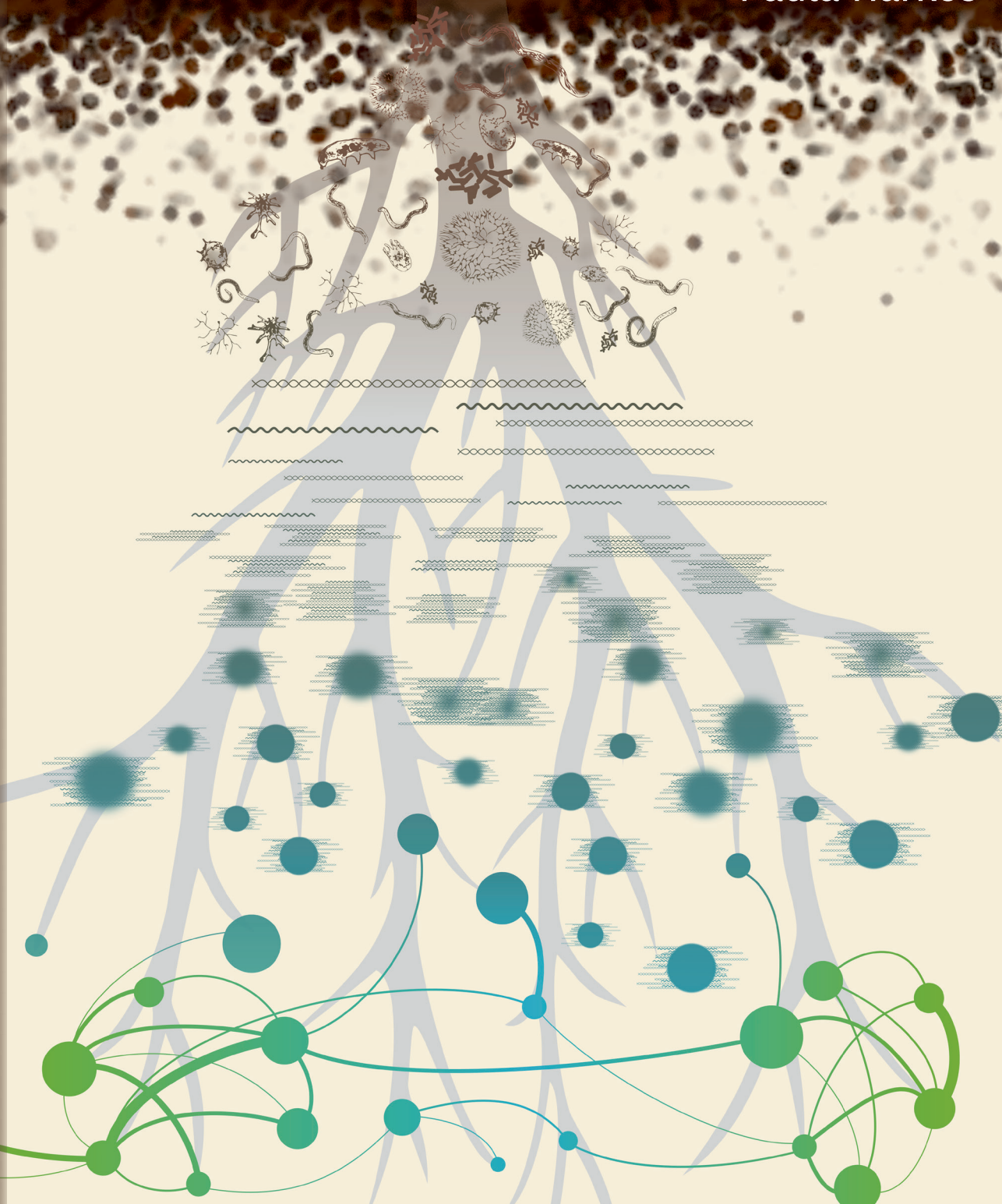
A leap towards unravelling the soil microbiome

Paula Harkes



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A leap towards unravelling the soil microbiome

Paula Harkes

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"*There's no limit to how much you'll know,*
depending how far beyond zebra you go."

— Dr. Seuss

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CHAPTER 1

General introduction

Paula Harkes

> "*The soil is the great connector of lives, the source and destination of all. It is the healer and restorer and resurrector, by which disease passes into health, age into youth, death into life. Without proper care for it we can have no community, because without proper care for it we can have no life.*"
>
> — Wendell Berry

Soil: *From Black box to Pandora's box*

When you take a walk outside and your feet touch the ground, you may not realize that you are standing on the most densely populated habitat on earth. Soil houses an enormous quantity and diversity of organisms. More organisms live in one gram of soil than there are people on this planet. This crowded gram is inhabited by up to 10 billion bacteria, meters of fungal hyphae, and a wide variety of nematodes, earthworms, protists, and arthropods (Bardgett and van der Putten 2014; Raynaud and Nunan 2014). For a long time, people referred to soil as being a black box, as we were unable to characterize the exact life that was present in it. The main reason for this obliviousness is that only 1–5% of the soil bacterial population is culturable and this percentage is estimated to be even lower for fungi (Bakken 1997; Janssen et al. 2002; van Elsas et al. 2000). The diversity of soil life contributes substantially to the functioning of terrestrial ecosystems (*e.g.* nutrient cycling, promoting plant productivity, water holding capacity etc.) (Neher 1999). The identification of soil communities is therefore perceived as an important challenge by many scientists.

With the introduction of DNA sequencing it became possible to identify the diversity of microbial communities. During the last decade, high-throughput sequencing has become less expensive and time consuming, making it currently the most frequently used technology in microbial ecology studies (Bartram et al. 2011; Fierer et al. 2012). Nowadays we can generate data in such a high turnover that the once described black box of soil – of which we did not know what it contained – can now be seen as Pandora's box, as we are challenged to distil useful information from the immense data pile in order to comprehend the myriad of relationships and interactions that occur in soil.

There's always a bigger fish: The soil food web

To visualize and better understand multidimensional relationships in soil, a soil food web approach can be used to link various trophic groups (Moore and de Ruiter

2012). Most soil food webs distinguish four trophic levels (TLs) in which each level includes organisms that feed on dwellers of a preceding level (Fig. 1). Organic material from soil and plant roots form TL0 and are considered the primary producers. TL1 contains the organisms that feed directly on living plant materials (*e.g.* plant-parasitic nematodes) and the primary decomposers that decompose organic substrates (*i.e.* bacteria and fungi). The second level (TL2) includes nematodes, protists and mites feeding on the primary decomposers. The highest level (TL3) are predacious organisms such as predatory mites, collembolans and nematodes.

The composition of food webs is highly variable both in terms of space and time. Small changes in the biomass of particular organisms can have a marked, disproportionate and asymmetrical effect on soil processes (Berg and Bengtsson 2007). Therefore, in order to better understand soil community dynamics, it would be desirable to monitor all trophic levels at once. However, due to technical and financial restrains, the majority of studies concerning soil biodiversity and ecosystem functioning focus on either the primary decomposers or on indicator groups rep-

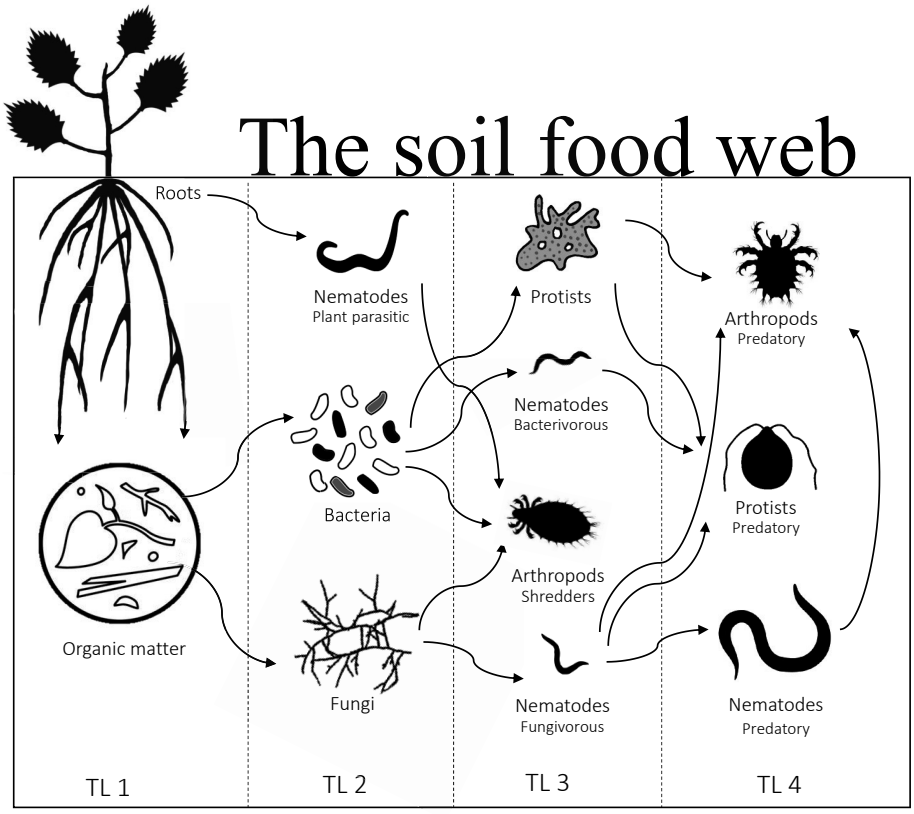

Figure 1: An example of a soil food web in which trophic connections are indicated by arrows. Arrows are pointed towards their consumers. TL = Trophic Level.

resented at different trophic levels – such as nematodes and earthworms (Griffiths et al. 2016). Even though soil microbes are the main players in nutrient cycling, focusing exclusively on the primary decomposers will preclude vital information, as the activity and biomass of bacteria and fungi is also affected by higher trophic levels (de Vries et al. 2013). For example, grazing mesofauna is able to enhance the rate of nutrient mineralization by 30% which, as a consequence, influences the productivity of plants. This illustrates that it may be too much of a conjecture to link plant growth solitary to microbes (Gebremikael et al. 2016; Postma-Blaauw et al. 2010).

Similar to studies centred on microbes, studies that concentrate solely on soil fauna can also be considered fragmentary in the context of improving our understanding of soil ecosystem functioning. For example, research on mesofauna has been mainly based on controlled greenhouse experiments, such as microcosms (Nielsen et al. 2011), and few studies have engaged on the effect of altered soil fauna community structure on processes across ecosystems (Sackett et al. 2010). Another limitation is the fact that indicator groups are often classified in trophic groups (*e.g.* bacterial feeders and fungal feeders). Although our insight in dietary preferences of nematodes is still fragmentary, research points at selective grazing (Hasna et al. 2007; Quist et al. 2014). Thereby making it more informative to investigate if particular changes in primary decomposer community are reflected in the next trophic level.

In conclusion, a holistic approach to monitoring the soil food web, that includes multiple trophic levels measured at a high taxonomic resolution, can provide us with more information on interactions and changes in soil communities.

The force awakens: Dormancy and activity in the rhizosphere

Being present in the soil is not the same as actively participating in the soil food web. Soil is a particularly heterogeneous ecosystem (Van Elsas et al. 2006). Abiotic factors such as moist, nutrient availability and temperature fluctuate vastly, creating temporal and spatial variation. To cope with these capricious circumstances, microorganisms have the ability to reduce their metabolic activity over an extended period of time (Guppy and Withers 1999; Kaprelyants et al. 1993). This state of reduced activity is often referred to as 'dormant', although 'quiescent' is a more accurate term since it refers to reduced activity resulting from the influence of solely external factors (Rao 2018).

The majority of soils are considered suboptimal for microbial growth, for instance because of lack of nutrients and/or energy sources, causing up to 80% of the cells to be dormant (Lennon and Jones 2011; Nannipieri et al. 2003). These

dormant communities, also referred to as microbial seedbanks, can be awakened when adequate environmental stimuli (*e.g.* organic substrates) are detected. Under such condition microbial hotspots can be formed (De Nobili et al. 2001). One important microbial hotspot is the rhizosphere; soil in the vicinity of plant roots, in which large amounts of organic compounds are released by plants (Sanaullah et al. 2016). This process of rhizodeposition allows plants to specifically tune the activity, abundance and composition of the local microbial communities to their advantage (Brimecombe et al. 2007).

In order to unravel which part of the microbial community is affected by a specific plant, it is important to distinguish between the active and total – also referred to as 'resident' – community. In combined community profiling, both the active and total fractions are analysed. This approach can provide useful insight into which microbial groups are positively or negatively affected by different external influences. In this way, revealing effects that otherwise may have been masked by a large dormant fraction (Freedman et al. 2015). While DNA is often used as a proxy for the total biodiversity, RNA is used as a marker of the theoretically active fractions. Recent research has emphasized the relevance of differentiation between the total and the metabolically active microbial fraction as both fractions respond differently to environmental factor such as drought and pH (Bastida et al. 2018; Nunes et al. 2018; Schostag et al. 2015). Analysing the total and active microbiome is imperative to diminish misinterpretations when considering soil ecological processes.

Illuminating the dark side: Exploitation of the soil microbiome

Now that we can map soil microbiomes at high resolutions with the use of high-throughput methods, we have new opportunities to look into the 'black box' and to deepen our understanding of plant-soil interactions in different contexts. This deeper understanding can allow us to better asses and mitigate the impact of human disturbances such as the spread of invasive plant species or the effect of different agricultural management types on the soil biodiversity.

The soil microbiome in invasive plant species

For centuries humans and animals have been relocating plants all over the world. Often, plants introduced into an area outside their native range, will not establish in non-native ecosystems because they are not adapted to the new environment. However, about one out of a hundred exotic plants is able to sustain and spread in its new environment and thereby considered an invasive plant species (Pyšek

et al. 2004; Richardson et al. 2000b). These species can have major effects on the invaded ecosystem and often cause the decline of local plant species (Hejda et al. 2009; Pakpour and Klironomos 2015; Vila et al. 2011).

The impact of invasive plant species on aboveground ecosystems has been studied for decades which has led to various theories as to why these species are so successful in their new environment. For instance, 'the enemy release hypothesis' assumes that a lack of coevolved natural enemies in their new habitat gives them an advantage over their native neighbours (Wolfe and Klironomos 2005). Another theory, 'the novel weapon hypothesis', presumes that more unique chemical compounds are produced by invasive species compared to their congeneric natives making invasive plants either less attractive or less edible (Callaway and Ridenour 2004; Schaffner et al. 2011). 'The enhanced mutualisms hypothesis' explains invasive success by the presence of strong mutualists involving pollination and seed dispersal (Richardson et al. 2000a). The importance of each theory differs per event and the success of invasive species may even be better explained by considering the combined effects these hypotheses (Mitchell et al. 2006).

All the aforementioned theories explaining invasiveness aboveground could also be applicable when considering the belowground effects of invasive plant species. Even though the impact is less well-studied as compared to aboveground effects, research on the effect of invasive plant species on soil communities has increased over the past years (Zhang et al. 2019). Several studies have demonstrated that invasive plants are capable of altering the soil microbiome to their own benefit. For example, the biomass of AMF in the rhizosphere of invasive plants was upregulated by 36% compared to native plants (Zhang et al. 2019). Another study showed that invasive plant species were better able to repel plant-parasitic nematodes (Wilschut et al. 2017) In order to further elucidate biological mechanisms that contribute to the success of invasive plant species, it is important to assess the multitrophic below-ground biotic impact.

The soil microbiome in agriculture

Over the past decades, the green revolution has increased crop yield immensely through fertiliser and pesticide application, improved crop varieties and enhanced water management (Tilman et al. 2002). Unfortunately, greenhouse emission, soil erosion and biodiversity loss are major negative consequences of this intensive management (Foley et al. 2005; Tsiafouli et al. 2014). Soils in intensively managed agricultural systems typically harbor half the biodiversity as compared to natural ecosystems (Tilman et al. 2002). As diverse soil life has shown to contribute to ecosystem stability and thereby resilience to extreme weather events, it has and will become increasingly important to understand how different agricultural soil

management can affect soil biodiversity when considering climate change (Bender et al. 2016; Brussaard et al. 2007; Lau and Lennon 2012; Philippot et al. 2013).

Organic farming has been the main, less intensive alternative to conventional farming management types and has been frequently shown to increase soil biodiversity (Francioli et al. 2016; Tuck et al. 2014; Wang et al. 2017). Nonetheless, yields are typically lower compared to conventional agriculture (Gabriel et al. 2013; Ponisio et al. 2015; Schrama et al. 2018). With an ever-increasing world population but not enough room to expand areas of cultivation, there is a growing need to understand the biological functioning of soil, in order to manage agricultural productivity and protect biodiversity in unison (Tilman et al. 2011).

Outline of this thesis

The central aim of the research presented in this thesis was to explore to what extent plants are able to influence the rhizosphere food web to their own benefit. Within this overarching aim, this thesis can be divided in two parts. The first two experimental chapters are focusing on invasive plants in natural systems and their effect on soil life, whereas the other three chapters are concentrating on food webs in the rhizosphere of agricultural crops. Also in terms of molecular techniques it can perceive it as an Old and New Testament of mapping soil biodiversity.

In the beginning of my thesis – **Chapter 2** – the differences between rhizosphere food web of an invasive and a native plant is studied. The rhizospheres of narrow-leaved ragwort (*Senecio inaequidens*) and the native tansy ragwort (*Jacobaea vulgaris*) were studied at high taxonomic level with specific quantitative (q)PCR primers for bacteria, fungi and nematodes. For both plant species, two life stages were taken into consideration. The results show subtle differences between the native and the exotic species, but also a consistent set of taxa that were negatively affected by increasing plant age for both ragwort species. This study suggests that plants are well capable of altering the soil rhizosphere food web to their benefit.

Research to invasive plant species in their natural habitat is continued in **Chapter 3**. Here, the result of the belowground impact of the giant goldenrod (*Solidago gigantea*) on two soil types is presented. Both biochemical and DNA-based markers were employed to investigate quantitative effects of giant goldenrod on the fungal community and on fungivorous nematodes. In invaded plots, elevated levels of fungal biomass were accompanied by the selective upregulation two out of three fungivorous nematode lineages present in these ecosystems. These results also imply that fungivorous nematode lineages may be characterized by specific and distinct food preferences.

In **Chapter 4**, the emphasis lies on the effects of agricultural management practices – organic vs conventional, and compost vs no-compost – on the soil food web of barley (*Hordeum vulgare*). With the use of high throughput sequencing, we mapped the active (rRNA) and the total (rDNA) bacterial, fungal, protozoan and metazoan communities. The results demonstrate that – next to DNA-based community characterisation – mapping of the active microbial community could provide essential insights on the effects of variables such as crop and soil management on soil microbial communities.

In **Chapter 5**, a specific fraction of the dataset used in Chapter 4 is further analysed to discover interkingdom relationships. Soil management using different fertilization regimes, such as the application of mineral or organic fertilizers, can influence the structure and composition of soil microbiota communities, which are important players in soil functioning. In general, organic amendments resulted in more complex interactions, with more inter- and intra-connections among primary decomposers and predators independent of soil compartments. It is proposed that organic fertilization selects bacterial orders with important interkingdom ecological interactions for survival, predation and cooperation.

Chapter 6 is the last experimental section of my thesis. The same soil management types as in Chapter 4 and 5 were applied but now we investigated the impact on an essentially distinct agricultural crop. We explored the abundance and activity of the microbiome of a legume, green pea (*Pisum sativum*), in association with different densities of the root-knot nematode *Meloidogyne chitwoodi*. This study revealed numerous microbial shifts that were associated with lower RKN densities as well as strong differences in microbial communities between soil compartments – bulk vs. rhizosphere. Seemingly, green pea is capable to select and boost its own microbial community to the extent that it exceeds soil management effects.

Finally, I close this thesis with a general discussion in **Chapter 7**. Here I discuss the main findings of the five experimental chapters and discuss a number of chapter overarching conclusions based on the findings presented in this PhD thesis.

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CHAPTER 2

The differential impact of a native and a non-native ragwort species (Senecioneae) on the first and second trophic level of the rhizosphere food web

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Abstract

Whereas the impact of exotic plant species on above-ground biota is relatively well-documented, far less is known about the effects of non-indigenous plants on the first and second trophic level of the rhizosphere food web. Here, rhizosphere communities of the invasive narrow-leaved ragwort (*Senecio inaequidens*) and the native tansy ragwort (*Jacobaea vulgaris*) co-occurring in three semi-natural habitats are compared. For both species, two life stages were taken into consideration. Quantitative PCR assays for the analyses of bacterial and fungal communities at a high taxonomic level were optimized, and it was investigated whether changes in the primary decomposer community were translated in alterations in bacterivorous and fungivorous nematode communities. In contrast to *J. vulgaris*, small but significant reductions were observed for Actinobacteria and Bacteroidetes (both $P < 0.05$) in case of the invasive *S. inaequidens*. More pronounced changes were detected for the overall nematode community density, and – more specifically – for the bacterivorous genus Anaplectus and the family Monhysteridae (both $P < 0.05$), as well as the necromenic Pristionchus ($P < 0.001$). At high taxonomic level, no differences were observed in fungal rhizosphere communities between native and non-native ragwort species. The impact of plant developmental stages on rhizosphere biota was prominent. The overall bacterial and fungal biomasses, as well as a remarkably consistent set of constituents (Actinobacteria, α- and β-Proteobacteria and Bacteroidetes) were negatively affected by plant stage for both ragwort species. Although later developmental stages of plants generally coincided with lower levels for individual nematode taxa, densities of the fungivorous genera Diphtherophora and Tylolaimophorus remain unaltered. Hence, even at a high taxonomic level, differential effects of native and non-native ragwort could be pinpointed. However, plant developmental stage has a more prominent impact and this impact was similar in nature for both native and non-native ragwort species.

Introduction

Due to intensified trading and travelling, the spreading of species outside of their native range has become one of the major drivers of changes in local biodiversity (Gurevitch and Padilla 2004; Pyšek and Prach 1995; Weber 1998). Most introduced species will not be able to establish themselves due to hostile and/or non-suitable biotic and abiotic conditions (Simberloff 2015). A relatively small subset of the organisms that is able to establish, has a measurable negative ecological or economic impact and is often referred to as 'invasive' (Kolar and Lodge 2001). An impression about the size of these fractions is given by the 'ten's rule' of Williamson (1996) developed for terrestrial plants, stating that approximately 1 out the 100 introduced species will become invasive. It is estimated that in Europe over 300 introduced plant species belong to this category (Vilà et al. 2010).

The majority of the non-native plant species in Europe has been introduced intentionally, mainly because of their useful or desirable traits in agriculture, in horticulture, or as an ornamental plant (Keller et al. 2011). To allow for a systematic comparison of the ecological impact of a wide range of non-native plants, Vila et al. (2010) defined 22 impact types divided over four categories. In this meta-analysis, invasive plants appeared to be highly diverse in the way they affect the surrounding ecosystem. Compared to other effects such as local changes in biodiversity, the impact of invasive plant species on nutrient cycling appeared to be minor (Vilà et al. 2010). In other words, no fixed set of characteristics can be defined that typify invasive plants.

To assess the ecological impact of invasive plant species, often pairs of phylogenetically related plants are selected; a congeneric pair of a native and an alien species (Gallagher et al. 2015). By doing so, a baseline is defined against which the ecological characteristics of the exotic plant species can be plotted. In the present study we selected two closely related *Senecioneae* species (a tribe within the Asteraceae): Tansy ragwort, *Jacobaea vulgaris* (syn. *Senecio jacobaea*) and narrow-leaved ragwort, *Senecio inaequidens*. *J. vulgaris* is a common native pioneer plant and can be found from Scandinavia to the Mediterranean, but is most abundant NW Europe. *S. inaequidens* on the other hand is considered to be an invasive species in Europe (Ernst 1998; Heger and Böhmer 2005; Schmitz and Werner 2001) which originates from South Africa, and was accidentally introduced in Europe by sheep wool transports (Werner et al. 1991). It was recorded in 1939 for the first time in the Netherlands and its range expanded mainly during the 1970's (Tamis et al. 2005).

While *S. inaequidens* is a perennial plant, *J. vulgaris* is (generally spoken) a biennial plant. *J. vulgaris* forms a rosette in its first growing season, a flowering stem

develops in the second growing seasons and it dies after seed production (Harper and Wood 1957). *S. inaequidens* has a life span of about five to ten years, it does not form a rosette, and flowers are formed from the first growing season onwards. Both species are characterized by the production of secondary metabolites known as senecionine-type pyrrolizidine alkaloids (PAs). These components play a role in the interaction between the plant and its (a)biotic environment. PAs can be found in all plant parts, and their distribution mainly depends on the developmental stage of the plant. Senecionine-type PAs are quite diverse (> 100 structures), and *J. vulgaris* is characterized by a subset that partly overlaps with alkaloid types found in *S. inaequidens* (Hartmann and Dierich 1998).

Most studies on the ecological effect of invasive plant species concentrate on above ground effects, as well as the impact of on the cycling of carbon and macronutrients such as N and P (Huang et al. 2015). Studies on belowground biotic effects are relatively rare (Chakraborty et al. 2012). Here, we assessed the below-ground biotic impact of the invasive narrow-leaved ragwort, by comparing it with the rhizosphere community of its native relative, tansy ragwort. The rhizosphere community is a niche showing large variation of microorganisms that interact directly or indirectly with the plant. The rhizosphere composition is plant species-specific (Grayston et al. 1998; Westover et al. 1997) and invasive success might be explained for instance by the lowered pressure of plant pathogens (enemy release hypothesis) or by the presence of strong mutualists (enhanced mutualisms hypothesis) (Kourtev et al. 2003; Kourtev et al. 2002a; Kourtev et al. 2002b; Reinhart and Callaway 2006; Moriën 2011). In the latter case, invasive species may recruit specific mutualists, or may more effectively boost general mutualists in comparison to its local relatives. It is noted that rhizosphere microbial communities change over time with plant development (Chaparro et al. 2014; Garbeva et al. 2004). Hence, two different grow stages of each species were selected in this study.

In order to get grip on biological mechanisms contributing to the success of invasive plant species, the impact of *S. inaequidens* and *J. vulgaris* on the rhizosphere community was investigated at two subsequent trophic levels. We hypothesized that the invasive *S. inaequidens* would induce fewer changes in the rhizosphere community as compared to its native relative *J. vulgaris* (*e.g.* Morrien and Putten 2013) as numerous plant generations are required for the selection of robust biochemical handles to manipulate a given microbial rhizosphere community for its own benefit. To test this we first concentrated on the primary decomposers, the bacterial and fungal communities directly surrounding the plant root. Secondly, we supposed that changes in the primary decomposer community will be reflected in major players in the next trophic level, bacterivorous and fungivorous nematodes. In case bacterivores or fungivores would have no preference with regard to the nature of their food

source, we should observe overall similar changes for all taxa. In case of distinct food preferences, we would expect to see taxon-specific responses. Although our insight in dietary preferences of nematodes is still fragmentary, available information points at selective grazing (*e.g.* Hasna et al. 2007; Quist et al. 2014; Shtonda and Avery 2006). Hence, plant-induced shifts in microbial soil communities might result in specific, quantitative shift in the rhizosphere nematode community. Hereto, we selected and adapted a set of quantitative PCR (qPCR)-based assays for analyses of bacterial and fungal communities in the rhizosphere. Subsequently, bacterivorous and fungivorous nematode assemblages were characterized as described in Vervoort et al. (2012). Hence, in this resaearch we mapped both direction and magnitude of shifts in the rhizosphere community of two closely related, indigenous and exotic plant species at two distinct developmental stages.

Materials and methods

Site description

Rhizosphere samples of *Jacobea vulgaris* and *Senecio inaequidens* were taken in November 2013, at three ex-arable fields in the central part of the Netherlands: De Mossel (52° 03' N, 5° 45' E) abandoned since 1995, Telefoonweg (51° 59' N, 5° 45' E) abandoned since 2002 and Plantage Willem III (51° 58' N, 5° 31' E) abandoned since 1994 (Van de Voorde et al. 2011). The locations were selected based on their similarity in terms of vegetation. *Agrostis capillaris*, *Holcus lanatus* and *Jacobea vulgaris* were the most dominant species at all three locations (for a complete vegetation overview see Supplementary material, Table A1).

Sampling and experimental design

At each location, two sampling sites of 16 m^2 (4.0 x 4.0 m) were selected based on similarity in the distribution patterns of the two plant species. The two sampling sites were about 100 meters apart. Sampling sites were divided into four plots of 4 m^2 (2 x 2 m). From each plot, four composite rhizosphere-enriched soil samples were taken for two developmental stages of both *J. vulgaris* and *S. inaequidens*. Each composite sample consisted of rhizosphere soil from three randomly chosen individual plants. In total, we collected 3 (locations) x 2 (plant species) x 2 (stages) x 8 (samples per location) = 96 samples.

Rhizosphere-enriched soil of 2nd year or older *S. inaequidens* plants was reached by lifting the whole plant. Subsequently, about 400 g soil was collected from the center of the root system. Afterwards perennial *S. inaequidens* plants were returned

to their original site. First growing season *S. inaequidens* and both stages of *J. vulgaris* plants were sampled by lifting the compete root system, soil adhering to the outside of the root system was carefully removed as it might have been affected by roots of neighboring plant species. The remaining soil was transferred in plastic bags. All samples were sieved over a 5 mm grid, mixed thoroughly and stored at 4°C 100 g of each composite sample was used for nematode analysis, 60 g was used for determining moisture content and pH and approximately 5 g was stored at -20°C prior total DNA extraction, in order to prevent DNA degradation.

Soil acidity, humidity and organic matter content

For determining moisture content and pH a subsample (60 g) of each sample was used. Soil moisture was measured by determining the weight loss after 72 hours incubation at 65°C. Dried soil was sieved with a mesh of 2 mm and 10 gram was diluted in 25 mL demineralized water for soil pH measurements using a gel-electrolyte electrode (Sentix 21, WTW, Weilheim, Germany). Organic matter content was determined by measuring weight loss after 3 hours incubation at 550°C.

Fungal and bacterial primer selection

To characterize primary decomposer communities at a high taxonomic level, quantitative PCR assays were developed. 16 bacterial and 8 fungal primer combinations were selected from literature, supplemented with 5 newly designed fungal primer combinations (see Supplementary material Appendix 1 Table A2). In a first selection round, the specificities of each of the 29 primer combinations was verified (primers from literature) or tested (newly designed primers) on target DNA, and to all available non-target DNAs (= DNAs of all other test strains) at a relatively low annealing temperature (55°C). Target DNA was obtained by lysing pure cultures of bacteria or fungi with a Maxwell blood and tissue purification kit (Promega, Madison USA). For this initial selection round, nine soil-relevant bacterial and nine fungal strains were used (Supplementary material Appendix 1 Table A2). Sterile, demineralized water (Milli-Q quality) was used as negative control.

Subsequently, temperature gradients from 55 to 70°C were performed in order to select primer combinations with an optimal annealing temperature of around 60°C. In this way, a single qPCR temperature profile could be use for all taxon-specific primer combinations.

In a next step, specificities of selected primer combinations were tested at 60°C (Supplementary material Appendix 1 Table A3). Testing was performed in an end volume of 20 µl containing 3 µl of 1,000 times diluted template, 1µl of each primer (final concentration: 10 ng/µl), 5µl of Milli-Q water and 10 µl iQ SYBR

Green Supermix (BioRad). The following temperature profile run on a Biorad iQ5 thermal cycler: 95°C, 3 min followed by 40x (95°C, 30 s; 60°C, 30 s; 72°C, 30 s). Afterwards, the nature of the amplicon was checked by its melting characteristics (stepwise temperature increase from 72 to 95°C, increment per cycle: 0.5°C, 10 s per temperature).

To establish the relationship between Ct values and the concentration of target bacterial or fungal DNA (ng/µl), a calibration curve was made for each primer combination using a dilution series of the corresponding DNA as a template. The concentration of DNA was measured using a NanoDrop ND-1000 UV–VIS spectrophotometer. (For a complete methodological approach see Supplementary material Appendix 1 A4).

Fungal primer development

Detection assays for Chytridiomycota and Zygomycota were designed on the basis of an 18S rDNA molecular framework. Relevant sequences were retrieved from the Silva SSU database (http://www.arb-silva.de), and aligned in ARB (Ludwig et al. 2004). A subsection with all available fungal groups and several non-fungal groups was selected in order to verify the specificity of the developed primers. Primers were designed at an optimal T_a of 60°C (calculated by on Mobyle Pasteur (http://mobyle.pasteur.fr/cgi-bin/portal.py) and tested on various fungal templates (target DNA as well as non-target DNA). Specificity and temperature profile of the primers was determined following procedures as described before.

Quantitative PCR on microbial community DNA

DNA was extracted from each frozen composite soil sample (0.25 g) using the PowerSoil™ DNA isolation kit (MOBIO, Carlsbad, CA, USA). As an internal standard, 20 µl of mammalian DNA (21 ng/µl) was included after the bead beading step, and fixed amounts were used when transferring the supernatant. The obtained DNA was diluted 250 times in order to serve as a template for qPCR. For each reaction 3 µl of the 250 times diluted template, 1 µl of each primer (final concentration: 10 ng/µl), 1.5 µl of Milli-Q water, 3.5 µl of PCR enhancer and 10 µl iQ SYBR Green Supermix (BioRad) was used.

Nematode community analysis

100 g soil of each sample was used for nematode extraction, using an elutriator (Oostenbrink 1960). DNA was extracted and purified from the nematode suspensions as described by Vervoort et al. (2012). 20 µl of mammalian DNA

(21 ng/µl) was included in the lysis buffer to function as an internal standard. To determine the nematode biodiversity of the samples, a mixture of the purified DNA was made per field (32 samples), which served as a template for 32 nematode taxon-specific primer combinations. On the basis of this biodiversity check, primer sets of the most abundant nematodes were selected.

Data analysis

One-Way ANOVA was used to test whether the abiotic characteristics of soil samples from the three sampling locations were different ($P < 0.05$). In case differences were observed, it was followed by a post-hoc comparison using Fisher's Least Significant Difference test ($\alpha = 0.05$).

The variation in taxon abundances was investigated by principal component analysis (PCA). The identified principal components were investigated for the likely causal factor: site dependent differences, plant differences, and stage differences. Statistical analysis was performed using R (version 3.1.2). To determine the explained versus residual variation, the adonis function in the vegan package of R (Oksanen 2016) was used. Adonis represents a multivariate analysis of variance that allows simultaneous testing of multiple factors and covariates based on permutation tests. A Euclidian distance matrix was constructed, which was used in multivariate analysis. Variables (age, location, plot, plant species and subplot) and their interactions were found significant at $P < 0.05$.

Making use of linear relationship between primary qPCR output, Ct values, and the number of individuals, nematode concentrations expressed as individuals per 100 g of soil were calculated. As both the resulting nematode densities and the concentrations of bacterial and fungal DNA (ng per 0.25 g soil) didn't show a normal distribution, data were transformed. Primary counts (y) were log transformed ($\ln(y+0.1)$). A constant (0.1) was added to push data away from the lower bound zero. However after transformation, not all biotic data followed a normal distribution. Consequently, a rank-based, nonparametric test (Kruskal-Wallis H) was conducted to test for differences in rhizosphere community between plant stages followed by Mann–Whitney U test for pairwise comparison, which was also used to test differences between plant species. Data analysis was performed using R (version 3.1.2) and IBM SPSS Statistics for Windows, version 22.

Spearman's rank correlation matrix based on abundance data was created using R 3.1.2 and visualized in Cytoscape 3.4.0 (Lopes et al. 2010). A correlation network approach was used to visualize interactions between all measured components for the two different plant species (*J. vulgaris* and *S. inaequidens*). Positive and negative correlations between components of Spearman's rank ≥ 0.5 were visualized.

Results

Abiotic characteristic between rhizosphere-enriched soils from native and exotic Senecioneae species

All three sampling locations are characterized by mildly acidic sandy to loamy sandy soils (pH range: 5.7–6.4), and slight but significant differences were observed between the three locations (Table 1). In essence, the same holds for the soil moistures contents (range: 14%–18%); they all fell in the field capacity range of these soils. In one location ('Telefoonweg'), the water content was higher as compared to the other two locations. The relatively low organic matter content (range: 4.4%–6.3%) is typical for this type of semi-natural locations. Also here, small but significant differences were observed between the three locations (Table 1).

Subsequently, it was investigated whether an effect of plant species and/or plant stage on abiotic characteristics of rhizosphere-enriched soil could be detected. As can be seen in Table 2, only in one case a significant difference was observed between the two plant species (soil moisture content in older plants). On the contrary, plant developmental stage had a substantial effect on these parameters. Except for pH in

Table 1: Abiotic characteristics of rhizosphere-enriched soil for the three different sampling locations. Each value is the average of 32 measurements (± standard error). Different letters indicate significant differences (P< 0.05).

	Soil pH	Soil moisture	Organic matter
Location	value ± (SE)	% ± (SE)	% ± (SE)
De Mossel	6.36 ± 0.05^{a}	14.62 ± 0.39^{a}	5.21 ± 0.16^{a}
Telefoonweg	5.73 ± 0.06^{b}	17.67 ± 0.36^{b}	6.28 ± 0.15^{b}
Plantage Willem III	5.99 ± 0.05^{c}	13.87 ± 0.35^{a}	4.40 ± 0.11^{c}

Table 2: Abiotic characteristics of rhizosphere-enriched soil from *Jacobaea vulgaris* and *Senecio inaequidens* for the different plant stages: *J. vulgaris* one year (JV 1), *J. vulgaris* two years or older (JV ≥ 2), *S. inaequidens* one year (SI 1) and *S. inaequidens* two years or older (SI ≥ 2). Each value is the average of 24 measurements (± standard error). Different letters indicate significant differences (P< 0.05).

	Soil pH	Soil moisture	Organic matter
Species	value ± (SE)	% ± (SE)	% ± (SE)
JV 1	6.17 ± 0.06^{a}	16.67 ± 0.43^{a}	5.71 ± 0.22^{a}
JV ≥2	6.04 ± 0.06^{a}	14.91 ± 0.44^{b}	5.06 ± 0.19^{b}
SI 1	6.13 ± 0.08^{a}	16.83 ± 0.48^{a}	5.81 ± 0.21^{a}
SI ≥2	5.77 ± 0.09^{b}	13.12 ± 0.42^{c}	4.61 ± 0.18^{b}

case of *J. vulgaris* (JV 1 and JV ≥ 2), all parameters differed significantly between the two developmental stages (Table 2). When data from the two life stages of each plant species were lumped and re-analyzed for species-specific impact on pH, moisture and organic matter content, no differences were observed (Supplementary material, Table A5).

Quantitative detection of primary decomposers at a high taxonomic level – essential tool development

A range of quantitative PCR-based assays have been published that in essence could be used to characterize primary decomposer assemblages in soil at a high taxonomic level. However, the optimal annealing temperature (T_a), a key parameter in (quantitative) PCR-based detection, differed substantially between the published assays (ranging from 53°C to 61.5°C; for full overview see Supplementary material, Table A2). We selected a broad range of primer combinations from literature and after testing a temperature range, an optimal annealing temperature of 60°C was selected. In a next final step the specificities of the selected primer combinations were examined. Details on the procedure that was followed to select the most optimal primer combinations, as well as the protocol used to determine the parameter values that define the relationship between primary qPCR output (Ct values) and target DNA concentration ('A' and 'B' in Table 3) are given in Supplementary material Table A3, A4 and Figure A4). Finally, 15 primer combinations were selected, targeting ten bacterial and five fungal taxa at T_a= 60°C. For all of the qPCR assays, there was a linear relationship between the ^{10}log of DNA concentration and the measured threshold cycle (R^2> 0.975 in all cases) (Table 3).

Published assays on the quantitative detection of the Chytridiomycota and Zygomycota did not fulfill our requirements and therefore new primers were designed based on phylum-characteristic 18S rDNA sequences. Molecular phylogenetic data suggest the Zygomycota to be non-monophyletic (Bruns et al. 1992; Tanabe et al. 2004; Tanabe et al. 2005). Therefore, two separate assays were developed for the Mortierellales and the Mucorales (Table 3). These are two large orders of the suborder Mucoromycotina, covering about one third of the Zygomycota.

