE	group	cover crop	response	SE	group
OSR_T	3133.8	1712.8	d	OSR_T	2582.7	2386.6	b
OSR_A	268.1	146.9	abc	OSR_A	1493.9	1380.8	b
OSR_R	638	349	bcd	OSR_R	2682.6	2478.9	b
PHA	87.6	48	a	PHA	14.6	13.6	a
VE	458.3	250.9	abc	VE	309.6	286.2	ab
BO	1316.8	719.9	cd	BO	407	376.5	ab
BO.OSR_R	275.5	151	abc	BO.OSR_R	963.1	890.2	b
BO.OSR_T	163.2	89.5	ab	BO.OSR_T	435.6	402.6	ab
PHA.OSR_T	145.9	98.1	ab	PHA.OSR_T	749.9	848.9	ab
VE.OSR_T	170.6	93.6	ab	VE.OSR_T	253.1	234	ab
RKN: Medium				RKN: Medium			
cover crop	response	SE	group	cover crop	response	SE	group
OSR_T	312.7	121.2	bc	OSR_T	656.3	429.3	ab
OSR_A	496.1	192	cd	OSR_A	1011.1	660.8	ab
OSR_R	212.4	82.6	bc	OSR_R	102.9	67.4	a
PHA	49.7	21.1	a	PHA	427.6	279.6	ab
VE	370.3	143.4	bc	VE	734.1	479.8	ab

BO	1530.8	591.8	d	BO	1510.1	986.8	ab
BO.OSR_R	267.6	103.7	bc	BO.OSR_R	536.4	384	ab
BO.OSR_T	161.7	62.7	abc	BO.OSR_T	818	534.8	ab
PHA.OSR_T	110.2	47.8	ab	PHA.OSR_T	401.7	287.7	ab
VE.OSR_T	471.1	182.2	cd	VE.OSR_T	2212	1445.4	b
RKN: High				RKN: High			
cover crop	response	SE	group	cover crop	response	SE	group
OSR_T	3516.6	1922.1	c	OSR_T	5820.3	5378.2	b
OSR_A	220	120.6	b	OSR_A	65	60.3	a
OSR_R	416.3	280.1	b	OSR_R	983.9	909.4	ab
PHA	28.7	15.9	a	PHA	46.1	42.8	a
VE	571.2	312.4	bc	VE	728.7	673.5	ab
BO	380.4	208.2	b	BO	1192.3	1101.9	ab
BO.OSR_R	207.2	113.6	b	BO.OSR_R	555.7	513.8	ab
BO.OSR_T	847.7	463.8	bc	BO.OSR_T	2608.1	2410.1	b
PHA.OSR_T	347.1	190.1	b	PHA.OSR_T	559.1	527.5	ab
VE.OSR_T	1088.2	595	bc	VE.OSR_T	12101.3	11182	b

Fusarium

DNA				RNA			
RKN: Low				RKN: Low			
cover crop	response	SE	group	cover crop	response	SE	group
OSR_T	1584	525	a	OSR_T	865	318	a
OSR_A	1485	494	a	OSR_A	637	285	a
OSR_R	2199	731	a	OSR_R	985	363	a
PHA	2360	789	a	PHA	1093	402	a
VE	3828	1269	a	VE	2436	896	a
BO	3048	1017	a	BO	1929	864	a
BO.OSR_R	2090	699	a	BO.OSR_R	1340	495	a
BO.OSR_T	2752	913	a	BO.OSR_T	1622	596	a
PHA.OSR_T	4406	1783	a	PHA.OSR_T	3903	1811	a
VE.OSR_T	5849	1940	a	VE.OSR_T	1467	540	a
RKN: Medium				RKN: Medium			
cover crop	response	SE	group	cover crop	response	SE	group
OSR_T	1202	300	ab	OSR_T	1457	539	a
OSR_A	2105	527	abcd	OSR_A	1244	376	a
OSR_R	2858	713	cd	OSR_R	1491	437	a
PHA	1021	254	a	PHA	1170	436	a
VE	3308	823	d	VE	1171	432	a
BO	3931	973	d	BO	3044	819	a
BO.OSR_R	2214	548	abcd	BO.OSR_R	1844	540	a
BO.OSR_T	2277	562	bcd	BO.OSR_T	1554	419	a
PHA.OSR_T	1416	378	abc	PHA.OSR_T	1009	299	a
VE.OSR_T	2350	583	bcd	VE.OSR_T	1000	298	a
RKN: High				RKN: High			
cover crop	response	SE	group	cover crop	response	SE	group
OSR_T	1541	514	bc	OSR_T	1580	585	c
OSR_A	2956	984	c	OSR_A	2066	922	c
OSR_R	2019	674	bc	OSR_R	2069	766	c
PHA	345	115	a	PHA	375	168	ab
VE	2848	953	c	VE	985	364	bc
BO	3651	1226	c	BO	1312	597	bc
BO.OSR_R	868	289	ab	BO.OSR_R	1864	833	c
BO.OSR_T	2154	719	bc	BO.OSR_T	897	401	abc
PHA.OSR_T	1728	575	bc	PHA.OSR_T	218	95	a
VE.OSR_T	2007	667	bc	VE.OSR_T	916	338	bc

Haptocillium

DNA				RNA
RKN:				no model, prevalence too low
RKN	response	SE	group	
Low	9.89	6.56	a	
Medium	25.4	7.12	a	

High	132.05	33.33	b

Hirsutella

DNA	RNA
n.s.	no model, prevalence too low

Mortierella

DNA	RNA			
n.s.	RKN:			
	RKN	response	SE	group
	Low	104	19.9	a
	Medium	167	24.1	b
	High	213	45.3	b

Nematoctonus

DNA				RNA
All RKN:				All RKN:
cover crop	response	SE	group	no model, prevalence too low
OSR_T	9.52	2.85	a	
OSR_A	70.74	24.83	c	
OSR_R	35.58	12.75	bc	
PHA	27.15	9.64	abc	
VE	11.47	4.63	ab	
BO	8.49	2.73	a	
BO.OSR_R	18.64	5.8	ab	
BO.OSR_T	15.38	4.72	ab	
PHA.OSR_T	18.7	4.08	ab	
VE.OSR_T	17.39	4.28	ab	

Penicillium

DNA				RNA
All RKN:				
cover crop	response	SE	df	n.s.
OSR_T	635	132.1	bc	
OSR_A	520	108.3	abc	
OSR_R	368	77.3	abc	
PHA	578	124.7	abc	
VE	1503	313.4	d	
BO	525	110.1	abc	
BO.OSR_R	302	63.2	a	
BO.OSR_T	347	72.2	ab	
PHA.OSR_T	752	174.2	cd	
VE.OSR_T	640	133.3	bc	

Trichoderma

DNA				RNA			
All RKN:				RKN: Low			
cover crop	response	SE	group	cover crop	response	SE	group
OSR_T	388	139.6	ab	OSR_T	266.2	1.663.078	a
OSR_A	282	104.7	ab	OSR_A	61.5	526.739	a
OSR_R	363	137.9	ab	OSR_R	272.4	1.662.404	a
PHA	338	129.3	ab	PHA	437.5	2.712.154	a
VE	666	239.1	b	VE	153.7	792.173	a
BO	353	126.8	ab	BO	111.5	653.167	a
BO.OSR_R	195	71.3	a	BO.OSR_R	262.4	1.333.852	a
BO.OSR_T	214	77.4	a	BO.OSR_T	424.9	2.620.196	a
PHA.OSR_T	245	93.1	ab	PHA.OSR_T	515.2	316.467	a
VE.OSR_T	373	134.9	ab	VE.OSR_T	242.4	1.259.103	a
				RKN: Medium			
				cover crop	response	SE	group
				OSR_T	1496.3	615.684	b
				OSR_A	351	1.473.155	ab
				OSR_R	798.6	4.847.013	ab

PHA	309.2	1.648.237	ab
VE	536.6	2.436.023	ab
BO	414.1	1.917.833	ab
BO.OSR_R	106.6	663.555	a
BO.OSR_T	489.6	2.207.116	ab
PHA.OSR_T	185.3	946.004	a
VE.OSR_T	470.2	2.145.878	ab

RKN: High			
cover crop	response	SE	group
OSR_T	524.6	2.678.484	ab
OSR_A	1214.9	6.237.241	b
OSR_R	591.4	4.978.063	ab
PHA	87	540.437	a
VE	306.2	159.621	ab
BO	137.4	840.664	ab
BO.OSR_R	0	0.0002	ab
BO.OSR_T	187.3	163.096	ab
PHA.OSR_T	328.8	194.742	ab
VE.OSR_T	194.7	1.189.346	ab

Bacteria

Bacillus

DNA	RNA
n.s.	All RKN :

cover crop	response	SE	group
OSR_T	436	81.2	cd
OSR_A	268	50.6	abc
OSR_R	350	65.7	bcd
PHA	186	39.6	ab
VE	326	63.1	abcd
BO	205	38.4	ab
BO.OSR_R	171	35.7	a
BO.OSR_T	292	54.8	abcd
PHA.OSR_T	295	59.7	abcd
VE.OSR_T	513	97.6	d

Lysobacter

DNA				RNA			
RKN: Low				All RKN			
cover crop	response	SE	group		response	SE	group
OSR_T	402	76.6	a	OSR_T	498	61.4	a
OSR_A	523	99.5	ab	OSR_A	472	58.6	a
OSR_R	494	93.8	ab	OSR_R	563	69.2	a
PHA	596	113.3	ab	PHA	511	68.7	a
VE	532	101.2	ab	VE	1783	224.8	b
BO	454	86.3	a	BO	553	68.4	a
BO.OSR_R	511	97.2	ab	BO.OSR_R	466	57.2	a
BO.OSR_T	608	115.4	ab	BO.OSR_T	550	70.5	a
PHA.OSR_T	1097	254.1	b	PHA.OSR_T	543	72.7	a
VE.OSR_T	399	75.9	a	VE.OSR_T	522	64.7	a

RKN: Medium			
cover crop	response	SE	group
OSR_T	645	86.5	a
OSR_A	501	67.4	a
OSR_R	586	86.2	a
PHA	416	56.1	a
VE	566	76	a
BO	596	80	a
BO.OSR_R	556	74.9	a
BO.OSR_T	600	80.5	a
PHA.OSR_T	506	83.6	a
VE.OSR_T	536	78.8	a

RKN: High

cover crop	response	SE	group
OSR_T	389	74.2	ab
OSR_A	481	91.5	abc
OSR_R	669	126.9	bc
PHA	463	88	abc
VE	702	133	bc
BO	517	98.2	abc
BO.OSR_R	584	111	bc
BO.OSR_T	820	155.3	c
PHA.OSR_T	270	52	a
VE.OSR_T	376	71.8	ab

Pseudomonas

DNA

RKN: Low

cover crop	response	SE	group
OSR_T	6759	2381	a
OSR_A	4090	1441	a
OSR_R	1934	682	a
PHA	4282	1509	a
VE	2899	1021	a
BO	2287	806	a
BO.OSR_R	3986	1404	a
BO.OSR_T	3290	1159	a
PHA.OSR_T	1186	512	a
VE.OSR_T	5863	2065	a