Below-ground biotic effects of native versus exotic Senecioneae

A principle component analysis was used for a first exploratory analysis of biota (bacteria, fungi and nematodes) in the rhizosphere of tansy and narrow leaved ragwort. First, we investigated the impact of sampling location. This variable explained a small fraction of the variation: the location "Telefoonweg" is separated from the other two locations on principal component three (9.2% of the variance)

and "De Mossel" and "Plantage Willem III" are separated on principal component five (5.6% of the variance). Most of the variation (29.9%) is captured by plant stage, especially for *Senecio inaequidens* 2 years and older (Fig. 1).

In order to incorporate the proper error structure of our experimental design in the analysis, a separate multivariate permutation tests was performed. Also here plant age explained most of the overall variation (9.7%, P= 0.001). Location, plot and their interaction captured respectively 4.8%, 3.2%, and 17.4% of the variation (P= 0.001). Especially for bacteria and fungi, plant age was the main explaining variable. For nematodes it was the third explaining variable, capturing 9.8% (behind the interaction between location*plot and plot*subplot). It is noted that 34% of all variance remained unexplained (Supplementary material Appendix A6).

J. vulgaris versus *S. inaequidens* – bacteria and fungi in rhizosphere-enriched soil

Analysis of rhizosphere samples from *J. vulgaris* and *S. inaequidens* revealed no species-specific differences in overall DNA levels of bacteria and fungi (Table 4). Subsequently, we investigated whether a discriminative impact of plant species could be detected at a high taxonomic level. Two bacterial phyla, Actinobacteria and Bacteroidetes, were significantly more abundant in the rhizosphere of *J. vulgaris* as compared to *S. inaequidens* (P< 0.05). For all other primary decomposer taxa, no significant differences between the two plant species were detected. Hence,

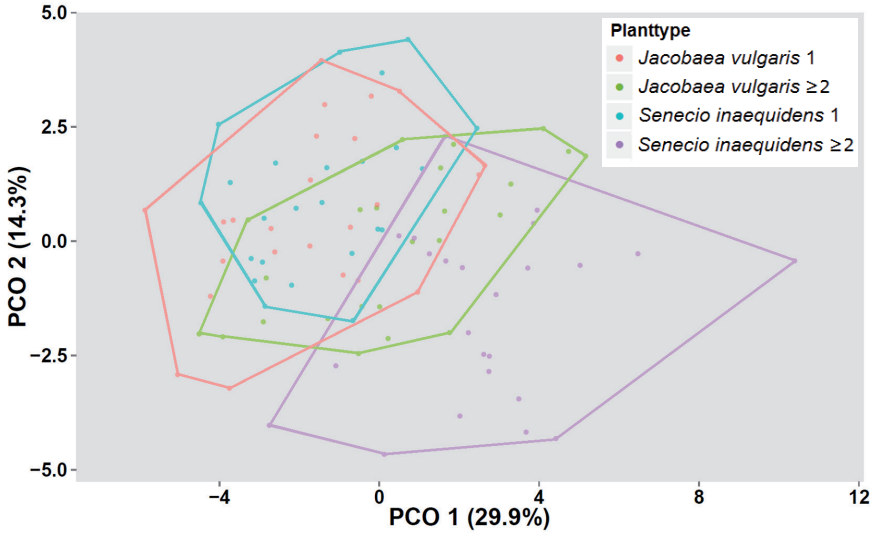

Figure 1: Principal Component Analysis (PCA), in which the vectors represent the abundance of all measured components (bacteria, fungi and nematodes). The dots represent the 96 measured results, categorized by the four plant types.

Table 3: Overview of primer combination used to quantitatively characterize bacterial and fungal rhizosphere communities. Specificity of bacterial primer combinations is based on taxon specific 16S ribosomal DNA motifs. Fungal primers are based on taxon-characteristic internal transcribed spacer (ITS) regions, occasionally combined with a more generic 5.8S forward or reverse primer.

Target	Primer sequence (5'–3') F: forward, R: reverse		Reference	T_a (literature)	T_a (this study)	Template DNA for specificity verification	Parameters defining the linear relationship between Ct and DNA concentration (Ct = a x ^{10}log [concentration] + b)		
							A	B	R^2
All bacteria	F	CCTACGGGAGGCAGCAG	López-Gutiérrez et al. (2004) Watanabe et al. (2001)	60°C	60°C	All bacterial stains	-3.62	16.58	0.997
	R	ATTACCGCGGCTGCTGGCA							
Acidobacteria	F	GATCCTGGCTCAGAATC	Barns et al. (1999) Muyzer et al. (1993)	60°C	60°C	*Streptomyces sp.*	-3.96	17.44	0.996
	R	ATTACCGCGGCTGCTGG							
Actinobacteria	F	TACGGCCGCAAGGCTA	De Gregoris et al. (2011)	61.5°C	60°C	*Acidicapsa ligni*	-4.71	18.10	0.975
	R	TCRTCCCCACCTTCCTCCG							
α-proteobacteria	F	CIAGTGTAGAGGTGAAATT	De Gregoris et al. (2011)	61.5°C	60°C	*Agrobacterium*	-3.93	16.05	0.994
	R	CCCCGTCAATTCCTTTGAGTT							
β-proteobacteria	F	ACTCCTACGGGAGGCAGCAG	Lane (1991) Overmann et al., (1990)	55°C	60°C	*Burgholderia sp.*	-3.65	15.96	0.986
	R	TCACTGCTACACGYG							
γ-proteobacteria	F	CMATGCCGCGTGTGTGAA	Mühling et al. (2008)	54°C	60°C	*Pseudomonas sp.*	-3.58	15.89	0.992
	R	ACTCCCCAGGCGGTCDACTTA							
Bacteroidetes	F	CRAACAGGATTAGATACCCT	De Gregoris et al. (2011)	61.5°C	60°C	*Flavobacterium sp*	-3.59	17.59	0.996
	R	GGTAAGGTTCCTCGCGTAT							

Target	Primer sequence (5'–3') F: forward, R: reverse	Reference	T_a (literature)	T_a (this study)	Template DNA for specificity verification	Parameters defining the linear relationship between Ct and DNA concentration (Ct = a x ^{10}log [concentration] + b)		
Firmicutes	F GCAGTAGGGAATCTTCCG R ATTACCGCGGCTGCTGG	Meier et al. (1999) Muyzer et al. (1993)	60°C	60°C	*Bacteriosis Paenibacillus sp.*	-3.47	13.51	0.995
All fungi	F TCCGTAGGTGAACCTGCGG R CGCTGCGTTCTTCATCG	White et al. (1990) Vilgalys and Hester (1990)	53°C	60°C	All fungal strains	-3.90	18.75	0.964
Ascomycota	F GGAAGTAAAAGTCGTAACAAGG R CGTTACTRRGGCAATCCCTGTTG	White et al. (1990) Nikolcheva et al. (2004)	58°C	60°C	*Aspergillus caespitosus* *Trichoderma harzianum* *Penicillium expansum*	-3.47	16.94	0.998
Basidiomycota	F CAGGAGACTTGTACACGGTCCAG R TCGATGAAGAACGCAGCG	Gardes and Bruns (1993) Vilgalys and Hester (1990)	55°C	60°C	*Bolbitius titubans* *Armillaria lutea*	-3.44	19.34	0.998
Chytridiomycota	F TGGTTTCTAGGACCGAAGT R CCGGCCAAGGTGAATT	This study	-	60°C	*Spizellomyces acuminatus* *Rhizophlyctis rosea*	-3.90	18.75	0.996
Zygomycota								
Mortierellales	F TGGTTTCTAGGACCGAAGT R CCTCAAACTTCCATTGACTAATA	This study	-	60°C	*Mortierella elongata*	-4.17	17.55	0.998
Mucorales	F CAGTTAAAACGTCCGTAGTCAA R CACCACCACCCATAGAATCTA	This study	-	60°C	*Rhizomucor miehei*	-3.75	20.60	0.997

Table 4: Mean (± SE) DNA levels of total bacterial and fungal communities. Targeted phyla are presented as a percentage of the total of all tested phyla. *P< 0.05 **P< 0.01 ***P< 0.001.

Target	*J. vulgaris*	*S. inaequidens*	Significance
All bacteria	7023 ± 251	6278 ± 320	NS
Acidobacteria	43.16%	43.36%	NS
Actinobacteria	22.52%	22.21%	*
α-proteobacteria	13.09%	13.22%	NS
β-proteobacteria	4.56%	4.65%	NS
γ-proteobacteria	5.68%	5.86%	NS
Bacteroidetes	10.28%	9.87%	*
Firmicutes	0.71%	0.84%	NS
All fungi	345 ± 21	333 ± 22	NS
Ascomycota	34.25%	29.33%	NS
Basidiomycota	45.49%	43.53%	NS
Chytridiomycota	2.80%	6.46%	NS
Zygomycota	17.46%	20.67%	NS

regarding the primary decomposer community in the rhizosphere only relatively subtle differences were detected between *J. vulgaris* and *S. inaequidens*.

J. vulgaris versus *S. inaequidens* – nematodes in rhizosphere-enriched soil

Based on local nematode biodiversity checks, a selection was made of 14 of the most abundant nematode taxa, distributed over three major trophic groups: 5 types of bacterivores, 4 fungivores, and 5 groups of predominantly plant-parasitic taxa (Supplementary material Table A7). One additional primers combination ("universal") was used to assess the overall nematode density. Comparison between the two plant species revealed significant differences for three nematode taxa, and well as the total nematode density (Table 5). The level of nematode accumulation in rhizosphere-enriched soil from the exotic *S. inaequidens* was significantly lower (P≤ 0.001) than for its native congener. This reduced accumulation was mainly explained by the decreased presence of the bacterivorous genus *Anaplectus* and the necromenic *Pristionchus*, and to a lesser extent by the decrease in density of the bacterivorous family Monhysteridae. It is noted that no significant differences were observed for any of fungivorous or herbivorous nematode taxa.

Effect of plant stage on bacteria and fungi in rhizosphere enriched soil

Rhizosphere communities change over time, and are strongly affected by plant development. In order to investigate this for these two members of the Senecioneae,

Table 5: Mean (± SE) number total nematodes and the targeted families in the two different plant species. *P< 0.05 **P< 0.01 ***P< 0.001.

	Target	*J. vulgaris*	*S. inaequidens*	Significance
	All nematodes	2682 ± 251	1625 ± 167	***
bacterivorous	*Anaplectus*	380 ± 46	296 ± 59	*
	Cephalobidae	796 ± 44	706 ± 64	NS
	Monhysteridae	18 ± 3.7	10 ± 2.0	*
	Plectidae - *Anaplectus*	134 ± 21	118 ± 29	NS
	Pristionchus	607 ± 112	107 ± 12	***
fungivorous	Aphelenchidae	280 ± 37	241 ± 27	NS
	Aphelenchoididae	19 ± 3.4	21 ± 4.6	NS
	Diphtherophoridae	59 ± 13	61 ± 13	NS
	Tylolaimophorus	4.32 ± 1.51	2.83 ± 1.18	NS
plant-parasitic	*Coslenchus*	714 ± 193	786 ± 243	NS
	Filenchus group 2	0.23 ± 0.074	0.61 ± 0.16	NS
	Filenchus group 3	18 ± 4.4	17 ± 3.8	NS
	Pratylenchus crenatus	44 ± 9.8	57 ± 10.7	NS
	Tylenchorhynchus	36 ± 11.6	37 ± 10.7	NS

samples were collected from two different growth stages of each plant species: first year plants and second year and older plants ('1' and '> 2' in Fig. 2).

As compared to the afore-mentioned differences in rhizosphere biota between the two congeneric plant species, the effect of plant stage was large. Both for *J. vulgaris* and *S. inaequidens* a decrease was measured in the bacterial and fungal DNA levels in older plants. This effect was more pronounced in the exotic narrow-leaved ragwort (SI; P< 0.001 both for total bacteria and fungi) than in the native tansy ragwort (JV; P< 0.05 both for total bacteria and fungi) (Fig. 2A and B). Analysis of the bacterial communities at division level revealed a remarkably consistent pattern between the two plant species. Acidobacteria, γ-Proteobacteria, and Firmicutes were not affected by plant stage, and this holds for both species. On the other hand, Actinobacteria, α-Proteobacteria, β-Proteobacteria, and Bacteroidetes decrease in the rhizosphere of older plants, and, again, this holds for both ragwort species. It is noted that decreased levels of individual bacterial divisions are more conspicuous in case of the exotic S. inaequidens (for α and β-Proteobacteria: P< 0.01, the other two divisions: P< 0.001) (Fig. 2A).

Also on case of the fungi, a larger decrease in fungal DNA levels was detected for the exotic narrow leaved ragwort (SI). This decrease could in part be attributed to a stronger decrease in Ascomycota (P< 0.001) compared to its native relative. Next to that, only a reduction of the presence of Chytridiomycota was measured in older *S. inaequidens* plants (P< 0.01) (Fig. 2B).

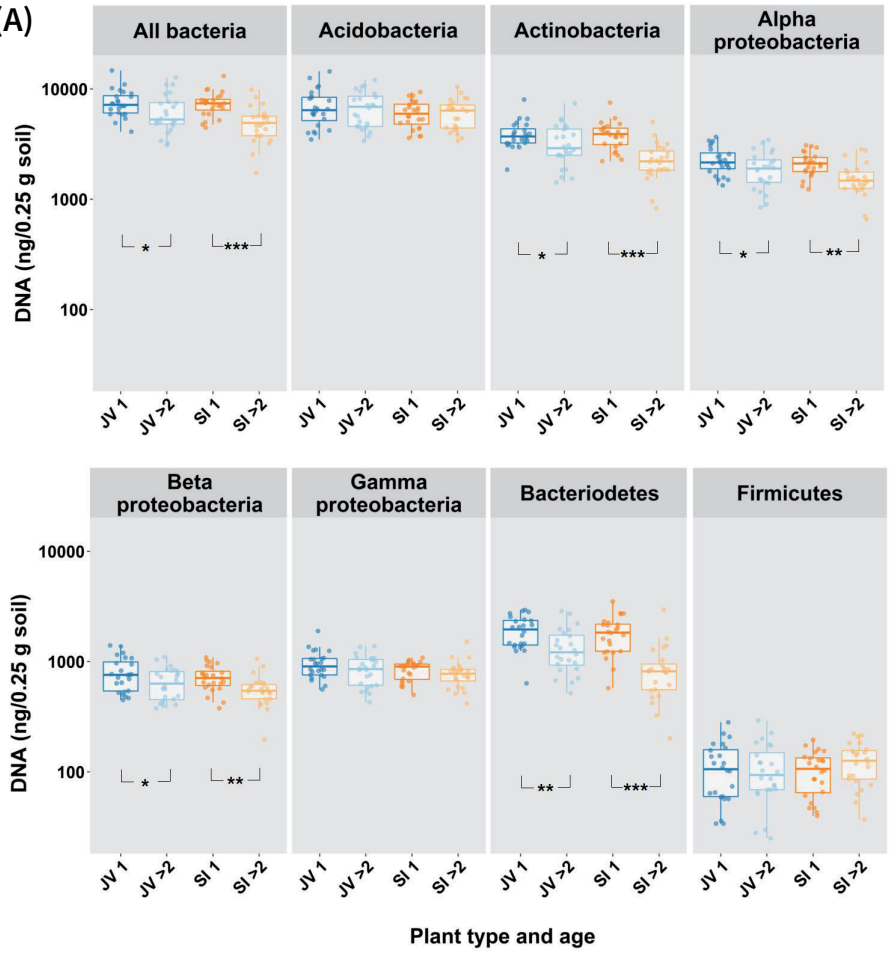

Figure 2: Mean (± SE) DNA levels of total bacterial (A) and fungal (B) communities and the targeted phyla in the different life stage of each plant species JV 1 = *Jacobaea vulgaris* one year JV > 2 = *Jacobaea vulgaris* 2 years SI 1 = *Senecio inaequidens* one year SI > 2 = *Senecio inaequidens* 2 year or older *P< 0.05 **P< 0.01 ***P< 0.001.

Effect of plant stage on nematodes in rhizosphere-enriched soil

Only in case of the exotic *S. inaequidens*, a strong negative effect of plant stage on nematode abundance was observed (P< 0.001) (Fig. 3). The differential impact of the two ragwort species on the rhizosphere nematode density was mainly explained by the clear decrease of the Cephalobidae density in case of narrow-leaved ragwort. In sandy terrestrial soil these nematodes are often the most abundant group of bacterivores. For the remaining bacterivorous taxa *Anaplectus*, Plectidae minus *Anaplectus*, and Monhysteridae aging of both ragwort species resulted in lower nematode densities in the rhizosphere. Only for *Pristionchus*, a necromenic bacterivore, an opposite impact was seen in case of *J. vulgaris*.

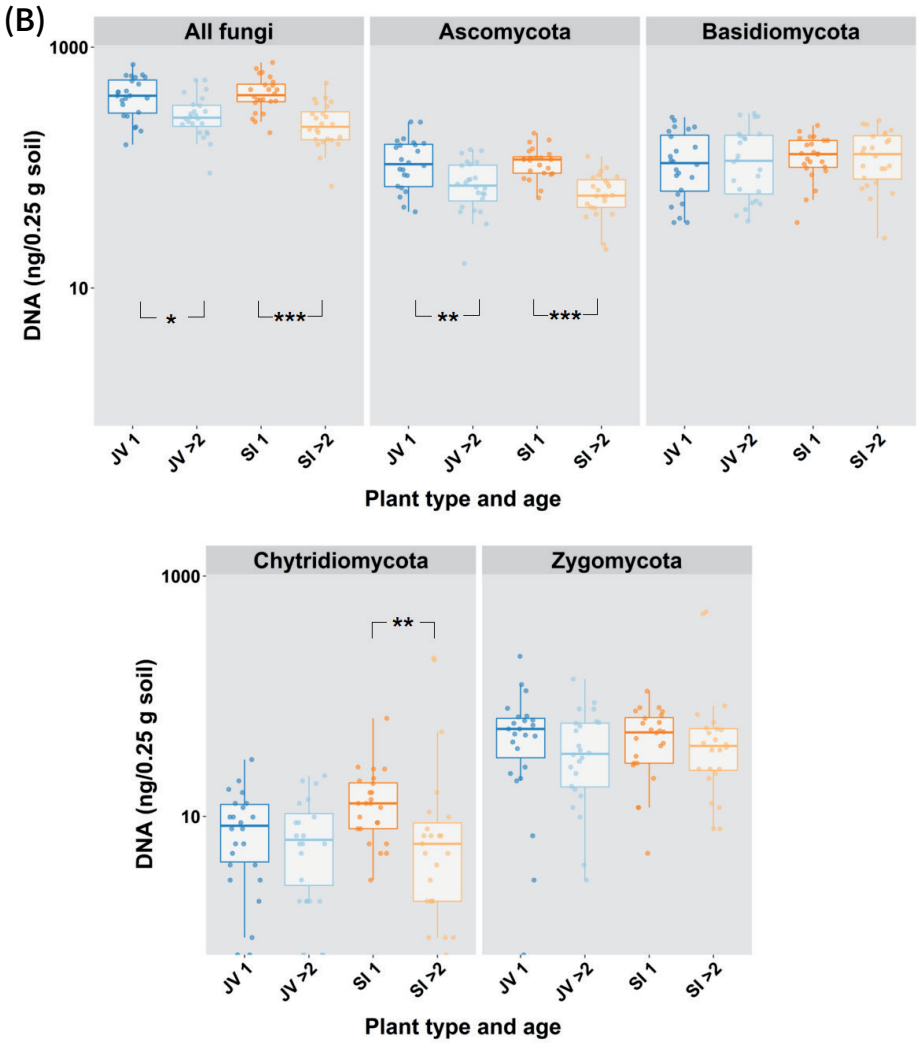

Figure 2: *Continued.*

A differential pattern was observed for the fungivorous nematodes; whereas *Aphelenchidae* and *Aphelenchoididae* abundances were negatively affected by plant stage, the presence of two other fungal feeding taxa, *Diphtherophora* and *Tylolaimophorus* was apparently unaltered by the increase in plant stage.

Regarding the plant-parasitic nematodes, one specific lesion nematode species showed higher densities in case of the non-native ragwort, *Pratylenchus crenatus*. *Coslenchus*, a genus of epidermal and root hair feeders, was the most abundant plant parasite in the rhizosphere of both ragwort species, and the abundances were unaffected by the developmental stage of their host plants.

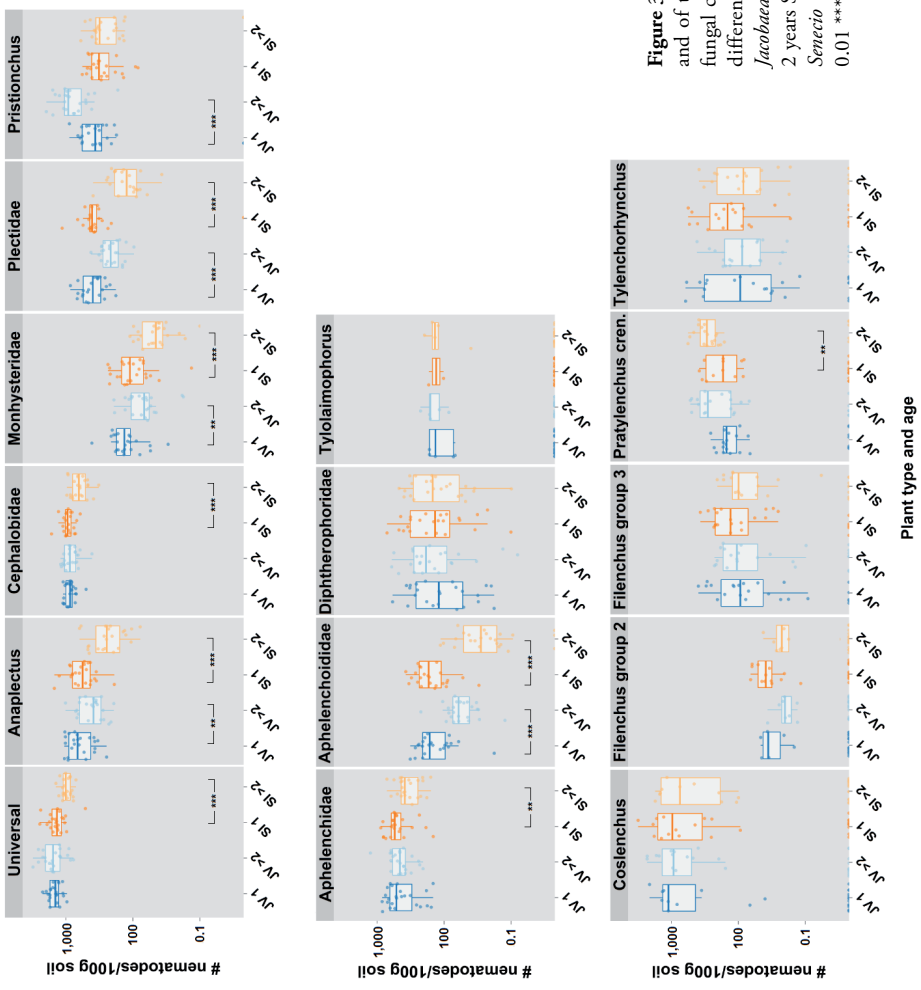

Figure 3: Mean (± SE) number of total nematodes and of the targeted families of total bacterial and fungal communities and the targeted phyla in the different life stage of each plant species JV 1 = *Jacobaea vulgaris* one year JV > 2 = *Jacobaea vulgaris* 2 years SI 1 = *Senecio inaequidens* one year SI > 2 = *Senecio inaequidens* 2 year or older *P< 0.05 **P< 0.01 ***P< 0.001.

Network structure of native versus exotic Senecioneae

Network visualization (Fig. 4) showed that there is an increase in the number of correlations (Spearman's rank ≥ 0.5) in *S. inaequidens* as compared to the native *J. vulgaris*. This increase is mainly due to stronger correlations between organismal groups. Less prominent changes were observed in the intra-organismal groups correlations (among bacteria, among fungi and among plant-parasitic nematodes). Moisture content, OM and pH also contributed to the observed network tightening in *S. inaequidens*.

Discussion

Comparison of the impact of a native and a non-native ragwort species, *J. vulgaris* and *S. inaequidens* respectively, on rhizosphere soil biota revealed subtle differences in the primary decomposer communities. Slightly stronger effects were observed among representatives of the next trophic level, bacterivorous and fungivorous nematodes. As compared to the differential impact of closely related indigenous and exotic ragwort species, plant developmental stage effects were more conspicuous. Not unexpectedly, the overall bacterial and fungal densities decreased with plant development, but only a few individual primary decomposer constituents were responsible for this decrease. This subset was similar for both the native tansy and the non-native narrow-leaved ragwort.

Plant species effects

Both above and below-ground secondary metabolites play a major role in the selection of attracting and repelling microbiota. Senecioneae constitute an extremely species-rich tribe with the plant family Asteraceae. A subset within this tribe, including the two ragwort species *S. inaequidens* and *J. vulgaris*, produce pyrrolizidine alkaloids (PA's). PA's are fairly general defense compounds against herbivory, produced by members of various plant families. Among the Senecioneae, the basic structure of PA, senecionine N-oxide (SO) is synthesized in the roots. A major part of the SO is transported to above-ground plant parts via the phloem and transformed into a wide range of alkaloid compounds. *S. inaequidens* and *J. vulgaris* can each be characterized by a qualitatively and quantitatively distinct set of PA's (Hartmann and Dierich 1998).

Rhizosphere community analyses showed that PAs in ragwort do not only play a role in protection against herbivory, but they also affect fungal assemblages just outside the plant root. Populations of *J. vulgaris* were shown to differ in their PA contents, and chemotypes with a high PA content displayed a lower fungal

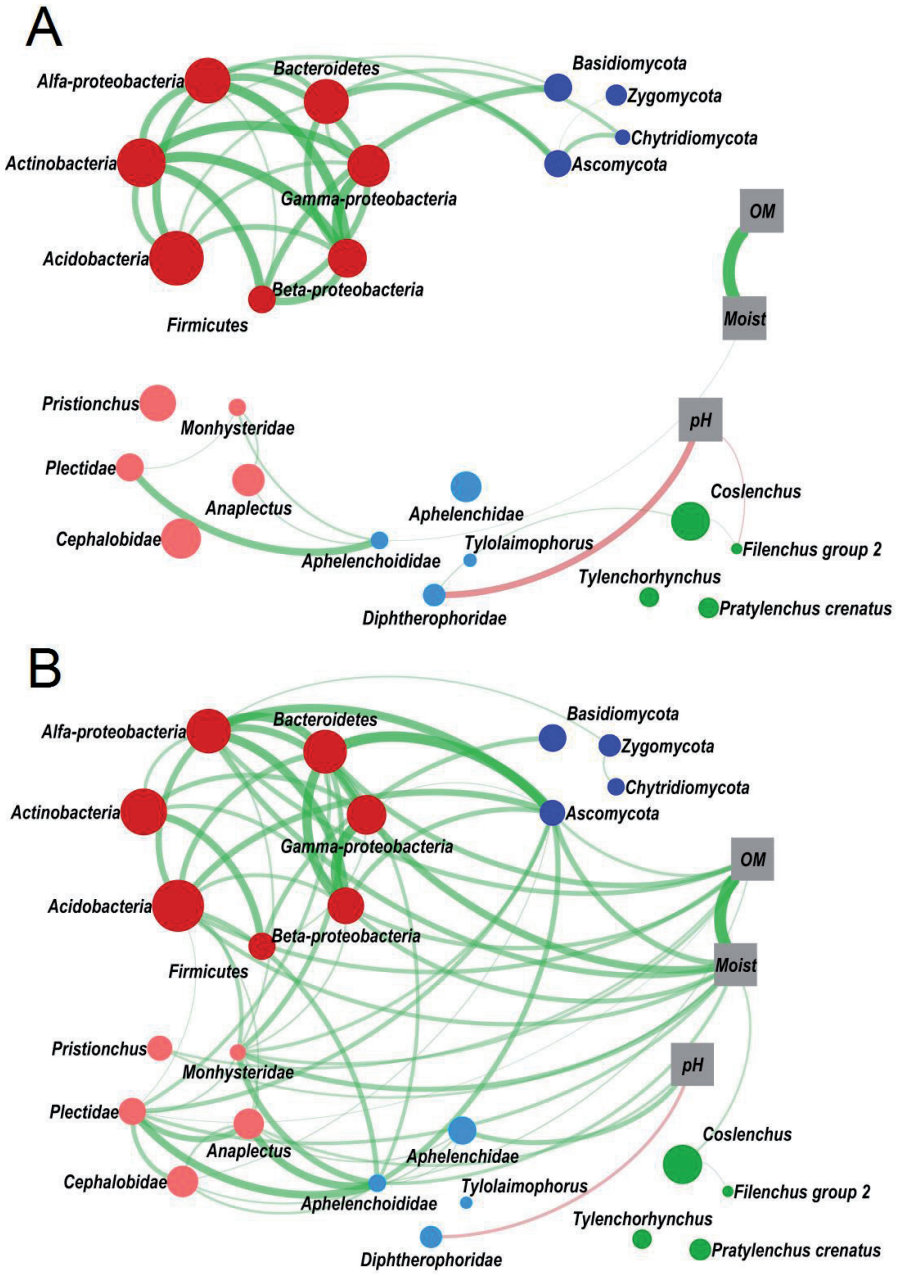

Figure 4: Network visualization. Interaction strength between the two species (A) *Jacobaea vulgaris* and (B) *Senecio inaequidens*. Spearman's rank correlations of the relative abundances of all measured components (bacteria, fungi, nematodes and abiotic measurements) were calculated. Positive and negative correlations between components of Spearman's rank ≥ 0.5 were visualized. Line color and transparency is proportional to the interaction strength. The size of the nodules is proportional to the abundance. Red filled nodes are bacterial groups, blue filled nodes are fungal groups, pink filled nodes are bacterivorous nematodes, light blue filled nodes are fungivorous nematodes and green filled nodes are plant parasitic nematodes.

diversity in their rhizosphere (Kowalchuk et al. 2006). The impact of PAs on several root-associated fungi was tested, and for most strains growth was negatively affected. However, for some fungi isolated from *J. vulgaris* roots growth stimulation was observed (Hol and Van Veen 2002). Hence, PAs in the rhizosphere mediate the formation of a plant-species specific fungal community surrounding ragwort roots. Little is known about the effect of PAs on bacterial communities under field conditions (Joosten and van Veen 2011). *In vitro* studies show an inhibition of various bacterial species in the presence of different PAs plant extracts suggesting an anti-bacterial effect (Jain et al. 1987; Marquina et al. 1989; Singh et al. 2002). It should be noted that these PAs were extracted from *Heliotropium subulatum*, a PA-producing plant species from yet another family (Boraginaceae).

As compared to the native *J. vulgaris*, our results showed a decrease of Actinobacteria and Bacteroidetes in the rhizosphere of the exotic *S. inaequidens*. If we hypothesize that the PA levels and composition in the rhizosphere are underlying this phenomenon, alkaloids present in substantial quantities in *S. inaequidens*, and absent in *J. vulgaris* could be responsible. Two alkaloids, retrorsine and florosenine, fulfil this requirement (Hartmann and Dierich 1998), and could be an explanation for the observed shift in the rhizosphere bacterial community.

From literature on the interaction between plant-parasitic nematodes and a range of *Senecio* the relevance of the intimacy of the interaction becomes clear. Although ample symptoms of infection can be found, root knot nematodes are unable to reproduce on *Senecio* (11 species including *Senecio jacobaea* (= *Jacobaea vulgaris*)). Contrary to these sedentary endoparasites, migratory endoparasites such as *Pratylenchus penetrans* and *Ditylenchus dipsaci* could parasitize and multiply well on various ragwort species (Thoden and Boppré 2010; Thoden et al. 2009). In another study, the impact of various PAs on the mobility of both free living and plant parasitic nematodes was tested (Thoden et al. 2009). It was remarkable to see that although mobility was 50% reduced at concentrations around 3 mg/ml, both cyst and root knot nematode were able to quickly recover. The effect of senecionine was not tested on free-living nematodes *Rhabditis* sp. and *Phasmarhabditis hermaphrodita*. However, other related N-oxides such as monocrotaline were shown to affect mobility of these free-living species at high concentrations only. These scattered observations are largely in line with our results. Despite differences in PA contents, densities of five different, migratory ecto- or endoparasitic nematodes in the rhizosphere were identical in both plant species. The same holds essentially for the four fungivorous taxa. Most striking was the decrease of two bacterivorous genera (*Anaplectus* and *Pristionchus*) and the family Monhysteridae. This decrease could be explained by a direct repellant effect of *S. inaequidens*-specific PAs on these nematodes. Alternatively, this decrease could be a reflection of the decrease

in density of the Actinobacteria and the Bacteroidetes. It should be noted that a preference of these bacterivorous taxa for members of these bacterial divisions has not been demonstrated. The alternative, top-down control, would require taxon-specific predation by *e.g.* nematophagous mites or predaceous nematodes. To the best of out knowledge, such a phenomenon has not been reported so far.

Plant life stage

Carbon (C) release in the rhizosphere in the form of carbohydrates, amino acids and organic acids is strongly life-stage dependent: in vegetative life stages up to 20% of the photosynthetically-fixed C is released by the roots, whereas in generative life stage the stream of metabolites is re-directed towards the shoots, in particular towards reproductive plant organs (*e.g.* Hutsch et al. 2002). In both ragwort species, a decrease in organic matter content was observed. Hence, the observed general decrease in bacterial and fungal DNA in the rhizosphere of both ragwort species is congruent with the change in translocation of metabolites that generally accompanies generative growth.

In *Arabidopsis thaliana*, plant life stage was shown to be a major determining factor with regard to a part of the rhizosphere microbial community. Four bacterial divisions responded to plant developmental stage, namely: Acidobacteria, Actinobacteria, Bacteroidetes, and Cyanobacteria. For example, relatively high level Actinobacteria were associated with vegetative life stages, whereas sexual life stages were accompanied by an increase of the Bacteroidetes (Chaparro et al. 2014). Other major bacterial groups were unaffected by plant development. A very similar phenomenon was observed for the two ragwort species under investigation here: Actinobacteria, α and β-Proteobacteria, and Bacteroidetes responded to plant developmental stage, whereas the other phyla did not. It is noted that Cyanobacteria were not included in our study. Moreover Chaporro and co-workers (2014) treated the Proteobacteria as a single group, whereas the α, β, and γ subdivisions were determined individually here. Hence, for the above-mentioned plant species, only a subset of the rhizosphere bacterial community was affected by plant development, and apparently the composition of this subset is plant-species dependent.

Most likely, the observed effect of ragwort life stage on the rhizosphere microbial community is determined by the blend of phytochemicals released by the plant during these life stages. Although detailed analyses have been published in changes PA composition in flowers and leaves during the growing season for *J. vulgaris* (Carvalho et al. 2014), no data could be found on the impact of life stage on the release of PAs in the rhizosphere of *J. vulgaris* and *S. inaequidens*. During development of a related member of the Senecioneae, *Senecio vulgaris* (common name: groundsel), the PAs concentration in the roots was more or less constant at

around 0.17 mg per g fresh weight (fw). Only in the S4 stage (senescent plant) the concentration dropped to about 0.09 mg per g fw. In the senescent stage, the PA concentration in the roots decreased relatively fast (Hartmann and Zimmer 1986). Hence, as compared to above-ground vegetative plant parts, *S. vulgaris* invests notably in a high and stable PA concentration in its root system till senescence. We hypothesize this might hold for the two ragwort species under investigation here as well. Based in this information, we are inclined to suggest that the more pronounced reduction of several microbial groups, such as Actinobacteria, α-Proteobacteria and Ascomycota, relates to qualitative differences in PA blend released by older *J. vulgaris* as compared to *S. inaequidens* plants. An alternative hypothesis explaining the more distinct decrease of microbial groups in case of *S. inaequidens* relates to its life cycle. While *J. vulgaris* is a biennial plant, *S. inaequidens* is a perennial. Hence, there is a longer period for *S. ineaquidens* to establish its own microbial community. However, additional experimentation would be required to disentangle the effects plant stage and phytochemical nature.

Remarkably, a significant decrease of the overall nematode density in the rhizosphere was observed for *S. inaequidens* only. This decrease can be attributed mainly to a decrease of the Cephalobidae. In sandy soils, this bacterivorous family is often numerically dominant. Based on our data, it is tempting to suggest the Acidobacteria, the γ-Proteobacteria or the Firmicutes are not the preferred food source of the Cephalobidae.

Concerning fungi and their nematode grazers, an observed correlation between Ascomycota and fungivorous Aphelenchoididae ($r= 0.43$ $P< 0.01$) suggest that it is more likely for Aphelenchoididae to have a trophic interaction with Ascomycota than with Basidiomycota or Zygomycota. The only supporting literature on this subject is a food experiment performed by Ruess et al. (1999). After seven days, *Aphelenchoides* preferred the food compartment containing an ascomycete over the option of a basidiomycete. In this way, plant-induced local shifts in primary decomposers communities in combination with quantitative data on their main consumers can be used to formulate hypotheses about trophic preferences of bacterivorous and fungivorous nematodes taxa.

Network structure

Although only 6 out of the 28 biotic parameters were shown to be significantly different between the two Senecioneae species, co-occurrence networks of the two related plant species were remarkably distinct. Network visualization showed that *S. inaequidens* has an increased connectedness between the organismal groups (bacteria, fungi, nematodes and abiotic components) as compared to *J. vulgaris*. Differences in the level of connectedness within the organismal groups were less obvious.

Morriën et al. (2017) recently showed an increased tightening of the networks of soil biota in later successional stages. Declined nutrient availability was suggested as one of the explanation for the observed phenomenon. The ragwort species sampled in this study were co-occurring in all sampling sites (16 m^2 each, two sampling sites per location). The very close physical distance between the two ragwort species in each of these sites makes site-specific differences in overall soil nutrient status unlikely. A species-specific difference nutrient cycling could underlie the observed tightening of the nutritional network. Being a perennial, *S. inaequidens* could have lowered the nutrient availability around its roots to a stronger extent than its biennial relative *J. vulgaris*. This might be an explanation for the higher level of inter-organismal group connectivity for *S. inaequidens*.

This visualization suggests that an invasive species has a rhizosphere network structure that is more attuned. This is in line with the prevailing hypothesis that invasive plant species are well capable of altering the soil positive feedback in their benefit (Klironomos 2002; Agrawal et al. 2005). However, keeping in mind the distinct life strategies of the two ragwort species, no strong statements can be made in this respect.

qPCR assays for the detection of shifts in bacterial and fungal taxa

Monitoring of changes in primary decomposer communities at high taxonomic level can be remarkably informative (De Gregoris et al. 2011; Turner et al. 2013). Quantitative PCR is a relatively simple and accurate approach to pinpoint shifts in primary decomposer communities in soil, and a series of qPCR assays that can be run under the same temperature profile was selected and/or developed. Seven bacterial and four fungal phyla were targeted, and we are planning to extend this set with a few other ecologically-relevant phyla such as Cyanobacteria, Verrucomicrobia, Gemmatimonadetes, Planctomycetes, and Glomeromycota. It should be noted that densities of primary decomposers were expressed as amounts of DNA per gram of soil. Whereas translation of DNA into bacterial biomass is relatively straightforward for bacteria, it is not for fungi. Fungal cells may show variation in number of rDNA gene copies, nuclei may be distributed highly unevenly over fungal tissues, and numbers of nuclei may fluctuate between fungal cells (Packter 1995; Yuill 1950). Despite of these reservations, Tellenbach and coworkers (2010) stated that there is no better method than qPCR to quantitatively characterize fungal communities. Hence, calibration curves defining the relation between Ct value and DNA concentration should be read as such. Translation of DNA concentrations into fungal biomass non-trivial, and observed shifts cannot be directly translated into changes in biomass. It is also noted that calibration curves presented in Table 3 are by definition approximations based on a limited number of representatives only.

This might be one of the reasons why the sum of the DNA levels shown in Fig. 2 adds up to only 40 to 52% for each soil. These limitations need to be appreciated when using this approach for the assessment of microbial community structure.

Conclusions

In this study it is shown that closely related native and exotic plant species may cause differential shifts at high taxonomic level in the microbial rhizosphere community, and these changes are reflected in the next trophic level. In a comparison between rhizosphere communities of the native *J. vulgaris* and the exotic *S. inaequidens* subtle difference were observed. Plant developmental stage appeared to be a more dominant determinant of the rhizosphere community. In next steps, a high throughput sequencing approach will be used to pinpoint the nature of the observed quantitative shifts. This could also generate more detailed insight in the trophic relationship between major consumers of the primary decomposer community, bacterivorous and fungivorous nematode taxa, and specific parts of this microbial community.

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Supplementary information

Supplementary information can be found on http://www.oikosjournal.org/appendix/oik-04530

Table A1: Floristic composition of semi-natural grasslands. 1: present, D: dominant and D! very dominant.

Table A2: Overview of all primer combination selected from literature used to quantitatively characterize bacterial and fungal rhizosphere communities. Specificity of bacterial primer combinations is based on taxon specific 16S ribosomal DNA motifs. Fungal primers are based on taxon-characteristic internal transcribed spacer (ITS) regions, occasionally combined with a more generic 5.8S forward or reverse primer.

Table A3: Specificity of the selected primers (Ct values) tested at T_a = 60°C. Yellow boxes indicate the value of the reaction of the primer with the corresponding DNA and should contain a reasonably lower Ct values compared to non-corresponding DNA samples (white).

Fig. A4: Procedure followed to determine annealing characteristics and specificity of bacterial or fungal primer combinations. A. Taxon-specific primers with an annealing temperature (T_a) around 60°C were selected from literature. B. Annealing characteristics of primer combinations were tested at various temperatures. C. Specificity of the two primer combinations was tested with target DNA, as well as seven non-targets DNAs (and MilliQ water as negative control). In red: ΔCt between target and closest non-target below 10 cycles. D. The linear relationship between Ct value and the concentration of target DNA for primer combination α1 at a T_m of 60°C.

Table A5: Abiotic characteristics of rhizosphere-enriched soil from *Jacobaea vulgaris* and *Senecio inaequidens*.

Table A6: Ct values of nematode taxa tested with qPCR to determine the abundance of 32 nematode taxa at the three locations. From this biodiversity check, the final 15 primersets are chosen based on their appearance and abundance at the three locations and are displayed in bold.

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CHAPTER 3

The habitat- and season-independent increase in fungal biomass induced by the invasive giant goldenrod is asymmetrically reflected in the fungivorous nematode community

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Abstract

Giant goldenrod (*Solidago gigantea*), an invasive perennial herb, has previously been shown to locally induce an increase in fungal biomass. We investigated whether this phenomenon is structural by comparing multiple locations, growth seasons and years. Moreover, we examined how fungal community manipulation is reflected in the next trophic level. To this end, composite soil samples were collected from 52 plot-pairs from eight different locations characterized by sandy or river clay soils. Both biochemical and DNA-based markers were used to investigate quantitative effects of giant goldenrod on the fungal community, and on fungivorous nematode lineages. Ergosterol measurements demonstrated a local increase of fungal biomass in both habitat types. Real-time PCR assays detecting fungal DNA as well as an Illumina-based characterization of fungal communities did not reveal overall quantitative differences between invaded and un-invaded plots. This apparent discrepancy might be attributable to a local change in DNA:biomass ratio. Further investigation about the nature of the giant goldenrod-induced shift in the fungal community revealed an increased abundance of the fungal families Cladosporiaceae and Glomeraceae paralleled by a decrease in Cucurbitariaceae. This shift was reflected in the next trophic level; fungivorous nematode lineages Aphelenchidae and Aphelenchoididae were elevated in plots invaded by *S. gigantea*. Hence, invasive giant goldenrod causes a local increase in fungal biomass that is not accompanied by a similar increase in fungal DNA, and fungal families underlying this shift could be identified. Outside its native range, giant goldenrod can manipulate locally the soil fungal community. It remains to be determined whether this manipulation has contributed to the invasiveness of this plant species.

Introduction

Deliberately or by accident, humans have been transferring plants all over the world for centuries. Most of the time, introduced plants will not invade native ecosystems because they are sub optimally equipped for the new environment. Exotic plants are considered naturalised once they are able to sustain self-replacing populations for at least ten years in their new non-native growth area (Pyšek et al. 2004). A subset of naturalised plants is able to spread widely and may reach high densities in their new environment. Such plants are referred to as invasive plants (Pyšek et al. 2004; Richardson et al. 2000) and can have a major impact on the invaded ecosystem's structure and processes (Vilà et al. 2011).

Aboveground observations have often shown that invasive plants induce a decrease in species richness of the native plant community (Hejda et al. 2009). Belowground, invasive plants can change physical conditions and the composition of soil biota. Japanese barberry (*Berberis tunbergii*) is an example of an invasive shrub that changed the local soil function due to its easily degradable litter, which has a high nitrogen content (Ehrenfeld et al. 2001). A second example is the Australian legume *Acacia dealbata* that forms densely patches in Northwestern Spain. In various ecosystems, the presence of this invasive plant resulted in a local increase in N, exchangeable P and overall organic matter content (Lorenzo et al. 2010). Exotic plant species have also been reported to induce changes in soil microbial community. *Chromolaena odorata*, a perennial herb from Mexico that became highly invasive in China, gave rise to a local increase in fungal biomass (Xiao et al. 2014). Comparable changes were observed for *Solidago gigantea* and *Solidago canadensis*, two *Solidago* species from Northern America that established throughout Europe and Asia. *S. gigantea* was shown to boost the local fungal community (Quist et al. 2014), whereas *S. canadensis* was demonstrated to induce a qualitative change in the local soil fungal community (Wang et al. 2018).

Invasive plants may even negatively affect the soil biological conditions for the native plant community, rendering the restoration of the original vegetation more difficult. The non-mycorrhizal *Brassica nigra* (black mustard), is invasive in North America and was shown to negatively affect mycorrhizal symbiosis. Thereby, making it more difficult for mycorrhizal plants – the vast majority – to establish in its vicinity (Pakpour and Klironomos 2015). Similarly, the non-mycorrhizal garlic mustard (*Alliaria petiolata*), an invasive species in North American forests, has a strong negative effect on native mycorrhizal communities, whereas in its native range (Europa) this effect is much milder (Callaway and Ridenour 2004).On the contrary, the mycorrhizal *S. canadensis* releases secondary metabolites in the rhizosphere that promote the growth of its own arbuscular mycorrhiza in the invaded area (Yuan et al. 2014).

To study belowground effects of invasive plants, it is advantageous to select rhizomatous perennial herbs. Rhizomes are subterranean stem parts that give rise to new stems. This mode a vegetative reproduction gives rise to dense, genetically uniform stands. Perennials are preferred as shifts in microbial communities might accumulate over years (Harkes et al. 2017). Giant goldenrod (*S. gigantea*; Asteraceae) is rhizomatous perennial herb native to North America (Weber and Jakobs 2005). After its introduction in Europe as an ornamental in the 18th century (Weber 1998), it became a widespread invasive plant. *S. gigantea* can survive under a broad range of light intensities, soil moistures, temperatures, nutrient conditions and pH (Vanderhoeven et al. 2006). In its natural range, *S. gigantea* is colonized by mycorrhizal fungi (Wardle et al. 2004). Zubek et al. (2016) showed that giant goldenrod interacts with AMF outside its native range, and the mycorrhizal frequency was higher in invaded as compared to neighbouring non-invaded plots. Being a rhizomatous perennial herb that forms well-nigh monoculture stands in various habitats, giant goldenrod is an auspicious species to study the effect of invasive plants on soil biota.

In previous studies on the belowground effects of the invasive giant goldenrod, a local increase in the overall fungal biomass was detected, both in a mesocosm experiment (Scharfy et al. 2010), and under semi-natural conditions (Quist et al. 2014; Stefanowicz et al. 2016). The total fungal biomass was assessed by ergosterol, a biochemical marker for higher fungi, or by PLFA 18:2ω6. Ergosterol is a valid marker for major fungal groups such as Ascomycota and Basidiomycota, but is should be noted that some fungal groups such as the Glomeromycota and the Chytridiomycota lack this sterol in their cell membranes (see *e.g.* Weete et al. (2010)).

Invasive plant-induced changes in the fungal community might be mirrored among fungivorous metazoan commity. Fungivorous nematodes are informative in this context as they are present at high densities in nearly any soil habitats, and as their ability to feed on fungi arose multiple times independently (Holterman et al. 2017) resulting in lineages with distinct preferences (Baynes et al. 2012; Okada and Kadota 2003). Previously, it was shown that a giant goldenrod-induced boost in fungal biomass was translated into an increase of a subset of the fungivorous nematode lineages (Quist et al. 2014).

The relevance of soil type and location for the impact of *S. gigantea* on fungal biomass was underlined by (Stefanowicz et al. 2016). They investigated 16 *S. gigantea*-invaded sites with two adjacent paired-plots (2 m x 2 m) at each site either in or outside a river valley. A local increase of fungal phospholipid fatty acids (PLFA) was observed in the *S. gigantea*-invaded plots, and this effect was more prominent in areas next to the river – directly exposed to fluvial processes – than in the areas just outside the river valley.

Here we investigated the impact of invasive *S. gigantea* on local fungal communities in more detail. First, we verified whether the *S. gigantea*-induced increase in fungal biomass was transient or long lasting. Therefore, soil samples were collected at the end of the growing season (November), whereas Quist et al. (2014) mapped this phenomenon in September, and Stefanowicz et al. (2016) in August. Second, ribosomal DNA-based markers were used next to ergosterol to characterize changes in the fungal community. The use of two independent markers for fungal biomass could provide a more solid basis for our findings, and fungal division-specific markers would allow us to characterize the impact qualitatively. Whereas the biomass marker ergosterol pointed at a stimulation of at least a major part of the fungal community, a general rDNA marker for fungi as well as markers for major constituents of the fungal community showed no effect of the presence of invasive *S. gigantea*. To further investigate these apparently contradictory results, DNA amplicons were sequenced in order to investigate which fungal family was indicative for invaded plots. In addition, we checked whether representatives of the next trophic level, fungivorous nematodes, were affected. Two out of the three nematode lineages present on these sites were stimulated in the presence giant goldenrod. Possible explanations for these interesting but paradoxical results are discussed.

Material and methods

Sampling sites

The belowground *S. gigantea* invasion effects were examined at eight sites in the Netherlands, located in either of the distinct semi-natural habitats, namely riparian zones (rive clay soil) and semi-natural grasslands (sandy soil). To allow for a comparison with results presented by Quist et al. (2014), the same sampling sites were used. 'Millingerwaard', 'Ewijkse Plaat' and 'Blauwe Kamer' were the selected riparian zone sites (Table 1). The other five sites, 'Dennenkamp', 'Plantage Willem III', 'Hollandseweg', 'Scheidingslaan' and 'Reijerscamp', are located in semi-natural grassland habitats on Pleistocene sandy soils (Table 1). For all invaded plots, the coverage by *S. gigantea* was scored as a 9 on a modified Braun-Blanquet scale (Barkman et al. 1964; Leps and Hadincova 1992) implying a 75–100% coverage. Non-invaded plots were dominated by native plant species and fell in category 2 which means that at most 2–5 *S. gigantea* were found in the control plots. More information on the floristic composition of these sites can be found in Quist et al. (2014).

Soil sampling

In total, 104 composite soil samples were collected from 52 plot-pairs in November 2014. Eight plot-pairs were selected per site for Millingerwaard, Ewijkse Plaat, Blauwe Kamer, Dennenkamp and Plantage Willem III. Four plot-pairs were sampled at the sites Hollandseweg, Scheidingslaan and Reijerscamp due to limited number of *S. gigantea* patches at these sites (see also Supplementary Table 1). Each plot-pair consisted of two directly neighbouring 2 x 2 m plots to minimize possible differences in soil type and structure. To average microscale variation, 12 soil cores (depth: 25 cm, ø 1.5 cm) were randomly collected within each plot and mixed thoroughly. Sampling material was thoroughly cleaned between plot-pairs in order to limit cross contamination. At the day of sample collection composite soil samples were split into two subsamples (200 g and 5 g). The 200 g subsample was stored at 4°C for subsequent nematode extraction (100 g) and the determination of abiotic soil characteristics (60 g). Nematodes were extracted within one week after sample collection. The other subsamples (5 g) were stored at -20°C to prevent DNA degradation prior to total DNA extraction, which was completed within three weeks after sample collection.

Abiotic soil characteristics

Per composite sample, subsamples were taken for the analysis of abiotic and biotic soil characteristics. Moisture content, pH, organic matter (OM) content, total carbon (C) content, total nitrogen (N) content and C:N ratio were determined. The total amount of C and N, determined with a composite sample of invaded and a composite sample of uninvaded plots per sampling site, was performed by BLGG AgroXpertus (Wageningen, The Netherlands).

Soil moisture content was measured per sample by determining the weight loss after 20 hours at 105°C. Dried soil was sieved with a mesh of 2 mm and 10 g was added to 25 ml demineralized water for soil pH measurements using a gel-electrolyte electrode (Sentix 21, WTW, Weilheim, Germany). Organic matter content was determined by measuring weight loss of 20 g of sieved soil after 5 hours at 550°C.

Fungal and bacterial extraction and community analysis

Fungal and bacterial DNA was extracted from 0.25 g subsamples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). Slight changes were made to the manufacturer's protocol. PowerBead Tubes were placed in a Qiagen Tissue Lyser for 7 minutes instead of 10 minutes to compensate for the high shaking frequency (30 Hz), and 20 µl of internal control DNA (20.6 ng/µl calf thymus DNA) was added to each sample in order to monitor DNA losses

during extraction and purification. To further reduce the impact of soil-derived PCR-inhibiting components, purified lysates were diluted 100 times. Diluted samples were stored at 4°C until further use. Undiluted purified samples were stored at -20°C. Microbial communities were analysed using real time PCR assays targeting total fungi (ITS1F/5.8 s), total bacteria (16S ribosomal RNA) as well as three fungal phyla: Ascomycota, Basidiomycota and Chytridiomycota (based on taxon-characteristic ITS regions). PCR primers, PCR conditions, and slope and intercept values describing the relationship between Ct-values and concentration of target bacterial or fungal DNA (ng/µl) are essentially according to Harkes et al. (2017) and details can be found in Supplementary Table 2.

Ergosterol measurements

Ergosterol, a biochemical marker for higher fungi frequently used in soil ecology, was extracted from 1 g of soil using the alkaline extraction method as described by Bååth (2001). In a mixture alkaline methanol and cyclohexane, ergosterol accumulated in the cyclohexane phase. After phase separation, the cyclohexane was removed by evaporation, and ergosterol is re-dissolved in methanol. Subsequently, high-performance liquid chromatography (HPLC) with photodiode array detection (peak identification is based on retention time and UV-spectrum) was used to separate and quantify the ergosterol contents of the samples as described by De Ridder-Duine et al. (2006).

Nematode extraction and community analysis

Per composite sample, nematodes were extracted from a 100g subsample using an Oostenbrink elutriator (Oostenbrink 1960). DNA extractions of the total nematode suspensions were performed as described by Vervoort et al. (2012). At the start of this extraction procedure, 25 µl of calf thymus DNA (20.6 ng/µl) was added to each sample to be able to quantify DNA loss after extraction and purification. After purification, each sample was diluted 10 times and stored at -20°C until further use. Diluted DNA extracts served as a template for the real time PCR-based determination of the total nematode density and the densities of the three major fungivorous nematode lineages present on these locations, Aphelenchidae, Aphelenchoididae and *Diphtherophora*. This qPCR detection method is based on taxon-specific SSU rDNA sequence motifs as previously described by Vervoort et al. (2012).

Because of the substantial variation estimation in rDNA copy numbers in fungi, using only the ITS marker might not suffice in all fungal clades, therefore two

qPCR assays for single-copy protein coding genes were included in this study (beta-tubulin (tub2) and translation elongation factor 1-alpha (tef1)) as they are supposed to be less viarable and occure as a single copy in fungi (Raja et al. 2017).

PCR Amplification and sequencing of fungal 16S rDNA

The variable V7-V8 of fungal 18S was utilized as a target for the analyses of Illumina 18S rDNA sequencing. To prepare the samples for sequencing a twostep PCR procedure was followed as described in Harkes et al. (2019). In brief, a locus-specific primer combination extended with an Illumina read area and the appropriate adapter were used to produce primary amplicons – in triplicate for all samples. PCR 2 was conducted on 40x diluted amplicons of PCR1 to attach the Illumina index and the Illumina sequencing adaptor. Randomly picked products of PCR 1 and 2 were checked on gel to ensure amplification was successful. Finally, all PCR products were pooled and sent for sequencing. Sequencing was done at Bioscience – Wageningen Research, Wageningen, The Netherlands – using the Illumina MiSeq Desktop Sequencer (2*250nt paired-end sequencing) according to the standard protocols. The raw sequences were submitted to the NCBI Sequence Read Archive (SRA) database under study accession numbers PRJNA563313.

Combined analysis of abiotic characteristics and quantitative biotic data

The impact of *S. gigantea* invasion on abiotic soil properties and the densities of nematodes, fungi and bacteria was analysed by using mixed linear models (PROC MIXED, SAS software system version 9.2, see Littell et al. (2006). When residuals did not approximate normal distributions, transformed data were used. OM, total C, total N, nematode densities and densities of fungi and bacteria were log-transformed. A constant of 0.1 was added prior to the log-transformation to bypass any zero values. This was done for Aphelenchidae, Aphelenchoididae, *Diphtherophora* and Chytridiomycota.

A split-plot design was used for all ten study sites, with sampling sites forming the main plots, associated with the factor habitat type (riparian vegetation or semi-natural grassland), with multiple plot pairs (8 or 4) per site, and two subplots per plot pair, associated with the factor plant invasion. This design was represented in the mixed models with random effects for sites, plot-pairs and individual plots, forming the random part of the model. Main effects of habitat type, invasion and the interaction between both factors formed the fixed part of the model. Random effects for site, plot-pairs and individual plots formed the random part of the model. In this way, the total error variance was split into variance components for sites, plot-pairs within sites and for individual plots within plot-pairs. Regarding

pH, the mixed model took into account that variances were different for riparian vegetation habitats and semi-natural grasslands (as was noticed from residual plots). Hypothesis tests (with F-test statistics) for the significance of the main effects of habitat type, invasion and their interaction on the soil variables were performed. P-values < 0.05 were considered significant. Regardless the outcome of hypothesis tests on interaction and main effects, comparisons between invaded and uninvaded plots were made per habitat type, using F-tests. The results were presented as (back transformed) 95% confidence intervals for the estimated mean responses of the soil variables (obtained from 'least squares means' outputs) in invaded and un-invaded plots per habitat type. Moreover, invasion impacts on soil variables were presented as ratios between estimated means of invaded plots and un-invaded plots.

Bioinformatics framework and statistics

The composition of the fungal communities of the soil samples was analysed based on the sequencing data obtained from the Illumina MiSeq platform. Reads were sorted into the experimental samples according to their index combination, quality trimmed by BBDUK and then merged via VSEARCH (Bushnell 2018; Rognes et al. 2016). Unique sequences were then clustered at 97% similarity by using the usearch_global method implemented in VSEARCH and a representative consensus sequence per *de novo* OTU was determined (Rognes et al. 2016). The clustering algorithm also performs chimera filtering to discard likely chimeric OTUs with UCHIME algorithm in *de novo* mode (Edgar et al. 2011) implemented in VSEARCH. Sequences that passed quality filtering were then mapped to a set of representative consensus sequences to generate an OTU abundance table. Representative OTU sequences were assigned to a taxonomic classification via BLAST against the Silva database (version 12.8). Sequences not belonging to fungi were discarded from the 18S fungal dataset. Low-abundance OTUs (those with an abundance of <0.005% in the total data set) were discarded (Bokulich et al. 2013) prior to analysis. Samples were transformed using Hellinger transformation for all downstream analyses.

To investigate the indicator taxa involved in the differences in fungal communities between invasive and un-invasive, a linear discriminate analysis (LDA) effect size (LEfSe) was conducted in Microbiome Analyst (Dhariwal et al. 2017) to explore the differential microbial populations at family level (Segata et al. 2011). A significance level of $\alpha \leq 0.05$ was used in this study.

Results

Changes in abiotic soil characteristics upon *S. gigantea* invasion

To gain insight in the abiotic environment of the *S. gigantea*-invaded sites, the soil moisture content, pH, OM content, total C content, total N content and C:N ratio were analysed. Significant changes were observed in soil moisture content, pH and OM content between *S. gigantea*-invaded and un-invaded plots in riparian and semi-natural grasslands sites (Table 1). In contrast, no differences were observed between invaded and un-invaded plots for the total C content, total N content and the C:N ratio (Tables 1 and 2, Fig. 1).

Plots invaded by *S. gigantea* had a lower soil moisture content than un-invaded plots ($F_{1,50}$= 6.58, P= 0.0134; Table 1, Fig. 1). This overall effect could mainly be attributed to the slightly lower moisture content of invaded plots in the riparian vegetation habitats ($F_{1,50}$= 5.79, P= 0.0199; Table 2, Fig. 1).

Riparian vegetation habitats and semi-natural grasslands differed significantly in pH ($F_{1,6}$= 92.22, P< 0.0001; Table 1). Riparian vegetation sites had a slightly alkaline soil with pH 7.5, while semi-natural grasslands had a moderately acidic soil with pH 5.6 (see Table 2 for 95% confidence intervals). Overall, no effect of invasion of soil pH was detected (Table 1, Fig. 1). Splitting by habitat type however, showed that for both types the pH was slightly lower in invaded plots, but only for riparian sites this difference was significant, due to the lower variance in riparian plots ($F_{1,50}$= 5.81, P= 0.0197; Table 2).

A significant interaction between invasion and habitat was found for OM content ($F_{1,50}$= 4.74, P= 0.0341), indicating that the effect of invasion was habitat type-dependent (Table 2). In semi-natural grasslands, *S. gigantea*-invaded plots had a higher OM content as compared to un-invaded plots ($F_{1,50}$= 8.12, P= 0.0063; Table 2, Fig. 1), whereas no difference in OM content was detected between plot-pairs at riparian sites.

Invasive *S. gigantea* increase fungal biomass, but not the total fungal DNA

Using ergosterol as a biochemical marker for biomass of higher fungi, a strong overall effect of *S. gigantea* was detected ($F_{1,48}$= 21.97, P< 0.0001; Table 1). In giant goldenrod-invaded plots, a significant increase in ergosterol levels was observed for both habitat types (Tables 1 and 2). It is noted that ergosterol is an important constituent of the cell membranes of higher fungi, and as such it correlates fairly well with fungal biomass (*e.g.* Newell and Fallon (1991). Using real time PCR assays, the total bacterial and total fungal communities were assessed, and no significant differences in fungal and bacterial DNA concentrations were observed, neither

Table 1: Main effects of habitat type, invasion and their interaction for the different abiotic and biotic variables analysed. F-test F_{df} values and corresponding P-values obtained from the mixed models are shown for each variable. Total C and N contents are expressed in g/kg dry soil. Total nematode density, Aphelenchidae, Aphelenchoididae and *Diphtherophora* are expressed in numbers (#) per 100 g dry soil. Total fungal density, Ascomycota, Basidiomycota, Chytridiomycota and total bacterial density are expressed in µg DNA per 100 g dry soil. Fungal biomass expressed as mg ergosterol kg^{-1} soil. The degrees of freedom (Crowther et al.) for *Diphtherophora* are lower than for the other variables, since this taxon was not present at two study sites (Scheidingslaan and Reijerscamp). Regarding invasion and interaction effects, the df for total C, N and C:N ratio are lower since samples were pooled together per study site. Significant P-values (< 0.05) are indicated in bold.

	Habitat type			Invasion			Habitat type * Invasion		
	F_{df}		P-value	F_{df}		P-value	F_{df}		P-value
Abiotic variables									
Moisture content (%)	$F_{1,6}$	2.02	0.2052	$F_{1,50}$	6.58	**0.0134**	$F_{1,50}$	0.93	0.3391
pH	$F_{1,6}$	92.22	**< 0.0001**	$F_{1,50}$	2.48	0.1213	$F_{1,50}$	0.19	0.6661
OM content (%)	$F_{1,6}$	0.13	0.7343	$F_{1,50}$	2.87	0.0965	$F_{1,50}$	4.74	**0.0341**
Total C (g/kg)	$F_{1,6}$	1.38	0.2848	$F_{1,6}$	0.05	0.8281	$F_{1,6}$	0.00	0.9562
Total N (g/kg)	$F_{1,6}$	0.57	0.4803	$F_{1,6}$	0.15	0.7090	$F_{1,6}$	0.60	0.4692
C:N ratio	$F_{1,6}$	0.73	0.4267	$F_{1,6}$	0.43	0.5378	$F_{1,6}$	1.00	0.3568
Biotic variables									
Total nematode density (#)	$F_{1,6}$	26.69	**0.0021**	$F_{1,50}$	0.00	0.9758	$F_{1,50}$	3.24	0.0780
Aphelenchidae (#)	$F_{1,6}$	0.02	0.8962	$F_{1,50}$	9.96	**0.0027**	$F_{1,50}$	3.16	0.0814
Aphelenchoididae (#)	$F_{1,6}$	0.65	0.4507	$F_{1,50}$	8.44	**0.0054**	$F_{1,50}$	6.92	**0.0113**
Diphtherophora (#)	$F_{1,4}$	0.08	0.7930	$F_{1,42}$	0.02	0.9007	$F_{1,42}$	0.29	0.5945
Fungal biomass (mg erg / kg)	$F_{1,6}$	3.63	0.1055	$F_{1,48}$	21.97	**< 0.0001**	$F_{1,48}$	0.72	0.3990
Fungal DNA (µg)	$F_{1,6}$	0.75	0.4184	$F_{1,50}$	2.50	0.1203	$F_{1,50}$	0.17	0.6846
Ascomycota (µg)	$F_{1,6}$	0.06	0.8187	$F_{1,50}$	3.34	0.0738	$F_{1,50}$	0.71	0.4027
Basidiomycota (µg)	$F_{1,6}$	10.83	**0.0166**	$F_{1,50}$	0.00	0.9637	$F_{1,50}$	0.01	0.9370
Chytridiomycota (µg)	$F_{1,6}$	1.07	0.3405	$F_{1,50}$	1.25	0.2693	$F_{1,50}$	0.12	0.7340
Total bacterial density (µg)	$F_{1,6}$	0.05	0.8360	$F_{1,50}$	3.29	0.0759	$F_{1,50}$	0.02	0.8826

Table 2: Estimated mean response and associated 95% confidence intervals of the soil characteristics analyzed for plots invaded and un-invaded by *S. gigantea* in two habitat types. The estimated mean response (Est. mean) and lower and upper bounds of the 95% confidence interval are shown for each variable. Values were obtained from 'least squares means' outputs of mixed models fitted to the variables. For both habitat types, riparian vegetation and semi-natural grassland, est. mean responses are shown for plots invaded and un-invaded by *S. gigantea*. Riparian vegetation habitats contained 24 plot-pairs in total, while semi-natural grasslands contained 28 plot-pairs in total. Values for OM content, total C (g/kg dry soil), total N (g/kg dry soil), Aphelenchidae, Aphelenchoididae, *Diphtherophora*, total fungi, Ascomycota, Basidiomycota, Chytridiomycota and total bacteria were back transformed from logarithmic values to the original scale. Aphelenchidae, Aphelenchoididae and *Diphtherophora* are expressed in numbers (#) per 100 g dry soil. For *Diphtherophora*, 8 plot-pairs from semi-natural grasslands were excluded from analysis. Fungal biomass expressed as mg ergosterol per kg soil. Total fungal density, Ascomycota, Basidiomycota, Chytridiomycota and total bacterial density are expressed in µg DNA per 100 g dry soil. Significant P-values (<0.05) are indicated in bold.

	Riparian vegetation (n= 24 plot-pairs)							**Semi-natural grassland (n= 28 plot-pairs)**						
	Invaded (n= 24)			**Un-invaded (n= 24)**				**Invaded (n= 28)**			**Un-invaded (n= 28)**			
	Lower	**Est. mean**	**Upper**	**Lower**	**Est. mean**	**Upper**	**P-value**	**Lower**	**Est. mean**	**Upper**	**Lower**	**Est. mean**	**Upper**	**P-value**
Abiotic variables														
Moisture content (%)	15.9	**20.7**	25.6	16.7	**21.5**	26.4	**0.0199**	12.8	**16.6**	20.4	13.2	**17.0**	20.7	0.2445
pH	7.33	**7.46**	7.59	7.37	**7.50**	7.63	**0.0197**	5.21	**5.58**	5.96	5.28	**5.65**	6.02	0.3059
OM content (%)	4.2	**5.9**	8.3	4.2	**5.9**	8.4	0.7429	4.3	**5.7**	7.4	4.0	**5.3**	6.9	**0.0063**
Total C (g/kg)	17.0	**31.2**	57.0	16.8	**30.6**	56.0	0.9181	13.9	**22.1**	35.3	13.5	**21.5**	34.4	0.8243
Total N (g/kg)	0.9	**1.7**	3.1	1.0	**1.9**	3.4	0.4895	0.9	**1.5**	2.3	0.9	**1.4**	2.2	0.7663
C:N ratio	12.9	**19.1**	25.2	10.6	**16.8**	22.9	0.3366	10.5	**15.2**	20.0	10.9	**15.7**	20.5	0.7879
Biotic variables														
Aphelenchidae (#)	0.4	**1.6**	6.0	0.1	**0.5**	2.0	**0.0015**	0.4	**1.2**	3.6	0.3	**0.9**	2.7	0.3156
Aphelenchoididae (#)	1.4	**4.3**	13.0	0.3	**1.1**	3.6	**0.0004**	1.6	**4.0**	9.8	1.5	**3.7**	9.2	0.8404
Diphtherophora (#)	0.4	**0.9**	1.9	0.4	**0.8**	1.7	0.7621	0.4	**0.9**	2.0	0.5	**1.1**	2.4	0.6564
Fungal biomass (mg erg / kg)	1.07	**1.75**	2.85	0.54	**0.88**	1.43	**0.0199**	1.74	**2.66**	3.97	1.22	**1.65**	2.76	**<.0001**
Fungal DNA (µg)	220	**299**	405	191	**259**	351	0.1814	264	**340**	438	243	**313**	403	0.3925
Ascomycota (µg)	26.1	**40.2**	61.9	19.1	**29.3**	45.2	0.0748	26.9	**38.7**	55.6	24.0	**34.5**	49.5	0.4730
Basidiomycota (µg)	1.6	**2.8**	5.1	1.6	**2.8**	5.0	0.9818	5.4	**8.8**	14.3	5.5	**9.0**	14.5	0.9270
Chytridiomycota (µg)	0.4	**0.9**	1.8	0.6	**1.2**	2.4	0.3250	0.3	**0.6**	1.2	0.4	**0.7**	1.4	0.5709
Total bacterial density (µg)	3598	**4929**	6754	3303	**4526**	6201	0.1875	3654	**4696**	6034	3399	**4367**	5612	0.2263

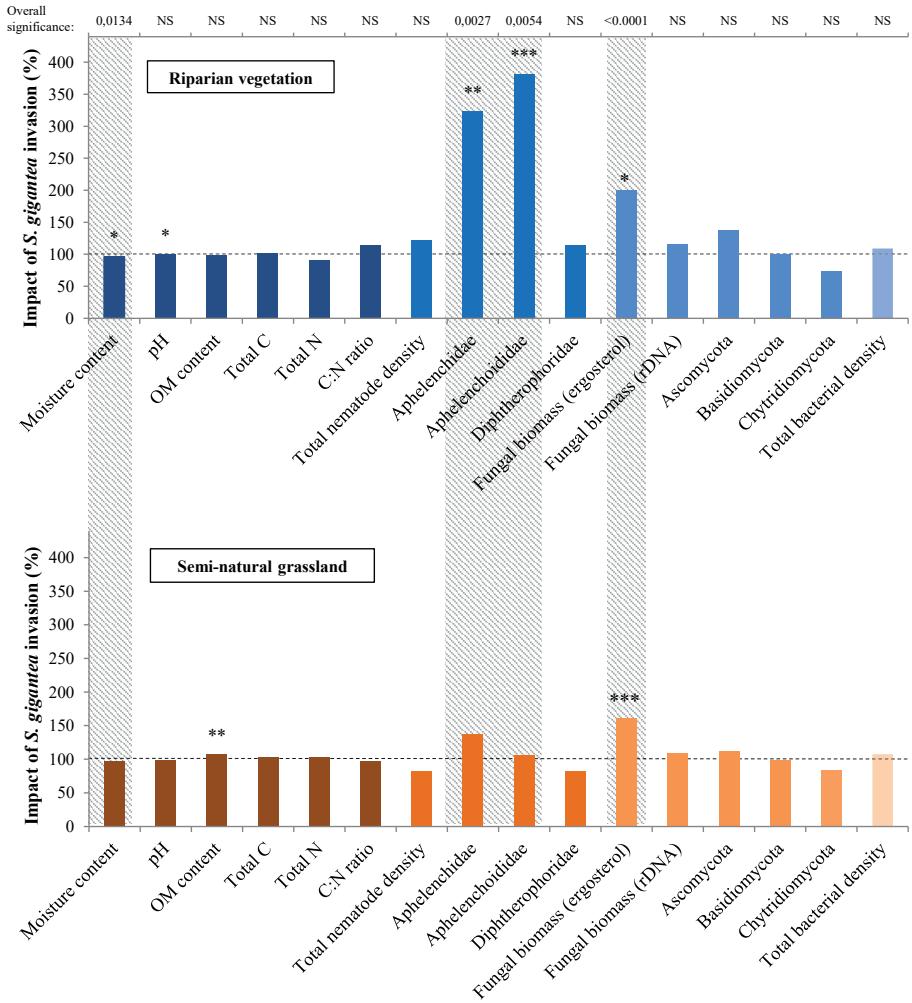

Figure 1: Impact of *S. gigantea* invasion in riparian vegetation (top) and semi-natural grassland habitat. The impact of *S. gigantea* invasion on the y-axis was calculated by dividing estimated means (Table 3) from invaded plots by estimated means from un-invaded plots and expressed as a percentage. Impacts are shown for the 6 abiotic variables, the total nematode density, densities of three fungivorous nematodes, total fungal density, densities of three fungal phyla and the total bacterial density (no invasion impact = 100%). Asterisks indicate significant differences (*P< 0.05, **P< 0.01, ***P< 0.001) between invaded and un-invaded plots per habitat type. Variables showing an overall significant invasion effect, for both habitats together, are indicated by a grey-shaded background. Corresponding P-values are shown at the top part of the figure (NS = not significant). Riparian vegetation habitats included 3 study sites and 24 plot-pairs, while semi-natural grasslands included 5 study sites and 28 plot-pairs.

between invaded and non-invaded habitats, nor between the two habitat types (Table 2). Also, the two single-copy fungal protein coding genes included in this study (tub2 and tef1) did not show any significant differences between invaded and uninvaded plots (data not shown).

Keeping in mind that ergosterol measurements predominantly reflect the presence of Ascomycota and Basidiomycota, representatives of two major distal clades within the kingdom Fungi (Weete et al. 2010), these phyla were quantified separately. In the riparian vegetation habitats, a trend was observed of Ascomycota having a higher DNA concentration in *S. gigantea*-invaded plots ($F_{1,50}$= 3.31, P= 0.0748; Table 2, Fig. 1). A similar invasion effect was observed when both habitats were analysed together ($F_{1,50}$= 3.34, P= 0.0738; Table 1. It is noted that the mean DNA concentration of Basidiomycota on sandy soils was about three times higher than the DNA concentration in the river clay soils ($F_{1,6}$= 10.83, P= 0.0166; Table 1). The DNA concentrations of Basidiomycota did not differ between invaded and un-invaded plots (Tables 1 and 2, Fig. 1). In addition, Chytridiomycota were measured, being a fungal phylum that is uses cholesterol instead of ergosterol as its major sterol, but no differences were observed between giant goldenrod-invaded and non-invaded plots. Comparison of Chytridiomycota between the two major habitats revealed no difference in DNA concentrations.

The overall bacterial DNA concentration tended to be slightly higher in *S. gigantea*-invaded plots ($F_{1,50}$= 3.29, P= 0.0759; Table 1, Fig. 1) but there were no significant effects of habitat type (Table 2, Fig. 1).

Two fungivorous nematode families benefitted from *S. gigantea*-induced increase in fungal biomass

The total nematode abundance, and density of the three fungivorous nematode taxa that were commonly present in both the Pleistocene sand and river clay locations were analysed to study the belowground impact of *S. gigantea* on the next trophic level of the soil food web. Representatives of the families Aphelenchidae, Aphelenchoididae and the genus *Diphtherophora* were used to determine whether and, if so, how the observed increase in biomass of higher fungi, and an unchanged fungal DNA levels are reflected in the local fungivorous nematode community.

Dominance by giant goldenrod did not affect the total nematode density (Table 1, Fig. 1). Total nematode density (per 100 g dry soil) only differed significantly between habitats. The riparian sites had an estimated mean nematode abundance about two times higher than in semi-natural grassland soils ($F_{1,6}$= 26.69, P= 0.0021; Table 1). Both Aphelenchidae ($F_{1,50}$= 9.96, P= 0.0027) and Aphelenchoididae ($F_{1,50}$= 8.44, P= 0.0054; Table 1, Fig. 1) were more abundant in *S. gigantea*-invaded plots than in un-invaded plots. A significant interactive effect between habitat type and invasion status ($F_{1,50}$= 6.92, P= 0.0113) was observed for Aphelenchoididae indicating that the response to invasion was dependent on habitat type (Table 2) whereas this interactive effect between habitat type and invasion status was not significant for Aphelenchidae ($F_{1,50}$= 3.16, P= 0.0814; Table 1).

For both fungivorous nematode taxa, the effect of *S. gigantea* was only seen in the riparian habitats. As compared to the un-invaded plots, Aphelenchidae densities were three times higher in invaded plots in riparian habitats ($F_{1,50}$=11.30, P= 0.0015; Table 2, Fig. 1). Similarly, the estimated densities of Aphelenchoididae were around four times higher in *S. gigantea*-invaded riparian plots as compared to the non-invaded neighbouring plots ($F_{1,50}$= 14.23, P= 0.0004; Table 2, Fig. 1). Giant goldenrod stands did not affect the abundance of representatives of the genus *Diphtherophora* (Tables 1 and 2, Fig. 1).

Fungal indicator taxa associated with invasive *S. gigantea*

Invasion by *S. gigantea* resulted in a local increase in fungal biomass, but not in total fungal DNA. This remarkable observation was investigated in more detail by comparing the composition of the communities. As a crude measure for fungal DNA content we compared the number of primary reads per sample. Whereas soil samples from uninvaded plots gave rise to ≈ 95,000 (SD 39,000) reads per samples, on average ≈ 102,000 reads (SD 37,000) were generated from samples from invaded plots. No significant difference in number of reads per sample were found between invaded and non-invaded plots. Although it is hard to compare qPCR data with Illumina reads, the sequencing data confirm the absence of a difference in fungal DNA contents between *S. gigantea*-invaded and non-invaded plots.

PERMANOVA on Bray-Curtis dissimilarity profiles identified 'habitat type' (riparian vegetation *vs.* natural grassland) as the main factor responsible for the difference in fungal composition (Table 3). This factor explained ≈ 27% of the overall variance. The second most informative variable was 'study site' with an R^2 value of 0.23. This is the variation in fungal communities between the various sampling sites within a habitat type. Against the substantial background variation caused by habitat type and study site, still a clear invasion effect could be discerned. Evidently, this effect explained a relatively low percentage of the overall variation, 1.7%, but this contribution was highly significant. As can be seen in Table 3, analyses of fungal communities for the two habitat types separately resulted in significant effects. It is noted that effect of plant invasion on fungal assemblages was more pronounced in the semi natural grasslands (P= 0.001, and P= 0.01 for riparian vegetation).

LEfSe (Linear discriminant analysis Effect Size) analysis allowed us to determine which fungal taxa contribute most to observed differences between *S. gigantea* invaded and un-invaded plots. With an LDA threshold of > 2 the families Cladosporiaceae, Teratosphaeriaceae (both Ascomycota), Glomeraceae (Glomeromycota) and Kondoaceae (Basidiomycota) were shown to be more abundant in plots invaded with *S. gigantea* (Fig. 2A). Analysis per habitat type

revealed that only the Cladosporaceae were present in higher densities in both habitat types in invaded plots (Fig. 2B and 2C). A higher abundance of members of the family Cucurbitariaceae (Ascomycota) was shown to be characteristic for the non-invaded plots.