RKN: Meddium

cover crop	response	SE	group
OSR_T	2345	584	b
OSR_A	4262	1062	bcd
OSR_R	5888	1467	cd
PHA	2953	736	bc
VE	450	112	a
BO	2623	654	bc
BO.OSR_R	8698	2167	d
BO.OSR_T	2627	655	bc
PHA.OSR_T	2070	632	b
VE.OSR_T	3477	866	bc

RKN: High

cover crop	response	SE	group
OSR_T	5797	2042	bcde
OSR_A	16687	5878	e
OSR_R	2247	792	bc
PHA	5090	1793	bcd
VE	454	160	a
BO	1868	658	b
BO.OSR_R	3142	1107	bc
BO.OSR_T	6390	2251	cde
PHA.OSR_T	6643	2340	cde
VE.OSR_T	10194	3591	de

RNA

RKN: Low

cover crop	response	SE	group
OSR_T	439	69.1	a
OSR_A	853	133.5	bc
OSR_R	586	91.4	ab
PHA	737	114.7	abc
VE	1242	192.8	c
BO	842	130.7	bc
BO.OSR_R	657	102.7	ab
BO.OSR_T	690	107.7	ab
PHA.OSR_T	602	114.9	ab
VE.OSR_T	713	111.3	abc

RKN: Meddium

cover crop	response	SE	group
OSR_T	846	93.3	bcd
OSR_A	613	67.9	ab
OSR_R	614	67.6	ab
PHA	802	88.5	bc
VE	1186	130.5	d
BO	769	84.6	bc
BO.OSR_R	490	54.1	a
BO.OSR_T	747	82.3	bc
PHA.OSR_T	990	119.5	cd
VE.OSR_T	720	79.3	bc

RKN: High

cover crop	response	SE	group
OSR_T	745	116.1	bc
OSR_A	273	43.6	a
OSR_R	794	123.6	bc
PHA	713	136	bc
VE	1160	180.3	c
BO	804	125.1	bc
BO.OSR_R	733	114.4	bc
BO.OSR_T	694	108	bc
PHA.OSR_T	816	127.1	bc
VE.OSR_T	512	80	b

Rhizobium

DNA

All RKN:

cover crop	response	SE	group
OSR_T	2297	246	ab
OSR_A	2527	267	ab
OSR_R	2452	262	ab
PHA	2171	230	a
VE	2437	258	ab
BO	2923	311	ab
BO.OSR_R	2602	275	ab
BO.OSR_T	3378	359	b
PHA.OSR_T	2377	281	ab
VE.OSR_T	3429	363	b

RKN

Low	2812	245	b
Medium	2309	164	a
High	2925	254	b

RNA

RKN: Low

cover crop	response	SE	group
OSR_T	2050	605	a
OSR_A	1493	441	a
OSR_R	1349	399	a
PHA	866	256	a
VE	904	267	a
BO	908	268	a
BO.OSR_R	1570	464	a
BO.OSR_T	1081	319	a
PHA.OSR_T	1323	479	a
VE.OSR_T	2842	839	a

RKN: Medium

cover crop	response	SE	group
OSR_T	1174	245	a
OSR_A	912	191	a
OSR_R	2898	605	b
PHA	1000	209	a
VE	879	184	a
BO	870	182	a
BO.OSR_R	1260	263	a
BO.OSR_T	1127	236	a
PHA.OSR_T	1027	235	a
VE.OSR_T	1053	220	a

RKN: High

cover crop	response	SE	group
OSR_T	1297	383	a
OSR_A	6418	1894	c
OSR_R	867	256	a
PHA	1647	596	ab
VE	896	265	a
BO	1427	422	a
BO.OSR_R	1711	505	ab
BO.OSR_T	1153	341	a
PHA.OSR_T	4327	1277	bc
VE.OSR_T	4424	1305	bc

Variovorax

DNA

All RKN:

cover crop	response	SE	group
OSR_T	1074	106.7	abcd
OSR_A	997	99.7	abcd
OSR_R	878	87.6	abc
PHA	1147	114.2	cd
VE	790	78.8	a
BO	1009	100.1	abcd
BO.OSR_R	1267	126.5	d
BO.OSR_T	1337	132.8	d
PHA.OSR_T	791	88	ab
VE.OSR_T	1083	107.7	bcd

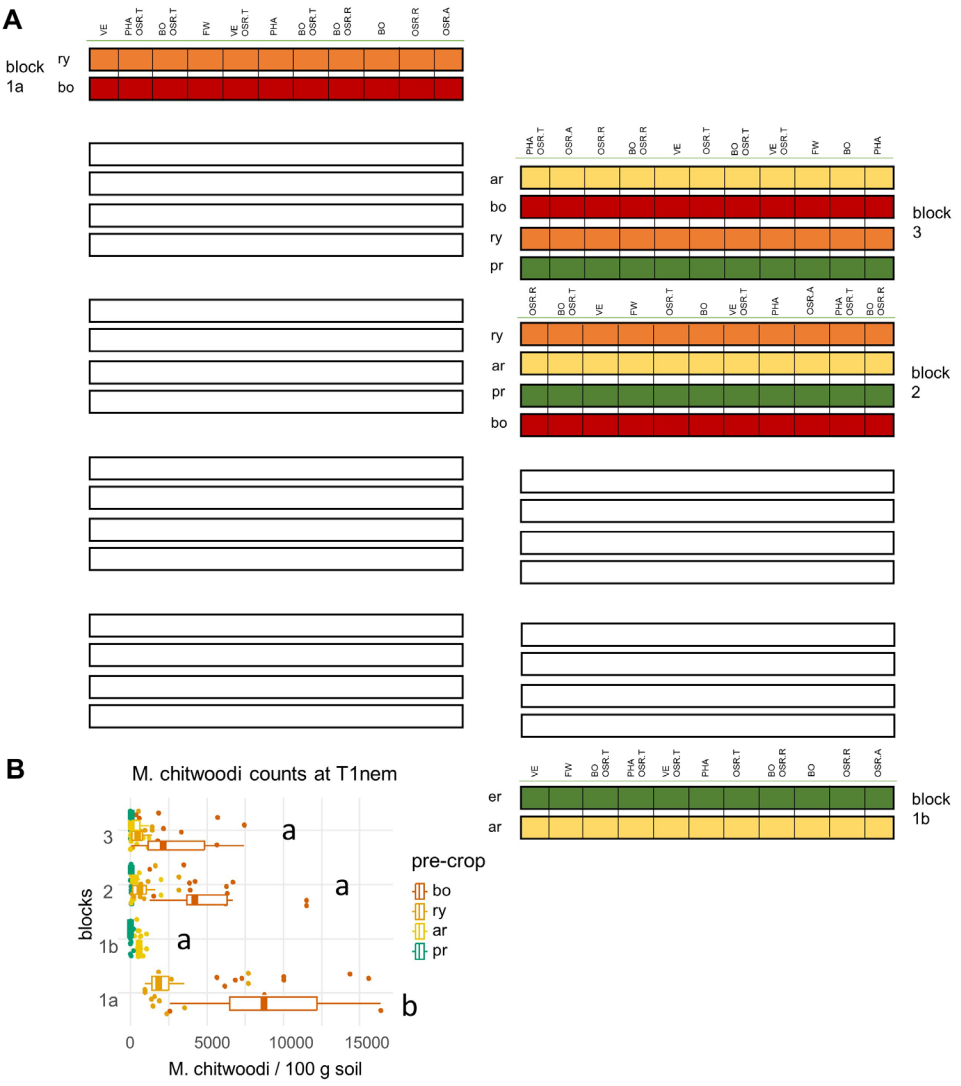
RKN

	response	SE	group
Low	1127	82.1	b
Medium	906	55.1	a
High	1085	78.1	b

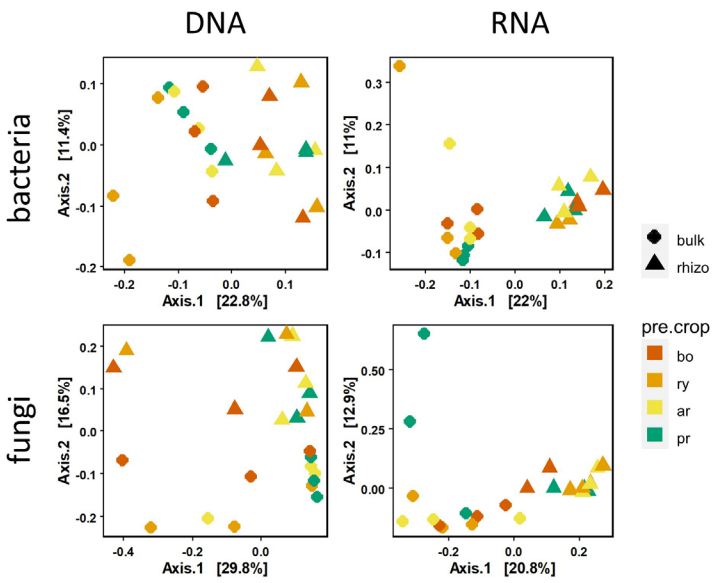
RNA

All RKN:

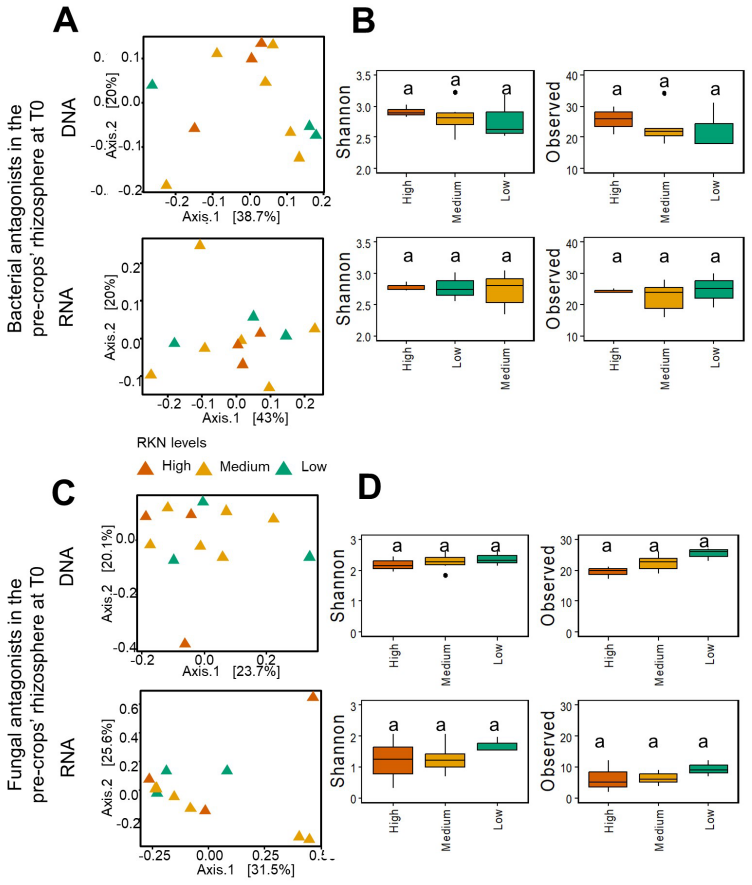
cover crop	response	SE	group
OSR_T	281	37.8	abc
OSR_A	211	29.8	a
OSR_R	296	41.3	abc
PHA	438	58.9	bc
VE	455	60.9	c
BO	343	46.1	abc
BO.OSR_R	325	43.3	abc
BO.OSR_T	309	41.2	abc
PHA.OSR_T	438	63.9	bc
VE.OSR_T	261	35.1	ab