Table 3: Summary PERMANOVAs on Bray-Curtis dissimilarity profiles of the fungal biome for the main effect and each habitat type. The effects of the following variables on the quantitative taxonomic composition of fungi were tested: Habitat type (Riparian vegetation/Semi natural grassland), Study site (RV= 3 SG= 5), Invasion (Invaded/ Uninvaded) and the interactions between Invasion and Habitat type or Study site. Differences are considered significant if P< 0.01. P = probability associated with the Pseudo F statistic. Significant P-values in bold.

	Main effect		**Riparian vegetation**		**Semi-natural grassland**	
	R^2	**P**	**R^2**	**P**	**R^2**	**P**
Habitat type	0.225	**0.0001**				
Study site	0.237	**0.0001**	0.257	**0.0001**	0.354	**0.0001**
Invasion	0.018	**0.0024**	0.037	**0.0050**	0.032	**0.0001**
Study site * Invasion	0.033	0.3964	0.032	0.4252	0.053	0.2181
Habitat * Invasion	0.009	0.0523				

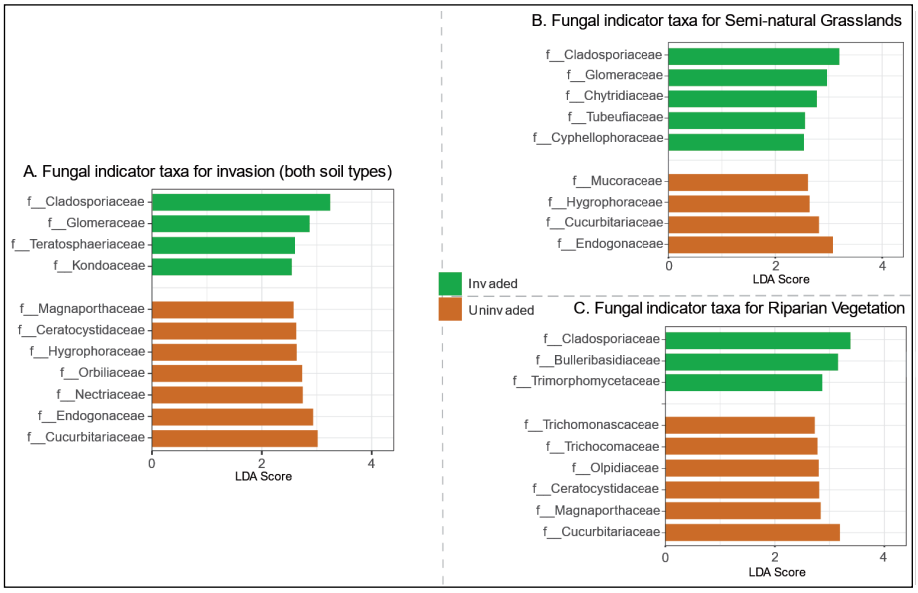

Figure 2: Discriminant fungal families indicated by LEfSe analysis (LDA threshold of 2) resulting from invaded (green) and un-invaded (brown) soils by *Solidago gigantea*.

Discussion

The aim of this research was to characterize quantitative and qualitative shifts in the fungal community brought about by the invasive plant species *S. gigantea*. To assess the impact of this invasive plant species on below-ground fungal biomass, two kinds of components of the fungal cell membranes (either ergosterol or the fatty acid 18:2ω6) have been used. Consistently, *i.e.* during multiple growth stages, over multiple years, and at multiple location invasive giant goldenrod was shown to induce a local increase in fungal biomass (Quist et al. 2014; Stefanowicz et al. 2016). However, both a qPCR-based approach and an Illumina-based characterisation of fungal communities pointed at the absence of a quantitative shift in total fungal biomass. The observed *S. gigantea*-induced local increase in fungal biomass combined with the unaltered presence of fungal DNA prompted us to suggest that invasion of *S. gigantea* locally induced an increase in fungal biomass:DNA ratio. Qualitative characterization of the fungal assemblages revealed that *S. gigantea* invasion was accompanied by a local increase in abundance of members of the families Cladosporiaceae and Glomeraceae, and a decreased presence of Cucurbitariaceae.

To further investigate these apparently contradictory results, we focussed on representatives of the next trophic level; three fungivorous nematode taxa that were commonly present in both soil types. The densities of the fungivorous Aphelenchidae and Aphelenchoididae increased in *S. gigantea*-invaded plots, while the abundance of the members of the fungivorous genus *Diphtherophora* did not change. Moreover, we suggest that distinct food preferences explain why only for two out of three commonly present fungivorous nematode lineages an increase in density was observed. Arguments underlying this interpretation of our results are presented below.

Apparent discrepancy between results from independent fungal biomass markers

Notably, the observed increase in ergosterol in *S. gigantea*-invaded plots was not accompanied by a comparable local augmentation of the total fungal DNA (Fig. 1). In case of the phylum Ascomycota, a trend towards more rDNA in invaded plots was detected (Table 2, P= 0.0748). With regard to the Basidiomycota, it is noted that the apparent low density (Table 2) might be an underestimation as relatively high rDNA copy representatives were used to generate the calibration lines (Harkes et al. 2017; Lofgren et al. 2019).

Ergosterol is a frequently used marker for the assessment of fungal biomass in soil. It is sterol found in all Ascomycota and most Basidiomycota. Several

representatives of the Zygomycota harbor ergosterol in their membranes as well, but this sterol is absent in the more basal fungal lineages (Weete et al. 2010). Using cultures of 6 non-basal fungal species (Montgomery et al. 2000) showed a tight correlation between ergosterol content and fungal biomass. Provided that local fungal community was dominated by later diverging divisions such as Ascomycota and Basidiomycota (as suggested by *e.g.* Hannula et al. (2017)), ergosterol could be a reliable marker for fungal biomass.

With regard to the use of rDNA as a marker for fungal biomass, it should be mentioned that a genome-based survey revealed considerable variation estimation in rDNA copy numbers. Nevertheless, some phylum-specific characteristics have been observed. The average number of rDNA copies for Ascomycota is around 50 and shows limited variation. Basidiomycota harbor about twice as many rDNA copies, and this is accompanied by substantial variation among its members (Lofgren et al. 2019). Hence, rDNA copy numbers can only be used to assess fungal biomass in case there are no major differences between the community composition of the samples, and rDNA-based estimation of the Ascomycota is likely to be more accurate than the estimation of the Basidiomycota biomass.

Hence, although both ergosterol and rDNA copy number have their limitations as fungal biomass markers in soil, comparison of data from adjacent plots from the same habitat are probably valid. Fungi are known to be more flexible with regard to the biomass:DNA ratio than many other organismal groups (*e.g.* Griffiths et al. (1997)). This is the result of the flexible cellular organization of fungi. The hyphal compartmentalization of fungi might be impaired by the partial or complete removal of septa, cross walls separating the fungal cells (Roper et al. 2011). Hence, growth of the mycelial network does not necessarily be accompanied by a comparable increase in the number of nuclei. Therefore, the difference in outcome between the two types of markers (biochemical or DNA-based) might be attributable to an increase in the fungal biomass:DNA ratio. Further research is required to investigate this hypothesis.

The habitat (in)dependent impact of *S. gigantea* on fungivorous nematode lineages

Due to the apparently contradictory results obtained by the two types of fungal biomass markers, the effects of *S. gigantea* on major representatives of the next trophic level, fungivorous nematodes, were checked. Two out of three lineages of fungivores present both in the riparian zone and in the semi-natural grassland sites, Aphelenchoididae and Aphelenchidae, were shown to be stimulated in the presence of giant goldenrod, whereas the third lineages, the genus *Diphtherophoridae*, was

unaffected by this invasive plant species. Moreover, the effect in the riparian habitats was much more pronounced than the effects in the sandy locations.

While the increase in Aphelenchoididae as a results of giant goldenrod invasion was found previously (Quist et al. 2014), the increase in Aphelenchidae was new. This difference may be explained by the seasonal fluctuations of nematode densities. Bulk soil concentrations of Aphelenchidae, Aphelenchoididae, and *Diphtherophora* were shown to have a distinct, taxon-dependent seasonality (Vervoort et al. 2012). In this research, samples were collected in late autumn, while samples analyzed in (Quist et al. 2014) were collected in early autumn. Hence it is conceivable that the *S. gigantea*-induced increase in Aphelenchoididae is only noticeable late in the season.

A pronounced boost of Aphelenchoididae and Aphelenchidae was observed in river clay soil, and a non-significant increase in sandy soils (*e.g.* Fig. 1). This difference in response might be explainable by the soil texture-dependent species representation for each of these two families. Within the family Aphelenchoididae, the genus *Aphelenchoides* is dominant, and it comprises >100 predominantly fungivorous species. At least 30 species have been described for the constituting genera of the family Aphelenchidae, *Aphelenchus* and *Paraphelenchus*. No data on soil texture preference are available for *Aphelenchus*, *Paraphelenchus* or *Aphelenchoides*. We hypothesize that the species composition of families Aphelenchidae and Aphelenchoididae differed between the two main habitats. Apparently, the *Aphelenchoides* species present in the river clay soil could benefit more from the local increase in fungal biomass, than the *Aphelenchoides* species present in the sandy soils. We propose the same line of reasoning for the Aphelenchidae genera *Aphelenchus* and *Paraphelenchus*.

Hence, apparently contradictory results with regard to the impact of the invasive plant species *S. gigantea* on the fungal community, translated in a paradoxical effect on the fungivourous nematode community. In fact, only members of the families Aphelenchoididae and Aphelenchidae in riparian habitats benefitted from the presumed increase in fungal biomass. Representatives of the genus *Diphtherophoridae* were unaffected by the presence of giant goldenrod. We hypothesize that this difference in response could be caused by a difference in food preference between the lineages (Li et al. 2014; Okada and Kadota 2003; Ruess et al. 2000).

It is noted that the fungivorous nematode densities reported in this study are relatively low. This could be a late season sampling effect. This effect has little impact on the current analyses as differences between uninvaded and invaded plots are considered rather than absolute changes.

Effect of habitat-characteristic abiotic differences between habitat-type

Despite the differences in floristic composition, soil type and land use history between the riparian zone and the semi-natural grasslands (Quist et al. 2014), the overall biotic impact of giant goldenrod induced similar overall invasion effects. Nevertheless, Basidiomycota were more abundant in semi-natural grasslands than in the riparian zones (P= 0.017, Table 1). This could relate to a substantial pH difference. Whereas riparian zones had a relative neutral pH of 7.5, semi-natural grasslands had a nearly 2 units lower pH. As compared to bacteria, pH windows for optimal growth are wider for fungi (Rousk et al. 2010). In a more recent study, Zhang et al. (2016) investigated fungal communities in arctic soils with a pH range of over 2.5 units. In the most acidic sites, Basidiomycota showed a higher relative abundance as compared to sites with more basic soils. It is noted that the higher abundance in Basidiomycota in semi-natural grasslands did not result in a significant change in either of the fungivorous nematode lineages.

Fungal indicator taxa related to invasion with *S. gigantea*

As shown in Fig. 2, the Ascomycete family Cladosporiaceae was one of the main families responsible for the *S. gigantea*-induced shift in fungal community composition. A closer look at the Cladosporiaceae OTUs revealed that *Cladosporium* was the dominant genus within this family.

Cladosporium is a fairly speciose genus, it comprises 189 species that are mostly saprotrophic but it also harbors some plant pathogens (Sandoval-Denis et al. 2016). Although in its native range leaves of showy goldenrod (*Solidago speciosa*) were shown to be infested by *Cladosporium* asterum causing brown rust pustules (website Missouri Botanical Garden (USA)), no information was found on Cladosporium being an important (root) pathogen of *S. gigantea* in Europe.

Recently Koyama et al. (2019) studied root-inhabiting fungi in a wide range of native and exotic plant species in Canada. The plant selection included *Solidago canadensis*, in this context a native plant species. Ascomycota dominated the root-associated fungal communities, and within this division the Dothideomycetes – a class that includes the family Cladosporiaceae – were identified as the second most dominant class. From the overview of 27 plant species, the Cladosporiaceae abundances were in general negatively correlated with plant abundances, and the authors identified *Cladosporium delicatulum* as an endophytic plant pathogen to a substantial fraction of the plant species under investigation. In our research we focused on another *Solidago* species, outside its native range. In accordance with Koyama et al. (2019) Cladosporiaceae was identified an abundant fungal family in the two habitat types under investigation. However, an opposite,

positive correlation between Cladosporiaceae and *Solidago gigantea* was observed in this study. Plant growth-promoting characteristics of a members of the genus *Cladosporium* could be marked as a possible benefit for *S. gigantea* associated with the fostering *Cladosporium* in its rhizosphere. Both *Cladosporium sphaerospermum* and *Cladosporium sp.* MH-6 were found to produced and release several types of gibberellins, which could explain their plant growth promoting characteristics (Hamayun et al. 2010; Paul and Park 2013).

Glomeraceae was the second most indicative family regarding the impact of *S. gigantea* on the local fungal community. Glomeraceae is a family of arbuscular mycorrhizal (AM) fungi and members of this family colonize the roots of a wide range of vascular plants including *S. gigantea* (Pirozynski and Dalpe 1989). Vallino et al. (2006) characterized the AMF colonization of *S. gigantea* outside its native range, and identified *Glomus*, a genus belonging to the Glomeraceae, the dominant root colonizing AMF. This result underlines that invasive *S. gigantea* can recruit local AMF, and establish such a successful interaction that it ends up as one of the main fungal taxa typifying the community shift that was brought about by the invasive plant species.

In both habitat types, uninvaded plots were characterized by an increased presence of Cucurbitariaceae, just like the Cladosporiaceae a family that belongs to the class Dothideomycetes.

Little is known about the ecology of Cucurbitariaceae. Its members are known as saprobes on relatively recalcitrant organic materials such as wood, bark and leaves (Jaklitsch et al. 2018).

High-throughput sequencing revealed multiple fungal families as indicative for invasion. In the previous section we hypothesize that this difference in response could be caused by a difference in food preference between the lineages. Interestingly Ruess et al. (2000) indicated *Cladosporium* as moderate feeding source for *Aphelenchoidides* sp, which is in line with our observations. Unfortunately, little is known about food preferences of different nematode taxa. Therefore, the indicator families observed in this research could be an interesting starting point for more targeted research to nematode feeding preferences. Especially the fungal families indicated for invasion in riparian habitat could be informative as Aphelenchidae and Aphelenchoididae showed a high significant increase in these soils.

Conclusion

This study shows that *S. gigantea* invasion has a structural impact on the below-ground soil community by increasing the fungal biomass independent of sampling

moment, sampling year or habitat. The increase of fungal biomass is reflected in the next trophic level by a boost of two independent lineages of fungivorous nematodes, Aphelenchidae and Aphelenchoididae. Notably, this effect is more pronounced in the river clay soils in riparian zone than in the soil soils under the semi-natural grasslands. Nematodes show strong preferences for certain soil textures, even at species level. Therefore, we hypothesize that the different response levels might be contributable to differences of the species composition of Aphelenchidae and Aphelenchoididae fungivores in the two major habitats. Another fungivorous family, *Diphtherophoridae*, did not benefit from the local, *S. gigantea*-induced increase in fungal biomass.

The ergosterol-based observation of the increase in the fungal biomass a *S. gigantea*, could not be confirmed by DNA markers. Both qPCR-based assessment of total fungal DNA as well as the characterization of the fungal communities on the basis of variable 18S rDNA regions did not reveal a difference in fungal DNA contents between *S. gigantea*-invaded and non-invaded plots. The apparent discrepancy might be attributable to a change in DNA:biomass ratio. High throughput sequencing of the variable 18S rDNA regions V7-V8 revealed an increased abundance of Cladosporiaceae and Glomeraceae, and a decrease of the Cucurbitariaceae. Further investigation of the nature of these community shifts could further elucidate the change fungal DNA-biomass ratio as potentially provoked by the invasive plant species *S. gigantea*.

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Supplementary information

Table S1: Eight study sites located in riparian zones and in semi-natural grassland habitats are indicated below. Although 'Blauwe Kamer' is one riparian study site, samples were collected from two distinct areas within the nature reserve (1 and 2). Riparian zones are characterized by river clay soils, whereas the semi-natural grassland sites were located on Pleistocene sandy soils. Coordinates and years of *S. gigantea* introduction were obtained from (Quist et al. 2014).

Habitat type	Study site	Soil type	Coordinates	Year of *S. gigantea* introduction	# of plots pairs
Riparian zone	Millingerwaard	River clay	51° 51' 58.11'' N 6° 00' 35.47'' E	~ 1950	8
	Ewijkse plaat	River clay	51° 52' 47.36'' N 5° 44' 52.17'' E	~ 1950	8
	Blauwe Kamer				
	West	River clay	51° 56' 40.22'' N 5° 36' 19.90'' E	after 1950	4
	East	River clay and sand	51° 56' 32.56'' N 5° 37' 09.54'' E	after 1950	4
Semi-natural grassland	Dennenkamp	Pleistocene sand	52° 01' 45.64'' N 5° 47' 53.50'' E	1982	8
	Plantage Willem III	Pleistocene sand	51° 58' 48.62'' N 5° 31' 08.47'' E	1995	8
	Hollandseweg	Pleistocene sand	51° 58' 49.89'' N 5° 40' 59.84'' E	before 2005	4
	Scheidingslaan	Pleistocene sand	51° 58' 28.60'' N 5° 41' 55.40'' E	unknown	4
	Reijerscamp	Pleistocene sand	52° 00' 47.49'' N 5° 46' 08.64'' E	2006	4

Table S2: Overview of primer combination used to quantitatively characterize bacterial and fungal rhizosphere communities. Specificity of bacterial primer combinations is based on taxon specific 16S ribosomal DNA motifs. Fungal primers are based on taxon-characteristic internal transcribed spacer (ITS) regions, occasionally combined with a more generic 5.8S forward or reverse primer.

Target	Primer sequence (5'–3') F: forward, R: reverse		Reference	T_a (l)	T_a (s)	Parameters defining the linear relationship between C_t and DNA concentration ($C_t = a \times {}^{10}\log[\text{concentration}] + b$)		
						A	B	R^2
All bacteria	F	CCTACGGGAGGCAGCAG	López-Gutiérrez et al. (2004) Watanabe et al. (2001)	60°C	60°C	-3.62	16.58	0.997
	R	ATTACCGCGGCTGCTGGCA						
All fungi	F	TCCGTAGGTGAACCTGCGG	White et al. (1990) Vilgalys and Hester (1990)	53°C	60°C	-3.90	18.75	0.964
	R	CGCTGCGTTCTTCATCG						
Ascomycota	F	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990) Nikolcheva et al. (2004)	58°C	60°C	-3.47	16.94	0.998
	R	CGTTACTRRGGCAATCCCTGTTG						
Basidiomycota	F	CAGGAGACTTGTACACGGTCCAG	Gardes and Bruns (1993) Vilgalys and Hester (1990)	55°C	60°C	-3.44	19.34	0.998
	R	TCGATGAAGAACGCAGCG						
Chytridiomycota	F	TGGTTTCTAGGACCGAAGT	Harkes et al. (2017)	60°C	60°C	-3.90	18.75	0.996
	R	CCGGCCAAGGTGAATT						

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CHAPTER 4

Conventional and organic soil management as divergent drivers of resident and active fractions of major soil food web constituents

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Abstract

Conventional agricultural production systems, typified by large inputs of mineral fertilizers and pesticides, reduce soil biodiversity and may negatively affect ecosystem services such as carbon fixation, nutrient cycling and disease suppressiveness. Organic soil management is thought to contribute to a more diverse and stable soil food web, but data detailing this effect are sparse and fragmented. We set out to map both the resident (rDNA) and the active (rRNA) fractions of bacterial, fungal, protozoan and metazoan communities under various soil management regimes in two distinct soil types with barley as the main crop. Contrasts between resident and active communities explained 22%, 14%, 21% and 25% of the variance within the bacterial, fungal, protozoan, and metazoan communities. As the active fractions of organismal groups define the actual ecological functioning of soils, our findings underline the relevance of characterizing both resident and active pools. All four major organismal groups were affected by soil management ($p < 0.01$), and most taxa showed both an increased presence and an enlarged activity under the organic regime. Hence, a prolonged organic soil management not only impacts the primary decomposers, bacteria and fungi, but also major representatives of the next trophic level, protists and metazoa.

Introduction

Intensification of food and feed production is often accompanied by increased nutrient inputs, intense pesticide applications, frequent tillage, and irrigation management (Tilman et al. 2011). The negative environmental implications of these practices include eutrophication, increased salinization, soil erosion and biodiversity loss (Foley et al. 2005; Tilman et al. 2001). Our knowledge of the consequences of agricultural intensification on belowground biodiversity is still limited and fragmented. It has been reported that larger-sized soil biota are more negatively affected by high-input agricultural practices than soil microbes (Postma-Blaauw et al. 2010), but is it widely acknowledged that more detailed studies are required to map the effects of soil management on soil microbiota (El Mujtar et al. 2019).

Soils harbour a quarter of the world's biodiversity and reside among the most complex habitats on earth (Bardgett 2005; Roesch et al. 2007). Soil biota plays a role in many essential soil functions such as nutrient cycling, carbon and water retention, soil texture formation and the interaction with the plant community (Henneron et al. 2015). Most intensive interactions between microbes and plants take place in the rhizosphere, where the plant is able to select and boost a subset of the microbial community by the release of rhizodeposits – a broad range of carbon-containing substances (*e.g.* root cells, mucilage, volatiles and exudates). The composition of the rhizobiome, the subset of the soil biota present in the rhizosphere, is co-determined by plant identity and age (Chaparro et al. 2014; Grayston et al. 1998; Inceoglu et al. 2011; Kuske et al. 2002; Schlemper et al. 2017). With the advent of affordable high throughput DNA sequencing techniques, the impact of plants on the identity and density of rhizosphere inhabitants can be mapped. Insight in this interaction could help to design soil management measures promoting a rhizobiome that would optimally support plant growth and improve crop yield (Knief 2014).

As in many other habitats, most soil inhabitants have to cope with unpredictable food availability (Morita 1997). In order to survive periods of food scarcity, various microorganisms can reversibly reduce their metabolic activity over an extended period of time (Blagodatskaya and Kuzyakov 2013). Such a condition is referred to as a state of dormancy (Stevenson 1978). In bulk soil, typically 80% of the cells and 50% of the operational taxonomical units (OTU's) are dormant. This so-called "microbial seedbank" (Lennon and Jones 2011) is alert in the sense that it can detect and respond to environmental stimuli (*e.g.* organic substrates) that are associated with favourable growing conditions (De Nobili et al. 2001). Plant roots produce and release a broad spectrum of environmental stimuli and, as such, the rhizosphere is a hotspot of microbial activity (Hinsinger et al. 2009; Reinhold-Hurek et al. 2015).

Given the typically high percentage of dormant microbiota in soil, it is relevant to discriminate between the resident and the active microbial community when considering soil ecological processes. The resident community refers here to all organisms present in a certain spatial unit of soil, whereas the active community comprises the fraction of the resident community that is metabolically active. Ribosomal (r)RNA is considered a representation of the active microbial community, while rDNA characterises the total microbial community (De Vrieze et al. 2016; Ofek et al. 2014). Hence, combined profiling of community rDNA and rRNA will provide insight in both aspects of local microbial assemblies. More specifically, such a characterisation will provide information about microbial fractions, whose activity is positively or negatively affected by any kind of external influence. Although a number of soil ecological studies considered both the active and the resident microbial community (Baldrian et al. 2012; Nunes et al. 2018; Schostag et al. 2019), large scale mapping of shifts in the active soil microbiome has been hampered so far by the low throughput nature and the costs of currently available kits for RNA extraction from soil. A combination of elements from various published protocols allowed us to develop a fast and affordable method for nucleic acid extraction from soil.

The aim of this study was to investigate the long-term impact of soil management regimes (including conventional and organic soil management) on resident (rDNA) and active (rRNA) microbial communities. To this end, we collected bulk and rhizosphere samples in two different growth stages of summer barley (*Hordeum vulgare*) grown on two distinct soil types – peaty and sandy soil – under the various types of soil management. Four major organismal groups were assessed: bacteria and fungi – representing the primary decomposers – and protists and nematodes – two major grazers on the bacterial and fungal communities. Specific variable ribosomal DNA regions were selected for the characterization of each of the four organismal groups. We hypothesise that (i) prolonged exposure to distinct soil management regimes will impact both the resident and active fractions of the primary decomposers (bacterial and fungal community), (ii) shifts in the primary decomposer community due to soil management will be translated into associated changes in the active fractions of major representatives of the next trophic level (protists and metazoa), (iii) exposure to rhizosphere will have a stronger stimulating effect on the active fractions of the primary decomposers (*i.e.* bacteria and fungi) than on protists and metazoa.

Results

Essentials on the characterisation of soil biota

Nucleic acids (total DNA and RNA) from 104 bulk soil and rhizosphere samples were extracted using a novel, lab-made protocol (Table 1). Contrary to bacteria, fungi and protists, the 2 g soil subsamples will not provide a proper representation of the metazoan community. We included metazoa in this study because this fraction of the soil microfauna was co-extracted with the microbial community, and therefore probably in close physical contact with this community. MiSeq sequencing of organismal group-specific 16S (bacteria) or 18S (fungi, protists, and metazoa) ribosomal DNA and cDNA fragments resulted in ≈ 31 million reads (15.5 million forward and 15.5 million reverse), and on average ≈ 75,000 reads per sample. After filtering, a total of 8,297,203 sequences were retained comprehending 724 samples for all taxa together. Comprehensive sampling of the microbial community was obtained for all treatments, with average sequence coverage of 63%, 70%, 96% and 97% for respectively bacteria, protozoa, fungi and metazoan as determined by Good's coverage estimator (Supplementary Table S3).

Contrasts between resident versus active fractions of four major soil food web constituents

PERMANOVA identified Nuclei Acid (*i.e.* rRNA and rDNA) as the main factor responsible for the differences between samples for all four organismal groups (Table 2). The factor explained 14 to 25% of the overall variance ($P < 0.01$). The second important explanatory factor of the observed variation was Location (6 to 13%), which is assumed to be attributable mainly to differences in soil type. 'Vredepeel' is characterized by sandy soils, whereas peaty soil typifies 'Valthermond'. For the primary decomposers, a higher percentage of the overall variation was explained by soil type (11% and 13% for bacteria and fungi, respectively) when compared to the representatives of the next trophic level (8 and 6% for protists and metazoans, respectively) (for all groups $P < 0.01$).

UniFrac, a method that uses phylogenetic distances as a measure for the comparison of microbial communities (Lozupone and Knight 2005), was used to verify the impact of individual variables. Both weighted and unweighted UniFrac demonstrated that all variables that were shown to have a significant effect on the bacterial community. This was in essence also true for fungi, although Sample Type was not significant in case of unweighted UniFrac ($P = 0.069$). For Protozoa, only the effect of treatment was not significant while using unweighted UniFrac ($P = 0.097$). In case of metazoa, the only non-significant variable was Time Point for the unweighted UniFrac ($P = 0.084$) (see Supplementary Table S4).

Table 1: Lab-made protocol for the direct extraction of DNA and RNA from soil developed by combining elements from several soil nucleic acid extraction methods (Anderson 2018; Braid et al. 2003; Miller et al. 1999). This protocol was optimized for nucleic acid extraction from sandy soils with a range of organic matter contents.

Step	Procedure
1.	Weigh 2 g of thoroughly mixed soil, transfer it to a 15 mL bead tube), and add 1.5 g of coarse silicon carbide powder (46 grit). (Keep bead tubes on ice from step 1–9)
2.	Add 2.5 mL of bead solution (181 mM disodium phosphate, 121 mM guanidinium thiocyanate), 0.25 mL of lysis buffer (150 mM NaCl, 4% (w/v) SDS, 0.5 M Tris), and 0.8 mL of a 120 mM ammonium aluminum sulfate dodecahydrate solution [28].
3.	Add 3.5 mL of phenol:chloroform:isoamyl alcohol (25:24:1, pH: 8.0, 4°C) and mix it manually to disintegrate the biphasic layer.
4.	Place the bead tubes for 10 minutes on a Digital Vortex genie 2 (SI-A256) with a SI-H512 horizontal 15 ml tube holder at maximum speed (2,850 rpm) at 4°C [29]. Note: place no more than 4 tubes in the tube holder as this would lower the rpm.
5.	Incubate the tubes horizontally at -20°C for 10 minutes. Thereafter: repeat step 4, and continue to step 6.
6.	Centrifuge the tubes for 10 minutes (2,500 x g) at 4°C to separate the soil particles from the lysate.
7.	Transfer 3 mL of the upper aqueous phase to a new 15 mL tube, and add 1.5 mL of ice-cold precipitation solution (an aqueous solution of 5 M NaCl, 22 mM citric acid anhydrous salt, and 29 mM trisodium citrate dihydrate)
8.	Centrifuge the tubes for 10 minutes (2,500 x g) at 4°C to separate the precipitate from the nucleic acids.
9.	Transfer 4 mL of supernatant to a new 15 mL tube containing 5 mL of isopropanol at room temperature (RT). Mix gently by hand for 5s and centrifuge the tubes for 15 minutes (2,500 x g) at 4°C to precipitate the nucleic acids.
10.	Discard the isopropanol, and air dry the pellet for 5 minutes at RT.
11.	Add 1mL binding solution (at RT) to the pellet (binding solution: an aqueous solution of 5 M guanidinium thiocyanate and 30 mM Tris-HCl (pH: 6.5) with 9% (v/v) isopropanol), and vortex to re-dissolve the pellet.
12.	Load 0.5 mL of solution to a silica spin filter (RP20 CommaPrep RNA extraction column, Biocomma, China) to bind the nucleic acids (DNA and RNA) [30], and spin for 30 s at 10,000 g.
13.	Discard the flow-through, add 0.75 mL of washing solution (10mM Tris-HCl, pH: 6.5), 100mM NaCl, and absolute EtOH final v/v 50%) to the spin filter, spin for 30 s at 10,000 g. Repeat this step 3 times.
14.	Air-dry the filter for 5 minutes, and transfer the spin filter to a clean collection tube.
15.	Add 0.2 mL of elution buffer (10mM Tris-HCl, pH: 8.0) to the spin filter, and spin for 30 s at 10,000 g.
16.	Collect the eluate (0.2 mL) and store it at -80°C until further use.

In addition to the PERMANOVA, we generated a principal coordinate analysis (PCoA) ordination of a Bray-Curtis dissimilarity matrix (Fig. 1). There is a clear differentiation visible between active (rRNA) and resident communities (rDNA). The resident communities cluster (blue and light blue) and are separated from the

Table 2: PERMANOVA analysis was used to test the effect of a number of variables on the composition of bacterial, fungal, protozoan and metazoan assemblages. The following factor were displayed: Nucleic Acid (cDNA/DNA), Location (Vredepeel/Valthermond), Treatment (ConSlu, ConMin, Org (Vredepeel), Comp and No-Comp (Valthermond)), Sample type (Bulk/Rhizosphere) and Time point (Vegetative/Generative) as factors. Differences are considered significant if P< 0.01. P = probability associated with the Pseudo F statistic.

Source	F	R^2	P
Bacteria			
Nucleic Acid	67.659	0.219	9.99-05
Location	32.604	0.106	9.99-05
Treatment	5.465	0.053	9.99-05
Sample Type	7.158	0.023	9.99-05
Time Point	3.700	0.012	0.00240
Residuals		0.410	
Fungi			
Nucleic Acid	48.045	0.144	9.99-05
Location	43.315	0.130	9.99-05
Treatment	11.086	0.100	9.99-05
Sample Type	7.174	0.022	9.99-05
Time Point	6.923	0.021	9.99-05
Residuals		0.410	
Protozoa			
Nucleic Acid	73.162	0.208	9.99-05
Location	27.792	0.079	9.99-05
Treatment	6.344	0.041	9.99-05
Sample Type	7.550	0.022	9.99-05
Time Point	4.788	0.018	9.99-05
Residuals		0.461	
Metazoa			
Nucleic Acid	76.172	0.245	9.99-05
Treatment	6.014	0.058	9.99-05
Location	17.300	0.056	9.99-05
Sample Type	12.655	0.041	9.99-05
Time Point	6.981	0.022	9.99-05
Residuals		0.429	

active microbial communities (red and ochre) for all four organismal groups. This separation between clusters was most obvious for Bacteria (Fig. 1A) and Protists (Fig. 1C). For Fungi (Fig. 1B) and Metazoans (Fig. 1D) this separation between active and resident communities was visible as well, but less pronounced.

Visualisation of the significant location effects that were demonstrated by the PERMANOVA for all four organismal groups resulted in two distinct patterns. Whereas a complete separation of clusters was observed for bacteria and protists (encircled in ochre and green in Fig. 1A, C), a less clear separation was seen for

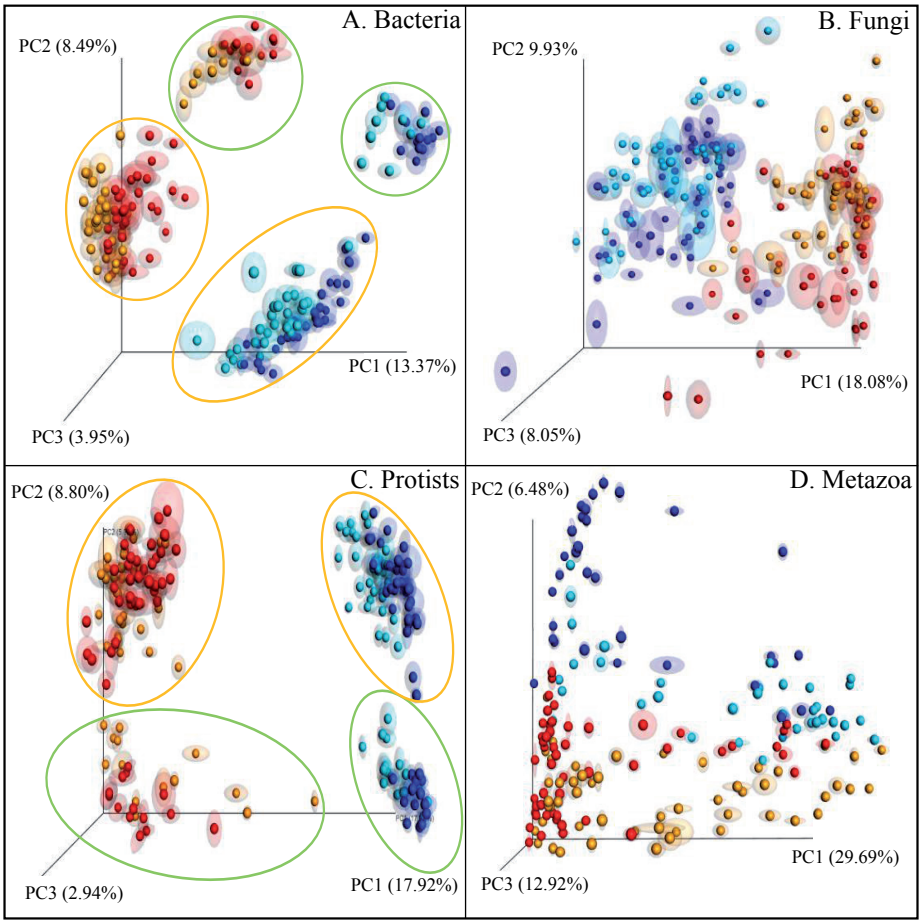

Figure 1: Principal coordinate analysis (PCoA) ordination of a Bray-Curtis dissimilarity matrix. Plots illustrate distances between communities (104 soil samples; for each sample both the resident (rDNA) and the active (rRNA) community were characterized) for each organismal group: A, Bacteria; B, Fungi; C, Protozoa, and D, Metazoa. Colours were used to distinguish between rRNA-bulk, rRNA-rhizosphere (ochre), and rDNA-bulk (dark blue), rDNA-rhizosphere (light blue). Locations are indicated by an ochre circle (Vredepeel, sandy soil) or a green circle (Valthermond, peaty soil).

fungal and metazoan communities (Fig. 1B, D). It appeared to be difficult to visualize the impact of all variables for the four organismal groups with a single set of graphic settings. In Supplementary Fig. S3 alternative settings were used to picture the location effect for fungi and metazoans.

Exposure to rhizosphere conditions resulted in a difference between both the resident and the active microbial community. For bacteria, the difference in community composition between bulk and rhizosphere soil is reflected at both rDNA and rRNA level (blue and light blue, and red and ochre, respectively) (Fig. 1A). For

protists, differences between bulk and rhizosphere soil were most pronounced at rDNA level (Fig. 1C). At rRNA level, protist communities only showed differentiation in peaty soils (location Valthermond). It is noted that the PERMANOVA pinpointed significant rhizosphere effects for all four organismal groups (Table 2).

Microbial taxa contributing to contrasts between resident and active communities

To pinpoint taxon-specific differences between resident and active communities and different soil management, we further analysed the samples of location 'Vredepeel'. Based on LEfSe with LDA thresholds for discriminative features set at ≥ 2 or ≤ -2, a total of 9 bacterial, 8 fungal, 11 protozoan, and 12 metazoan orders contributed significantly to the differences between the resident (rDNA-based) and active (rRNA-based) communities (Fig. 2).

Four bacterial orders were identified as highly active in both rhizosphere and bulk soil (Fig. 2A). For barley rhizosphere the active Sphingobacteriales were distinct, whereas Bacillales, Acidimicrobiales, Solirubrobacterales and Propionibacteriales were predominately found in bulk soil. It is noted that only Propionibacteriales showed a large contrast between bulk and rhizosphere samples, the other three bacterial orders had LDA scores just below 2 in rhizosphere soils (data not shown).

Analysis of the fungal community revealed four orders that were highly active in both bulk and rhizosphere soil. The order Glomerellales was present (DNA) but barely active in both soil compartments (LDA score below -2). The bulk soil was typified by active members of the orders Filobasidiales and Endogonales. Notably, the contrast between bulk soil and rhizosphere was subtle for Filobasidiales, and more substantial for members of the Endogonales (data not shown).

Protists, being a predominantly bacterivorous group in soil, were included as major representatives of the next trophic level. Colpodida, Philasterida and Haptoria were identified as protist orders with an enhanced metabolic activity in the barley rhizosphere.

Our analyses revealed active rotifers, mites, nematodes, and insects in the rhizosphere compartment. A pronounced difference (LDA distance of 8) between the activity of the bacterivorous nematode order Rhabditida in bulk and rhizosphere soil. Rhabditida are known as extreme opportunists (Bongers 1990). This ecological characteristic is reflected in Fig. 2.

Effects of soil management regimes on community structures

Based on our analysis, compost treatment at location 'Valthermond' had no significant impact on any of the analysed organismal groups (see Supplementary Table S5).

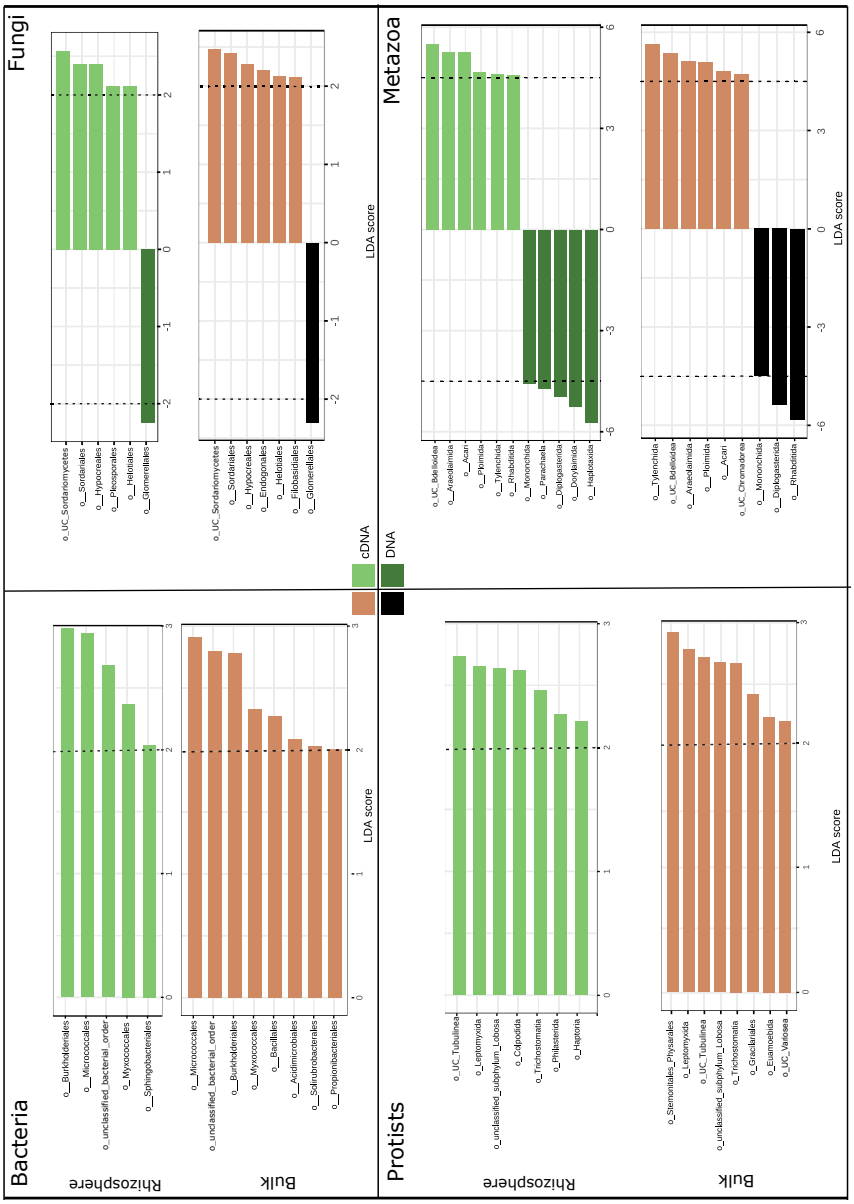

Figure 2: LEfSe analysis of bacterial, fungal, protists and metazoan OTUs identifying taxa for which a major part of the population was active in rhizosphere (light green) or in bulk soil (light brown) (LDA score > 2), or for which a major part of the population is dormant in rhizosphere (green) or in bulk soil (Brussaard et al.) (LDA scores < -2). UC = Unclassified.

In contrast, at location 'Vredepeel' the three soil management regimes (ConMin, ConSlu and Org) showed to have a significant effect on the microbial community structure. Both the PERMANOVA and a PCoA (Table 3, Fig. 3) indicated a distinct microbial community structure for Org fields as compared to communities found under ConMin and ConSlu management. This soil management effect was most evident for the active communities (rRNA) of Bacteria, Fungi and Protozoa. Results were verified with weighted and unweighted UniFrac. For both UniFrac variants the effect of soil management ('Treatment') was significant for all four organismal groups (Supplementary Table S6). A similar analysis on the resident community (rDNA) revealed a comparable, though less pronounced pattern (Supplementary Fig. S4). For Metazoans, no clear soil treatment effect was observed in both the active and the resident communities.

Table 3: PERMANOVA analysis was used to test the effect of the following factors: Nucleic Acid (cDNA/DNA), Sample Type (bulk soil /rhizosphere), Treatment (soil management regime: ConMin, ConSlu, or Org), and Time Point (vegetative and generative). Differences are considered significant if P< 0.01. P = probability associated with the Pseudo F statistic.

Source		F	R^2	P
Bacteria				
Nucleic Acid		92.223	0.35373	9.99-05
Sample Type		7.158	0.02745	0.0001
Treatment		9.906	0.07599	9.99-05
Time Point		7.588	0.02911	0.0004
	Residuals		0.40	
Fungi				
Nucleic Acid		56.589	0.22513	9.99-05
Sample Type		8.448	0.03361	9.99-05
Treatment		18.436	0.14669	9.99-05
Time Point		12.776	0.05083	9.99-05
	Residuals		0.40	
Protozoa				
Nucleic Acid		120.58	0.39706	9.99-05
Sample Type		6.588	0.02169	0.0014
Treatment		5.922	0.039	0.0002
Time Point		13.585	0.04473	1.00E-04
	Residuals		0.389	
Metazoa				
Nucleic Acid		41.323	0.19827	9.99-05
Sample Type		13.602	0.06526	9.99-05
Treatment		9.311	0.08935	9.99-05
Time Point		9.06	0.04347	9.99-05
	Residuals		0.50	

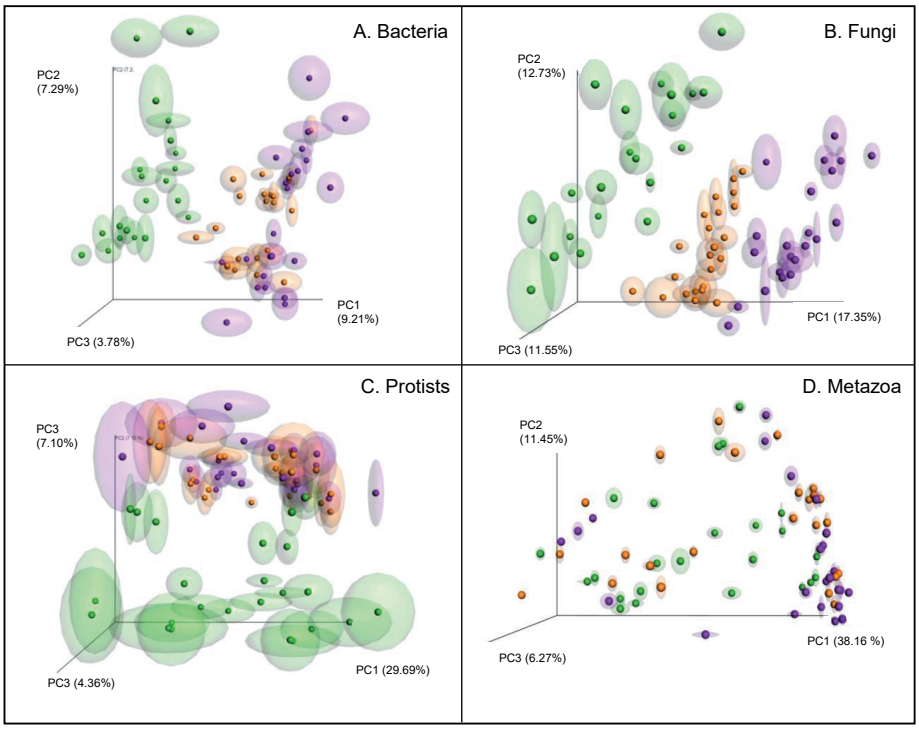

Figure 3: Principal coordinate analysis (PCoA) ordination of a Bray-Curtis dissimilarity matrix. Plots illustrating distances between the active fractions of communities at location Vredepeel (sandy soil) (n=72) for A: Bacteria, B: Fungi, C: Protozoa and D: Metazoa. Colors were used to indicate soil management regimes: ConMin (purple), ConSlu (orange), and Organic (green).

Bacteria - In general, prolonged organic soil management on sandy soil boosted the abundance of almost all bacterial orders rDNA level (Supplementary Fig. S5.1b). Out of the 38 bacterial taxa that significantly differed in abundance between ConMin and Org fields, 36 taxa were more abundant in the organic treatment. When considering the active fraction of the bacterial community, 47 taxa were significantly affected by soil management, of which 31 taxa were found to be more active in Org. Among the soil management-affected taxa, 16 showed a higher activity in ConMin fields.

As compared to two conventional soil treatments ConMin and ConSlu, prolonged organic soil management has boosted the activity (rRNA) of a range of bacterial orders. Desulfuromonadales, Clostridiales, Erysipelotrichales, Rhodocyclales, and Rhodobacterales had the highest LDA scores (LEfSe, LDA score > 2, Fig. 4), and their increased activity was confirmed by ANOVA (grey arrows in Supplementary Fig. S5.1). The orders Bacillales, Deinococcales, Micrococcales, Acidobacteriales, Kineosporiales, and Streptomycetales were identified as indicators

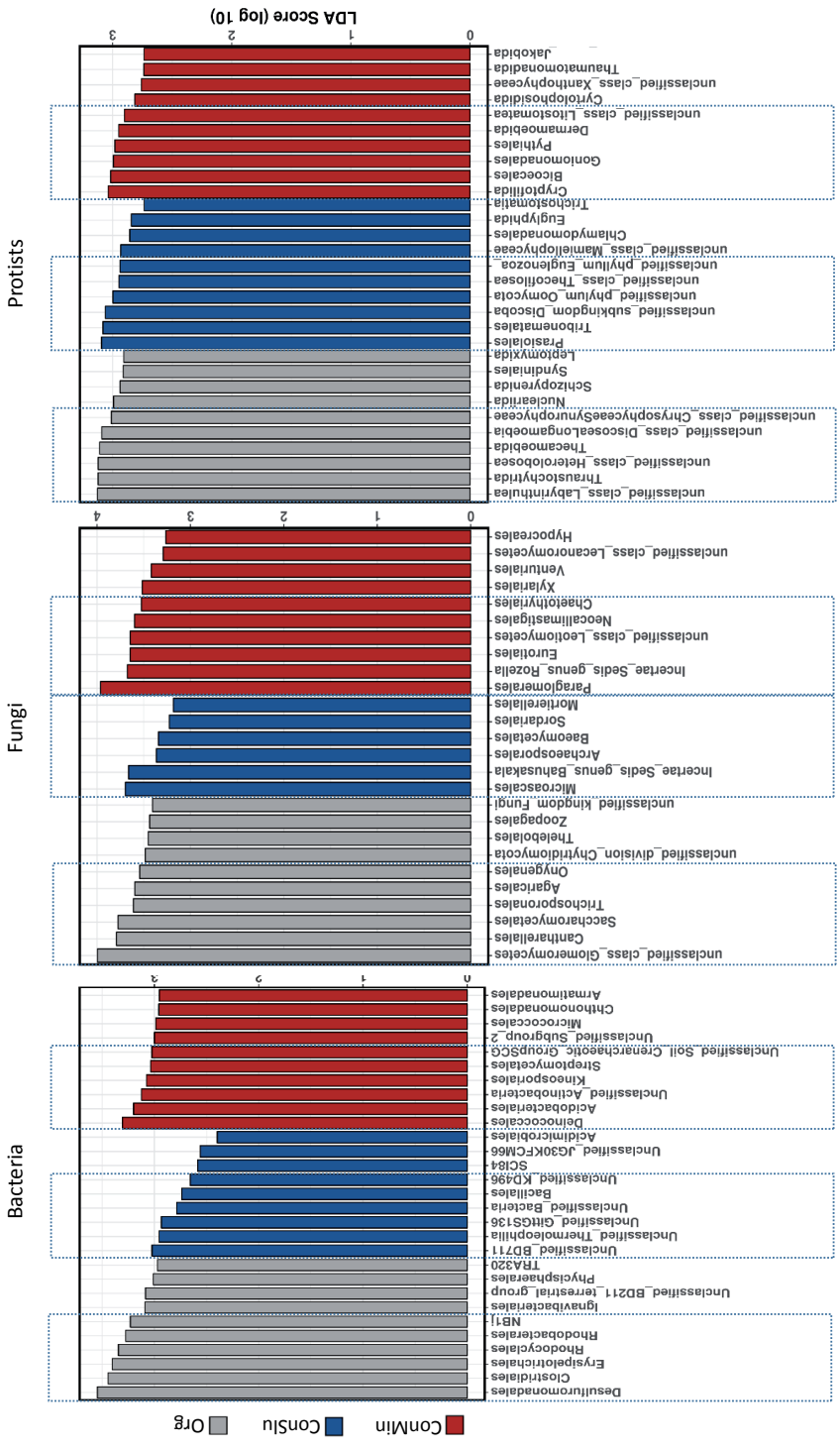

Figure 4: Discriminant active bacterial, fungal, and protozoan taxa indicated by LEfSe analysis resulting from distinct soil management types at location Vredepeel: ConMin (red), ConSlu (blue) and Org (grey). For each treatment and organismal group, six taxa with the highest LDA scores are delineated.

for conventional soil treatments. ANOVA did not confirm the status of the order Kineosporiales as indicator taxon for the ConMin treatment (red arrow 4 in Supplementary Fig. S5.1). It is noted that no further attention was paid to bacterial taxa without a formal systematic name.

Fungi - Out of the 30 fungal taxa that significantly differed in OTU abundance (rDNA), 22 taxa were more abundant in organic fields compared to conventional managed fields (Supplementary Fig. S5.2). When concentrating on the fungal orders of which the activity (rRNA) was affected by soil management type, 19 taxa were more active in organic soils and 10 were promoted under conventional soil management regimes (ConMin and ConSlu) (Supplementary Fig. S5.2).

LEfSe analysis of fungal rRNA sequences revealed the orders Glomeromycetes (unclassified class), Cantharellales, Saccharomycetales, Trichosporonales, Agaricales, and Onygenales as indicative for the organic regime (Fig. 4). In most cases, ConSlu-promoted taxa were also activated under the ConMin regime. The order Microascales was identified as a specific indicator for ConSlu-treated fields. ANOVA confirmed the indicator status of this order for the ConSlu treatment (blue arrow 1 in Supplementary Fig. S5.2). The orders Paraglomerales, Eurotiales, Neocallimastigales, and Chaetothyriales were identified as indicators for conventional farming system using mineral fertilizer only.

Protozoa - The total abundance (rDNA) of 28 protist taxa was upregulated in organic treatment, while 13 taxa were promoted under conventional soil management (Supplementary Fig. S5.3). The impact of organic soil management at rRNA level showed an opposite trend since only 12 out of 41 taxa showed a higher OTU abundance in the fields under organic management.

Thraustochytrida and Thecamoebida were identified as protist indicators for organic soil management. Unclassified members of the classes Labyrinthulea and Heterolobosea were characterized by higher densities and higher activity in Org fields (Fig. 4, Supplementary Fig. S5.3). Prasiolales, Tribonematales, Cryptofilida, Phytiales, Dermamoebida and Bicoecales were identified as indicator taxa for conventional soil management (ConMin and ConSlu) (Fig. 4). Notably, ANOVA did not confirm the indicator status of Tribonematales and Bicoecales (Supplementary Fig. S5.3).

Metazoa - The impact of the three soil managements regimes had little impact on the metazoan communities. Only a few orders were characterized as indicators by LEfSe analysis. Mononchida, an order of predatory nematodes, and the mollusc superfamily Nuculoidea were more actively abundant under organic farming. The nematode orders Dorylaimida and Areaolaimida were more active under the ConSlu regime, while Tylenchida and Monhysterida were indicative for ConMin. ANOVA gave non-corresponding results for a number of the aforementioned orders.

Discussion

Characterisation of both resident and active fractions of bacterial and fungal communities as well as their primary consumers, protists and metazoans, generated a holistic view on long-term effects of various soil management systems including organic farming. Analysis of 104 samples from fields with barley as main crop underlines the relevance of distinguishing active (rRNA) and resident (rDNA) communities. For all four organismal groups nucleic acid type was the most important explanatory variable. The long-term impact of soil management was significant for all four organismal groups and dozens of taxa were identified that showed an increased presence and an enlarged activity under the organic soil management regime.

A few aspects of the use of rRNA and rDNA as markers for active and resident soil biota require further attention. Ribosomal RNA is a highly abundant transcript, and rDNA is a multi-copy gene for which the number of copies varies enormously between organismal groups. Among bacteria, the number of rRNA operons is moderately diverse; typically, bacterial genes harbour 1 to 15 copies (Espejo and Plaza 2018). For protists, the number of rDNA copies is substantially higher. Focussing on a range of diatoms and dinoflagellates, individual species were shown to harbour rDNA copy number in the range of 100 to 10,000 (Godhe et al. 2008). For fungal taxa, a recent study estimated the number of rDNA copies to range between 14 and 1,400. This variation in fungal rDNA copy number could not be linked to trophic preferences or other easily observable ecological characteristics (Lofgren et al. 2018). Based on copy number variation, it is clear that rRNA and rDNA data from microbial communities should not be used for quantitative comparisons between organismal groups, neither should it be used for comparison of abundances or activities between high taxonomic level taxa within an organismal group. In this study, we infer rRNA to represent the active community. Nevertheless, dormant soil inhabitants may have ribosomes with functional rRNAs (Nanamiya et al. 2010) to allow organisms to resume activity as soon as condition are favourable again. However, it is reasonable to assume that the transition from a dormant to a physiologically active state will be accompanied by a substantial increase in rRNA level. Although it is appreciated that the use of rRNA/rDNA data for the characterisation of active and resident microbial communities in soil has a number of inherent constraints, within-taxon comparison of rDNA-based sequence data under various environmental conditions is likely to reveal robust and valuable information on the impact of these conditions.

Community characterisation by either of the two types of nucleic acids revealed that dormancy was a phenomenon relevant for all four organismal groups under investigation.

Bacteria - Several bacterial orders were shown to be active in both bulk soil and in the rhizosphere. Sphingobacteriales, as an exception, was indicated as specifically active in the rhizosphere (Fig. 2). Pfeiffer et al. (Peiffer et al. 2013) found a similar enrichment of this order in the rhizosphere of maize. A study by Haichar et al. (Haichar et al. 2008) showed Sphingobacteriales accumulation on roots of wheat, rape and barrel clover. This apparent general rhizosphere accruement is in line with the observed upregulated activity near the roots of barley. Propionibacteriales were identified as being specifically active in bulk soil. Propionibacteriales are known to contribute to both primary and secondary fermentations (Johnson and Cummins 1972). The observed activity of Propionibacteriales in bulk soil could therefore relate to the distinct fertilisation regimes.

Fungi - Both in bulk soil and in the rhizosphere representatives of the orders of Sordariales, Hypocreales, Helotiales were highly active (all belonging to the Ascomycota). This in accordance with a survey on four conventional arable fields in Austria that revealed the dominant presence of the same three fungal orders (Klaubauf et al. 2010). However, the high abundance of these Ascomycete orders is not specific for arable soils, Tedersoo et al. (Tedersoo et al. 2009) found similar orders associated with tree roots. Both these studies indicate high abundance (based on DNA data) of the three orders and our data demonstrates that these fungal orders are also highly active. Glomerellales (Ascomycota) showed a negative LDA score for bulk as well as rhizosphere, indicating dormancy. Drought has been shown to specifically decrease protein abundances of Glomerellales (Bastida et al. 2017). As sampling took place during a relatively dry period, this could explain the observed dormancy. Unfortunately, little is known about the ecology of Glomerellales.

Protozoa - Barley rhizosphere was characterized by active representatives of the orders of Haptoria, Colpodida and Philasterida. Haptoria are ubiquitous free-living predatory ciliates in soils (Vd'acny et al. 2014), whereas Colpodida are predominantly grazers of bacteria (Vdacny and Foissner 2018). The high bacterial density in the rhizosphere could explain the accumulation of active Colpodida. Philasterida are known to occur in terrestrial habitats, but additional information about their ecology is scarce. Bulk soil was typified by active Euamoebida, a supergroup that has been indicated active in grassland and forest mineral soils (Geisen et al. 2015). In the same study, the paraphyletic class Variosea was found to be a dominant active class in bulk soil collected from grassland. Our study confirms the presence of active members of Variosea in bulk soil from barley fields. This class was defined only recently (Cavalier-Smith et al. 2004), and little is known about their ecological role in soil. Physarales is suggested to play an important role in litter breakdown (Kamono et al. 2009) and therefore it is no surprise that they are specifically active in bulk soils.

Metazoa - As compared to bacteria, fungi and protists, metazoa showed the strongest compartment effect (R^2=0.041, Table 3 (Metazoa / Sample type)). This might point at a large difference in community composition between bulk soil and rhizosphere keeping in mind that the subsamples analysed in this study were too small to be representative for each of the compartments. Nevertheless, this result is in line with the highly density and activity of soil micro fauna in the immediate vicinity of plant roots that has been well reported for a range of systems (Chen et al. 2007; Griffiths 1994; Irshad et al. 2011). Our analysis indicated also a number of orders as dormant (LDA < -2). This category included the orders Haplotaxida (oligochaetes), Dorylaimida (nematodes) and Parachaela (tardigrades). For Haplotaxida and Dorylaimida the observed signals may have originated from unhatched eggs. A number of plant parasites reside within the order Dorylaimida, and these may remain unhatched until signals from a suitable host plant are perceived. Tardigrades including the Parachaela member *Hypsibius dujardini* are known for their ability to survive relatively dry conditions such as in the upper soil layers at the time of sample collection in a dormant state (Vasanthan et al. 2019). In case of bulk soil, the dormancy of the nematode order Rhabditida was most prominent. This is not unexpected as members of the Rhabditidae are highly opportunistic bacterivores that enter the dormant Dauer stage under unfavourable conditions (*e.g.* drought, food scarcity) (Treonis et al. 2000; Yeates et al. 1993). It is noted that due the relatively large size of soil metazoa, environmental DNA could have contributed to relatively low RNA to DNA ratio which could erroneously be interpreted as a signal for dormancy (Hu et al. 2016).

The effect of organic soil management on bacterial communities is relatively well documented (Hartmann et al. 2015; Lori et al. 2017; Lupatini et al. 2017; Suleiman et al. 2018). To the best of our knowledge there are no other studies investigating the effects of distinct soil management regimes on four organismal groups simultaneously.

Bacteria - At DNA level, ANOVA revealed that 44 out of the 48 bacterial taxa were more abundant under organic management (Supplementary Fig. 5.1). This observation is in line with earlier research on the effects of organic soil management (Mäder et al. 2002) . However, at activity level (as revealed by rRNA-based analysis) 'only' 1/3 of the 50 bacterial taxa were most active under organic soil management conditions (Supplementary Fig. 5.1). As microbial activity matters in terms of soil food web functioning, these results underline the relevance of taking – next to abundance data – activity data into account.

The following bacterial orders contributed most to the difference between organic versus conventional soil management regimes (in terms of OTU abundance and LEfSe score): Desulfuromonadales (δ-proteobacteria), Clostridiales

(Firmicutes), Rhodocyclales (β-proteobacteria), Rhodobacterales (α-proteobacteria). Desulfuromonadales, an order typifying Org fields, harbour a range of sulphate and sulphur reducing bacteria (Warren et al. 2016). Their enhanced activity could relate to the slightly higher S content of the Org fields (Org:247 mg S/kg soil, ConMin and ConSlu: 193 and 214 mg S/kg). Clostridiales are metabolically diverse but increased abundance of members of this order has been observed upon the addition of organic matter with a high recalcitrant C content (Goldfarb et al. 2011). The Org fields studied here received crop residues as green manure, and hard-to-degrade plant remains could have promoted the Clostridiales. Rhodocyclales and Rhodobacterales may act as denitrifiers under low oxygen conditions (Saito et al. 2008; Yoshida et al. 2009), but the background of their activation under the Org regime remains unclear.

Conventional fields were characterized by highly active Actinobacteria (Kineosporiales, Streptomycetales and Micrococcales). This was also observed in previous research on the impact of conventional and organic cropping systems on the microbial community. In a survey over 3 years, Orr et al. (Orr et al. 2015) detected a similar increase in Actinobacteria, but they also showed a strong sample year effect. For the interpretation of our data this should be taken into consideration.

Fungi - In total 70% of the significantly soil management-affected fungal orders were more abundant under the organic regime. In general, this higher abundance was accompanied by higher activity. The majority of the fungal OTUs were assigned to the Ascomycota. Among the Ascomycota, there are numerous decomposers of organic substrates (such as leaf litter, wood, and manure) and more studies reported them as the major fungal phylum present in agro-ecosystems (Francioli et al. 2016; Lienhard et al. 2014). Two Basidiomycetes (Agaricales, Cantharellales) were found to be abundant in organic fields. Both orders harbour numerous wood and litter decomposer taxa (Floudas et al. 2015), substrates that are added to these fields in relatively large quantities. Onygenales were rarely found in the conventional fields but highly active in the organic soils. This order is associated with animal dung (Doveri et al. 2012; Sugiyama et al. 2002). So, active members of the Onygenales are probably the result of the application of cattle manure in the Org fields.

The strongest fungal indicator for organic farming was an "unclassified class of Glomeromycetes'. Glomeromycetes are known to form arbuscular mycorrhizas and colonize the roots of vascular land plants including barley (Williams et al. 2017). In a recent study on the same experimental farm (Vredepeel), AM fungi were also found to be more abundant under organic soil management (Lupatini et al. 2019; Martinez-Garcia et al. 2018). AM fungi can stimulate the decomposition of recalcitrant organic matter, and makes nitrogen bioavailable (Hodge 2001). Hence, the distinct type of manure used under organic management might explain the specific stimulation of Glomeromycetes.

Paraglomerales, an order of the Glomeromycetes was predominantly found in conventional systems. This finding was corroborated by Dai et al. (Dai et al. 2014) who found *Paraglomus* to be positively associated with the conventional production of wheat. The relation between *Paraglomerales* and fertilization system would require further investigation.

Protozoa - In contrast to the primary decomposers, organic soil management decreased the activity of many protozoa. In parallel, we observed an increase in total abundance (rDNA) under the organic regime for a majority of the soil management-affected taxa. Increased densities of protozoa as a result of organic amendments have been reported before (Treonis et al. 2010). Under controlled greenhouse conditions, application of organic fertilizers increased bacterivorous and omnivorous protists, and strongly reduced the relative abundance of plant pathogenic protists (Xiong et al. 2018). We aren't aware of other studies on the impact of organic amendment to soil protist activity.

Metazoa - Organic soil management stimulated the activity an order of predatory nematodes Mononchida, and members of the mollusc order Nuculoidea. Predatory Mononchida feed on other nematodes, but this does not hold for all life stages. Larval stages are too small to capture other nematodes, and they feed on bacteria (Yeates 1987). The strongly enriched bacterial community under the organic regimes may have promoted the activity of the Mononchida. The impact of soil management on metazoa will not be discussed further, as the numbers of individuals present in the 2 g soil samples are relatively low (with some nematode taxa as an exception). Hence, sampling effects could easily obscure soil management effects.

Our results demonstrate that prolonged (> 15 consecutive years) exposure to distinct soil management regimes causes shifts in the primary decomposer assemblies (bacteria and fungi) as well as changes in primary consumer communities (protists and metazoa). It is concluded that organic management practices results soil microbial communities that are demonstrable distinct from the communities under the conventional regimes. However, our fragmentary knowledge about the ecology and food preference of soil microbiota limited our ability to link most of the observed shifts to desired soil-bound ecosystem services.

Methods

Study sites

Samples were collected from barley fields at two locations in The Netherlands: (1) WUR experimental farm 'Vredepeel' is located in the southeast of the Netherlands (51°32N and 5°51E) and is characterized by sandy soil (93.3% sand, 4.5% silt,

2.2% clay) and an organic matter (OM) content of 3–5%. Three different soil management strategies were applied from 2001 onwards: ConMin, ConSlu and Org. ConMin fields solely received mineral fertilizer and processed organic fertilizer without organic matter (liquid mineral concentrates), and ConSlu fields were supplemented with mineral fertilizer and pig/cow slurry. In case of organic soil management, farmyard manure and cow slurry were applied, and no pesticides were used. For further details of the set up and layout of the soil management experiments see additional research reports (de Haan et al. 2018a; b; Quist et al. 2016; Schrama et al. 2018). (2) WUR experimental farm in Valthermond is situated in the northeast of the Netherlands (52°50′N, 6°55′E) and characterized by sandy peat soil (90% sand, 7% silt, 3% clay) and a high OM content (up to 14%). At Valthermond, the effect of the application of compost was investigated (yearly application of 15 tons (green) compost per hectare) since 2013. For this study, we made a comparison between the control and compost plots.

Soil sampling

At both experimental farms, barley (*Hordeum vulgare*) is one of the main crops in the crop rotation system. Due to a slight latitudinal difference, development of the barley plants in 'Valthermond' was one week delayed as compared to 'Vredepeel'. Samples were collected at two time points in spring 2017 (during the vegetative and the generative stage of the crop, see also Supplementary Table S1).

At the 'Vredepeel', each of the three fields was divided in 6 subfields of 540 m^2 (Supplementary Fig. S1). In each subfield, a bulk soil and a rhizosphere sample were collected. Rhizosphere composite samples were taken by harvesting all barley plants within a vicinity of 20 x 20 cm. Excessive soil was removed from the root system by shaking the plants. Immediately thereafter, the plants were transferred to a field laboratory, and rhizosphere samples were collected by brushing off soil that adhered to the roots from 10 individual barley plants. For bulk soil, three cores were collected between the barley rows using an auger (ø 1.5 cm, depth approximately: 15 cm), and thoroughly mixed in pre-labelled bags.

In total 36 composite samples (18 rhizosphere and 18 bulk) were taken at each time point. Rhizosphere soil and bulk soil samples were frozen immediately after sampling in liquid nitrogen and transported on dry ice to the laboratory and stored at -80°C.

At the 'Valthermond' location, samples were taken in the first 2 meters of the subfield. In total 4 subfields of each treatment (Supplementary Fig. S2) were sampled resulting in 16 samples (8 rhizosphere and 8 bulk) at each time point. Barley rhizosphere samples were collected as described above. Hence, a total of 104 soil samples (72 from 'Vredepeel', 36 from 'Valthermond') were used for further analysis.

DNA/RNA extraction and cDNA synthesis

Both DNA and RNA were simultaneously extracted from soil samples, using a lab-made protocol based on a combination of published protocols. This protocol uses a subsample of 2 g from a thoroughly mixed soil sample as starting point (Table 1). Contrary to bacteria, fungi and protozoa, a subsample of 2 g will not give a proper representation of the metazoan community (Wiesel et al. 2015). Metazoan DNAs were co-extracted with DNAs from other microbiota, and the resulting data represent microfauna that was presumably living in the close physical vicinity of the observed microbiota. For this reason, metazoa were included in this study.

After precipitation of humic substances from the 2 g subsample by the chemical flocculant NH_4 Al $(SO_4)_2$ (Braid et al. 2003), and the removal of proteins by a standard phenol:chloroform:isoamyl alcohol mixture, samples were physically disrupted by bead beating using a Vortex Genie 2 with tubes attached to a horizontal tube holder. After a standard isopropanol precipitation, nuclei acids were re-dissolved in a binding solution. DNA and RNA were further purified using a silica-based RP20 CommaPrep RNA extraction column (Miller et al. 1999) (see Table 1 for technical details). Quality and quantity of the obtained RNA and DNA was measured with a Nanodrop and Qubit. Until further processing, nucleic acid eluates were stored in -80°C.

For synthesis of cDNA from extracted RNA the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, Thermo Fisher Scientific Inc., USA) was used according to the manufacturer's instructions. All individual DNA and cDNA samples were diluted to 1 ng/ul and 0.1 ng/ul respectively, to serve as a template for PCR amplification.

PCR amplification and sequencing

For the characterisation of bacterial, fungal, protist and metazoan communities, variable regions (V) of 16S or 18S ribosomal DNA were amplified. For bacteria, the V4 region was amplified, while for protozoa, fungi and metazoa respectively the V9, V7-V8, V5-V7 regions were targeted (for details see Table 4). To prepare the samples for Illumina sequencing, a two-step PCR procedure was followed. In the first PCR, locus-specific primers extended with an Illumina read area and the appropriate adapter (Table 4) were used to produce primary amplicons. Three µl of diluted DNA or cDNA template was used with the following temperature profile: 3 min 95°C, followed by 35x (95°C, 10 s; 55°C, 20 s; 72°C, 20 s) and a final extension step of 72°C of 5 min. This was done in triplicate for all samples and for each of the four organismal groups. The second PCR step was performed on 40x diluted amplicons of PCR step 1. This PCR 2 was conducted to attach the

Table 4: PCR1 primers with adaptor sequences (underlined), read area, and locus-specific part (bold)

Target	Primer	Adaptor	Target region	Reference
Bacteria	515F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGCCAGCMGCCGCGGTA**	V4	(Turner et al. 1999)
Bacteria	806R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACHVGGGTWTCTAAT**		(Caporaso et al. 2012)
Fungi	FF390.1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CGWTAACGAACGAGACCT**	V7-8	(Verbruggen et al. 2012)
Fungi	FR1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**AICCATTCAATCGGTAIT**		(Vainio and Hantula 2000)
Protozoa	1391F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTACACACCGCCCGTC**	V9	(Lane 1991)
Protozoa	EukBr	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TGATCCTTCTGCAGGTTCACCTAC**		(Medlin et al. 1988)
Metazoa	M1041F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGAGGTGAAATTCTTGGAYCGY**	V5-7	(Capra et al. 2016)
Metazoa	M1648R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ACATCTAAGGGCATCACAGAC**		(Capra et al. 2016)

Illumina index and the Illumina sequencing adaptor (3 min 95°C, followed by 10x (95°C, 10 s; 60°C, 30 s; 72°C, 30 s) and a final extension step of 72°C of 5 min). Products of PCR 1 and 2 were randomly checked on gel to ensure amplification was successful. Finally, all PCR products were pooled and sent for sequencing (Bioscience, Wageningen Research, Wageningen, The Netherlands) using the Illumina MiSeq Desktop Sequencer (2*300nt paired-end sequencing) according to the standard protocols.

Bioinformatics pipeline

The composition of microbial communities of the soil samples was analysed based on the sequencing data obtained from the Illumina MiSeq platform. Reads were first sorted into the experimental samples according to their index combination. Thereafter, they were sorted into the four organismal groups based on their locus-specific primer sequences (general run statistics can be found in Supplementary Table S2).

Sequences were processed with Hydra pipeline version 1.3.3 (de Hollander 2017) implemented in Snakemake (Köster and Rahmann 2012). Forward and reverse reads were paired only for bacteria and fungi while single-end sequences were analysed for protozoa and metazoa. The four taxonomical groups were quality trimmed by BBDUK and then merged via VSEARCH (Bushnell 2018; Rognes et al. 2016). Resulting unique sequences were then clustered at 97% similarity by using the usearch_global method implemented in VSEARCH and a representative consensus sequence per *de novo* OTU was determined (Rognes et al. 2016). The clustering algorithm also performs chimera filtering to discard likely chimeric OTUs with UCHIME algorithm in *de novo* mode (Edgar et al. 2011) implemented in VSEARCH. Sequences that passed quality filtering were then mapped to a set of representative consensus sequences to generate an OTU abundance table. Representative OTU sequences were assigned to a taxonomic classification via BLAST against the Silva database (version 12.8) for bacteria, fungi and metazoa, and against the Protist Ribosomal Reference database PR2 (Guillou et al. 2013) for protozoa using SINA (Pruesse et al. 2012). Sequences belonging to chloroplasts, cyanobacteria and mitochondria were discarded from the bacterial dataset, and sequences not belonging to Fungi and Metazoa were removed from the 18S Fungi and Metazoa datasets. The Protozoa dataset was filtered for Streptophyta, Metazoa, fungal and unclassified Opisthokonta sequences. Low-abundance OTUs (those with abundance of <0.005% in the total data set) were discarded prior to analysis (Bokulich et al. 2013). Samples were transformed using Hellingers' transformation for all downstream analyses.

Statistical analyses

Sampling effort was estimated by Good's coverage (Good 1953). For statistical analysis, we explored β diversity patterns by performing principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity using QIIME software (Caporaso et al. 2012). Permutational multivariate analysis of variance (PERMANOVA) was used to compare the microbial community structure between soil managements taken from different sites and with different plant growth stages for active and resident community for 4 different taxa. This was performed with 999 permutations using the adonis function, based on Bray-Curtis and UniFrac (weighted and unweighted) distances using the "vegan" package (Oksanen et al. 2015) in R. To investigate the indicator taxa involved in the differences between resident and active community, a linear discriminate analysis (LDA) effect size (LEfSe) was conducted in Microbiome Analyst (Dhariwal et al. 2017) to explore the differential microbial populations at the family level for the four different taxa (Segata et al. 2011). A significance level of $\alpha \leq 0.05$ was used for all biomarkers evaluated in this study.

Data availability

The raw sequences were submitted to the NCBI Sequence Read Archive (SRA) database under study accession number BioProject ID PRJNA543417.

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Supplementary information

Supplementary information can be found on www.nature.com/articles/s41598-019-49854-y#additional-information

Suppl. Table S1: Barley sampling dates of the two locations

Suppl. Table S2: General run statistics of MiSeq.

Suppl. Table S3: Number of sequences and coverage

Suppl. Table S4: Results of PERMANOVA based on UniFrac distances (weighted and unweighted). The following variables were analyzed: Nucleic Acid (cDNA/DNA), Location (Vredepeel/Valthermond), Treatment (ConSlu, ConMin, Org (Vredepeel), Comp and No-Comp (Valthermond)), Sample type (Bulk/Rhizosphere) and Time point (Vegetative/Generative). Differences are considered significant if $P < 0.05$.

Suppl. Table S5: Results of PERMANOVA testing samples of Valthermond for the effects of Nucleic Acid (cDNA and DNA), Treatment (compost and no compost), Sample Type (bulk soil and rhizosphere) and Time Point (vegetative and generative) on four soil-borne organismal groups.

Suppl. Table S6: Results of PERMANOVA analysis based on UniFrac distances (weighted and unweighted). The following variables were analyzed: Nucleic Acid (cDNA/DNA), Sample Type (bulk soil /rhizosphere), Treatment (soil management regime: ConMin, ConSlu, or Org), and Time Point (vegetative and generative). Differences are considered significant if $P < 0.05$.

Figure S1: Field set up location Vredepeel (sandy soils).

Figure S2: Field set up location Valthermond (peaty soils).

Figure S3: Coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity. Plots illustrating distances between communities in either DNA (n= 104) A and C or cDNA (n=104) B and D. For Fungi A and B and Metazoa C and D. Distinguishing between Valthermond (red) and Vredepeel (blue).

Figure S4: Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity. Plots illustrating distances between communities in all individual DNA samples from Vredepeel (n= 72) for Bacteria, Fungi, Protozoa and Metazoa. Distinguishing between treatments: ConMin (purple) ConSlu (orange) and organic (green).

Figure S5.1/5.2/5.3: Differences in bacterial OTU abundance between the different soil management types (ConMin=red, ConSlu=blue and ORG=grey) for active (a) and total (b) bacterial (5.1), fungal (5.2) and protist (5.3) communities. Asterisks represent statistically significant variance (ANOVA p-values; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) ANOVA. Arrows specify the order in which LefSe analysis categorises the indicative active orders.

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CHAPTER 5

Organic amendment strengthens interkingdom associations in the soil and rhizosphere of barley (*Hordeum vulgare*)

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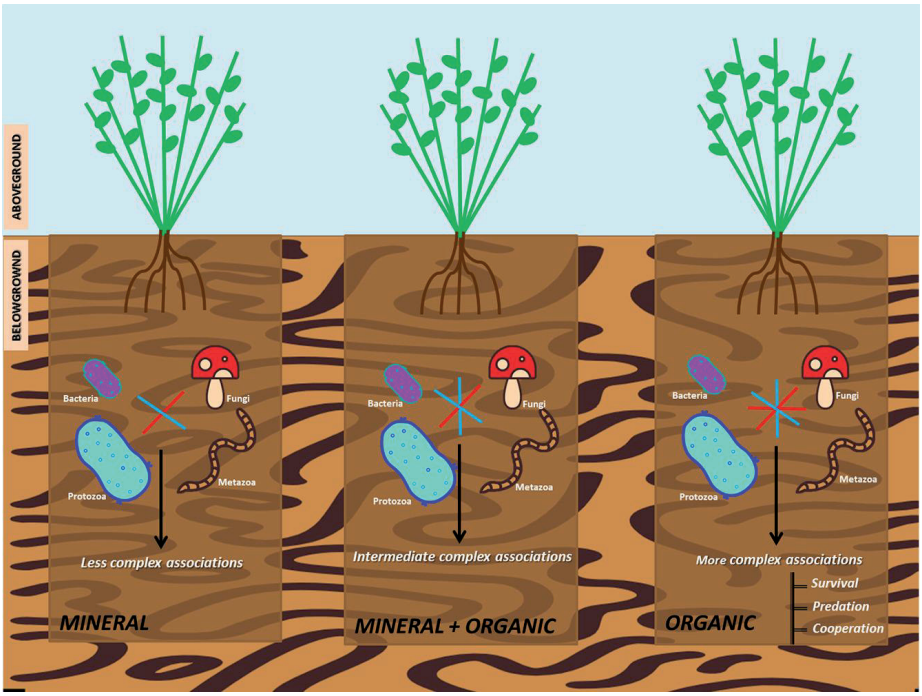
Abstract

Anthropogenic modification of soil systems has diverse impacts on food web interactions and ecosystem functioning. To understand the positive, neutral or adverse effects of agricultural practices on the associations of community members of soil microbes and microfaunal biomes, we characterized the effects of different fertilization types (organic, inorganic and a combination of organic and inorganic) on the food web active communities in the bulk soil and rhizosphere compartments in field conditions. We examined the influence of fertilization on (i) individual groups (bacteria, protozoa and fungi as microbe representatives and metazoans as microfauna representatives) and (ii) inter-kingdom interactions (focusing on the interactions between bacteria and eukaryotic groups) both neglecting and considering environmental factors in our analysis in combination with the microbial compositional data. Our results revealed different patterns of biota communities under organic versus inorganic fertilization, which shaped food web associations in both the bulk and rhizosphere compartments. Overall, organic fertilization increased the complexity of microbial–microfaunal ecological associations with inter- and intra-connections among categories of primary decomposers (bacteria and fungi) and predators (protozoa and microfauna) and differences in potential function in the soil food web in both the bulk and rhizosphere compartments. Furthermore, the inter-connections between primary decomposers and predators in bulk soil were more pronounced when environmental factors were considered. We suggest that organic fertilization selects bacterial orders with different potential ecological functions and interactions as survival, predation and cooperation due to more complex environment than those of inorganic or combined fertilization. Our findings support the importance of a comprehensive understanding of trophic food web patterns for soil management systems.