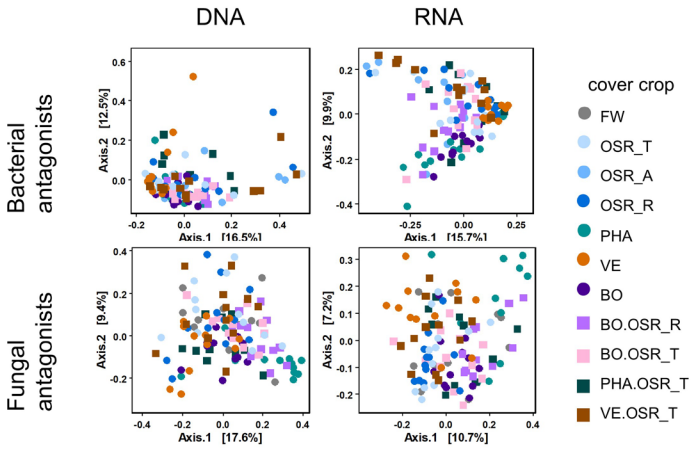
Supplementary Figure S5.1 | A) Lay out of the field experiment at Vredepeel. 'bo' = black oat, 'ry' = rye, 'ar' = annual ryegrass, 'pr' = perennial ryegrass pre-crops. Perpendicular to the longitudinal direction of the pre-crop strips 11 plots (each 6x3 m) were defined, and ten cover crop treatments were grown in them. VE= vetch, BO= black oat, PHA= phacelia, T= oilseed radish Terranova, R= oilseed radish Radical, A= oilseed radish Adios, BO OSR.T= black oat – oilseed radish T, PHA OSR.T= phacelia – oilseed radish T, VE OSR.T= vetch - oilseed radish T, BO OSR.R= black oat – oilseed radish R. FW = fallow, unplanted control. B) *M. chitwoodi* densities per block.



Supplementary Figure S5.2 | Principal Coordinate Analysis (PCoA) of CSS normalised ASV assigned to genera of putative nematode antagonists at T0. Dissimilarity matrix built on Bray-Curtis metric and plotted separating ASVs based on pre-crops (colours) and sample type (shapes). PERMANOVA indicated a non-significant effect of pre-crops, but a significant difference between rhizosphere ad bulk soil (Supplementary Table S5.2 A). The latter clearly separates samples along the principal PCoA axis.



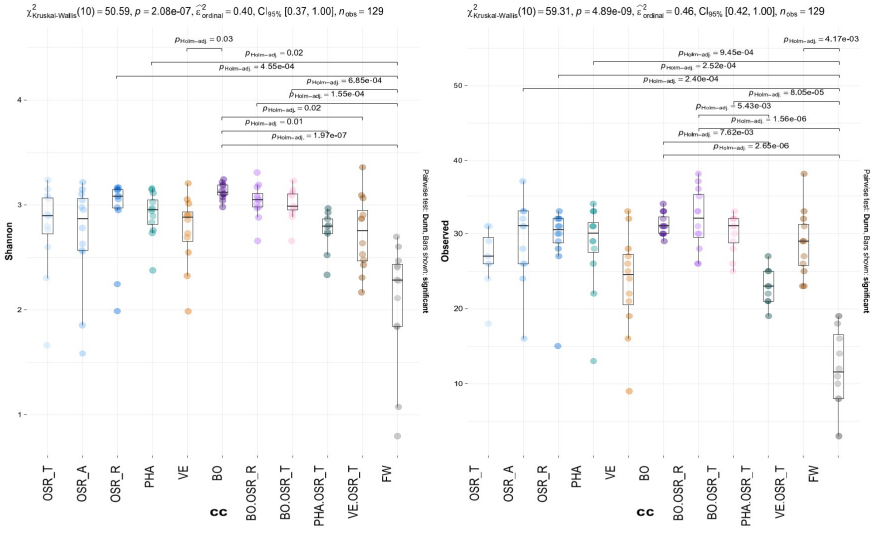
Supplementary Figure S5.3 | Diversity of putative nematode antagonists in pre-crops at T0, tested per RKN density. A and C: Principal Coordinate Analysis (PCoA) of CSS normalised ASVs assigned to bacterial and fungal putative nematode antagonists. Dissimilarity matrix built on Bray-Curtis metric and plotted separating ASVs based on RKN levels (colours). PERMANOVA indicated a non-significant effect of pre-crops. B and D: diversity (Shannon) and richness (Observed) of antagonists calculated on rarefied bacterial and fungal datasets. Differences among diversity indices per RKN level were calculated with the Kruskal-Wallis test and Holm adjustment for multiple testing.



Supplementary Figure S5.4 | Principal Coordinate Analysis (PCoA) of CSS normalised ASV assigned to putative nematode-antagonistic genera at T1. Dissimilarity matrix built on Bray-Curtis metric and plotted separating ASVs based on cover crops (colours) and monocultures or mixtures (shapes). PERMANOVA indicated a significant effect of cover crops on the DNA and RNA community of bacterial and fungal putative antagonists, and a small, but significant effect of RKN levels on the fungal fraction (Supplementary Table S5.3 B).