Graphical abstract



Introduction

Soil is a rich ecosystem comprising both biotic and abiotic factors and a multitude of intra- and inter-interactions that play an essential role in ecological cycles. Disturbances to the soil such as management practices or nutrient addition can alter these interactions and, in turn, impact important ecosystem functions, with consequences for soil fertility, soil quality, soil health and crop yields (Griffiths and Philippot 2013). The effects of increased nutrient input on the abundance and structure of microbial and micro-faunal communities have been widely studied in different soils (Jiang et al. 2017; Suleiman et al. 2018; Wang et al. 2017; Xiong et al. 2017). However, food webs are abstract representations of soil biota interactions, and the importance of soil trophic relationships in agriculture ecosystems remains to be elucidated. Bacteria and fungi, which are the most dominant microbial groups in terrestrial ecosystems and constitute the base of the soil food web, are referred to as primary decomposers. Bacteria feed on relatively easily degradable parts of organic matter, while fungi can decompose recalcitrant plant polymers as their main source of carbon and energy (Folman et al. 2005; Rinnan and Bååth 2009). Other organisms, such as protozoans and metazoans, the main predators of bacteria and fungi, also play important roles in shaping food web structures. Protozoans and metazoans perform diverse functions by recycling nutrients and promoting plant growth, and together they transfer energy to higher trophic levels in the soil food web (Jousset 2017; Trap et al. 2016). Despite microbes co-inhabit in a wide variety of environments, each group in the food web has different time to develop depending on the disturbance added to soil, such as fertilization. Protists can be key hubs in soil microbiome indicating that any alteration in protist community might substantially influence soil microbial associations (Sun et al. 2017).

The impacts of nutrient enrichment on soil microbial composition remain unclear, as divergent findings have been reported for microbial groups. Francioli and colleagues (Francioli et al. 2016) revealed that organic fertilization increased bacterial diversity and stimulated microbial groups known to prefer nutrient-rich environments in loam soil with total organic carbon (TOC) of 160 g kg^{-1} and based on a 4-year crop rotation, whereas Suleiman et al. (Suleiman et al. 2016) showed that organic amendments decreased microbial diversity and changed the microbial structure temporarily in a soil presenting 20.5 g kg^{-1} of TOC cultivated with a corn/cereal succession under a no-tillage system. Furthermore, most of these studies examined a single kingdom, mainly bacteria or archaea, while in nature microbial species exist in complex communities. A clear limitation of investigations of microbial ecological interactions is that it is generally not feasible to quantify the interactions in the soil biota community. To overcome this constraint of microbial

relationships, some researchers have proposed the use of co-occurrence frameworks to explore interactions between organisms and the effects of environment on coexistence within biological communities (Madeo et al. 2014). However, little is known about how soil management practices modify multi-trophic interactions (although see (Cassman et al. 2016; Kåhrström 2014)). Recent findings using next generation sequencing of resident community suggest that organic farming promotes higher fungal diversity and decreases root knot and lesion nematodes (*Meloidogyne* and family Pratylenchidae), whereas conventional systems increase the population of plant parasitic nematodes (Lupatini et al. 2019). Xiong et al. (2017) also indicated that organic amendment reduces plant pathogen *Fusarium oxysporum* and increases bacterivorous and omnivorous protists, with protists as the core group linking bacterial and fungal populations. The mentioned studies suggest different interactions occurring in soil promoted by organic systems. However, no study has simultaneously examined primary decomposers and next trophic level predators and how inter-kingdom communities are structured and associate with each other in different soil management systems.

The major compartments of soil are bulk soil and the plant rhizosphere, and diverse microbial associations are expected in each compartment. Soil type and factors support microorganism recruitment by plant roots in the rhizosphere (Schlemper et al. 2017a), and rhizosphere processes associated with microorganisms have inherent potential to improve crop nutrient uptake capability (Mendes et al. 2014; Pii et al. 2015). The rhizosphere differs substantially from the surrounding soil (bulk soil) due to changes in biogeochemistry by root exudates, which support microbial activity in exchange for enhancing plant mineral acquisition and pathogen protection (Dennis et al. 2010; Turner et al. 2013; Wang et al. 2002). In bulk soil the biota pool is the main source of species richness, of which a subset will colonize the rhizosphere (Armada et al. 2018; Li et al. 2018; Mendes et al. 2014; Yan et al. 2017). Different inorganic fertilizers strongly influence the structure of bulk soil microbial communities (Cassman et al. 2016; Leite et al. 2017; Pan et al. 2014), while organic amendments increase the co-occurrence complexity of microbial members within the community (Lourenço et al. 2018; Schmid et al. 2018). Studies focused on the effects of fertilization type on the structure and stability of the rhizosphere by integrating different soil communities are scarce. However, a complete picture of the effect of fertilization on prokaryotes and eukaryotes requires the elucidation of the heterogeneous inter-kingdom relationships and the ecological rules guiding multi-trophic community assembly in soil and the rhizosphere.

To gain insights on the ecological relationships between different trophic levels of soil biota, we investigated the effect of different fertilization types on soil inter-kingdom interactions, namely prokaryotes, as represented by bacteria and

archaea, and eukaryotes, as represented by fungi, protozoans and metazoans. To compare the co-occurrence of different eukaryotic groups (fungi, protozoans and metazoans) with bacteria under different agricultural practices, three amendment variants (mineral, organic and a combined of mineral and organic) were applied to soil under field conditions of barley cultivation. The microbial communities were analyzed by sequencing ribosomal genes of metabolically active bacteria (*16S rRNA*), fungi, protozoans and microfauna (referred to here as metazoans; *18S rRNA*) using RNA approach. We hypothesized (i) increased complexity of bacterial interactions under organic fertilization, with fewer associations with the main primary decomposers, fungi; (ii) increased associations of bacteria with the main predators, protozoans and metazoans, under mineral fertilization; (iii) a stronger effect of fertilization type on the overall associations among different groups in the rhizosphere than in bulk soil; and (iv) an additional driving force of environmental factors on taxa co-occurrence feedback.

Material and methods

Soil and sample collection

To assess the interkingdom relationships in the soil and rhizosphere, we collected samples from field experiment in Vredepeel experimental farm in The Netherlands (51° 32′ 24.958′ N, 5° 51′13,826′ E). The soils are sandy soils (93.3% sand, 4.5% silt, 2.2% clay) with organic matter (OM) content of 3–5% and were selected because they have different nutrient amendment cultivated with the same plant species barley. The physicochemical properties are shown in Table S1. The nutrients applications for the treatments of the field experiment were (1) Mineral (M): 80 kg N ha^{-1} (ammonium nitrate), 43 kg P ha^{-1} (triple superphosphate) and 66 kg K/ha (potassium chloride); (2) Mineral in combination with organic (M + O): 80 kg N ha^{-1} (ammonium nitrate), 81 kg K/ha (potassium chloride) and pig manure in rate of 19,850 Kg ha^{-1} yr^{-1}; and an adjacent site with (3) Organic (O): 4500 kg organic matter ha^{-1} (cow manure). The nutrients of the treatments (M), (M + O) and O were applied to the soil once before sowing barley seeds.

Samples were collected at maturity stage of the barley (*Hordeum vulgare*) cultivar "Summer" crop in 2017. The bulk soil samples were obtained at a depth of 0–20 cm from space between barley plants. The rhizosphere soils from barley roots were sampled by collecting soils with brush that were adhered to the root hairs. The experimental design had three treatments representing nutrient amendments (M, O + M, O) x 2 compartments (bulk soil, rhizosphere) x 6 replicates. Each replicate of bulk soil was composed by 10 multiple random soil cores and each replicate of

rhizosphere soil was composed by 10 random plants from random places in each plot. The bulk and rhizosphere soils were directly frozen in liquid nitrogen in the field and stored at -80°C for molecular analyses in laboratory. Another fraction was air-dried for soil physicochemical characterization.

Nucleic acid extraction

Total RNA was extracted from each homogenized soil sample as described in Harkes et al. (Harkes et al. 2019). Briefly, 2 g of soil was vortexed in 15 ml bead tube containing silicon carbide powder in 2.5 ml bead solution, 0.25 ml lysis buffer, 0.8 ml of a 120 mM ammonium aluminium sulfate dodecahydrate solution supplemented with 3.5 ml of phenol:chloroform:isoamyl alcohol (25:24:1). The concentration of the extracted nucleic acids was determined by spectrophotometer NanoDrop (ND-2000, Thermo Fisher, USA) and Qubit (fluorometric quantitation), and its integrity and size were confirmed using 1.0% agarose gel electrophoresis. Maxima First Strand cDNA Synthesis Kit and random hexamer primers (Fermentas, Thermo Fisher Scientific Inc., USA) were used for cDNA synthesis (Harkes et al. 2019).

PCR and sequencing

The variable V4 region of bacterial 16S rRNA gene V4, V7-V8, V5-V7 regions for protozoa, fungi and metazoan 18S rDNA sequencing, respectively were utilized as targets for the analyses of Illumina 16S rRNA gene sequencing cDNAs were normalized to 0.1 ng/µl for all PCR reactions. The 16S rRNA gene universal bacterial primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), and 18S rRNA gene protozoan primers Euk1391F (5′-GTACACACCGCCCGTC-3′) and EukBr (5′-TGATCCTTCTGCAGGTTCACCTAC-3′), fungal primers FF390.1 (5′-CGWTAACGAACGAGACCT-3′) and FR1 (5′-AICCATTCAATCGGTAIT-3′) (Verbruggen et al. 2012). Protozoan V9 variable region was amplified with primers 1391f (Lane 1991) and EukBr as described by (Medlin et al. 1988) and for metazoan, primers M1041F (5′-AGAGGTGAAATTCTTGGAYCGY-3′) and M1648R (5′- ACATCTAAGGGCATCACA GAC-3′) (Capra et al. 2016) were used for PCRs amplifications.

The different libraries preparation is detailed in Harkes et al. (Harkes et al. 2019). Finally, the mixture of PCR products was subjected to paired-end sequencing using a MiSeq Reagent Kit v3 on the Illumina MiSeq Sequencing platform at the Bioscience division of Wageningen Research (Wageningen, The Netherlands).

Bioinformatic and statistical analysis

The composition of microbial communities of the soil samples were analyzed based on the raw sequencing data obtained from the Illumina MiSeq platform. Sequencing reads were sorted into each sample according to the corresponding indexes combination. The sequences were quality filtered for expected errors >0.5 or a length shorter than 100 bp eliminated. For bacteria and fungi, paired-end sequences were overlapped to assemble amplicon regions with VSEARCH (Rognes et al. 2016) while single-end sequences were analyzed for protozoa and metazoan. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity using the usearch_global method implemented in VSEARCH and the singletons across the data set were discarded (Rognes et al. 2016). Sequences were screened for chimeras using de novo mode in UCHIME (Edgar et al. 2011) and the resulting chimeric sequences were removed. A representative sequence was chosen from the most abundant sequence for each OTU, and taxonomy was assigned using BLAST against the Silva database (version 128) for bacteria, fungi, and metazoan and PR2 database (Guillou et al. 2013) for protozoa using SINA (Pruesse et al. 2012).

Downstream analyses were done with an OTU table normalized with Hellinger's transformation to minimize double-zeros and reduce the influence of large abundances (Borcard et al. 2011). We removed sequences belonging to Chloroplasts and Mithocondrial from 16S rRNA amplicon dataset, discarded the sequences not belonging to Fungi and Metazoa for 18S Fungi and Metazoa datasets, respectively and filtered Streptophyta, Metazoa, fungal and unclassified Opisthokonta sequences for Protozoa dataset. After filtering, a total of 3.301,980 sequences were retained for all taxa together comprehending 36 samples (bulk soil and rhizosphere) for each taxon totalizing 144 samples (Supplementary Table S2). The six replicates sampled for the different treatments were combined for each bulk and rhizosphere to simplify the representation for each system. Species that occur in low quantities across a study site (few occurrences and low abundance) were removed as they generate a large number of zeros in data matrices. To alleviate the bias of zero-inflated data, we reduced data tables for bacteria, protozoa and fungi removing reads below than 10. Similar rationalization was done by Mamet et al. (Mamet et al. 2017). After discarding reads, a total of 617,746 bacteria, 1,601,848 protozoa, 332,956 fungi, and 749,430 metazoan reads were used in the analyses for both soil compartments (bulk and rhizosphere). Ordination of the *16S rRNA* and *18S rRNA* sequences were investigated using a discriminant analysis of principal components (DAPC) implemented in the 'adegenet' R package (Jombart et al. 2010). Permutational multivariate analysis of variance (PERMANOVA) was used to assess statistical differences in community composition among treatments. Factors in the PERMANOVA were treatments, compartment and their interaction using

the 'vegan' package with 10,000 permutations (Oksanen et al. 2017). Unlike other ordination methods, DAPC can optimize cluster variation as this analysis postulate defined previous groups to construct the plot based on different factors. Individual indicator microbial orders associated within the organic and conventional treatments were identified using 'random forest' analysis (Breiman 2001) and 'boruta' (Kursa and Rudnicki 2010) R packages. The selected orders from aforementioned analysis were plotted on heatmap based on square-root transformation, scaled and centered from the raw data to improve normality and homogeneity of variances.

The relationship between pairs of taxa within treatments was tested using co-inertia analysis. The analysis was performed using the 'coinertia' function in the 'ade4' package (Dray and Dufour 2007) and significance of coefficients was tested with the 'randtest' function of the same package. Briefly, this method calculates the covariance of two taxa tables and plots a common ordination space of co-inertia axes in a principal component analyses (PCA) aiming to maximize the concordance between the data sets. The generated RV coefficient is a multivariate generalization of the Pearson correlation coefficient that provides a measure of the consistency of the two data sets and takes a value between 0 and 1 (1 being identical). Bacterial-fungal, bacterial-protozoan and bacterial-metazoan community structure co-variance scores were given by COIA analysis. Siginificative orders responsible for such co-variance had higher score than the 95% of sample normal distribution. This was calculated similarly to the approach used in (Schlemper et al. 2017b). The top 20 co-variant results for each axis were selected in order to infer the strength of the relationships between bacterial and the other analyzed group for each soil compartment. Co-occurrence of bacterial-fungal, bacterial-protozoan and bacterial-metazoan were performed with residue correlation analysis in a latent multivariable model to investigate correlation among groups. The analyses were performed in R using the packages "mvabund" (Wang et al. 2012) and "boral" (Hui 2016). We also used redundancy analysis (RDA) to identify significant covariates among microbial communities for bulk soil samples, physicochemical characteristics and aboveground biomass (roots and shoots biomass) to investigate a range of potential mechanisms underlying soil managements. For each taxonomical group for every RDA analysis, we included alpha diversity data (observed-species and Shannon) of the 3 other taxonomical to represent the influence of the different groups in the analyzed microbial group. Both observed-species and Shannon indices were calculated using a rarefied OTU table accordingly with the sample with less number of reads with QIIME software (Caporaso et al. 2012). The above-ground barley plants biomass data also included in the RDA analysis were cut at the soil surface and shoots and grains fresh weights for spring barley, recorded. After rhizosphere sampling, the plant roots were gently washed and root fresh weights measured.

Shoots and roots were oven-dried at 60°C until a constant weight was achieved, and dry weights measured. RDA analysis and Monte Carlo permutation tests (Braak and Smilauer 2002) were computed using Canoco 4.53.

Results

Responses of individual microbe-microfauna groups in the bulk soil and rhizosphere compartments

We investigated bacterial, protozoan, fungal and metazoan communities in bulk soil and rhizosphere under different fertilization amendments (inorganic, a combined inorganic and organic, and organic). Significant increases in kingdom richness and not in diversity (Shannon) were observed in the bulk soil while the opposite was found for the rhizosphere compartment under organic fertilization (Fig. S1). For each compartment, the organic fertilization samples clustered separately from the mineral and the combined fertilization samples, indicating a different community structure (Fig. 1; Fig. 2). Permutational multivariate analysis of variance

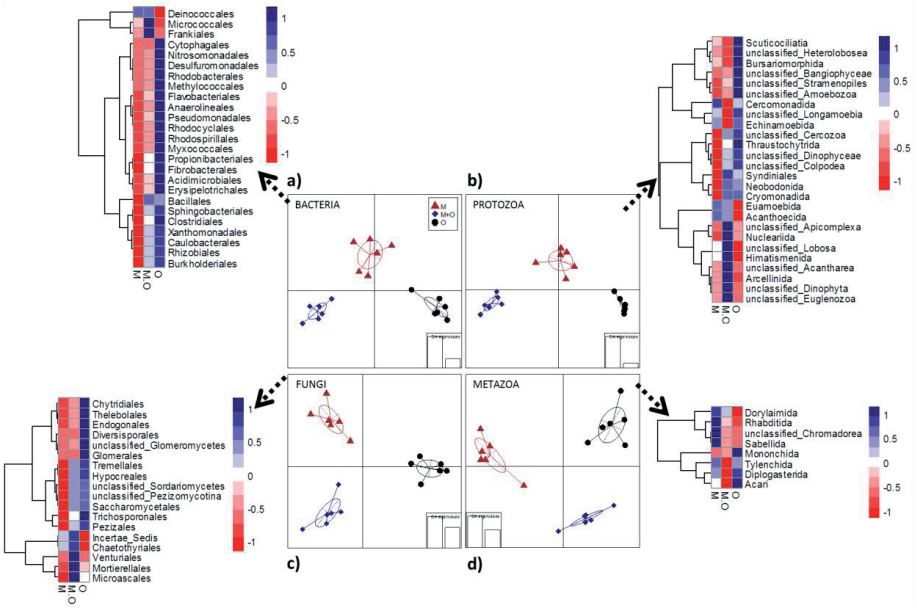


Figure 1: Discriminant analysis of the principal components (DAPC) of different kingdoms (a) bacteria, (b) protozoa, (c) fungi and (d) metazoan in the barley rhizosphere under different fertilization (M = mineral; M + O = a combined of mineral and organic; O = organic). The heatmaps of each DAPC display the relative abundance (Hellinger's transformation) of different taxonomic orders selected by random forest analysis with Boruta feature selection.

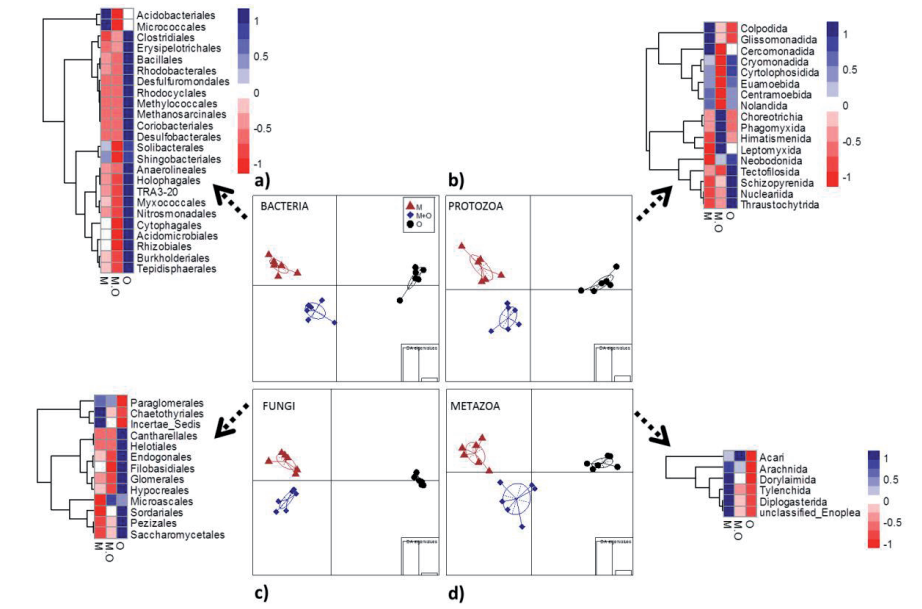


Figure 2: Discriminant analysis of the principal components (DAPC) of different kingdoms (a) bacteria, (b) protozoa, (c) fungi and (d) metazoan in the bulk soil under different fertilization (M = mineral; M + O = a combined mineral and organic; O = organic). The heatmaps of each DAPC display the relative abundance (Hellinger's transformation) of different taxonomic orders selected by random forest analysis with Boruta feature selection.

(PERMANOVA) confirmed the DAPC clustering pattern (P< 0.05; Table 1). Of the variation, 2–3% was explained equally by treatment and soil compartment for bacteria and protozoans, while for fungi and metazoans, 6–8% was explained by treatment factor and 3% by soil compartment.

Furthermore, we checked for the microbes responsible for the differences found among treatments visible in the DAPC analysis (Fig. 1, Fig. 2). In the rhizosphere, 21 OTUs were highly enriched in the organic treatment (Fig. 1a). Only OTUs from Deinococcus-Thermus (order *Deinococcales*) and Actinobacteria (orders *Frankiales* and *Micrococcales*) decreased significantly in abundance under organic amendment. In the bulk soil compartment, organic addition significantly increased the abundance of most bacterial orders (23); the exceptions were Acidobacteria (order *Acidobacteriales*) and Actinobacteria (order *Micrococcales*) (Fig. 2a). Common orders that were enriched in both the bulk soil and rhizosphere compartments under organic treatment are present in both Fig. 1, Fig. 2.

For protozoa, in the rhizosphere compartment, 13 of 26 total protozoan OTUs were highly enriched in the organic treatment (Fig. 1b). By contrast, in the bulk soil compartment, 8 of 17 selected protozoan OTUs were enriched solely by organic

Table 1: Results of PERMANOVA testing the effects of fertilization treatment (Mineral, Organic and their combinations), compartment (bulk soil and rhizosphere), and their interactions on soil microbial community

Kingdom	Effect	Pseudo-F value	Variance explained	P value
Bacteria	Treatment (T)	**3.6716**	0.04	**0.001**
	Compartment (C)	**2.7922**	0.03	**0.001**
	Interactions	0.92481	0.009	0.529
Protozoa	Treatment (T)	**2.8061**	0.028	**0.004**
	Compartment (C)	**2.9101**	0.0291	**0.008**
	Interactions	0.9418	0.0094	0.444
Fungi	Treatment (T)	**7.7368**	0.08	**0.001**
	Compartment (C)	**3.5084**	0.03	**0.004**
	Interactions	0.7643	0.007	0.714
Metazoa	Treatment (T)	**6.0416**	0.06	**0.001**
	Compartment (C)	**3.5535**	0.03	**0.001**
	Interactions	0.80162	0.008	0.699

amendment (Fig. 2b). Under organic treatment, Alveolata (orders *Scuticociliatia*, unclassified *Colpodean*, *Bursariomorphida* and *Syndiniales*), Excavata (unclassified *Heterolobosea* and *Neobodonida*), Stramenopiles (unclassified *Stramenopiles* and *Thraustochytrida*) and Rhizaria (*Cryomonadida* and unclassified *Cercozoa*) were enriched in the rhizosphere, while mostly Excavata (*Neobodonida*, *Schizopyrenida* and *Heterolobosea*) were enhanced in the bulk (Fig. 1b and 2b). Many protozoans, namely orders of Alveolata (*Colpodida*) and Rhizaria (*Cercomonadida* and *Glissomonadida*), decreased under organic fertilization.

Similar patterns of differences between compartments were observed for fungi and metazoans. Of the 18 fungal OTUs, 13 increased in the rhizosphere in the organic fertilization treatment, while 5 were more abundant in the rhizosphere in the combined treatment (Fig. 1c). In bulk soil, 9 of 13 fungal OTUs increased in the organic treatment, and 3 were more abundant in the mineral treatment (Fig. 2c). Under organic fertilization, the phyla Zygomycota (order *Endogonales*), Glomeromycota (order *Glomerales*), and Ascomycota (order *Pezizales*) increased while the phyla *Incertae sedis* (*Rozella*) and Ascomycota (order *Chaetothyriales*) decreased in the bulk soil and rhizosphere compartments. Fewer metazoan OTUs were selected, and this group showed the greatest contrast between the rhizosphere and bulk soil. In the rhizosphere compartment under organic treatment, OTUs from the nematodes *Mononchida*, *Tylenchida*, and *Diplogasterida* and Acari were more abundant, whereas OTUs from the orders *Dorylaimida* and *Rhabditida* were less frequent (Fig. 2d). Interestingly, the addition of organic fertilizer to soil significantly decreased the selected metazoan orders only in bulk soil (Fig. 2d).

Identification of microbe-microfauna paired associations in the bulk soil and rhizosphere compartments neglecting environmental factors

The associations between different microbes and microfauna groups were highly significant in both the bulk soil and rhizosphere compartments as indicated by co-inertia analysis (CoIA) (Fig. 3; Fig. 4). The treatments were clearly separated for all inter-kingdom variances in both compartments as revealed by the high non-random covariance of RV rhizosphere = 86–92% and RV bulk soil = 79–96% for all possible combinations ($P < 0.05$). Inter-kingdom comparisons refer to the associations of bacteria, representing prokaryotes, with eukaryotic groups (protozoans, fungi and metazoans). For each pairwise inter-kingdom combination, we assessed the top 40 significant representatives (top 20 of each axis) of the rhizosphere and bulk

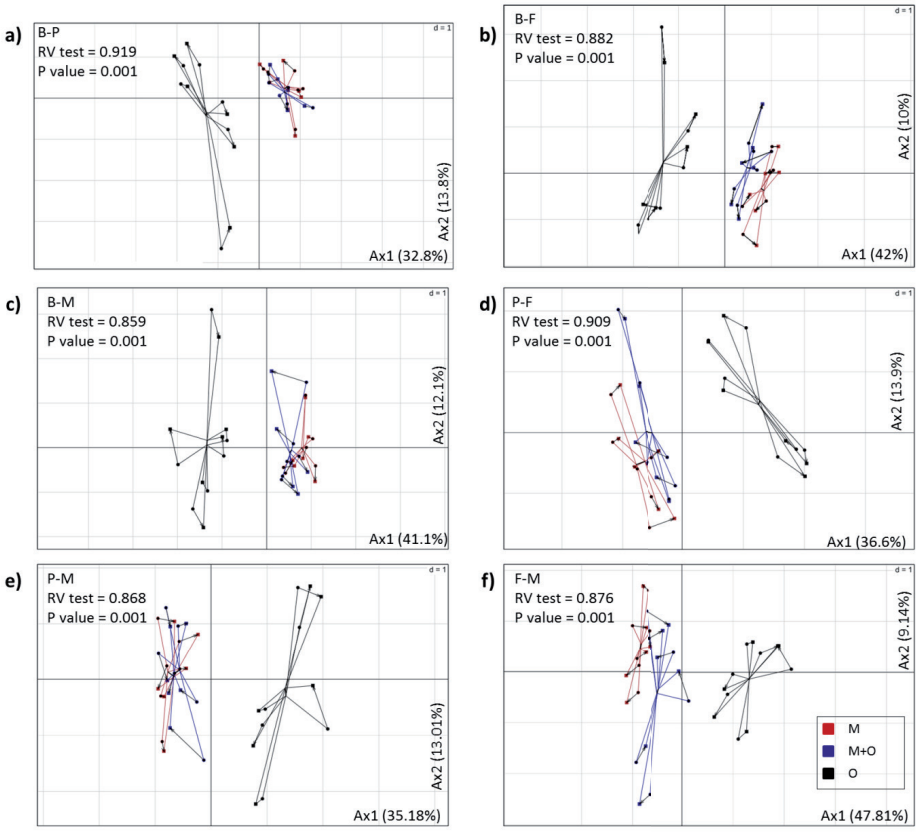


Figure 3: Co-inertia analyses of rhizosphere inter-kingdom interactions between (a) bacteria and protozoa (B-P), (b) bacteria and fungi (B-F), (c) bacteria and metazoa (B-M), (d) protozoa and fungi (P-F), (e) protozoa and metazoa (P-M), and (f) fungi and metazoan (F-M) along different fertilization (M = mineral; M + O = a combined mineral and organic; O = organic). The large boxes contain pairwise comparisons plots colored by treatments, where arrows link kingdom diversity. Arrow length indicates the strength of the association between matrices: short arrows indicate strong concordance; long arrows indicate weak concordance.

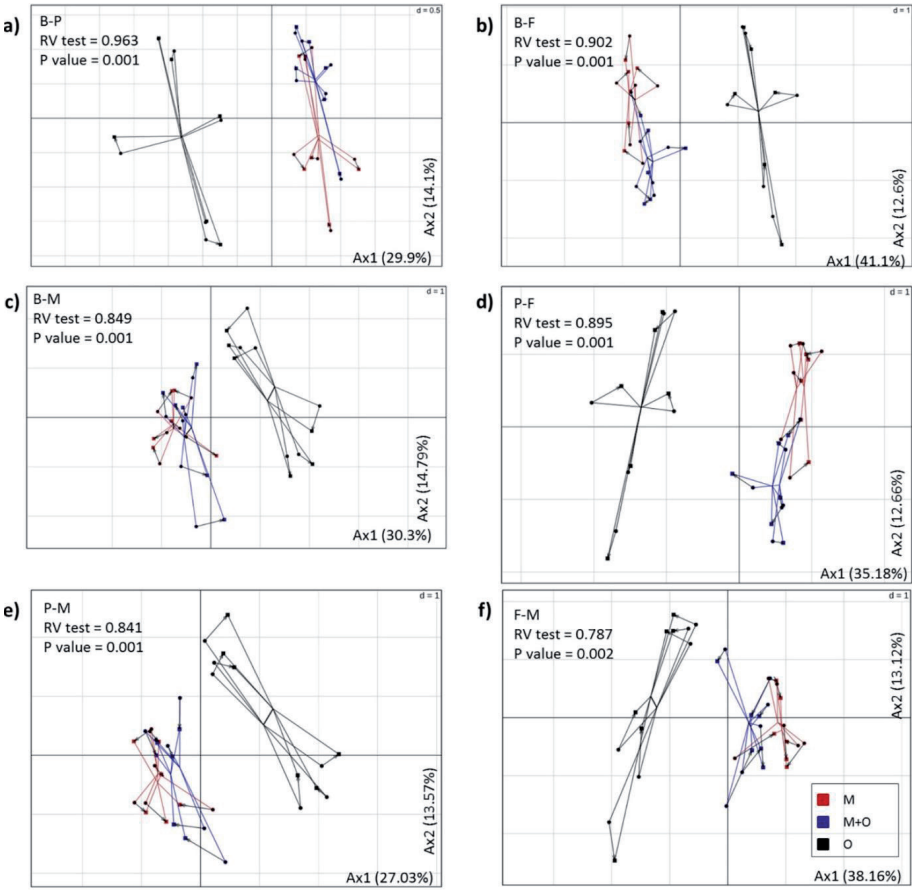


Figure 4: Co-inertia analyses of bulk inter-kingdom interactions between (a) bacteria and protozoa (B-P), (b) bacteria and fungi (B-F), (c) bacteria and metazoan (B-M), (d) protozoa and fungi (P-F), (e) protozoa and metazoa (P-M), and (f) fungi and metazoan (F-M) along different fertilization (M = mineral; M + O = a combined mineral and organic; O = organic). The large boxes contain pairwise comparisons plots colored by treatments, where arrows link kingdom diversity. Arrow length indicates the strength of the association between matrices: short arrows indicate strong concordance; long arrows indicate weak concordance.

soil community groups responsible for the co-variance of each co-inertia axis (Supplementary Tables S3 and S4). Typically, the first two axes of the association between different inter-kingdom combinations represented 40.6–56.95% of the variance (considering bulk and rhizosphere compartment plots) (Fig. 3; Fig. 4). To confirm this directional pairwise co-variation, we assessed whether the abundances of the individual bacterial OTUs selected for in one pairwise co-inertia were similar to the other inter-kingdom variances, regardless of the effects of soil management (Table S3; Table S4).

Strength of selected paired inter-kingdom associations in the bulk soil and rhizosphere compartments neglecting environmental factors

After extracting the paired selected OTUs that co-varied together from Supplementary Tables S3 and S4, we investigated the strength of these possible interactions between bacteria and three other kingdoms, excluding possible intra-kingdom co-occurrences, for each treatment and for each compartment. For bacteria-protozoans (B-P), we found only positive interactions under mineral fertilization in the bulk

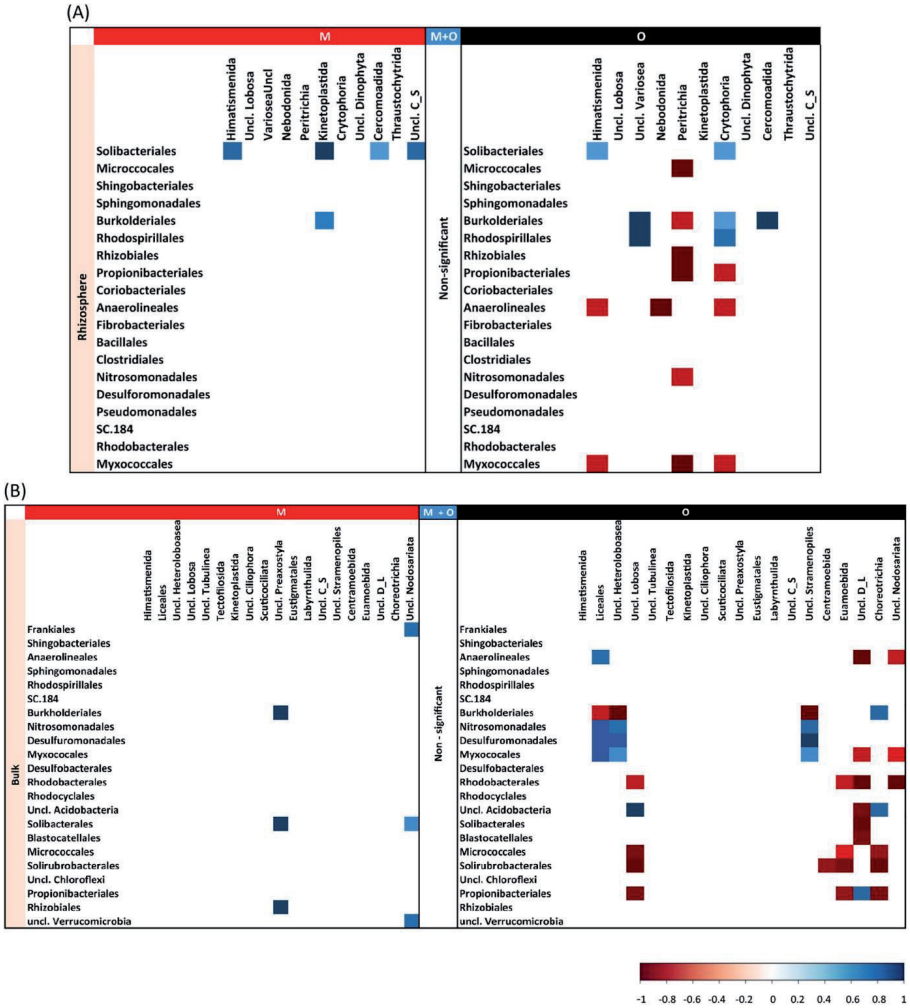


Figure 5: Diagram of correlations between bacterial and protozoan (BP) communities in rhizosphere (A) and bulk soil (B) compartments under different fertilizations (M = Mineral, M + O = a combined mineral and organic; O = Organic). Squares approaching blue color represent positive correlations while squares approaching red color represent negative correlations. White squares are non-significant relationships. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and rhizosphere compartments, while under organic fertilization, we identified a more complex environment, with 7 positive and 12 negative interactions in the rhizosphere and 14 positive and 24 negative interactions in the bulk compartment (Fig. 5). In general, different interactions were selected in the bulk and rhizosphere; we therefore focused on individual orders selected in random forest analysis (Fig. 1; Fig. 2). In the organic treatment, some bacterial orders showed positive and negative correlations depending on the protozoan order. For example, *Burkholderiales* (Betaproteobacteria) correlated positively with *unclassified Variosea* (Amoebozoa), *Chyrtophoria* (Alveolata) and *Cercomonadida* (Rhizaria) in the rhizosphere and with *Choreotrichia* (Alveolata) in bulk soil. In addition, *Burkholderiales* correlated negatively with *Pertrichia* (Alveolata) in the rhizosphere and with *Liceales* (Amoebozoa), *unclassified Heterolobosea* (Excavata) and *unclassified Stramenopiles* in the bulk soil compartment. By contrast, no significant co-occurrence between the bacterial and protozoan groups was observed in the combined treatment.

The associations between bacteria and fungi (B-F) showed only positive correlations in bulk soil in the mineral treatment, while positive and negative correlations were found in both compartments in the organic treatment (Fig. 6). The bulk soil of the combined treatment also showed complex interactions comprising positive and negative correlations; however, none of the associations involved the individual orders selected in the random forest analysis. Furthermore, in M + O and M treatments there were no significant interactions in rhizosphere and bulk compartments, respectively. The strongest positive correlations were between the bacterial order *Propionibacteriales* (Actinobacteria) and the fungal orders *Filobasidiales* (*Basidiomycota*), *Sordariales* and *unclassified Chaetothyriales* (Ascomycota), and *Endogonales* (*Zygomycota*) in the bulk soil in the mineral treatment. In the same compartment (bulk), the order *Propionibacteriales* (Actinobacteria) had a similar positive correlation with *Sordariales* but negative correlations with *Tremellales*, *unclassified Leotiomycetes* and *Chaetothyriales*. We also found positive correlations between *Rhodobacterales* and *Pleosporales* (Ascomycota) and negative correlations between *unclassified Leotiomycetes* and *Chaetothyriales* (Ascomycota). The bacterial orders *Desulfuromonadales* and *Micrococcales* presented similar positive correlations with *Cantharellales*, *unclassified Sordariomycetes*, *Agaricomycetes* (Basidiomycota), and *Dothideomycetes* (Ascomycota) in bulk soil, while *Bacillales* correlated positively with *Glomerales* (Glomeromycota) and *Saccharomycetales* (Ascomycota) in the rhizosphere.