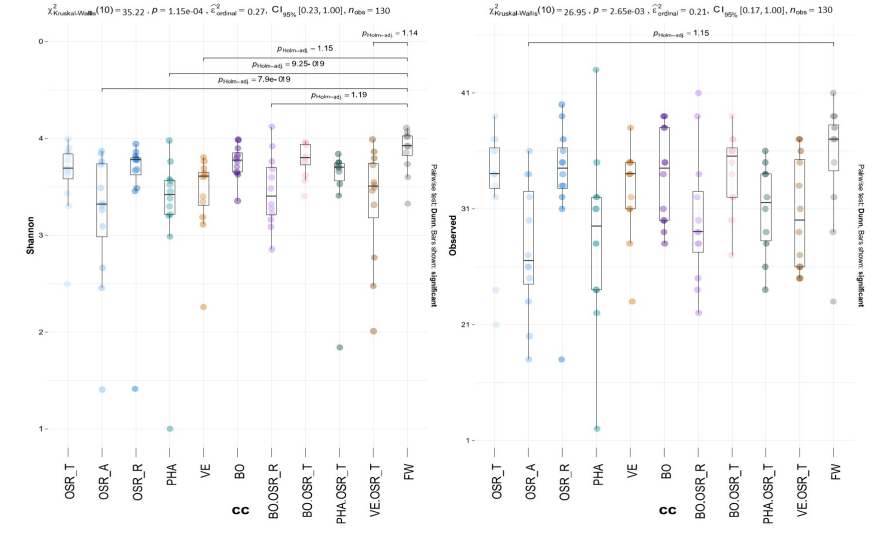
A

Bacterial antagonists - DNA

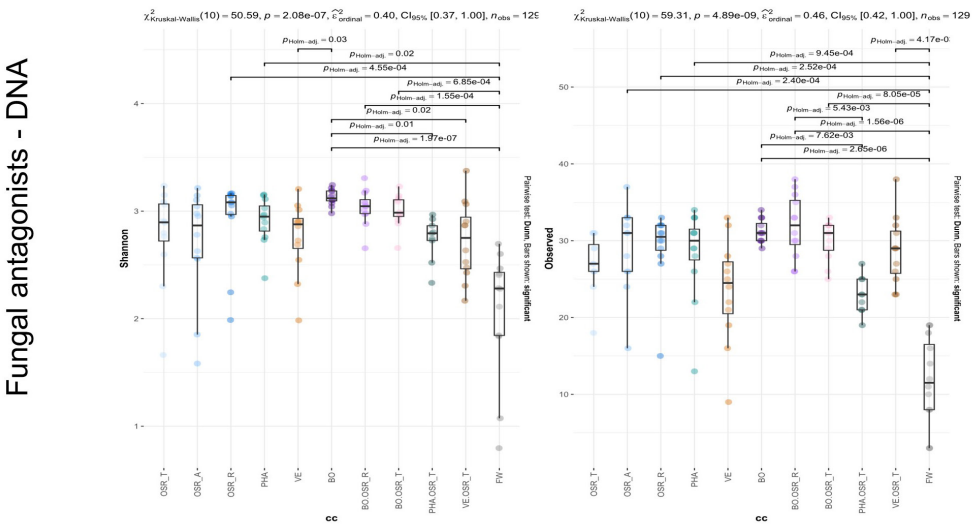


B

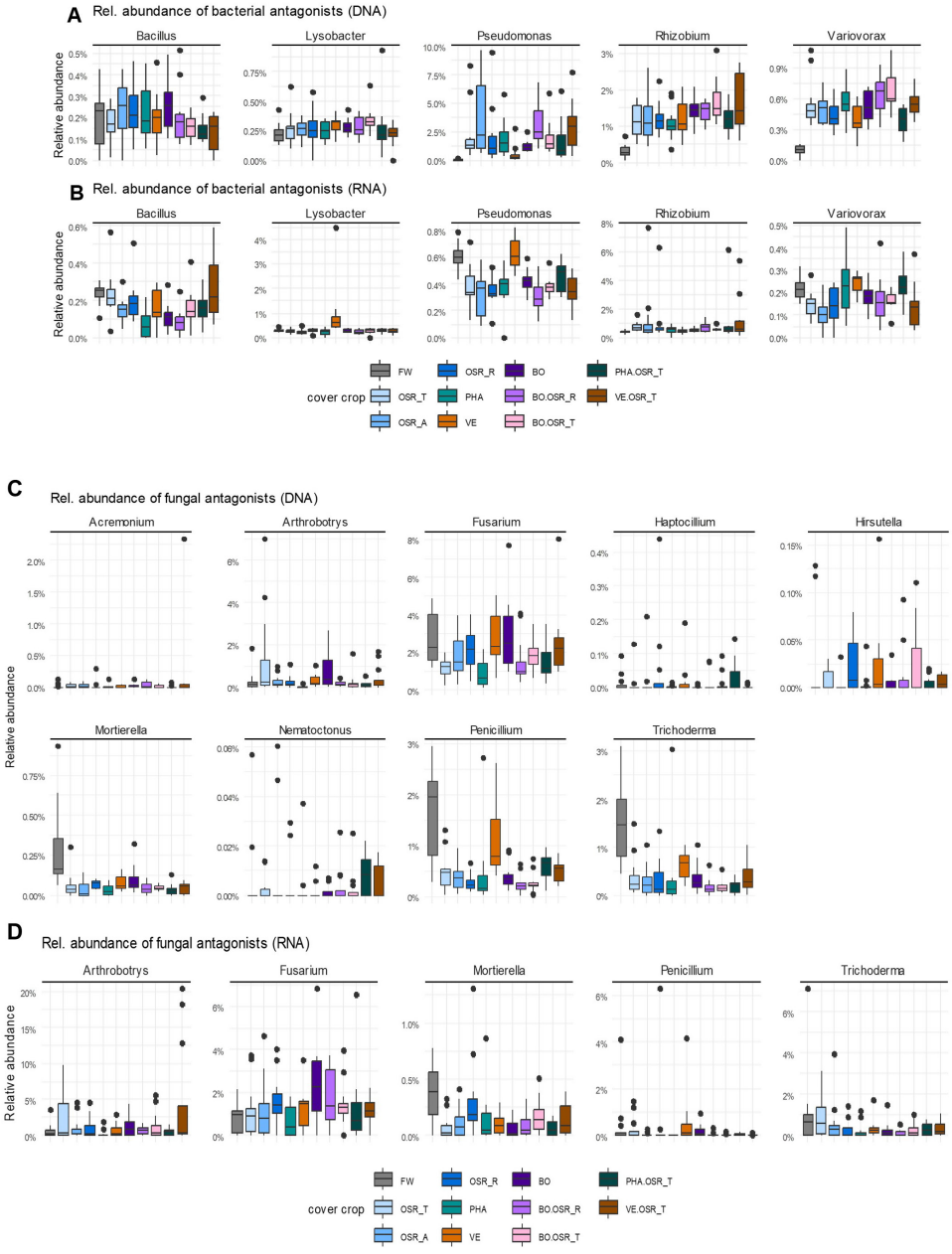
Bacterial antagonists - RNA



C



Supplementary Figure S5.5 | Diversity (Shannon) and richness (Observed) of putative nematode antagonists in the rhizosphere of cover crops and fallow at T1. A) putative bacterial antagonists at the DNA level, B) putative bacterial antagonists at the RNA level, C) putative fungal antagonists at the DNA level. No significant effect of cover crops was found on the putative fungal antagonists at the RNA level. Differences among diversity indices per cover crop treatment were calculated with the Kruskal-Wallis test and Holm adjustment for multiple testing.



Supplementary Figure S5.6 | Relative abundance of putative nematode antagonists in cover crops rhizosphere.

Chapter 6

General Discussion

Sara Giulia Cazzaniga

The preservation and promotion of healthy and resilient soils is a central objective within European soil conservation initiatives (EU soil strategy for 2030, https://environment.ec.europa.eu/topics/soil-and-land/soil-rategy_en). Soil health, as defined by Doran and Zeiss (2000), refers to the 'sustained ability of soil to function as a vital living system within ecosystem and land-use boundaries' and encompasses the maintenance of biological productivity, preservation of air and water quality, and promotion of plant, animal, and human well-being (Doran and Zeiss, 2000). This conceptual framework acknowledges the fundamental role played by soil microbial communities in delivering essential ecosystem services, including nutrient cycling, improvement of soil structure and stability, and disease suppression (Lehman et al., 2015). Being a primary source of carbon and nutrients for microbes, plants can shape microbial communities by sustaining microbial growth and activity. Consequently, crop management practices can be used to manipulate the soil microbial communities within agricultural systems. Among others, the introduction of cover crops in rotation with main crops to limit the periods of fallow represents a potential strategy for enhancing the physical, chemical, and biological properties of managed soils (Blanco-Canqui et al., 2015; Blanco-Canqui and Ruis, 2020). Although extensive research has demonstrated the overall general impact of including cover crops in crop rotations on soil life (*e.g.,* increased overall diversity, activity and abundance of soil microorganisms as summarized by Kim et al. (2020) in a metanalysis), our understanding of the contribution of cover crops on soil biology for soil health is limited. Especially, information on cover crop-species-specific interactions with the different components of the soil microbiome and the resulting outcome in agronomic settings is lacking.

This thesis addresses the existing knowledge gap by examining the species-specific impacts of a range of cover crops on key components of the soil microbial communities, including bacteria, fungi, protists, and nematodes, using DNA and RNA high-throughput sequencing techniques. Following a systematic roadmap approach (Figure 6.1), the research aimed to advance understanding of cover crops' effect on the soil microbial communities by 1) identifying cover crops-species specific footprints on the rhizosphere microbial communities (**Chapter 2**) and evaluating their persistence over time in soil (**Chapter 3**), and 2) investigating the impact of distinct cover crops on plant-parasitic nematode populations (**Chapter 4**) and their antagonists (**Chapter 5**). This research allowed for the accumulation of knowledge and provided valuable insights within the framework of soil health and potential disease control. In this final chapter, the integration and discussion of the main results from this thesis provide a foundation for guiding future investigations and advancing the understanding of the contribution of cover crops to improve certain parameters of soil health.

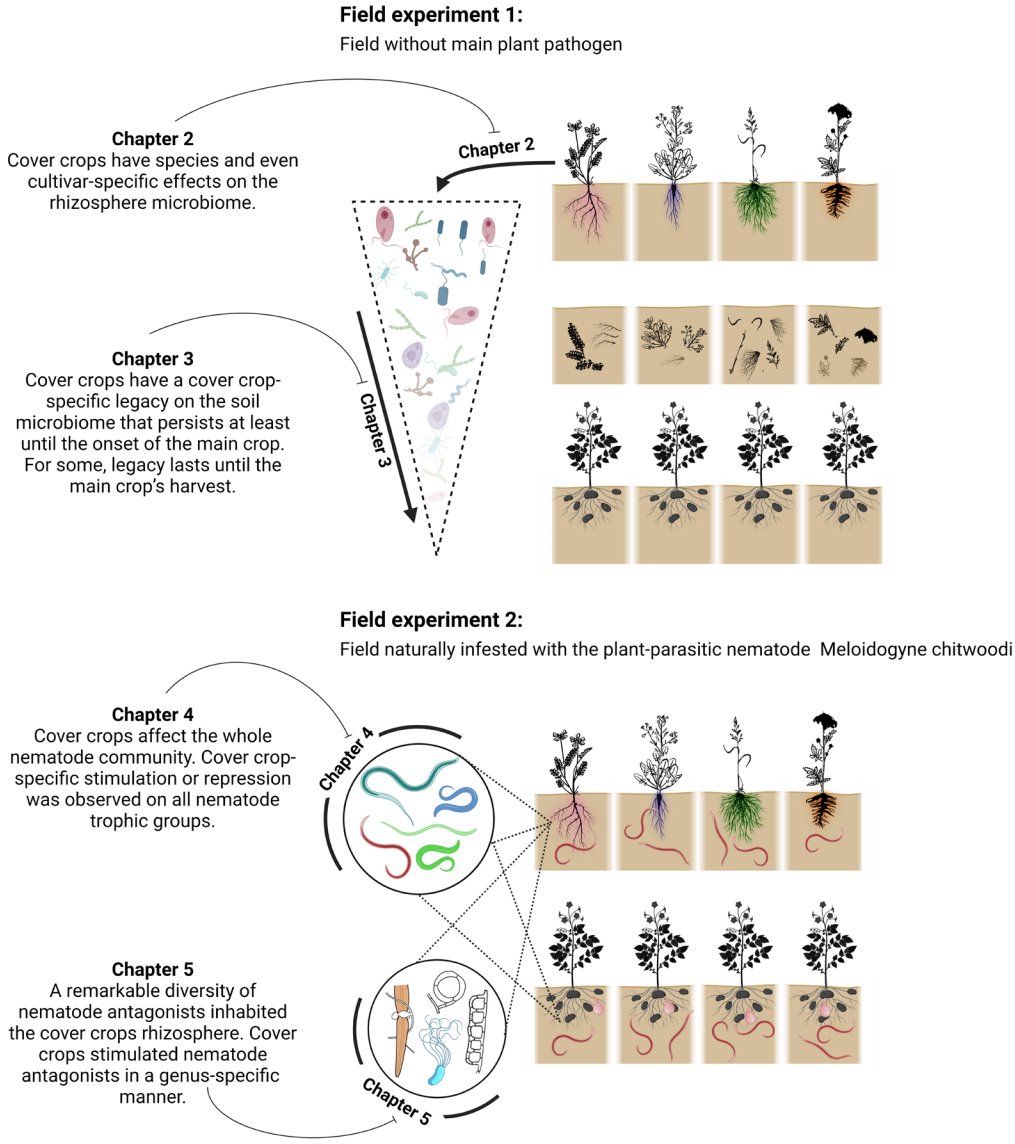


Figure 6.1 | Conceptual overview of the experimental chapters presented in this thesis and the main results. The research presented in the chapters aimed to advance understanding of cover crops' effect on the soil microbial communities by 1) identifying cover crops-species specific footprints on the rhizosphere microbial communities (**Chapter 2**) and evaluating their persistence over time in soil (**Chapter 3**), and 2) investigating the impact of distinct cover crops on plant-parasitic nematode populations (**Chapter 4**) and their antagonists (**Chapter 5**).

6.1 Identification of cover crop species-specific effects on the soil microbiome

Cover crops can manipulate the soil microbial community by increasing and differentiating the carbon and nitrogen sources available to soil microorganisms (Barel et al., 2018; Martínez-García et al., 2018). Such manipulations happen, essentially, in two different moments during cover cropping: during cover crop growth (Finney et al., 2017), due to the active and passive release of secondary metabolites from roots (Berg and Smalla, 2009), and after their incorporation into the topsoil, through litter decomposition (Liu et al., 2021a). The identification of the species-specific effects of cover crops is essential due to the unique characteristics and interactions each cover crop species may have with the soil microbial communities at different times over a cropping season (Finney et al., 2017). By deciphering these specific effects, it could become possible to tailor cover crop selection to improve soil health by promoting beneficial microbial dynamics. In practical terms, this knowledge could allow for more targeted and effective interventions in agricultural systems, leading to enhanced soil fertility, nutrient cycling, disease suppression, and overall more sustainable agricultural practices.

In **Chapter 2** and **Chapter 3**, I examined the species-specific effects of ten cover crop species (and cultivars), growing in monocultures on the resident and the potentially active fractions of the native soil microbial communities under field conditions. In **Chapter 2**, I investigated the direct effects of living cover crops by characterising the cover crops' rhizosphere microbiome. In **Chapter 3**, I investigated whether the cover crops-induced changes observed in the rhizosphere would extend beyond cover crop termination and manifest in the bulk soil before and after the growth of a cash crop (potato in this case).

Results of these chapters reveal that cover crops had unique selection strengths on the pool of native soil microorganisms and all developed rhizosphere microbiomes diverging from the microbiome of the fallow (unplanted) soil (**Chapter 2**). Community-wide effects of cover crops on the soil microbiome persisted until the onset of the main crop for most of the cover crop treatments, and even after the main crop's harvest in the case of oilseed radish cover crops (**Chapter 3**). Notably, treatments with oilseed radish displayed the most robust and persistent effects on the microbiome at all times. In the rhizosphere and bulk soil beyond the termination, oilseed radish strongly repressed a wide range of native taxa, including the most repressed bacterial family Planococcaceae, and promoted the abundance and activity of specific taxa, such as members of the Pseudomonadaceae family. Although the strong repressive effect of oilseed radish on the microbiome could be partly explained

by the release of glucosinolates and their biocidal breakdown products (Choesin and Boerner, 1991; Bressan et al., 2009) the strong stimulation of the remainder taxa, is likely to be attributed to other (unknown) species-specific effects (Pathan et al., 2020). Lentil, vetch, and marigold showed a generally positive effect on fungal diversity in the rhizosphere and stimulation of several fungal taxa, including those from the Glomerales order which lasted beyond crop termination and even up to the main crop's harvest. Borage, and to a lesser extent, phacelia, caused considerable shifts in the native microbial communities in their rhizospheres. However, only a small minority of taxa were subjected to cover crops' legacy beyond termination, which could be related to the small amount of biomass produced (Barel et al., 2018). On the other hand, black oat and hybrid ryegrass had comparatively milder impacts on the rhizosphere microbiome. Nonetheless, they exhibited a strong and lasting stimulating effect on specific bacterial families such as Oxalobacteraceae and Sphingobacteriaceae.

The combined results of **Chapters 2** and **3** indicate that cover crop-specific effects on the soil microbial communities not only occur in the rhizosphere but can also persist over time, potentially influencing the establishment and growth of the main crop. However, the cover crops' legacy can be more or less pronounced and more or less targeted (affecting a wide range of taxa vs a few selected ones) based on the cover crop species chosen. This implies that the selection of the cover crop to use in rotation with main crops is relevant in agronomic settings, and could have repercussions on the growth of the following main crop.

6.2 Identification of cover crop species-specific effects on pathogens and pathogens' antagonists

Having established that cover crops have a considerable effect on the composition and activity of soil microbial communities (in **Chapters 2**, **3**), a compelling question arises as to whether cover crops can be used to stimulate specific groups of microbes. Of particular interest was the stimulation by cover crops of the 'enemy of the enemy', *i.e.* the antagonists of plant pathogens (Rasmann et al., 2005; Friman et al., 2021).

To address this question, a second field experiment was conducted on a field specifically chosen for its infestation by the plant-parasitic nematode *Meloidogyne chitwoodi*. After altering the initial amount of the nematode density to create a high, medium and low population density, six cover crop monocultures and four mixtures thereof, were grown in the field. For this study,

M. chitwoodi served as the main pathogen and cover crops were selected knowing their host suitability towards the nematode.

Considering, however, that cover crop species can act as good hosts for certain nematode species and poor hosts for others (Wang et al., 2004), it was crucial to identify and quantify all plant-parasitic nematodes present in the field. In **Chapter 2**, an initial attempt to characterise the nematode community through the analysis of the whole Metazoa group yielded limited results. The DNA and RNA sequencing method utilised to characterise members of this organismal group (short-read sequencing with MiSeq Illumina) gave insufficient taxonomic resolution due to the limited length of the sequenced reads. Moreover, another limitation came from the limited amount of soil material the nucleic acids were extracted from (2 g of soil). Therefore, in **Chapter 4**, a novel methodology utilizing Oxford Nanopore long-read sequencing was employed to comprehensively assess the overall nematode community composition at the species level and examine the dynamics of plant-parasitic nematodes in response to different cover crop species. To overcome the limitations of the available soil material, the nematode DNA was extracted from nematodes in suspensions (previously separated from the soil matrix through elutriation). The results revealed that while *M. chitwoodi* was the most abundant pathogen in the field, there were an additional 13 species of plant-parasitic nematodes present in lower abundances. Notably, among these, the densities of three species of root-lesion nematodes (*Pratylenchus* spp.) and the free-living *Tylenchorhynchus dubius* were directly affected by cover crops in a cover crop-specific way. *Tylenchorhynchus dubius* abundance was decreased by black oat, phacelia, oilseed radish cv. R and its mixture with black oat. *P. crenatus* was also decreased by black oat, while *P. neglectus* was decreased by all oilseed radish cultivars and increased by phacelia and its mixture with oilseed radish. *P. fallax* was increased after the cover crop treatments with phacelia and its mixture with oilseed radish. Cover crop-specific stimulation or repression was also observed on trophic groups other than plant-parasitic nematodes, indicating a significant effect of cover crops on the overall nematode soil community.

Once the community of plant-parasitic nematodes was assessed, in **Chapter 5** I focused on identifying and mapping bacterial and fungal taxa with known antagonistic potential to plant-parasitic nematodes in the cover crops rhizosphere. The overall bacterial and fungal rhizosphere communities were sequenced through short-read NovaSeq Illumina sequencing. Then, to study the diversity of nematode antagonists in the field and their response to cover crops, nematode antagonists were identified among the taxa classified at a low taxonomic level (genus or species) through literature research. Results revealed that a remarkable diversity of nematode antagonists inhabited the

cover crops rhizosphere. Cover crops had a significant impact on the community structure, activity and diversity of this group of microbes as compared to the fallow. Notably, the abundance of one genus of nematode facultative parasites, *Mortierella*, and one genus of nematode obligate parasite, *Haptocillium*, were significantly influenced by cover crop treatments, irrespectively of the cover crop species, becoming more abundant in the presence of high density of the nematode. *Arthrobotrys* and *Pseudomonas*, on the other hand, were most abundantly found in the rhizosphere of two cultivars of oilseed radish, namely Adios and Terranova, respectively.

The combined results of **Chapters 4** and **5** indicate that cover crop-specific effects occur also in relation to pathogens, in this case, plant-parasitic nematodes, and to a lesser extent, on their antagonists. This finding strengthens the idea that the selection of the cover crop to use in rotation with main crops matters. It is also shown that the introduction of cover crops in the otherwise fallow periods can have an overall positive effect on the nematode antagonists in the field. However, to make informed decisions and maximize the potential benefits of cover crops, cover crop choice needs to be preceded by an investigation of potential pathogens in the field.

6.3 Cover crop mixtures and monocultures

The use of cover crop mixtures offers a promising approach to harness synergistic interactions among diverse cover crop species, capitalizing on their positive attributes for enhanced soil health parameters (Cong et al., 2014). Multispecies cover cropping might provide a compelling substitute for external agricultural inputs (Isbell et al., 2017; Drost et al., 2020; Wang et al., 2020), as it could significantly increase soil microbial abundance, richness and activity (Carrera et al., 2007; Buyer et al., 2010; Lange et al., 2015; Kim et al., 2020) and reduce the negative effects of pathogens by decreasing the abundance of the host plants (Mommer et al., 2018; Ampt et al., 2019) and increasing the diversity of pathogen antagonists (Latz et al., 2012).

In the experiment presented in **Chapter 3**, a commercial mixture of eight cover crops, including oilseed radish, was grown next to eleven cover crop monocultures, which also included oilseed radish. Analysis of the impact of cover crop mixtures and monocultures on the bulk soil microbiome after the cover crops termination showed a resemblance between the legacy effects of the mixture and those of oilseed radish monocultures, indicating a dominant influence of oilseed radish within the mixtures. Similarly, in the experiment presented in **Chapter 5** simple cover crop mixtures of two plant species were grown under field conditions next to monocultures of each species. Cover crop mixtures used in the study leveraged complementary traits, such as the deep

root structure of oilseed radish, the nitrogen-fixing abilities of Fabaceae, the substantial biomass production of grasses, and the weed suppression and pollinator support provided by phacelia. Results of **Chapter 5** revealed that within the mixtures, one particular oilseed radish cultivar, namely Terranova, played a dominant role in shaping the assembly of the rhizosphere microbiome among the two species. Conversely, mixtures containing another oilseed radish cultivar, Radical, resulted in significantly distinct rhizospheric microbial communities compared to both monocultures. These results emphasize the crucial role of cover crop composition in determining soil microbiome assembly. Notably, different mixtures may either result in additive or averaging effects, like in the case of the mixtures with oilseed radish Radical, or demonstrate a singular dominant influence, as in the case of oilseed radish Terranova.

In an additional experiment not included in this thesis (Maciá-Vicente et al. 2023, MS in preparation), cover crop mixtures comprising five and eight species did not lead to enhanced microbial diversity in the bulk soil when compared to monocultures. This suggests that the impact of cover crop mixtures on the soil microbiome can vary significantly based on the soil compartment being examined. In cases involving intricate mixtures, collecting samples from the rhizosphere is challenging due to the complex interwoven nature of root systems. While opting to sample bulk soil might seem more practical, it is important to recognize that the outcomes yielded by these two sampling methods could diverge. As a result, a comprehensive approach that takes into account both bulk and rhizosphere soil sampling is essential for a thorough understanding of the effects of cover crop mixtures on the soil microbiome. Such research will enable a more informed selection of multi-species cover crop strategies, ultimately contributing to improved agricultural practices and sustainable land management.

6.4 Evaluating the contribution of cover crops to soil health

The use of cover crops in agricultural settings is generally associated with improved soil health (Blanco-Canqui et al., 2015). Based on the concept that they can influence and steer microbial communities, cover crops can provide beneficial effects and contribute to the creation of ‘healthy’ microbiomes and crops (French et al., 2021). However, the relationship between cover crop-mediated microbiomes and the improvement of soil health is not straightforward and can be evaluated on several parameters. In this section, I discuss how the findings of the previous experimental chapters can help to evaluate the contribution of cover crops-mediated microbiomes to soil health in the specific contexts of crop productivity and sustainable disease control.

6.4.1 Cover crop-mediated microbiomes for productivity

In **Chapter 3**, cover crops were found to have a positive legacy on taxa known for their disease-suppressive and plant-growth-promoting potential. Among others, these groups are generally connected to soil health and they are a proxy for 'healthy' soils (Finney et al., 2017; Fierer et al., 2021). For instance, radish cover crops persistently stimulated the Pseudomonadaceae family, which is associated with nematode suppressiveness (Adam et al., 2014) and plant growth promotion (Kloepper et al., 1980; Lugtenberg and Kamilova, 2009; Qessaoui et al., 2019). Grasses stimulated Oxalobacteraceae, suggested to play a role in the control of fungal soil-borne pathogens (Gómez Expósito et al., 2017). Several cover crops stimulated Kaistiaceae and Cladosporiaceae families, which are suggested to be associated with the suppression of the fungal pathogen *Fusarium oxysporum* (Fujiwara et al., 2016) and plant-parasitic nematodes (Giné et al., 2016). Furthermore, oilseed radish promoted the symbiotic nitrogen-fixing bacteria, Rhizobium (Muthusamy et al., 2023), while Fabaceae cover crops, in particular, lentil (Xiao et al., 2019), stimulated the families of arbuscular mycorrhizal fungi (AMF), Glomeraceae and Claroideoglomeraceae. AMF are considered to play a vital role in enhancing nutrient uptake, protecting host plants against pathogens, and increasing host plant tolerance to environmental stresses (Bonfante and Genre, 2010; Veresoglou and Rillig, 2011; Cavagnaro et al., 2015). Although the cover crop-mediated stimulation of beneficial microbial groups lasted until the harvest of the main crop, they did not bring any significant positive effect on the main crop (potato) performance or yield in this study (**Chapters 2** and **3**). It is important to note that the experiment of **Chapters 2** and **3** was carried out on well-fertilized soil in the absence of major soil-borne pathogens, suggesting relatively optimal soil conditions for plant growth. Previous studies reported a negative relationship between nutrient (especially nitrogen) availability and the beneficial effects of cover crops on the main crop yield (Porter and Sisson, 1991; Sincik et al., 2008). Presumably, also the potential influence of beneficial microbial groups stimulated by cover crops might have been overshadowed by the presence of optimal growth conditions and absence of a pathogen.