The associations between bacteria and metazoans (B-M) revealed positive and negative relationships in bulk soil in the mineral treatment and only negative correlations in the rhizosphere in the organic treatment (Fig. 7). Besides, the organic treatment showed non-significant associations for rhizosphere. In the rhizosphere

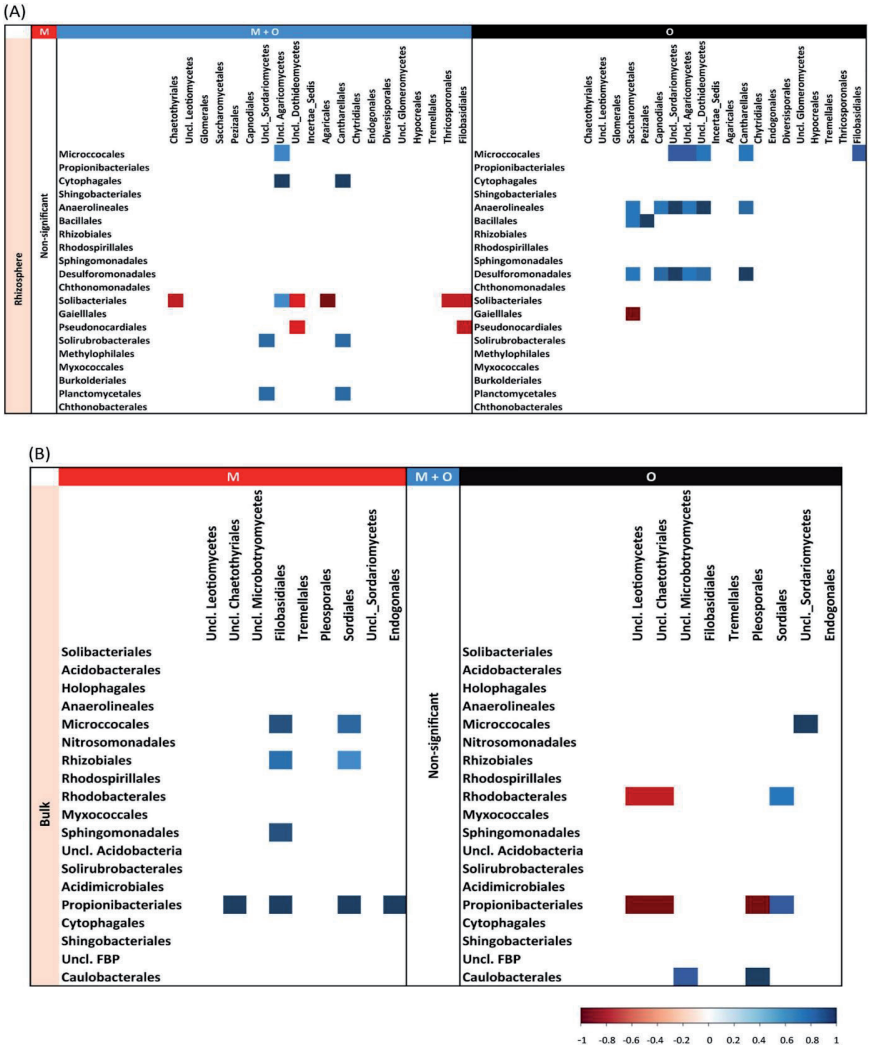


Figure 6: Diagram of correlations between bacterial and fungal (BF) community in rhizosphere (A) and bulk soil (B) compartments under different fertilizations (M = Mineral, M + O = a combined mineral and organic; O = Organic). Squares approaching blue color represent positive correlations while squares approaching red color represent negative correlations. White squares are non-significant relationships. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compartment in the mineral and combined treatments, both *Burkholderiales* and *Bacillales* correlated positively and negatively, respectively, with the same metazoan order, Haplotaxida. In bulk soil, the correlations differed between treatments and compartments (Fig. 7). In the organic treatment, both *Coriobacteriales* and *Myxococcales* were linked negatively with *Rhabditida* and unclassified *Chromadorea*.

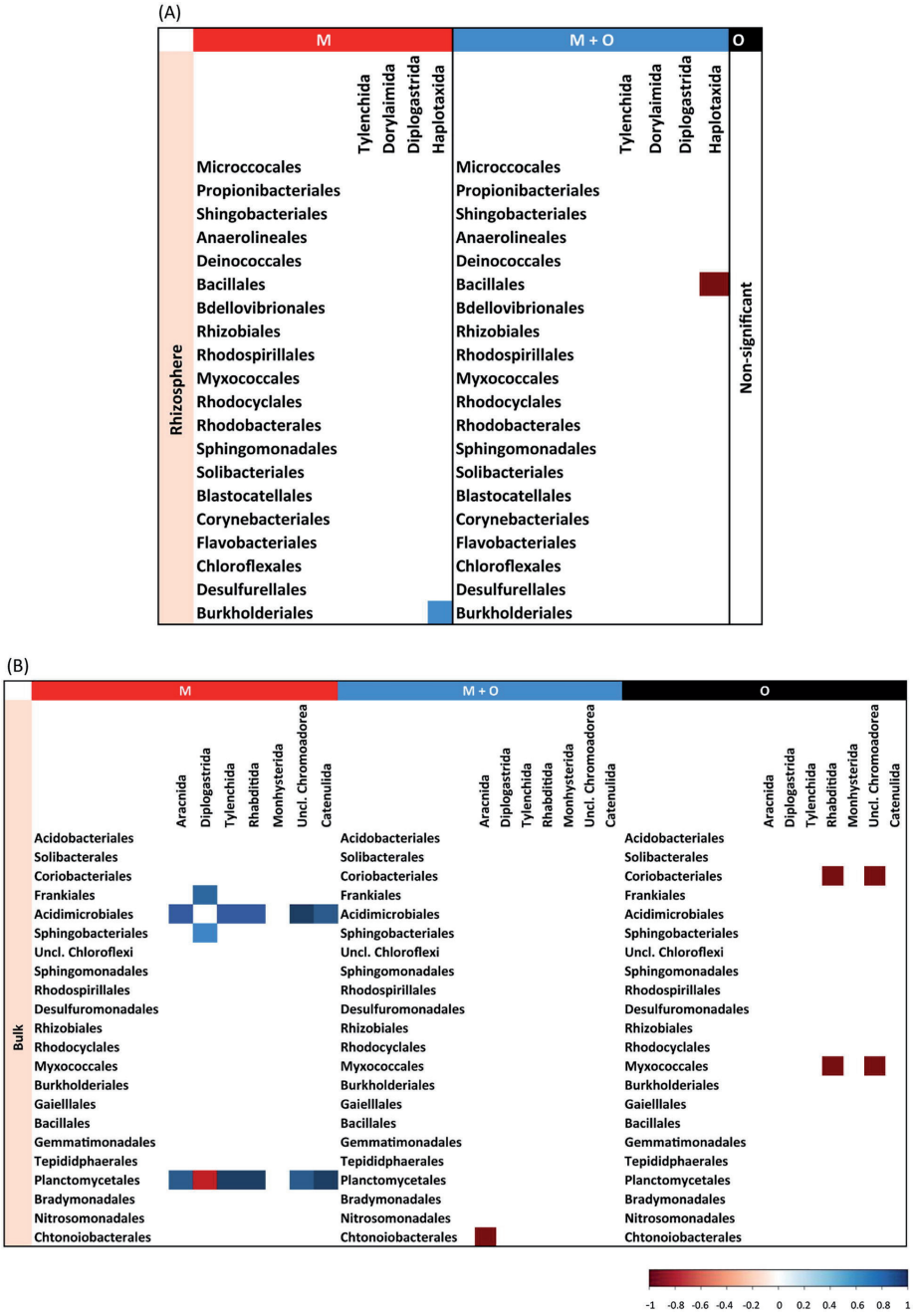


Figure 7: Diagram of correlations between bacterial and metazoan (B-M) community in rhizosphere **(A)** and bulk soil **(B)** compartments under different fertilizations (M = Mineral, M + O = a combined mineral and organic; O = Organic). Squares approaching blue color represent positive correlations while squares approaching red color represent negative correlations. White squares are non-significant relationships.

Inter-kingdom associations in the bulk compartment considering environmental factors

Redundancy analysis (RDA) revealed compositional differences for all kingdoms, mainly in the organic fertilization treatment; this variance appeared to be driven by different factors unique to each kingdom, but shared factors also varied considering pairwise kingdoms (Table 2; Supplementary Table S5; Fig. S2a to S2d). We identified only significant belowground and inter-kingdom correlations in the bacterial and metazoan communities (P< 0.05) (Table 2; Fig. S2a; Fig. S2d). Interestingly, bacteria influenced protozoan diversity in the mineral and combined treatments and abundance in the organic treatment, while metazoans influenced protozoan richness and bacterial diversity in the organic treatment (although not vice versa as inferred from the RDAs). For fungi (36.3% of the total variance in the matrix) and protozoans (6.9%), we found correlations related only to soil factors (Table 2; Fig. S2b; Fig. S2c). No significative correlation was found for barley biomass representing aboveground interactions with soil microbes. The biomass data of roots and shoots of barley are shown Table S1.

Table 2: Redundancy analysis (RDA) of soil biota belonging to different kingdoms in bulk soil compartment

Kingdom	Covariate	Variance explained (%)	F	P-value
Bacteria	Na-plant available	35.4	4.4	0.002
	pH	13.5	1.8	0.018
	Organic matter	11.8	1.6	0.034
	Diversity-Protozoa	10.3	1.5	0.05
	S-plant available	12	1.8	0.008
	OTUs-Protozoa	12	1.9	0.006
Protozoa	Organic matter (%)	14.9	2.8	0.002
	Total N stock	7.4	1.4	0.016
	P-plant available	7	1.4	0.022
	C/N ratio	6.6	1.3	0.048
Fungi	K-plant available (kg K/ha)	19.6	3.9	0.002
	N-supplying capacity (kg N/ha)	8	1.7	0.05
	S-plant available (kg S/ha).6	8.7	1.9	0.038
Metazoa	K-plant available (kg K/ha)	17	3.3	0.002
	Diversity-Bacteria	9	1.8	0.022
	S-plant available (kg S/ha).5	7.5	1.6	0.014
	Richness-Protozoa	7.6	1.7	0.022

Discussion

Intensive soil management practices effects soil physical and chemical characteristics, especially soil microbial community structure and diversity (Kuramae et al. 2012; Navarrete et al. 2013; Pan et al. 2014), which in turn affect vital soil ecosystem services such as nutrient cycling (Moore et al. 2004; Power 2010). The effect of organic amendment on bacterial community composition and structure is well-known (Leite et al. 2017; Lourenço et al. 2018; Lupatini et al. 2019; Soares et al. 2016; Suleiman et al. 2018), but has been less studied for other soil biota (Lupatini et al. 2019). Furthermore, most studies focus on evaluation of the total microbial community and fail to determine its active fraction, RNA-based community (Blagodatskaya and Kuzyakov 2013) which can be directly connected to soil disturbances. No study has simultaneously examined the effects of different fertilizations, environmental factors, plant biomass, and active multi-kingdom biota communities in the bulk and rhizosphere compartments. We hypothesized that organic fertilization would affect soil kingdom communities, with a stronger association between different trophic levels in the rhizosphere than in bulk soil. By contrast, we found strong interactions between all kingdoms in both the bulk soil and rhizosphere compartments. The background for such hypothesis is that resource competition and predation are the two major driving forces underlying the dynamic changes of species composition in the biological community (Chesson and Kuang 2008) and this would most probably occur in different trophic levels. For example, Bonkowski et al. (2000) accompanied high production rates of protozoa suggesting effects on nutrient mineralization due to grazing on bacteria. Similarly, Jiang et al. (2017) showed positive correlation between the most dominant bacterivores nematode *Protorhabditis* and the ALP-producing keystone 'species' *Mesorhizobium*. In the same direction, Koller et al. (2013) found protozoans as improving foraging efficiency of arbuscular mycorrhizal fungi in soil to the benefit of host plants.

The influence of the organic fertilization treatment on the structure of all of the soil kingdom communities in the bulk soil and barley rhizosphere clearly differed from that of the other treatments (Fig. 1, Fig. 2). Some studies demonstrated that inorganic fertilizer application decreased the bacterial community diversity (Wang et al. 2017), while organic fertilizer amendment increased the bacterial community diversity (Bonilla et al. 2012). In this study, inorganic and organic fertilizers amendments rarely altered the microbial community alpha diversity index.

Our results revealed taxonomic orders exclusively to one compartment (bulk or rhizosphere) for all kingdoms and many orders were shared between them (Fig. 1, Fig. 2). This could be explained as rizospherical microorganisms are a subset of the soil biota (Armada et al. 2018; Barbosa Lima et al. 2015; Turner et al. 2013) and

rhizosphere-exclusive taxa are considered the ones under detection limit in the soil and highly enriched by root activity. Among bacteria, not surprisingly, we perceive a trend for specific organisms under organic fertilization, that is, organisms associated with the N and C cycles. In general, orders of Bacteroidetes, Actinobacteria and Proteobacteria increased with organic fertilization, as they play roles in organic matter turnover and carbon cycling (Suleiman et al. 2016). *Cytophagales* and *Sphingobacteriales* (phylum Bacteroidetes), which were found in both soil compartments, metabolize a wide array of carbon sources and complex organic molecules, such as cellulose, chitin, and pectin (Kirchman 2002; Reichenbach 2006). In particular, *Sphingobacteriales* harbor carbon metabolism genes associated with di- and oligosaccharide degradation and fermentation, genes involved in the utilization of xylose, and other plant-associated genes (Hester et al. 2018). Similarly, *Myxococcales* (class Deltaproteobacteria) are cellulolytic organisms that produce secondary metabolites with antibacterial and antifungal activities and act as micro-predators in the soil, thereby actively affecting the microbial food web (Gaspari et al. 2005; Lueders et al. 2006; Proksch et al. 2003; Reichenbach and Dworkin 1992; Weissman and Müller 2009; Zhou et al. 2014). *Acidimicrobiales* (phylum Actinobacteria) participate in C decomposition by secreting glycoside hydrolases and carbohydrate esterases (Barabote et al. 2009). However, the Actinobacteria order *Micrococcales* responded negatively to organic fertilization alone in the bulk soil and rhizosphere while *Anaerolineales* increased with organic fertilization as reported by Chen et al. (Chen et al. 2017). Alpha- and Betaproteobacteria are typically rhizosphere and root colonizers (Bulgarelli et al. 2012; Schlemper et al. 2017a); however, in agreement with a previous study (Wang et al. 2016), members of these classes also increased in bulk soil with organic addition, including the nitrogen-fixing bacteria *Rhodobacterales* and *Rhizobiales* (class Alphaproteobacteria), *Rhodocyclales* and *Burkholderiales* (class Betaproteobacteria), which can be involved in denitrification, and *Nitrosomonadales* (class Betaproteobacteria), which are involved in the nitrification process via autotrophic conversion of NH_4^+ to NO_3^-.

These results demonstrate that the addition of organic fertilizer increases the activity and abundance of microorganisms participating in the N cycle (Lourenço et al. 2018; Suleiman et al. 2018) and might have served as an inoculum for introducing taxa as *Erysipelotrichales* and *Bacillales* (Suleiman et al. 2016). *Desulfuromonadales* (class Deltaproteobacteria), another order that increased with organic amendment, are involved in sulfur metabolism pathways and include several humus-reducing microorganisms (Martinez et al. 2013; Warren et al. 2016). Firmicutes are a fast-growing group that was enriched under organic amendment (Hayden et al. 2012; Lupatini et al. 2017; Pitombo et al. 2016; Sharmin et al. 2013).

It is important to emphasize the merits of using RNA to characterize eukaryotic soil microorganisms because of its ability to target the metabolically active community. Organic fertilization primarily alters different protozoan groups in the bulk soil and rhizosphere (Guo et al. 2018), but we also found differences in protist community members between the soil and rhizosphere. In general, organic fertilization had the greatest effect on the abundance of groups considered predators of other taxonomic groups, such as Stramenopiles, Alveolata, Rhizaria and Excavata in the rhizosphere and mostly Excavata and Stramenopiles in bulk soil. Within Stramenopiles, we found an increase in the saprophytic osmo-heterotrophic protist group *Thrautochytrida* in bulk soil and the rhizosphere. This group can contribute to the degradation of highly refractory organic compounds (Bongiorni et al. 2005). Our results showed that organic addition increased groups belonging to *Ciliophora*, which are useful as bioindicators of the soil environment (Lara and Acosta-Mercado 2012). In the rhizosphere, (Clarholm 1981; Ekelund and Ronn 1994) showed that organic compounds released by plants can stimulate bacterial and ciliate activity in the root zone, leading to mineralization of organic soil nitrogen and assimilation by living plants. Rhizaria include agriculturally important plant pathogens; in particular, Cercozoa, which were more enriched in the rhizosphere, contain a variety of groups that mainly specialize in the predation of other taxonomic groups, such as bacteria, fungi and nematodes, suggesting a potential role in structuring soil food webs in agroecosystems (Bass et al. 2009; Bulman and Braselton 2014; Harder et al. 2016; Howe et al. 2009). Excavata also increased in bulk soil and the rhizosphere with organic amendment. Within Excavata, *Euglenozoa*, which include free-living and parasitic protists capable of moving with flagella, were selected in the rhizosphere, while *Heterolobosea*, which were selected only in the bulk compartment, appear to have a ubiquitous distribution, including in extreme environments (Amaral Zettler et al. 2002; De Jonckheere 2006; De Jonckheere et al. 2011; Park and Simpson 2011). By contrast, Amoebozoa were notably abundant in the combined inorganic and organic treatment and decreased only in the rhizosphere in the organic treatment, despite their sensitivity to environmental changes such as fertilization (Krashevska et al. 2014; Smirnov et al. 2008). In summary, soil management strategies such as organic amendments can greatly alter the soil protist community, which encompasses a wide range of functions such as predators, saprophytes, and nutrient mineralization and thus has potential consequences for the soil food web in agricultural systems.

Similar to Protozoa, organic amendment had different effects on groups of fungi in bulk soil and the rhizosphere. The phyla Zygomycota (order *Endogonales*), Glomeromycetes (order *Glomerales*), and Ascomycota (order *Pezizales*) increased while the phyla Incertae Sedis (*Rozella*) and Ascomycota (order *Chaetothyriales*)

decreased in both compartments in the organic treatment. Our results suggest that agricultural activities steer habitat preference for fungal arbuscular mycorrhizae (AMF) to facilitate plant growth through symbiosis (Sikes et al. 2010). Fungal species belonging to *Endogonales* and the *Glomerales* are plant symbionts (Bidartondo et al. 2011; Field et al. 2015) that decreased under inorganic fertilization, probably due to the presence of nutrients that are readily available to plants (Johnson et al. 2010). The application of organic matter can also enhance the growth of fungi that antagonize pathogenic species and suppress the growth of pathogenic fungi (Cwalina-Ambroziak et al. 2010; Saxena et al. 2015). Consistent with these patterns, *Pezizales*, which are known to be saprotrophic (Hansen and Pfister 2006) were enriched in the organic treatment. Moreover, in the mineral fertilization treatment, we observed an increase in *Incertae Sedis*, which are considered parasites of other fungi and protozoans (Lara et al. 2010; Wurzbacher et al. 2011). These results suggest that parasitism could be a driver of food web stability in the mineral treatment. More harmful fungal taxa with known pathogenic traits were also overrepresented under the mineral regime, such as the order *Chaetothyriales*, well-known as metazoan opportunistic pathogens (Arzanlou et al. 2012; Maharachchikumbura et al. 2015).

Microfauna also control soil organisms, including pests and pathogens, through grazing, predation or parasitism (Costantini et al. 2018). Contrasting relationships between soil metazoan groups and different fertilization regimes were also observed for the rhizosphere and bulk soil. Nematodes that feed on fungi and bacteria tend to increase in organic soils (Lupatini et al. 2019), and we speculate that nematode colonization increased in the rhizosphere due to their potential bacterial vectors. An increase in bacterivorous nematode family such as Cephalobidae or Plectidae could be associated with organic matter decomposition by saprophytic microbes (Ekschmitt et al. 2001). *Tylenchida* nematodes besides plant parasitic they are also classified as possibly fungal feeders (Yeates et al. 1993), and remarkably, this group decreased in bulk soil in the organic treatment. *Diplogasterida* are predators that feed on nematodes and bacteria and are generally abundant in decomposing organic manure (Khan and Kim 2005). Interestingly, some orders (*Acari* and *Tylenchida*) that were selected in the rhizosphere compartment were also associated with bulk soil under mineral fertilization. Wang et al. (Wang et al. 2015) showed that inorganic and organic fertilizers increase the abundance of all soil micro-arthropods and bacterivorous Acari. The difference in metazoan abundances between the rhizosphere and bulk soil could be related to an increase in microbial activity due to high root exudation under organic fertilization. Consequently, microfauna could have increased in the rhizosphere to support multi-trophic interactions by selectively grazing bacteria and fungi to benefit rhizosphere ecological functions (Chen et al. 2017; Chen et al. 2007).

Based on these results, we can summarize many possibilities for different associations among soil biota. We found inter and intra-connections among primary decomposers (bacteria and fungi) and predators (protozoa and metazoans) independent of the soil compartment (Fig. 3, Fig. 4). When environmental factors of bulk soil were taken into account, the inter-connections between primary decomposers and predators became more pronounced (Table 2). *Burkholderiales*, which were more abundant in the organic treatment, presented positive correlations with unclassified *Variosea*, *Chytrophoria* and *Cercomonadida* in the rhizosphere and unclassified *Longamoebia* in bulk soil. *Burkholderiales* also showed negative correlations with *Pertrichia* and *Liceales* in the rhizosphere and with unclassified *Heterolobosea* and *Stramenopiles* in bulk soil. Furthermore, in the rhizosphere compartment, *Burkholderiales* and *Bacillales* correlated positively and negatively, respectively, with the metazoan order *Haplotaxida* in the mineral treatment.

Several possible mechanisms can account for the facilitation of *Burkholderiales* species in protozoan and metazoan communities. Numerous facultative bacterial intracellular pathogens survive as endosymbionts in free-living amoebae, suggesting that growth in the amoebic intracellular environment assists these bacteria in adapting to survival in cells (Bozue and Johnson 1996; Cirillo et al. 1994). There is also increasing evidence suggesting that other bacterial species of the orders *Burkholderiales* (Marolda et al. 1999) and *Bacillales* (Huws et al. 2008) can evade digestion within amoebae, which may increase the survival capacity of these bacteria in the environment. Interestingly, species of *Burkholderiales* are associated with the intestines and casts of earthworms (Singleton et al. 2003), and a previous study showed similarity of an uncultured 16S rRNA gene clone isolated from a cast of *Lumbricus rubellus* with *Burkholderiales*. However, although earthworm casts are composed primarily of soil organisms, it is not clear whether these bacteria are specifically associated with earthworms.

Grazing by heterotrophic protists is a major source of bacterial mortality in soil (Ekelund et al. 2002). Protozoa are the main consumers of bacteria and consequently perform a key role in regulating bacterial biomass and nutrient recycling (Gude 1985; Sherr and Sherr 2002). Our data suggest that bacteria that participate in the N cycle, such as *Burkholderiales*, *Rhizobiales*, *Micrococcales* and *Nitrosomonadales*, have negative interactions with *Peritrichia* (phylum Alveolata) in the rhizosphere. Previous studies in water systems and activated sludge have shown that ciliates, including *Peritrichia*, are important bacterivores (Königs and Cleven 2007) that can release metabolic substances that affect bacterial nitrification (Ratsak et al. 1996). Moreno et al. (2010) showed that peritrich ciliates dominated the acquisition of carbon from bacteria with access to CO_2 under ammonia-oxidizing conditions, while there was no evidence of specific grazing on acetate consumers under denitrifying conditions,

which might partly explain the negative correlation found in the present study. It has also been suggested that protozoan grazing on bacteria led to the development of bacterial protozoan-resistance strategies, which in turn served as drivers for the evolution of pathogenic bacteria (Gong et al. 2016). Protozoan resistance could explain the negative correlations of *Micrococcales* (order Actinobacteria) and *Lobosea* with *Centroamoebida* (Amoebozoa) and *Choreotrichia* (Alveolata) in bulk soil. The ecological mechanisms underpinning the selection of protozoan resistance in bacteria are poorly characterized (Andersson et al. 2018), but it has been suggested that actinobacterial strains cannot be digested by nanoflagellates due to the presence of the protective S-layer (paracrystalline protein surface arrays) in the cell wall, which protects cells from enzymatic degradation in the food vacuoles of ciliates (Apple et al. 2011; Boenigk et al. 2001; Simek et al. 2013; Zwirglmaier et al. 2009). Interestingly, we found positive correlations of the bacterial order *Micrococcales* with different fungal orders (mainly *Sordariales* in different compartments and different treatments). Many members of Sordariomycetes and Actinobacteria are involved in plant decomposition (Kuramae et al. 2013) and thus might cooperate with each other in this soil activity. Another order that deserves attention is *Myxococcales*, which was negative correlated with Amoebozoa (*Himatismenida*), Excavata (*Neobodonida*) and Alveolata (*Crytophoria*) in the rhizosphere and with the nematodes *Rhabditida* and *Monhysterida* in bulk soil. Although protozoans are traditionally considered the main microbial predators of bacteria, the micro-predators Myxobacteria could compete with protozoans and nematodes for nutrient and carbon cycling (Koller et al. 2013). Recently, Petters et al. (2018) showed that Myxobacteria are a keystone taxon with prey-dependent characteristics similar to protozoan dynamics during colonization.

These analyses of the individual microorganisms associated with each kingdom and their co-occurrences reinforce the message that organic fertilization offers a more complex environment than inorganic fertilization (Lupatini et al. 2019). The organic system drives selective grazing, survival strategies, and cooperation in multi-trophic interactions. The inorganic system provides substantial nutrients in readily available forms to plants and biota, and thus no further processing is expected. By contrast, organic fertilizer is a complex nutrient matrix that must be processed and degraded by different biota functional groups into available nutrient forms for plant and biota uptake, and thus positive and negative interactions are expected in such a system. Co-occurrence analysis provides tools for understanding the soil and rhizosphere biota relationships in a system and how these network structure relationships influence soil quality and crop productivity and health. We are aware of the obstacles for translating the results of this study into soil management strategies. One challenge is that the co-occurrence of two groups may not reflect a

real biological interaction. Numerous approaches for discovering connections have been used, such as correlations (Edwards et al. 2015), but each method affords distinct perspectives; therefore, interpretation of co-ocurrences requires cautious consideration of the specific data types and processing steps for a given method. Nevertheless, inferences about interactions are an important starting point for hypothesis development and directions for future studies. In each scenario, the aim is to recognize taxa that may have roles in the soil under different management systems and therefore play crucial roles in soil ecosystem functioning.

Conclusion

Soil management using different fertilization types, such as inorganic or organic, can greatly influences the structure and composition of soil microbiota communities, which are important players in soil functioning. Organic amendments affect microorganisms associated with nutrient cycles and the interactions between soil food web organisms in bulk soil and the rhizosphere. Organic amendment, in general, resulted in more complex interactions, with inter- and intra-connections among primary decomposers and predators independent of soil compartment. The changes in bacterial abundance under organic fertilization selected orders with different potential associations. These associations are most likely related to survival strategies and predation for protozoans and metazoans, while fungal-bacterial associations are most likely mediated by cooperation in decomposition. Future studies should continue the exploration of interactions among different kingdoms while simultaneously targeting their functions to advance our understanding of appropriate soil management and plant health based on soil food webs.

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Availability of data and materials

The raw sequences were submitted to the European Nucleotide Archive (ENA) under study accession number PRJEB31292.

Supplementary information

Supplementary information can be found on: www.sciencedirect.com/science/article/pii/S0048969719338355#s0090

Figure S1: Chao and Shannon diversity indices of all assembled kingdoms in bulk soil (A, B) and rhizosphere (C, D) compartments. OTUs were clustered at 97% similarity level. The different alphabet denotes significance at P= 0.05 (ANOVA; Post-Hoc test: Tukey HSD; n= 6). (M = Mineral; M+O = a combined of mineral and organic; O = Organic).

Figure S2: Biplots of RDAs for each kingdom (a) bacteria, (b) protozoa, (c) fungi and (d) metazoan with significant explanatory variables (red blue arrows within biplots; $P \leqslant 0.05$). The variables are aboveground (plant shoots), belowground (root shoots and soil properties), and inter-kingdom influences on soil biota communities in the bulk soil compartment under different fertilizations (M = Mineral; M+O = a combined of mineral and organic; O = Organic).

Table S1: Physicochemical properties parameters of soil (0 to 20 cm) and plants biomass under different fertilization (M = mineral; M+O = a combined of mineral and organic; O = organic). The number from 1 to 6 represents the different replicates for each treatment.

Table S2: Numbers of good quality sequences and the good's coverage per sample of each kingdom in bulk soil and rhizosphere compartments. (M = Mineral; M+O = a combined of mineral and organic; O = Organic).

Table S3: Selected significant taxa inter-kingdom interactions between bacteria and protozoa (B-P), bacteria and fungi (B-F) and bacteria and metazoan (B-M) in the rhizosphere under different fertilizations.

Table S4: Selected significant taxa inter-kingdom interactions between bacteria and protozoa (B-P), bacteria and fungi (B-F) and bacteria and metazoan (B-M) in bulk soil under different fertilizations.

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CHAPTER 6

Mapping shifts in the active rhizobiome that might underlie low *Meloidogyne chitwoodi* densities in fields under prolonged organic soil management

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Abstract

Plants manipulate their rhizosphere community in a species and even a plant life-stage dependent manner. In essence plants select, promote and (de)activate directly the local bacterial and fungal community, and indirectly representatives of the next trophic level, protists and nematodes. By doing so, plants enlarge the pool of bioavailable nutrients and maximize local disease suppressiveness within the boundaries set by the nature of the local microbial community. MiSeq sequencing of specific variable regions of the 16S or 18S ribosomal DNA (rDNA) is widely used to map plant-based selection and promotion of soil microbiota. As current RNA extraction procedures are time-consuming and expensive, the rRNA-based characterization of active microbiota is taken along less frequently. Recently, we developed a relatively fast and affordable protocol for the simultaneous extraction of rDNA and rRNA from soil. Here we investigated the impact of three soil management regimes on bulk as well as rhizosphere soil in fields with pea (*Pisum sativum*) as a main crop. These fields were naturally infested with the Columbian root-knot nematode *Meloidogyne chitwoodi*. For all soil samples, large differences were observed between resident (rDNA) and active (rRNA) microbial communities. Together with the variable 'sample type' (bulk soil *versus* rhizosphere), nucleotide type (rDNA *versus* rRNA) was shown to be the main explanatory variable. Depending on organismal group, these variables accounted for respectively 6.3–51.3% and 10.4–44.0% of the total variation. Prolonged organic soil management was accompanied by significantly lower *M. chitwoodi* densities as compared to the two conventional systems, and we investigated whether this phenomenon could be associated with bacterial or fungal families that are known to harbor nematophagous members. Active Orbiliaceae (Ascomycota), Olpidiaceae (Olpidiomycota), and Pseudomonadaceae (γ-Proteobacteria) were shown to be present, and a more detailed look at the community data revealed that a local accumulation and activation of nematophagous members of the genus *Pseudomonas* could be seen as a possible explanation for the observed low infestation levels of *M. chitwoodi* in the fields under a prolonged organic soil management regime.

Introduction

For decades, conventional soil management has resulted in consistent and high level of crop production by external inputs such chemical fertilizers and pesticides. However, it is widely acknowledged that intensive monocropping has a number of downsides including soil degradation, leaching of nutrients, and biodiversity loss (Tsiafouli et al. 2014). Organic farming, an umbrella term for a wide range of management regimes having the abstinence of the use of mineral fertilizers and chemical pesticides in common, is a possible alternative that might alleviate the negative impact of crop production on soil ecosystems. In organic farming, most often organic manure is used to replenish the nutrient levels in the top soil and to maintain or increase the overall soil organic matter content. In addition, grain legumes are frequently part of the crop rotation because of their nitrogen binding capacity. However, especially in Europe a wider application of grain legumes is currently hampered, by the relatively high level of variability in yield. This variation is thought to be due to the sensitivity of these crops to biotic and abiotic stressors (Cernay et al. 2015).

One of the key characteristics of sustainable soil management regimes should be the preservation of a relatively high level of soil biodiversity. In terms of biomass, bacteria and fungi are the most important biotic constituents of soils. Depending on soil type, cultivated soils typically harbor 0.2–0.7 mg of bacteria per g of dry soil, whereas the fungal community is represented by 0.01–0.2 mg per g (Kaczmarek 1984). Protists and nematodes are major consumers of bacteria and fungi in soil ecosystems. Although the biomass of protists and nematodes is small compared to the primary decomposers (Bar-On et al. 2018), their impact on the turnover of bacteria and fungi is enormous. Protists alone are typically consuming >50% of the bacterial (Foissner 1999). Though it is a simplification of the biological reality, one could argue that the bacterial and fungal communities are shaped by (1) the quantity and nature of external C and energy inputs into the soil ecosystem, and (2) the activity of protist and nematode communities.

Being present in the soil ecosystem does not imply that a given organism is actively participating in the soil food web. On the contrary, many soil inhabitants are able to reduce their metabolic activity when unfavorable conditions occur, such as food scarcity or drought. This is especially relevant for bulk soils, where typically 80% of the cells, and 50% of the Operational Taxonomic Units (OTUs) are inactive (Lennon and Jones 2011). Hence, it is essential to take both the resident and the active fractions into account when assessing the biological functioning of a soil ecosystem. A range of studies underlined the relevance of the distinction between resident and active soil biota (Baldrian et al. 2012; Nunes et al. 2018; Schostag et

al. 2019). For taxonomic profiling, 16S or 18S ribosomal DNA (rDNA) is often used as molecular marker. Ribosomal RNA is frequently used to map the active microbial fractions (Ofek et al. 2014; De Vrieze et al. 2016). By the molecular characterization of both the resident and the active fractions of the bacterial, fungal, protist and metazoan community, it is possible to assess the impact of soil management regimes on the soil food web (Harkes et al. 2019).

The rhizosphere of plants creates a center of high metabolic activity in soils. At the interface between the plant root and soil, the plant releases primary and secondary metabolites (Hinsinger et al. 2009; Reinhold-Hurek et al. 2015). With this blend of plant-derived components, the plant boosts a specific fraction of the soil biota. In return, stimulated microbiota increase the bioavailability of plant nutrients and/or they may contribute to the protection of the plants against pathogens (Lugtenberg and Kamilova 2009; Berendsen et al. 2012; Turner et al. 2013). Especially in agricultural soil, the microbial community structure was shown to be distinct from the surrounding bulk soil (Sharma et al. 2005). Due to the application of fertilizers, roots exudation is enhanced which on its turn affects the microbial community in the rhizosphere (Zhu et al. 2016).

Next to bacterivores and fungivores, the nematode community harbors a wide range of plant parasites. Most of them are relatively harmless root hair feeders and ectoparasites. Only a small subset may have a high impact in crop production. Root-knot nematodes (RKN), members of the genus *Meloidogyne*, are number one in terms of global crop damage by phytonematodes (Jones et al. 2013). The highly polyphagous Columbian root-knot nematode *Meloidogyne chitwoodi* has a global distribution in temperate climate zones. In this study we investigated the long-term effects of three soil management regimes, conventional, integrated and organic, on the soil microbiome in fields naturally infested with *M. chitwoodi*. The legume *Pisum sativum* was used as main crop in these fields. Illumina MiSeq sequencing was used to characterize the active (rRNA) as well as the resident (rDNA) communities of bacteria, fungi, protozoans and metazoans both in bulk soil and in the rhizosphere. The main objectives of this study were to i) characterize the resident and active microbial community in the rhizosphere of pea ii) compare the active bulk and rhizosphere microbial community in different soil management systems, and iii) identify microbial taxa associated with various infestation levels of the root-knot nematodes *M. chitwoodi*.

Materials and methods

Study sites

Samples were collected at the WUR experimental farm 'Vredepeel' which is located in the south east of the Netherlands (51°32N and 5°51E). Experimental plots were situated on sandy soils (93.3% sand, 4.5% silt, 2.2% clay) with an organic matter (OM) content of 3–5%. Three different soil management regimes were continuously applied from 2001 onwards: 'ConMin', 'ConSlu' and 'Org'. 'ConMin fields' solely received mineral fertilizer and processed organic fertilizer (liquid mineral concentrates), whereas 'ConSlu fields' were supplemented with mineral fertilizer and slurry (pig/cow). In case of organic soil management ('Org fields'), farmyard manure and cow slurry were applied, and no pesticides were used. For further details of the set up and layout of the soil management experiments see the research reports (de Haan et al. 2018a;b).

Soil sampling

Pea (*Pisum sativum*) is one of the main crops in the crop rotation system. Sampling was executed twice in spring 2018, during the vegetative stage (7th of May) and during the generative stage (31st of May). Each of the three fields (ConMin, ConSlu and Org) was divided in 6 subfields of 540 m^2 (Suppl. Fig. S1). In each subfield, a bulk soil and a rhizosphere sample was taken. Rhizosphere composite samples were taken by harvesting all pea plants within a rectangle of 20 x 20 cm. Excessive soil was removed by shaking the plants and whole plants were transported to the laboratory at the field site. Bulk soil was collected by combining three individual cores in the close vicinity of the rhizosphere sampling spot. This was done in between the pea rows with the use of an auger (ø 1.5 cm, depth approximately: 15 cm). In total 36 samples (18 rhizosphere and 18 bulk) were taken at each time point, making a total of 72 samples.

At the field laboratory, the remaining soil that adhered to the roots was brushed off from 10 individual pea plants. Rhizosphere soil and bulk soil samples were frozen in liquid nitrogen and transported on dry ice to the laboratory, and stored at -80°C until further processing.

For nematode extraction a composite sample was collected in each field (ConMin, ConSlu and ORG). In total 12 soil cores (ø 1.5 cm, depth approximately: 15 cm) per field were collected, mixed and stored at 4°C. Two days after collection, nematodes were extracted from 100 g soil, using an elutriator (Oostenbrink 1960). This was done *in duplo* for each field resulting in 6 nematode suspensions.

DNA/RNA extraction and cDNA synthesis

Both DNA and RNA were simultaneously extracted from soil samples (2 g each), using a lab-made protocol based on phenol-chloroform-isoamylacohol extraction (Harkes et al. 2019). Quality and quantity of the obtained RNA and DNA was measured with a Nanodrop and Qubit. The nucleic acid eluate was stored at -80°C until further processing. For synthesis of cDNA from extracted RNA, the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, Thermo Fisher Scientific Inc., USA) was used according to the manufacturer's instructions. All individual DNA and cDNA samples were diluted to 1 ng/µl and 0.1 ng/µl respectively, and used as template for PCR amplification.

Nematode suspensions, as a result from the 100 g composite samples, were concentrated and lysed according (Vervoort et al. 2012). This resulted in 100 µl purified DNA, which served as a template for quantitative PCR (qPCR).

PCR amplification and sequencing

The variable V4 region of bacterial 16S rRNA gene was utilized as target for the analyses of Illumina 16S rDNA sequencing and the V9, V7-V8, V5-V7 regions were utilized as targets for protozoa, fungi and metazoan 18S rDNA sequencing, respectively. To prepare the samples for sequencing, a two-step PCR procedure was followed as described in (Harkes et al. 2019). In short, locus-specific primer combinations extended with an Illumina read area and the appropriate adapter were used to produce primary amplicons. This was done in triplicate for all samples and for each of the four organismal groups. PCR 2 was conducted on 40x diluted amplicons of PCR1 to attach the Illumina index and the Illumina sequencing adaptor. Products of PCR 1 and 2 were randomly checked on gel to ensure amplification was successful. Finally, PCR products of fungi, protozoa and metazoa were pooled and sent for sequencing. Bacterial PCR products were sent separately in order to improve the sequencing resolution. Sequencing was done at Bioscience – Wageningen Research, Wageningen, The Netherlands – using the Illumina MiSeq Desktop Sequencer (2*300nt paired-end sequencing) according to the standard protocols.

For analysis of the obtained nematode DNA from the 100 g subsamples, 12 nematode taxa were selected for qPCR. 11 primer sets to asses a various set of plant parasitic nematodes – including *M. chitwoodi* – and one primer set to measure the total nematode density (see Supplementary Table S1)

Bioinformatics framework

The composition of microbial communities of the soil samples was analysed based on the sequencing data obtained from the Illumina MiSeq platform. Reads were sorted into the experimental samples according to the unique combination of two index sequences. Thereafter, reads were sorted into the four organismal groups based on their locus-specific primer sequences.

Forward and reverse reads were paired for bacteria and fungi, while single-end (forward) sequences were analysed for protozoa and metazoan. The four taxonomical groups were quality trimmed by BBDUK and then merged via VSEARCH (Rognes et al. 2016; Bushnell 2018). Resulting unique sequences were then clustered at 97% similarity by using the usearch_global method implemented in VSEARCH and a representative consensus sequence per *de novo* OTU was determined (Rognes et al. 2016). The clustering algorithm also performs chimera filtering to discard likely chimeric OTUs with UCHIME algorithm in *de novo* mode (Edgar et al. 2011) implemented in VSEARCH. Sequences that passed quality filtering were then mapped to a set of representative consensus sequences to generate an OTU abundance table. Representative OTU sequences were assigned to a taxonomic classification via BLAST against the Silva database (version 12.8) for bacteria, fungi, and metazoan and PR2 database (Guillou et al. 2013) for protozoa using SINA (Pruesse et al. 2012). Sequences belonging to chloroplasts, cyanobacteria and mitochondria were discarded from the bacterial dataset; sequences not belonging to Fungi and Metazoa were removed for 18S Fungi and Metazoa datasets, respectively and Streptophyta, Metazoa, fungal and unclassified Opisthokonta sequences were filtered for Protozoa dataset. Low-abundance OTUs (those with abundance of < 0.005% in the total data set) were discarded prior to analysis (Bokulich et al. 2013). Samples were transformed using Hellinger transformation for all downstream analyses.