6.4.2 Cover crop-mediated microbiomes for sustainable disease control

Chapter 5 focused on gaining a comprehensive understanding of how cover crops influence microbial groups associated with nematode antagonism, specifically in the presence of the plant-parasitic nematode *M. chitwoodi*. Results of this study indicate that cover crops influence the community of nematode antagonists and shift abundances of fungi that can parasitise

nematodes, but not necessarily in a species-specific way. Facultative nematode-parasitic fungi can offer a natural and environmentally friendly approach to managing plant-parasitic nematode populations (Bell et al., 2016; Jiang et al., 2017a). Therefore, being able to stimulate and manipulate the abundance of naturally occurring antagonists could prove the contribution of cover crops to enhancing soil health in relation to disease control. The direct impact of the cover crop-mediated microbiome on disease control could not be established in this study, as no apparent relationship was identified between the abundance of nematode antagonists in the rhizosphere of cover crops and *M. chitwoodi* densities. In some cases, however, nematode antagonists responded to high levels of *M. chitwoodi* only in the presence of cover crops, indicating that cover crops can influence the relationship between nematode-trapping fungi and nematodes to a certain extent (as observed for organic amendments by Jaffee, 2004).

As nematode-parasitic fungi do not necessarily feed on plant-parasitic nematodes, but on nematodes in general, further analyses should be conducted to reveal if a more direct correlation exists between nematode antagonists and the total amount of nematodes (Oka, 2010) or any other trophic group identified in **Chapter 4**. It is also important to note that nematode-parasitic fungi primarily exhibit saprophytic lifestyles and are not uniquely dependent on nematodes as a food source, especially in nitrogen-rich environments (Oka, 2010; Niu and Zhang, 2011). Hence, simply identifying the effects of cover crops on nematode antagonists' abundance might be insufficient for drawing definite conclusions about the contribution of cover crops to soil health for disease control. A better approach would be to determine which and if cover crops can promote the predatory behaviour of nematode antagonists and in which conditions.

The study reported in **Chapter 5** shows that using cover crops to stimulate the communities of antagonists can constitute a potential approach for nematode control. Further studies, though, would need to unravel the relationship between the predatory behaviour of the antagonists, cover crop identity and soil nutrient availability to maximise the potential of this approach.

6.4.3 Cover crop effect on predatory nematodes for sustainable disease control

The study in **Chapter 4** provided a complete overview of the nematode community in the experimental field. All trophic groups of nematodes were represented, including one of predatory nematodes which feed on small soil organisms, including nematodes (Devi and George, 2018). Although some members of this group delivered promising results in reducing populations of

plant-parasitic nematodes (Rasmann et al., 2005; Caccia et al., 2013), the potential of this group for disease control is highly understudied (Li et al., 2023a). Among others, members of the genus *Mononchoides*, found in the experimental field, have been identified as potential biological control agents against plant-parasitic nematodes and in particular endoparasitic pests (Khan and Kim, 2007). In **Chapter 4**, several cover crops were found to indirectly influence the non-plant-parasitic nematodes via, hypothetically, changes in the microbiome composition. Interestingly, however, no significant effect of cover crops on the abundance of predatory nematodes, including *Mononchoides* members was observed. This may indicate that the use of cover crops to influence the nematode predatory community is not a viable option. However, previous studies reported that plants can recruit predatory nematodes, for example, in response to the attack by herbivores (Rasmann et al., 2005; Ali et al., 2012; Li et al., 2015) in a mechanism known as 'cry for help' (Dicke and Baldwin, 2010). It is therefore plausible that cover crops, especially good hosts for *M. chitwoodi*, could have had an effect on the group of predatory nematodes, but this was not measurable in the study. It is important to note that the predatory activity of nematodes such as *Mononchoides* is typically highest in the plant rhizosphere, where the nematode densities are higher (Jiang et al., 2017b; Li et al., 2023b), whereas the study in **Chapter 4** focused on bulk soil samples at the termination of the cover crop growth.

6.5 Challenges of using the soil microbiome as a soil health indicator

Cover crops offer a valuable tool to stimulate soil health through manipulations of the members of the soil microbiome. However, their contribution to soil health is difficult to assess. This is mainly because the microbiome *per se* is a difficult parameter to be used as a soil health indicator (Fierer et al., 2021). In the absence of a stressor, the identification of an enhanced microbiome relies on general assumptions that the presence of certain taxa has favourable effects (Shade, 2017) on soil health. This, however, might not be coupled with concrete, measurable, outcomes (see **Chapter 3**). When a stressor is present, more targeted analyses can be conducted on the fraction of the microbiome that most likely responds to the stressor of interest (Hammarlund and Harcombe, 2019). As presented in **Chapter 5**, in the case of plant-parasitic nematodes as biotic stressors, one could study the effect of cover crops on the community of nematode antagonists. However, to implement this approach for a wide range of stressors, several things need to co-occur. First of all, one has to be aware of all stressors affecting the field (abiotic or biotic), which may require a significant investment in time and resources. Second, knowledge of the problem should be coupled with knowledge of the microbial taxa that could

offer a solution. Although the contribution of certain microbial species to various aspects of soil health is known or is being investigated (as an example, in their review Banerjee and Van Der Heijden (2023) provide a list of soil microbes and their tested contribution to several soil health parameters) the understanding of the overall impact of the soil microbiome on soil health remains limited. It is also important to acknowledge that most of the studies are primarily centred around bacteria and fungi, leaving a notable gap in understanding the contributions of other organismal groups that constitute the soil microbiome. Soil protists (Xiong et al., 2020), nematodes, viruses, and archaea are key components of the soil ecosystem (Fierer et al., 2007), yet their roles and functions in relation to different aspects of soil health remain largely understudied.

To advance soil health management, it is essential to first identify members of the microbiome related to particular soil conditions or processes, and only then, try to manipulate them through agricultural management.

6.6 Methodological insights for microbiome analysis

High-throughput sequencing has emerged as the primary tool for studying microbiomes, mainly due to its growing affordability and continuous improvements in sequencing speed and data quality (Di Bella et al., 2013; Liu et al., 2021b). Among the most widely used sequencing approaches is metabarcoding (or amplicon sequencing), which involves the sequencing of variable regions of ribosomal marker genes previously amplified by PCR (amplicons). This method, used throughout this study, enables the identification of members within soil microbial communities and their dynamics in response to various environmental conditions, all in a time- and cost-efficient manner (Kothari et al., 2017).

In this section, I discuss the methodological insights I gained while conducting microbiome studies presented in this thesis and I attempt to highlight advantages, pitfalls and the direction in which microbiome studies should progress.

6.6.1 Integrating DNA- and RNA-based analyses enables a better data interpretation

Previous works described that a significant portion of cells (approximately 80%) and microbial taxa (about 60%) in bulk soil are dormant (Lennon and Jones, 2011), which implies that they are not actively participating in plant-soil-microbiome interactions (Bastida et al., 2008). Additionally, relic DNA (Carini et al., 2017) and necromass (Buckeridge et al., 2022) can persist in

soils for extended periods, ranging from weeks to years, which can influence the reliability and interpretation of genetic information derived from soil samples. In the context of my research, it was observed that cover crops generally had a more pronounced effect on the active rather than the resident soil microbiome (**Chapter 2**). This suggests that RNA-based analyses might provide a better reflection of the impact of environmental influences on the assembly of the microbiome community (Bay et al., 2021). In both **Chapters 2** and **3**, the impacts of various cover crops on DNA and RNA communities revealed similar overall patterns, but changes observed in the DNA-based microbial composition did not always correspond to similar changes at the RNA level. These findings provided valuable insights and allowed to put results into perspective. For instance, the notable stimulation of the potentially beneficial Pseudomonadaceae family by oilseed radish at both DNA and RNA levels justified further interest in this taxon. Conversely, the stimulation of the pathogenic fungal family Olpidiaceae by the same cover crop was observed only at the DNA level and not accompanied by activation at the RNA level. This suggested that it may not be an immediate cause for concern as the pathogen was presumably inactive. However, the presence of survival structures detected at the DNA level might raise concerns when considering the long-term legacy of oilseed radish. These results exemplify the importance of mapping the RNA community in relation to cover crops, and more in general to environmental changes (Ofek et al., 2014).

6.6.2 Increased taxonomic resolution with longer sequencing reads

Currently, the most used metabarcoding method uses short-read sequencing of 16S, 18S and ITS variable regions, resulting in theoretical read lengths of up to 600 bp for paired-end methods. While this approach is effective in classifying reads at the phylum level and, to a lesser extent, at the family and genus levels, it lacks accuracy when identifying species (Ranjan et al., 2016). This constitutes a limitation in microbiome studies, particularly considering that different species and strains within the same genus often serve distinct ecological functions (Spiers et al., 2000; Benitez-Malvido et al., 2021) (see *e.g.* **Chapter 5**). It was demonstrated that targeting the entire 16S gene (~1,500 bp) with long-read sequencing can achieve much better taxonomic resolution even reaching strain level (Johnson et al., 2019). The use of long-read sequencing is also particularly relevant for the 18S gene used to identify eukaryotes (~1,700 bp). In our study, the latest Oxford Nanopore platform with mini flow cells (MinIon™) was employed in **Chapter 4** to study the soil nematode community through full-length 18S sequencing. This approach provided a comprehensive overview of all trophic groups present in the

experimental field and enabled the characterization of nearly all nematodes at the genus or species level. Furthermore, comparison between microscopic counts and DNA reads of the main pathogen in the field, *M. chitwoodi*, revealed highly similar quantitative contrasts, indicating the value of this methodology for semi-quantitative studies. The recently improved accuracy of Oxford Nanopore sequencing (https://nanoporetech.com/accuracy) has made it a viable option for studying complex microbial communities and a useful tool that combines easy use and portability of MinIon™ with improved taxonomic resolution (De Corato, 2020). However, more runs of Oxford Nanopore implemented on MinIon™ will be needed to reach a read coverage similar to the one offered by Illumina platforms. For instance, in **Chapter 5**, approximately 400 samples featuring bacterial and fungal amplicons were sequenced in a single Illumina NovaSeq run, while **Chapter 4** entailed sequencing 40 nematode amplicon samples per MinIon™ run. The partitioning of samples across multiple sequencing runs may potentially result in biases between these runs. Nonetheless, this issue can be readily addressed and accounted for through the appropriate randomization of the samples and bioinformatic tools.

6.6.3 Beyond taxonomy, toward functionality

Amplicon sequencing offers several significant advantages: 1) cost-effectiveness, 2) data analysis using established pipelines, and 3) the availability of a substantial body of archived reference data (Di Bella et al., 2013; Ranjan et al., 2016). However, this method also has limitations, such as its dependence on PCR amplification and primer selectivity, which can introduce biases in the results (Forney et al., 2004). Furthermore, amplicon sequencing typically focuses on specific marker genes, limiting the breadth of information obtained.

Opposed to metabarcoding, metagenomic sequencing, or whole genome shotgun (WGS), is independent of PCR amplification and primer selectivity, making microbial surveys less biased (De Corato, 2020). WGS involves the random (shotgun) sequencing of broad regions of the entire genomic content of microbial communities, enabling the classification of many reads at the species level. Moreover, WGS can simultaneously identify organisms from different kingdoms and groups, providing a holistic view of the various co-occurring organisms in the soil without being constrained by the use of different primers (Cruz-Silva et al., 2023).

One of the most crucial advantages of WGS is the ability to identify genes related to functions within the microbiome. This enables to not only identify the presence of different microbial species but also to explore the functional

capabilities of these microorganisms. Given the phenomenon of horizontal gene transfer (Maheshwari et al., 2017), the high genetic variability and plasticity in microbial populations (Roper et al., 2011), and the existence of numerous microbial species and strains with diverse and shifting ecological roles, relying solely on the taxonomic classification of microbiome members often cannot provide a complete overview of the potential of microbiomes for soil health. The ability to identify functional genes within the microbiome through WGS is crucial for gaining a more comprehensive understanding of the microbial community's role in ecosystem functioning and soil health. This method, however, also comes with important limitations, which are generally related to the costs of the high-coverage sequencing, the computational power and the bioinformatic training needed to perform data analyses (Chen and Pachter, 2005; Di Bella et al., 2013). Furthermore, as discussed in 6.6.1, when not coupled with RNA analyses (in this case metatranscriptomics), WGS can only provide an overview of the functional potential of the microbiome, rather than a description of the functions actively carried out by the microbiome. Therefore, the combination of multiple 'omics approaches, validated, when possible, by conventional methods, will be more powerful than any single approach (Mauchline et al., 2018).

6.7 Implications for the applications of cover crops on the soil microbiome

The research conducted in this thesis advanced our comprehension of the effect of cover crops on soil life and provided an initial demonstration of cover crops' ability to manipulate different organismal groups within the soil microbiome. In **Chapter 2**, cover crop-specific effects on the soil microbiome were identified at the level of the rhizosphere, while in **Chapter 3** cover crop-specific legacies were observed on several microbial taxa after the main crop had been harvested, suggesting that effects of cover crops on the microbiome last longer than they grow in the field (Cazzaniga et al., 2023b). The combined results of **Chapters 2** and **3** thus lay the foundation for the recognition of long-lasting cover crop-specific effects on the soil microbiome. A noteworthy outcome of the experiments described in these chapters is the robust and persistent stimulation of fungal community members, including arbuscular mycorrhizal fungi (AMF), by Fabaceae cover crops such as lentil and vetch. These findings suggest that incorporating these cover crops into rotations can facilitate the establishment of AMF in subsequent crops (Higo et al., 2019). Additionally, oilseed radish cultivars were found to perform a strong selection on their rhizosphere microbes, which involved strong repression of several microorganisms and the pronounced stimulation of a minority, notably the beneficial *Pseudomonas* spp. (Cazzaniga et al., 2023a). Grasses tested in this

thesis generally performed a mild selection on the microbes in their rhizosphere, but significant variation among species was observed which suggests the need for broader research on species-specific microbial signatures of grasses. In conclusion, although cover crops are grouped under an umbrella term, it is crucial to recognise that the species of cover crop to be used in the field matters for steering the soil microbial community and will likely influence the establishment and growth of the next cash crop.

This thesis gave initial evidence that the species and cultivars composing cover crop mixtures determine the overall impact of such mixtures on the soil microbiome. As shown in **Chapters 3** and **5**, one cultivar of oilseed radish emerged as the dominant driver of the soil rhizosphere microbiome assembly among the species composing the mixtures. In **Chapter 5**, a mixture including another cultivar of oilseed radish showed 'averaging' effects on the rhizosphere microbiome assembly, resulting in significantly distinct rhizospheric communities compared to both monocultures. These results indicate that cover crop mixtures may have a variety of effects on the soil microbiome based on the composing species and cultivars. More informed use of cover crop mixtures can enable the exploitation of their specific effects to achieve desired microbiome steering results depending on starting field conditions.

Given that some species of cover crops can be hosts for pathogens, their uninformed use could result in substantial pathogen build-up, which in turn could result in serious damage to the following cash crop. To effectively avoid pathogens propagation by cover crops, a prior assessment of their presence in the field must be undertaken. Molecular methods that allow for high taxonomic resolution of the microbial community, including nematodes (as proposed in **Chapter 4**), can enable fast, cost-effective and reliable field surveys, enabling to map potential threats. Once the pathogen threats are pinpointed and quantified, an informed decision on the cover crop monoculture or mixture to use in the field can be made based on their host status toward the pathogen.

In conjunction with plant pathogens, sequencing-based field surveys can also assess the presence of pathogen antagonists in the field, thereby evaluating the potential for pathogen control (Rao et al., 2012). **Chapter 5** delineates the ability of cover crops to stimulate and modulate the abundance of nematode antagonists. Notably, oilseed radish was found to have the most positive effect on the antagonists' community, in a cultivar-dependent manner. The observation that different cultivars of the same oilseed radish had different effects on the microbiome and pathogen antagonists suggests that breeding for microbiomes (Wei and Jousset, 2017) is possible. A generally high level of genetic diversity exists in the cover crops' germplasm (Tani et al., 2017), which offers a possibility to breed for 'enhanced' cover crop-associated microbiomes.

Such advancements in cover crop breeding would strengthen the cover crop-mediated effects on the soil microbiome for soil health.

To be able to exploit the full potential of cover crops, other agricultural practices have to be adjusted as well (Chaparro et al., 2012; Hallama et al., 2019). By reducing fertilization rates, it may be possible to strengthen plant-microbiome relationships and enhance the benefits provided by cover crops-mediated microbiomes (Lehman et al., 2015; Hammarlund and Harcombe, 2019; Huang et al., 2019). In the same way, reducing tillage (Lehman et al., 2015; Wittwer et al., 2017; Schmidt et al., 2019) and pesticide use (Walder et al., 2022) can also complement the benefits of cover crops and their associated microbiomes. A general trend in agriculture is that of decreasing agricultural input, aiming to accelerate the transition to a sustainable food system, minimising biodiversity loss and agricultural inputs ("Farm to Fork Strategy", food.ec.europa.eu/horizontal-topics/farm-fork-strategy_en). Within this framework, growing cover crops in suboptimal soil conditions can present an opportunity to exploit cover crops to their full potential.

6.8 Conclusions

This thesis demonstrates that cover crops induce significant shifts in the soil microbial community (**Chapter 2**) that can last, depending on the cover crop species, until the onset or the harvest of the main crop (**Chapter 3**). Cover crops can potentially impact the main crop growth and productivity by promoting beneficial microbial groups involved in plant growth and disease suppression, but also potentially influencing the relationships between pathogens (**Chapter 4**) and the endogenous pathogens' antagonists (**Chapter 5**). I could, therefore, conclude that cover crops hold the potential to influence the soil microbiome in agricultural settings, which can lead to enhanced soil health. However, the specific outcomes will likely vary based on the chosen cover crop species and the soil health parameters under consideration. Moreover, other soil management parameters, arguably most importantly fertilisation rates, will define the contribution of cover crops-mediated microbiomes to soil health. To comprehensively evaluate the effects of cover crop-mediated microbiomes on soil health, further research is needed, which uses sequencing methodologies that go beyond the taxonomic identification of microbial taxa and focus on their functionalities. To obtain more tangible results, studies should consider the impacts on soil health in relation to various abiotic and biotic stressors, as well as the contingent effects of other management practices such as fertilisation, tillage, and pesticide use in conjunction with cover cropping. Such studies will help identify specific mechanisms by which cover crops influence the microbiome to achieve desired soil health outcomes. When properly integrated into existing cove crop support

tools, such as the nematode and pathogen Best4Soil databases (https://www.best4soil.eu/database), this information will enable farmers and agricultural practitioners to make informed decisions, maximizing the potential benefits of cover cropping for crop productivity, soil health, and overall sustainability in agriculture.

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Summary

Soils play a crucial role in supporting life on Earth, as they deliver nutrients, store water and carbon, harbour an immense biodiversity. Soils are home to an array of microorganisms that contribute to these essential ecosystem functions. However, the intensive agricultural practices employed to meet the increasing global food demand have led to significant soil degradation. As an estimate 60-70% of soils in Europe are considered degraded. Excessive use of chemical fertilizers, pesticides, and monoculture farming has disrupted the delicate balance of soil ecosystems, leading to soil erosion, decreased fertility and loss of biodiversity. Therefore, it is imperative to couple the need for agricultural production with the need of preserving, supporting and optimising soil functions and, intrinsically, soil health. Soil health management involves and envisions practices which – amongst others - enhance the soils biological functioning through the fostering of the soil microbiome. One way to achieve this is the addition of cover crops into rotation schemes of main crops to limit the periods of fallow. Although extensive research has demonstrated the overall general impact of including cover crops in crop rotations on soil life (e.g. increased overall diversity, activity and abundance), our understanding of the contribution of cover crops on specific groups within the microbiome is limited. This thesis contributes to deciphering the effects of cover crops on different groups of soil microorganisms, including bacteria, fungi, protists and nematodes under field conditions. The aim is to evaluate the contribution of cover crops to effective and sustainable microbiome engineering.

Chapter 1 provides the general background of important concepts such as soil health, soil life, soil microbiome and cover crops; introduces the two main field experiments, and provides the outline of the thesis.

Chapter 2 investigates how different cover crop species affect the rhizosphere microbiome. DNA- and RNA-based Illumina sequencing methods were used to compare the bacterial, fungal, protists and metazoan communities in the rhizosphere of ten cover crops belonging to five plant families to the fallow control. Results showed that all cover crop species significantly impacted the resident (i.e. DNA) and the potentially active (i.e. RNA) microbial communities in their rhizospheres by exerting distinct selection strengths on the native microbial communities. Oilseed radish (Brassicaceae family) was shown to provoke the strongest microbial shifts, in part attributable to the significant promotion of the bacterial family Pseudomonadaceae and the repression of Microascaceae in the rhizosphere. Lentil (Fabaceae family) induced a widespread stimulation of fungal taxa, including Trichocomaceae and fungal members of the Glomerales order, whereas black oat and hybrid ryegrass (both Poaceae family) gave rise to relatively mild changes in the soil microbial communities compared to fallow. Analyses of rRNA-based rhizobiome data revealed that, except for phacelia, all cover crops induced an increase in microbial network complexity as compared to the fallow control. The results of this chapter suggested that different cover crops can steer

the rhizosphere microbiome in a predictable direction to promote soil health and sustain healthy crop growth.

Chapter 3 examines whether cover crop-induced changes in the soil rhizosphere persist after cover crop termination. We sampled additional samples in the same experiment as **Chapter 2**. Bacterial, fungal and protist communities of the bulk soil were sampled before planting and after harvesting the main crop (potato), and analysed using DNA- and RNA-based Illumina sequencing. Data showed that each of the ten cover crop treatments resulted in significant microbial changes that lasted at least until the planting of the main crop. As in **Chapter 2**, also later in the growing season, the two oilseed radish cultivars had the most persistent effect on the microbial community as shifts were even observable after the harvesting of the main crop (potato). Furthermore, each cover crop treatment resulted in qualitatively distinct microbial footprints. Most notably, oilseed radish treatments significantly and consistently boosted the presence and activity of members of the family Pseudomonadaceae, known for plant-growth-promoting and disease-suppressive potential. Other crop treatments had a significant effect on the abundance and activity of other microbial taxa related to enhanced soil health, including the mycorrhizal groups Glomerales and Claroideoglomeraceae by lentil and vetch, and Oxalobacteraceae by black oat. Analyses of the soil communities at the DNA and RNA levels showed similar trends. However, for several taxa, the promotion or repression at the DNA level was not accompanied by similar changes at the RNA level and *vice versa*. This finding exposes the importance of combining DNA and RNA analyses to gather a comprehensive view of the community shifts in relation to environmental and/or experimental factors. Our study demonstrates that cover crops can be used to manipulate the soil microbiome and promote beneficial microbes in a cover crop-specific manner. A significant part of these changes can persist in the topsoil until at least the onset of the main growing season. This, although not proven in this study, has the potential to influence the establishment and productivity of the following crop.

Chapter 4 studies the effect of cover crops on the nematode community, and in particular on the group of plant-parasitic nematodes. Six cover crop monocultures and four mixtures thereof were grown in a field infested with the plant-parasitic nematode *Meloidogyne chitwoodi.* As both microscopy- and short-read DNA-based methods are unable to provide accurate, up-scalable and affordable nematode community analyses, a novel sequencing methodology, based on Oxford Nanopore sequencing of relatively long DNA amplicons (≈ 1,700 bp) was used in this chapter to identify nematodes at a low taxonomic level and in a semi-quantitative manner. This sequencing technique provided a complete overview of all nematode trophic groups (*i.e.* bacterivorous, fungivorous, omnivorous, predators and plant-parasitic) and allowed to track their dynamics in response to cover crops at genus and species level. The comparison between microscopic counts and DNA-based counts (in terms of sequencing reads) of the main field pathogen, *M. chitwoodi*, revealed highly similar quantitative contrasts, proving the validity of this method in quantifying

plant-parasitic nematodes. While *M. chitwoodi* was the most abundant plant-parasitic nematode in the field, this methodology allowed to identify an additional 13 species of plant-parasitic nematodes present in lower abundances. Notably, among these, the abundance of four species of root-lesion nematodes (*Pratylenchus* spp.) and the free-living *Tylenchorhynchus dubius* were directly influenced by cover crops. Also other nematode trophic groups, especially omnivorous, fungivorous and bacterivorous nematodes, were influenced by cover crops, indicating a possible indirect effect of cover crops through manipulations of the microbiome. **Chapter 4** presents a workflow that could promote 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 highly abundant and speciose soil inhabitants such as bacteria, fungi and protists.

Chapter 5 explores the ability of cover crops to stimulate the native antagonistic potential of soils. Using the same experimental set-up described in **Chapter 4**, this chapter studies the effect of cover crop identity in combination with different densities of the plant-parasitic nematode, *M. chitwoodi*, on the bacterial and fungal communities in the soil using DNA- and RNA-based Illumina sequencing. Among the taxa which received a taxonomic classification, five bacterial and 15 fungal genera of putative nematode antagonists were identified in the rhizosphere of cover crops. These included genera of obligate nematode parasites (*Haptocillium* and *Hirsutella*) and genera of facultative nematode parasites (or nematode-trapping fungi), such as *Arthrobotrys, Nematoctonus* and *Monacrosporium*. More generalist fungal and bacterial genera that harbour species known to control nematodes (*e.g. Penicillium*, *Mortierella*, *Acremonium*, *Pseudomonas*, *Variovorax*) were also identified and included in the study. Cover crops generally had a stronger impact than the different *M. chitwoodi* densities on the soil microbial communities and the putative nematode antagonists. Cover crops also induced shifts in the abundance and activity of some nematode antagonists in a genus-specific way. Interestingly, some putative nematode antagonists responded to higher densities of the plant-parasitic nematode only when cover crops were introduced, irrespectively of the cover crop species. This study gives a first indication that cover crops have the potential to be used as tools to increase suppressiveness against nematodes in nematode-infested agricultural fields. Moreover, it proves that changes in the community of nematode antagonists can be mapped with affordable high-throughput sequencing methods, providing compelling evidence for considering microbiome composition as a promising indicator of soil health for pathogen control.

Chapter 6 summarises and incorporates results obtained from the previous chapters to discuss the contribution of cover crops to different parameters of soil health, including crop productivity and disease control. Considerations on the use of soil microbiome as an indicator to evaluate soil health and reflections on the technical aspects of microbiome analyses are also presented.

This thesis demonstrates that cover crops induce significant shifts in the soil microbial community (**Chapter 2**) that can last at least until the onset or the harvest of the main crop (**Chapter 3**). Cover crops can potentially impact the main crop growth and productivity by promoting beneficial microbial groups involved in plant growth and disease suppression, but also potentially influencing the relationships between pathogens (**Chapter 4**) and the native pathogens' antagonists (**Chapter 5**). I could, therefore, conclude that cover crops hold the potential to specifically influence some groups of the soil microbiome in agricultural settings, which can lead to enhanced soil health. Because of the intrinsic complexity of the soil environment and the interplay between microorganisms and soil physicochemical properties as well as crop management practices, further research is needed to comprehensively evaluate the effects of cover crop-mediated microbiomes on soil health. To obtain tangible results, studies should consider the impacts on the soil microbiome in relation to various abiotic and biotic stressors, as well as the contingent effects of other management practices such as fertilisation, tillage, and pesticide use in conjunction with cover cropping. Yet, this thesis advances our understanding of the effect of cover crops on the soil microbiome providing a baseline for future cover crop and soil microbiome studies.

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Now, it's time to give a special shout-out to my two outstanding paranymphs. You both have been there, supporting and encouraging me (not to mention the friendly 'bitching' sessions) nearly every day of my PhD journey, and I'm grateful to have crossed paths with you and to have had the opportunity to spend numerous occasions together. Manouk, we immediately connected when I started at Nema. I immediately loved your warmth, friendliness and kindness. As for Alex, you're not only a great friend and a trustworthy person, but you also have that unique cabrón edge that makes you the person I like so much. It's truly an honour to have you by my side as I defend my PhD <3

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> *"…Ti vuole bene chi conosce i tuoi punti deboli, ma non li tocca, chi ti dà voce quando non l'hai in bocca, chi quando affondi a prua, ti bilancia stando in poppa e ti tiene su; senza sentirsi simili ai tipici tipi di complicità ma (a)tipici e riderci su ci porta i problemi fin laggiù…"*
>
> Olly, Menomale che c'è il mare

About the author

Sara Giulia Cazzaniga was born in Milano, Italy, in 1992. She discovered her love for the world of biology, and that of microbes specifically, at the beginning of her BSc studies in Industrial and Environmental Biotechnology at the University of Milan. Having experienced the power of plant genetic engineering hands-on during an Erasmus traineeship at the University of Valencia, she enrolled in the Plant Biotechnology MSc programme at Wageningen University with the goal of better understanding and strengthening plant-microbe interactions to help solving current real-World problems.

During her MSc thesis, she investigated the role of plant-microbe interactions on plant performance and heavy metal accumulation in heavy metal-hyperaccumulating plants, with the intention to advance soil bioremediation practices. In the MSc internship, she deepened her knowledge of plant-microbe interactions by working on microbial amendments for improved resilience to drought in a shared project between the Ecology and Biodiversity group of Utrecht University, and the leading corporate group in the substrate industry, Klasmann-Deilmann GmbH.

Following her graduation *cum laude* from the MSc programme, she began a PhD project at Wageningen University. Working between the Laboratory of Nematology, Plant Ecology and Nature Conservation Group, and Biointeractions and Plant Health Business Unit, she explored the potential use of cover crops as natural tools to stimulate and steer the microbial communities of agricultural soils, with a particular focus on their use in controlling soil-borne diseases. The thesis she published is the result of extensive microbiome studies, long reflections on the uncertainties embedded within the field of microbial ecology, support from beloved friends, and long walks with her gorgeous dog Pepa.

> *"Ignorance is bliss" - but, that the bliss be real, the ignorance must be so profound as not to suspect itself ignorant*
>
> Edgar A. Poe, Fifty Suggestions

PE&RC Training and Education Statement

With the training and education activities listed below, the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC), which comprises a minimum total of 32 ECTS (= 22 weeks of activities).

Review/project proposal (4.5 ECTS)
- The use of cover crops as drivers to steer the soil microbiota

Post-graduate courses (8.3 ECTS)
- International PhD and postdoc one health summer school microbiomes in soil, plant, animal and human health; One Health, Bern University (2019)
- Multivariate analysis; PE&RC (2019)
- Tidy data transformation and visualization with R; PE&RC (2020)
- Generalised linear models; PE&RC (2021)
- Linear models; PE&RC (2021)
- Mixed models; PE&RC (2021)
- Microbial ecology; PE&RC (2022)

Competence strengthening/skills courses (4.1 ECTS)
- Introduction to latex; WGS (2019)
- Linux basic course; FB-IT and SRF (2020)
- Present your research with impact; RZSB Royal Belgian Zoological Society (2020)
- How to remain sane during your PhD; RZSB Royal Belgian Zoological Society (2020)
- School on the analysis of microbial time series data; KU Leuven (2021)
- Scientific publishing; WGS (2022)
- Research data management; WGS (2022)
- Career orientation; WGS (2022)
- Writing propositions for your PhD; WGS (2023)
- Last stretch of the PhD programme; WGS (2023)

Scientific integrity/ethics in science activities (0.3 ECTS)
- Ethics in plant and environmental sciences; WGS (2019)

PE&RC Annual meetings, seminars and the PE&RC weekend (0.9 ECTS)
- PE&RC Day (2019, 2020, 2021)

Discussion groups/local seminars or scientific meetings (10.2 ECTS)
- EPS Annual meeting (2019)

- Plant-soil feedbacks symposium (2019)
- 7th Plant genomics & gene editing congress; Rotterdam (2019)
- Groenbemesters open day at Vredepeel-WUR experimental farm (2019)
- Monday root lunch with PEN group (2019-2022)
- KNPV Working group soilborne pathogens and soil microbiology (2019-2022)
- Plant microbiome discussion group (2020-2021)
- Annual ecology meeting (2021)

International symposia, workshops and conferences (8 ECTS)

- LXIII Annual Congress of the Italian Society of Agricultural Genetics; poster presentation; Naples (2019)
- International symposium on soil and plant analysis; oral presentation; Wageningen (2019)
- Spotlight on young farmers symposium; Wageningen (2019)
- Virtual nematology conference; online (2021)
- miCROPe Symposium; oral and poster presentation; Vienna (2022)

Societally relevant exposure (0.6 ECTS)

- Groenbemesters open day at Vredepeel-WUR experimental farm (2019)
- Abstract on KNPV Gewasbescherming journal (2021)

Committee work (5 ECTS)

- PE&RC-PhD Council Cocial Committee (2019-2021)
- PE&RC-PhD Council Buddy System Committee (2019-2020)
- Wageningen PhD Council Committee (2020-2021)

Lecturing/supervision of practicals/tutorials (5 ECTS)

- Ecological Aspects of Biointeractions (2019-2021)
- Host-Parasite Interactions (2020)
- WEBs of terrestrial diversity (2021)

BSc/MSc thesis supervision (6 ECTS)

- The rhizosphere of cover crops, first insights
- The cover crops' microbiome, monocultures vs mixtures
- Do cover crops stimulate naturally present microbial antagonists?
- Developing primers to assess microbial antagonistic species stimulated by cover crops in the field

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