Processing and analysis of nematode specific sequences

For the nematode specific analysis, metazoan reads were blasted against a nematode database after quality trimming of the reads. Trimmomatic v.0.35 (Bolger et al. 2014) was used to trim poor quality bases (4 base sliding window with a 13 (P= 0.05) average phred score cut-off), remove the locus specific primer and filter out short reads (< 50 bases). The Blast database was based on the dataset of Holterman et al. (2017). Forward and reverse reads were blasted separately as the sequences did not overlap. Each read had one or multiple families assigned to it based on the best blast hit and any additional hits that differed by no more than 1 base pair from the best blast hit. Reads with less than 92 percent identity to the sequences

in the database were considered not to be nematodes. Reads between 92 and 95 percent identity were counted as nematodes, but no family name was assigned to them. After this the results for the forward and reverse sequence of each mate pair was compared. Where possible, the results of both reads were combined to refine the family assignment. If the family assignments of both reads of a mate pair were in complete disagreement, the reads were discarded. In some cases, the amplified SSU fragment did not allow for the distinction between certain families, and the reads had to be pooled into a larger taxonomic unit, *e.g.* all members of the order Dorylaimida or members of the families Bastianiidae and Prismatolaimidae.

Statistical analysis

Good's coverage was assessed (Good 1953) in order to estimate what percent of the total species is represented in each sample. We explored β diversity patterns by performing principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity using QIIME software (Caporaso et al. 2012). Permutational multivariate analysis of variance (PERMANOVA) was used to compare the microbial community structure between soil managements taken from different sites and with different plant growth stages for active and resident community for 4 different taxa. This was performed with 1,000 permutations using the adonis function, based on Bray-Curtis distances using the "vegan" package (Oksanen et al. 2015) in R. In order to compare microbial community diversity in a phylogenetic context, UniFrac was performed with 1,000 permutations via the "phyloseq" package in R. To assess variation in both relative abundance and presence/absence, we analysed both weighted and unweighted UniFrac distances (Lozupone et al. 2007). To investigate the indicator taxa involved in the differences between resident and active community, a linear discriminate analysis (LDA) effect size (LEfSe) was conducted in Microbiome Analyst (Dhariwal et al. 2017) to explore the differential microbial populations at the family level for the four different taxa (Segata et al. 2011). A significance level of $\alpha \leq 0.05$ was used for all biomarkers evaluated in this study.

To assess differences between family read abundances of nematodes, a Kruskal–Wallis test was conducted, followed by a Dunn's Test to test for significance between each of the three management types (SPSS).

Data availability

The raw sequences were submitted to the NCBI Sequence Read Archive (SRA) database under study accession numbers PRJNA561075 for bacteria, and PRJNA561072 for fungi, protozoa and metazoa.

Results

The long-term impact of organic soil management on four major soil organismal groups was monitored in experimental field where pea was grown as main crop. The bacterial and fungal communities were mapped as main primary decomposers, whereas protists and metazoa (mainly nematodes) were included as representatives of the next trophic level. For each the four organismal groups resident and the active communities were characterized in (1) bulk and rhizosphere soil, (2) with three types of soil management and (3) at two time points representing the vegetative and the generative growth stage of pea.

General analyses of the sequencing data

Total DNA and RNA was extracted from 72 bulk soil and rhizosphere samples. MiSeq sequencing was performed on ribosomal DNA and cDNA fragments (16S for bacteria or 18S for fungi, protists, and Metazoa). After filtering, a total of 22 million sequences with an average length of 250bp were retained, comprehending 288 samples for all taxa together. Comprehensive sampling of the microbial community was performed for all treatments, with average sequence coverage of 95%, 93%, 99% and 99% for bacteria, protozoa, fungi and metazoan, respectively determined by Good's coverage estimate.

Difference between resident and active communities in bulk and rhizosphere soil at organismal group level

To investigate whether contrasts could be observed between resident and active fractions of the individual organismal groups, and between bulk and pea rhizosphere soil, principal coordinate analysis (PCoA) ordinations on Bray-Curtis dissimilarity matrices were conducted (Fig. 1). The effect of the variable Nucleic Acid (rRNA for active and rDNA for the resident community) is clearly visible. For all four organismal group there are distinct clusters for rRNA (red and orange) and rDNA (blue and light blue) although this is somewhat less pronounced in case of Fungi. The effects of sample type (bulk *versus* rhizosphere) were easily observable as well. Especially for bacteria there is a clear separation of the two soil compartments (Fig. 1).

To determine whether soil organismal groups were significantly affected by the four main variables included in this study three distinct methods to compare communities were used: Bray-Curtis dissimilarity, and weighted and unweighted UniFrac. Results of the PERMANOVAs are shown in Table 1. The R^2 values indicate how much of the observed variation is explained by each of the individual variables.

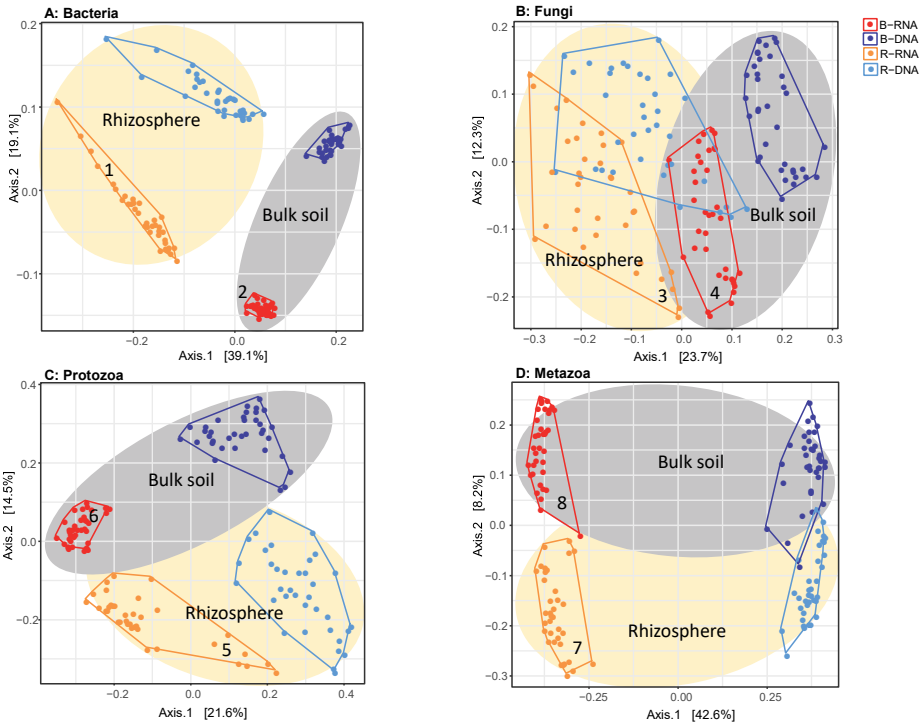

Figure 1: Principal coordinate analysis (PCoA) ordination of a Bray-Curtis dissimilarity matrix. Plots illustrate distances between communities (72 soil samples; for each sample both the resident (rDNA) and the active (rRNA) community were characterized) for each organismal group: **(A)**, Bacteria; **(B)**, Fungi; **(C)**, Protozoa, and **(D)**, Metazoa. Colours were used to distinguish between rRNA-bulk (red), rRNA-rhizosphere (orange), and rDNA-bulk (dark blue), rDNA-rhizosphere (light blue). For all organismal groups: grey ellipses for bulk and ivory ellipses for rhizosphere.

Sample Type (bulk *versus* rhizosphere soil) was the dominant explanatory variable for the observed shifts in the bacterial communities. The large difference between the relevant R^2 values resulting from the Unifrac analyses (unweighted 16.4%, weighted 51.3%), points at a quantitative rather than a qualitative shift. For fungi, Sample Type was most important in the Bray Curtis analysis only. Phylogeny-based distance methods identified Nucleic Acid – the difference between the resident and the active fungal community – as the most dominant variable explaining 12.6 and 25.0% of the observed variations. For the protozoa and the metazoa, Nuclei Acid was identified as the main explanatory factor as well (except for the weighted Unifrac in case of Metazoa).

Table 1: The impact of four variables on four organismal groups in fields with three soil management regimes with pea as main crop. Summary of the PERMANOVA for Bray-Curtis dissimilarity distances as well as phylogenetic distances (UniFrac). This analysis tests differences in quantitative taxonomic composition of Bacteria, Fungi, Protozoa and Metazoa assemblages taking Nucleic Acid (cDNA/DNA), Sample type (Bulk/Rhizosphere), Treatment (ConSlu, ConMin, Org) and Time point (Vegetative/Generative) as main variables. Differences are considered significant if P< 0.01.

	Bray-Curtis		**UniFrac - Unweighted**		**UniFrac - Weighted**	
	R^2	**P**	**R^2**	**P**	**R^2**	**P**
Bacteria						
Nucleic Acid	0.231	0.001	0.137	0.001	0.261	0.001
Treatment	0.076	0.001	0.086	0.001	0.042	0.001
Sample Type	**0.295**	0.001	**0.164**	0.001	**0.513**	0.001
Time Point	0.040	0.001	0.025	0.001	0.021	0.001
Residuals	0.357		0.589		0.163	
Fungi						
Nucleic Acid	0.108	0.001	**0.126**	0.001	**0.250**	0.001
Treatment	0.134	0.001	0.102	0.001	0.091	0.001
Sample Type	**0.172**	0.001	0.066	0.001	0.177	0.001
Time Point	0.045	0.001	0.028	0.001	0.039	0.001
Residuals	0.542		0.679		0.443	
Protozoa						
Nucleic Acid	**0.192**	0.001	**0.305**	0.001	**0.434**	0.001
Treatment	0.082	0.001	0.088	0.001	0.047	0.002
Sample Type	0.125	0.001	0.099	0.001	0.106	0.001
Time Point	0.091	0.001	0.039	0.001	0.083	0.001
Residuals	0.510		0.469		0.329	
Metazoa						
Nucleic Acid	**0.440**	0.001	**0.104**	0.001	0.072	0.001
Treatment	0.040	0.001	0.089	0.001	0.046	0.001
Sample Type	0.063	0.001	0.100	0.001	**0.216**	0.001
Time Point	0.022	0.001	0.023	0.001	0.059	0.001
Residuals	0.440	0.001	0.104	0.001	0.072	0.001

Active taxa contributing to the difference between bulk soil and rhizosphere

LEfSe (Linear discriminant analysis Effect Size) was used to determine which active taxa have the highest contribution to the observed differences between bulk and rhizosphere. A LDA (Logarithmic Discriminant Analysis) threshold of > 2.5 was set, which resulted in 28 bacterial, 20 fungal and 14 protozoan orders that gave rise to the differences between bulk and rhizosphere communities (Fig. 2). It is noted that metazoans are not displayed. Soil samples of 2 g were analysed, and the sample size is too low to give a genuine impression about the composition of the micro and mesofauna. Metazoa were nevertheless taken along as co-extraction of their DNA is indicative for spatial association of microbial taxa and the detected metazoans.

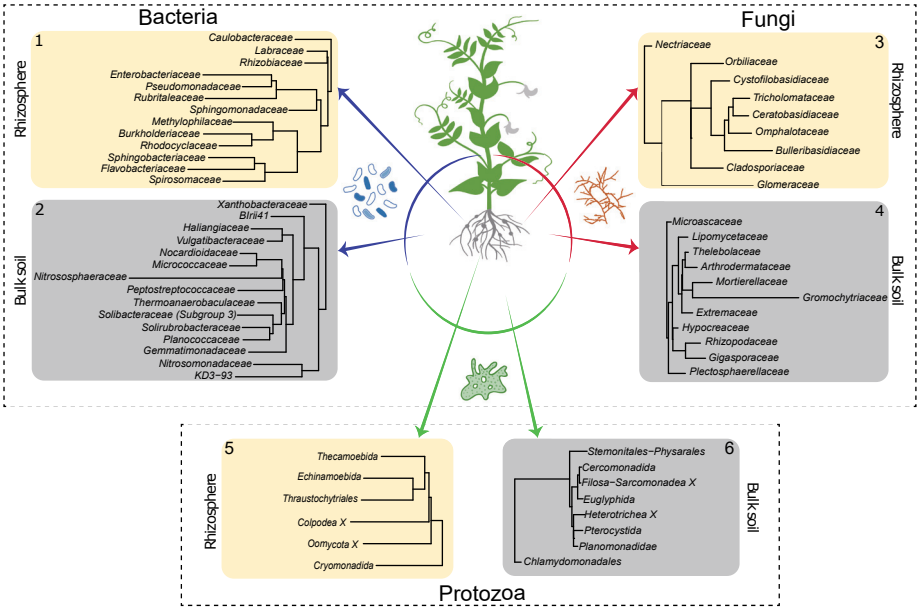

Figure 2: LEfSe analysis of the active bacterial, fungal and protists OTUs. Identifying taxa for which a major part of the population was active in rhizosphere (ivory square) or in bulk soil (gray square) (LDA score > 2.5).

The elevated activities of members of Rhizobiaceae and Labraceae (both Rhizobiales) were detected in the rhizosphere of pea. As *Pisum sativum* is nodulated by the N_2–fixing *Rhizobium leguminosarum* an upregulation of the Rhizobiaceae was expected (Fig. 2, panel 1). Moreover, increased activity was observed for number of bacterial families that harbour P-solubilizing members such as Rhizobiacea (including the genus *Rhizobium*), Enterobacteriaceae (including *Serratia*), Pseudomonadaceae (including *Pseudomonas*), Burkholderiaceae (including *Burkholderia*) (Pii et al. 2015). In the bulk soil we observed a relatively high activity of the Nitrosophaeroceae (Archaea, Thaumarchaeota). Representatives of this family are known as ammonia oxidizers (Stieglmeier et al. 2014). In addition, a bacterial family that harbours members that initiate the oxidation of ammonia to nitrite, the Nitrosomonadaceae, showed enhanced activity.

Regarding fungal families with upregulated activity in the rhizosphere at least two observations are noteworthy (Fig. 2, panel 3). The Orbiliaceae harbour numerous nematophagous fungi, and this could affect the root-knot nematode *Meloidogyne chitwoodi* present in these experimental fields. Differential activity of two members of the Glomeromycota were observed in the two compartments. Whereas Glomeraceae (order Glomerales) showed enhanced activity in the

rhizosphere, elevated levels of Gigasporaceae (order Diversisporales) activity were detected in the bulk soil (Fig. 2, panels 3 and 4). This observation suggest that pea might interact with a member of the order Glomerales.

Members of the order Thecamoebida (Fig. 2, panel 5) are known as large protists and voracious predators of bacteria and other protozoans (Melton et al. 2019), which explains their elevated activity in the rhizosphere. Another representative of the naked amoebae, the Echinamoebida, was highly active in the rhizosphere. Terrestrial Colpodea, small bacterivorous ciliates, are known as extreme r-strategists (Lüftenegger et al. 1985), and they can easily cope with fluctuating environmental dynamics, this might explain their enhanced activity in the rhizosphere (Foissner 1993). The detection of active Pterocystida in bulk soil (Fig. 2, panel 6) is remarkable as they belong to the heliozoan protists that are normally found in freshwater and marine environments, and only occasionally in soil. Also, the enhanced presence of active Chlamydomonadales, an order of green algae, is worth noting. We assume that these photosynthesizing protists were present at the very top layer of the bulk soil.

Difference between resident and active communities under three distinct soil management regimes at organismal group level

To determine the level at which the soil management regimes ConMin, ConSlu and Org had an effect on the four organismal groups, we analysed the rDNA sequence data separate from the rRNA data. As can be seen in Table 2, soil management ('Treatment') had a significant effect on both the composition of the communities, and their levels of activity for all four organismal groups (in all cases $P < 0.001$). These analyses also showed that the compartment effect, the contrast between rhizosphere or bulk soil, is consistently larger than the treatment effect.

Principal coordinates analysis (PCoA) was used to visualize the effect of prolonged exposure to three distinct management regimes in bulk soil and rhizosphere on the active and resident communities of each organismal groups. As can be seen in Fig. 3, soil management had a major impact on all four organismal groups. 'DNA bulk' shows the resident communities in absence of the main crop, and the communities in the fields under organic management were distinct from two conventional treatments, ConMin and ConSlu. In case of 'RNA bulk', the same trend was observed. Another interesting shift was observed within the two conventional treatments (ConMin in red and ConSlu in blue). Whereas the soil communities in bulk soil were fully separated in all three different management types, the two conventional treatments tend to overlap in their communities in the rhizosphere. It is noted that the highest percentage of the variation explained

Table 2: The impact of three variables on the resident and the active fractions of four organismal groups in fields with three soil management regimes with pea as main crop. This summary of the PERMANOVA for Bray-Curtis dissimilarity distances shows the impact of Treatment (ConSlu, ConMin, Org), Sample type (Bulk/Rhizosphere), and Time point (Vegetative/Generative) on four categories of soil inhabitants. Differences are considered significant if P< 0.01. For Unifrac-based analyses see Suppl. Table S2.

		rDNA		rRNA	
Source		**R^2**	**P**	**R^2**	**P**
Bacteria					
Treatment		0.101	0.001	0.103	0.001
Sample Type		**0.378**	0.001	**0.428**	0.001
Time Point		0.048	0.001	0.063	0.001
	Residuals	0.472		0.406	
Fungi					
Treatment		0.154	0.001	0.165	0.001
Sample Type		**0.218**	0.001	**0.208**	0.001
Time Point		0.047	0.001	0.063	0.001
	Residuals	0.581		0.565	
Protozoa					
Treatment		0.139	0.001	0.132	0.001
Sample Type		**0.241**	0.001	**0.159**	0.001
Time Point		0.136	0.001	0.120	0.001
	Residuals	0.484		0.589	
Metazoa					
Treatment		0.138	0.001	0.101	0.001
Sample Type		**0.168**	0.001	**0.200**	0.001
Time Point		0.064	0.001	0.067	0.001
	Residuals	0.630		0.632	

by the two axes was observed for 'RNA rhizosphere' with an average of 44.4% for the four organismal groups.

Active rhizosphere taxa contributing most to the observed difference between the three soil management regimes

Fig. 4 show the results of LEfSe analyses that revealed the taxa that contributed most to observed differences in the active pea rhizosphere communities under the three soil management systems. Regarding the bacterial community, families harbouring P-solubilizing members such as the Burkholderiaceae, the Enterobacteriaceae, and the Pseudomonadaceae showed high levels of activity in the organic fields. Members of the Nocardioidaceae showed elevated levels of activity under the ConMin regimes, the family was recently identified as being associated with the domestication of a pea relative, the common bean *Phaseolus vulgaris* (Perez-Jaramillo et al. 2017).

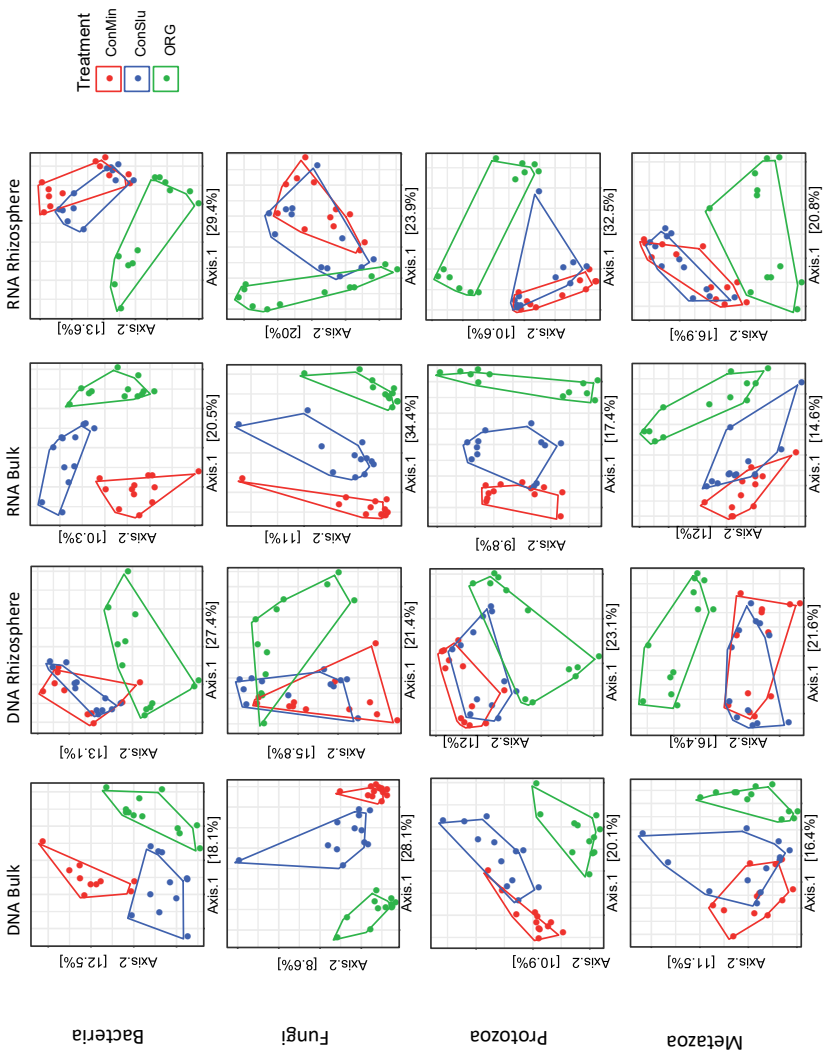

Figure 3: Principal coordinate analysis (PCoA) ordination of a Bray-Curtis dissimilarity matrix. Plots illustrate distances between communities for each of the organismal groups under three different soil management types (ConMin (red), ConSlu (blue), and Organic (green)). Split plots per organismal group for DNA bulk, DNA rhizosphere, RNA bulk and RNA rhizosphere.

Both Actinobacterial families Intrasporangiaceae and Nocardioidaceae showing a high level of activity in the conventional soil treatments ConSlu and ConMin (Fig. 4). Both families are known as glucose-degraders under oxic conditions (Schellenberger et al. 2010). This observation might suggest that pea roots released more glucose under conventional soil management.

The families Nectriaceae and Chaetomiaceae were identified as fungal indicators for fields with the most conventional treatment (ConMin). The high activity of Nectriaceae might be related to the application of mineral fertilizer. A similar phenomenon was observed in tropical rain forest plots treated with mineral fertilizer (Kerekes et al. 2013). The other upregulated member of the Sordariales, the family Chaetomiaceae, is known for its cellulolytic members (Wilhelm et al. 2017)

The Tubulinea orders Euamoebida and Nolandida were metabolically active in the organic fields. The first family was found in high relative abundances in grasslands and forest mineral soils, whereas the Nolandida is a relatively rare protist order in soils (Geisen et al. 2015). Xantophyceae, consisting of stramenopilan photoautotrophs, typify the ConMin fields. Other phototrophs showing enhanced activity in the ConMin fields were members of the green algae order Chlorellales, and the red algae order Cyanidiales.

Soil management-related shifts in nematode communities

Microscopic nematode density counts, and qPCR nematode density data showed no significant differences in overall nematode abundances between the three soil management types (Table 3). By means of rDNA sequencing, taxonomic shifts in nematode communities were detected at family level (Fig. 5). Five out of six bacterivorous families were found to be more abundant in Org fields. In case of the two fungivorous families Aphelenchidae and Aphelenchoididae, small but significant trends towards lower densities in fields under organic management were observed. The predator family Mylonchulidae was specifically upregulated in the ConSlu fields. With regard to the plant parasites, lower infestation levels for the families Telotylenchidae, Heteroderidae and Meloidogynidae were observed in the organic fields.

Table 3: Mean nematode density and *M. chitwoodi* abundance (individuals per 100 g soil) in the pea fields with three distinct soil management regimes. Different letters indicate significant mean difference (Post-hoc Bonferroni P< 0.05). Samples were analyzed microscopically (m) or with quantitative PCR (q).

	ConMin	ConSlu	Org
Nematode density [m]	3100	3210	2880
Nematode density [q]	1551	1622	1440
M. chitwoodi [q]	58[a]	65[a]	2[b]

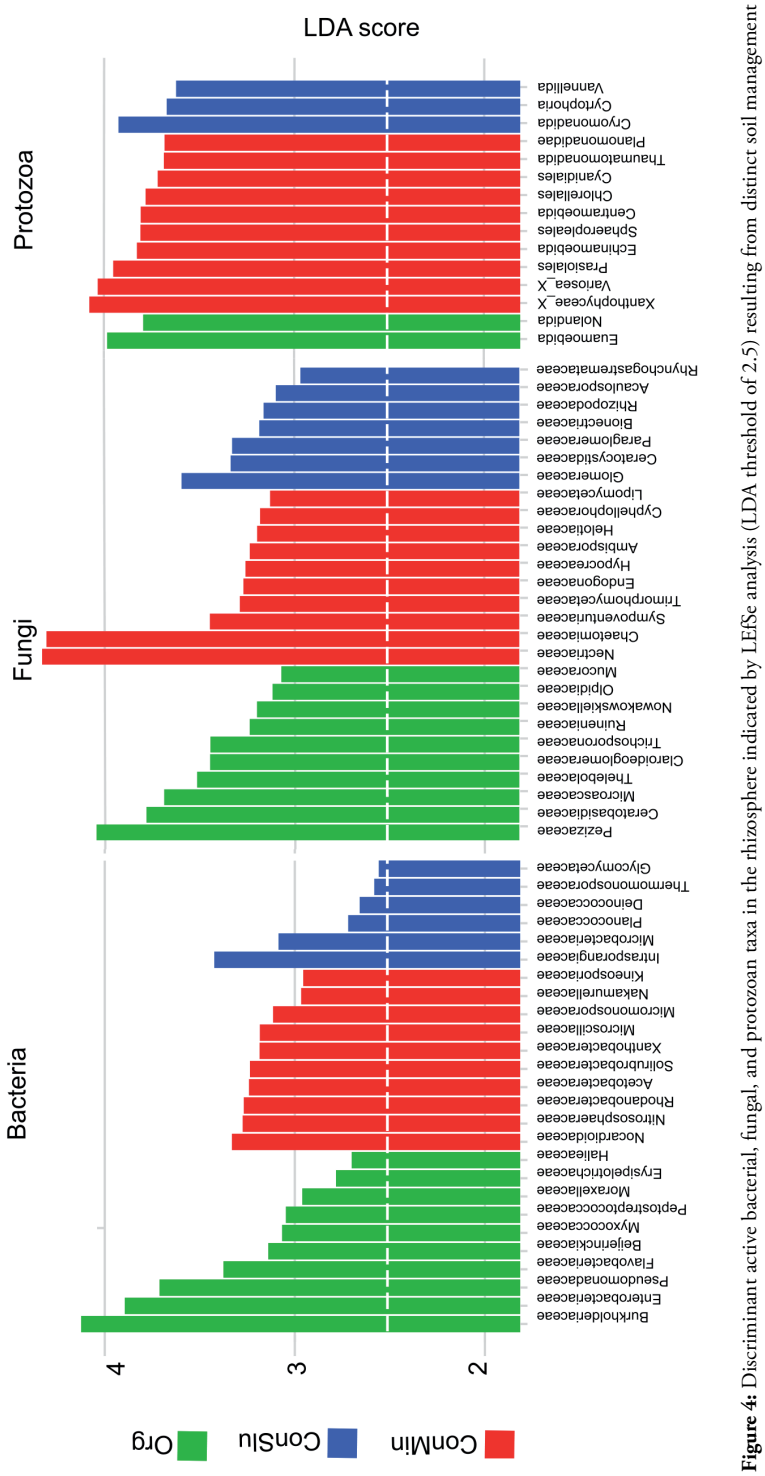

Figure 4: Discriminant active bacterial, fungal, and protozoan taxa in the rhizosphere indicated by LEfSe analysis (LDA threshold of 2.5) resulting from distinct soil management types at location Vredepeel: ConMin (red), ConSlu (blue) and Org (green).

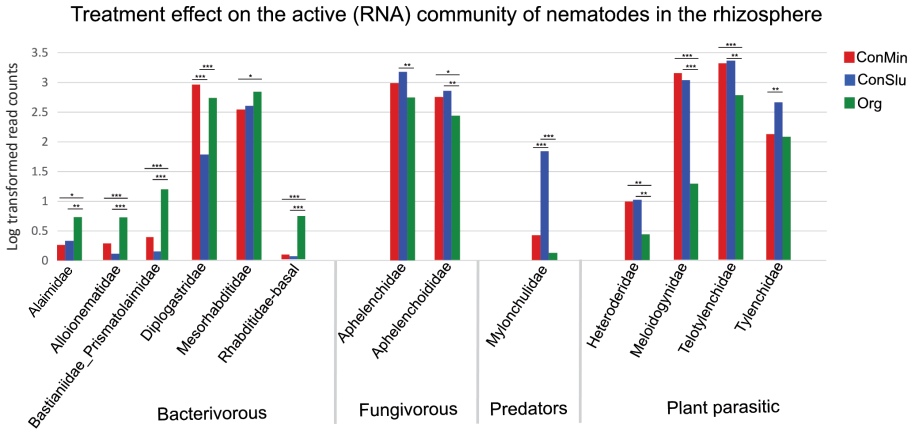

Figure 5: Taxonomic shifts in the active nematode communities in the rhizosphere for each of the three management types: ConMin (red), ConSlu (blue) and Org (grey). Kruskal-Wallis test, followed by Dunn's Test. (*P< 0.05, **P< 0.01, ***P< 0.001).

MiSeq nematode community analysis does not allow for the detection of nematodes at species level, and therefore a species-specific qPCR assay was run to pinpoint the observed downregulation of members of the genus *Meloidogyne*. Quantitative PCR analysis revealed that the *Meloidogyne chitwoodi* were significantly lower in the Org fields as compared to the two conventional treatments (P< 0.05). Soil samples were checked for the presence of other *Meloidogyne* species (Supplementary Table S1), and these were not present or at very low levels only. Therefore, we conclude that the observed difference in Meloidogynidae levels in Fig. 5 can be attributed predominantly to the Columbian root-knot nematode *M. chitwoodi*.

Commonly used nematode extraction protocols exploit the mobility of nematodes to separate the roundworms from the soil matrix. Hence, non-active nematodes will not be extracted by these methods. Direct extraction of nematode DNA and RNA from soil doesn't include this selection step, and therefore we compared rRNA-based results (Fig. 5) with results with rDNA (Supplementary Fig. S4). Both figures show significant lower abundances of the monogeneric family Meloidogynidae in the organic fields. Hence, all three approaches, rDNA or rRNA-based sequencing and species-specific qPCR assays, demonstrate significantly lower root-knot nematode levels in Org fields.

Discussion

Mapping of resident and active fractions of the primary decomposer community – bacteria and fungi – as well two major primary consumer groups – protists and nematodes – under three distinct soil management regimes revealed that pea exerts a large effect on the soil microbiome. Below we'll discuss (1) how the current characterisation of the pea rhizobiome relates other studies, (2) how our observations regarding the effect of soil management relate to previous findings, and (3) whether we can find plausible biological explanations for the observed sharp decline in root knot nematode densities in fields under prolonged organic soil management.

How does the current characterisation of the pea rhizobiome relate other studies?

As exemplified by the impact of pea, lentil and chickpea, legumes have been shown to exert a large influence on the soil microbiome as compared to other crops such as cereals (Turner et al. 2013; Hamel et al. 2018). N rhizodeposition shown to comprise 13% of the total plant N for pea (Mayer et al. 2003), and presumably this has contributed to this large impact. The large overall effect of legumes could be corroborated by comparing the current study with a recent study on the barley rhizobiome that made use of the same experimental fields (Harkes et al. 2019). In case of barley, the compartment effect (bulk *versus* rhizosphere) explained a smaller percentage of the observed variation than impact of the soil management regime. For pea, on the contrary, all four organismal groups indicated the compartment effect to be larger than the soil treatment effect. Hence, under similar experimental conditions the compartment effect on the soil microbiome induced by pea (a legume) is stronger than the effect induced by barley (a cereal).

In a previous study in an agricultural field near Norwich (UK) the pea rhizosphere showed elevated activity of the bacterial genera *Masillia* (Oxalobacteraceae), *Dyadobacter* (Cytophagaceae), *Flavobacterium* (Flavobacteriaceae) and *Streptomyces* (Actinomycetaceae) (Turner et al. 2013). In our study, we could only confirm the increased activity of Flavobacteriaceae (Fig. 2, panel 1). Difference in pH might have contributed apparent discrepancy. The pH of the Vredepeel experimental fields is around 6.0, about 1.5 units lower than pH at the Norwich site. This difference is meaningful as soil bacterial communities are very sensitive to pH (Fierer and Jackson 2006). Regarding eukaryotes, Turner and co-workers (2013) also reported a strong selection by pea of the protists *Bresslaua* (Colpodea) and *Dimastigella* (Bodonida) and the plant-parasitic nematode *Meloidogyne*. Elevated presence of Colpodea and *Meloidogyne* were observed in our experiment as well, the activation of *Dimastigella* not.

More recently, the effect of various frequencies pulse crop cultivation (including pea) on resident soil bacterial communities was mapped (Hamel et al. 2018). Increased frequency of pulse cultivation resulted in higher abundances of α-Proteobacteria in the rhizosphere, and a decrease in γ-Proteobacteria (although the latter was accompanied by an increased presence of *Pseudomonas*). Keeping in mind that the Rhizobiales (in our study Rhizobiaceae and Labraceae) belong to the subclass α-Proteobacteria, an overall increase of this subclass was to be anticipated. Moreover, Hamel et al. (2018) detected in an increase *Pseudomonas* read in rotations involving pea. This might correspond to the increased activity of Pseudomonadaceae we observed in the pea rhizosphere (Fig. 2, panel 1). This could be caused by an accumulation of *P. syringae*, the causal agent of bacterial blight in pea (Hamel et al. 2018). We could not confirm the increased presence of Actinobacteria in the pea rhizosphere as observed in rotation systems with frequent inclusion of pulses (referred to as '3-pulse systems'). This phenomenon might only be observable after repeated cultivation of legumes.

We conclude that a number of parallels can be discerned between studies on the effect of pea on the rhizobiome. It is noted that differences in experimental approach (focus on resident or active soil biota) and set up (soil type, soil management practices) complicates the identification of generic effects of legumes on the soil living community.

How does the current characterisation of the effects of soil management on the soil microbiome relate other studies?

In a long-term (> 10 years) greenhouse experiment the effect of organic, integrated and conventional farming systems on the soil rhizobiome was investigated (Li et al. 2019). The authors identified a bacterial hub, a small number of highly interconnected taxa, consisting of *Bacillus* (Bacillaceae), *Sporosarcina* (Planococcaceae), *Hyphomicrobium* (Hyphomicrobiaceae), *Gaiella* (Gaiellaceae), as well as *Pirellula* and *Blastopirellula* (both Planctomycetaceae) that were significantly more abundant in soil from the organic management regime (Li et al. 2019). Another hub comprising of the genera *Rhizobium* (Rhizobiaceae), *Sphingobium* (Sphingomonadaceae), *Pseudoxanthomonas* (Xanthomonadaceae), and *Dyadobacter* (Cytophagaceae) was present in higher densities in the conventional and the integrated treatments. These findings show very little resemblance with the bacterial taxa that were shown to be activated under the organic or one of the two conventional soil managements systems in the present study (Fig. 4). From this we conclude that the plant effects can be stronger than the effect of soil management (variable 'treatment' in Tables 1, 2). Moreover, it is noted that the active bacterial community can be quite distinct from the resident bacterial communities mapped by (Li et al. 2019) (Fig. 1).

In another long-term field experiment (running for ≈ 15 years at time of sampling) fields were continuously exposed to either conventional or organic farming practices, and the impact of the practices on bulk soil were determined (Bakker et al. 2018). These authors showed a remarkable contrast between bacterial phyla with regard to the extend by which they were affected by the contrasting farming practices. More taxa showed in higher abundances in organic as compared to conventional farming. Moreover, some bacterial phyla such as Chloroflexi, Firmicutes and Gemmatimonadetes seemed to be unaffected by farming practice while others such as Proteobacteria, Acidobacteria and Verrucomicrobia were. Our data only partly support this observation. The families Burkholderiaceae and Hyphomicrobiaceae (Proteobacteria), and the Peptostreptococcaceae (Firmicutes) were both more abundant and more active in bulk soil in organic fields (Supplementary Fig. S3). Peptostreptococcaceae are one of the dominant family in the gut microbiome of earthworms (see *e.g.* Zeibich et al. 2018), and as such this result could point at an elevated presence of earthworms in fields under organic management. In our analysis of resident bacterial community in bulk soil under the organic regime also showed an increase of members of the Acidobacterial family Blastocatellaceae (Supplementary Fig. 3). In the most conventional soil management system (ConMin), the Verrucobacterial family Pedosphaeraceae was both abundant and highly activated. At high taxonomic level, this is in line with the observations presented by (Bakker et al. 2018).

Hence, despite the fact that plant identity may have a stronger effect on the rhizobiome than soil management practices, the effect of these practices could be pinpointed at taxon level. Our data suggest that the working hypothesis saying that only a subset of the soil bacterial phyla is affected by conventional or organic soil management practices might be correct.

Can we pinpoint nematophagous bacterial or fungal taxa that might underlie the *M. chitwoodi* decline in fields under organic management?

In this study we investigated the soil microbiome of pea in fields naturally infested with *M. chitwoodi* under three different soil management regimes, conventional, integrated and organic. *M. chitwoodi* is a highly polyphagous plant parasite infecting numerous mono- and dicotyledonous crops, including pea (*Pisum sativum*) (OEPP/EPPO 1991), and it has a reputation as a major pest in potato. In all studied fields here, *M. chitwoodi* was already present for multiple years (Visser et al. 2014). As potato – a highly suitable host – was the main crop in the previous growing season, we expected the *M. chitwoodi* population to be physiologically fit at the onset of the pea growing season.

At the end of the growing season, *M. chitwoodi* densities in the two conventional soil management systems harbours ≈ 60 individuals per 100 g soil, whereas about 2 individuals per 100 g were detected in the organically managed system. We investigated whether a biological explanation could be found for this difference. Within the bacterial and fungal rhizosphere communities, families were detected that are known to harbor multiple nematode-trapping species. As shown in Fig. 2 (panel 3), the Orbiliaceae were shown to be active in the pea rhizosphere. This family comprises genera such as *Arthrobotrys*, *Dactylella*, and *Monacrosporium*. These genera are essentially saprophytic fungi but can become predatory under *e.g.* low nutrient conditions. As a response, the fungi will form traps (*e.g.* constricting rings, adhesive networks) which allow them to prey on nematodes. We verified whether Orbiliaceae activity was upregulated in the fields under organic management. This was not the case, and even a non-significant trend towards lower activity in organic fields was observed (Supplementary Fig. 2). Elevated activity of another fungal family, the Olpidiaceae, typified fields under organic soil management. A member of this family, *Olpidium vermicola*, has been reported to parasitize eggs and females of endoparasitic nematodes such as *M. chitwoodi* (Esser and Schubert 1983; Askary 2015). However, other *Olpidium* species are virus-transmitting plant pathogens. Zoospores of *Olpidium virulentus* colonize roots of various plant species, and were demonstrated to accumulate in crop rotation with multiple pulses including pea (Niu et al. 2018).

The bacterial family Pseudomonadaceae was also identified as an indicator species for organic farming (Fig. 4). Further analyses identified the genus *Pseudomonas* as main contributor to the indicator status of Pseudomonadaceae. The *Pseudomonas* species *P. aeruginosa*, *P. fluorescens*, *P. protegens* and *P. chlororaphis* belong to ecologically most relevant nematophagous bacteria in soil (Li et al. 2014). *Pseudomonas* species produce toxins which may inhibit hatching, survival and *M. chitwoodi*'s ability to penetrate plant roots (Thiyagarajan and Kuppusamy 2014; Nandi et al. 2015; Kang et al. 2018).

The increased densities of bacterivorous nematode families might form an indirect explanation for the downregulation of *M. chitwoodi* in organic soil management systems (Fig. 5). As bacteria-grazing nematodes in the immediate vicinity of plant roots could locally improve nutrient availability via the excretion of easily uptakeable N and P. Plants could benefit from this in terms of improved growth and vitality, possibly making them less susceptible to plant-parasitic nematodes (Thoden et al. 2011).

Presumably multiple factors have contributed to significantly lower *M. chitwoodi* levels in the organic fields. This might have included nematode parasitic members of fungal genus *Olpidium* and/or the elevated activity of members of

the Pseudomonadaceae. These results should be seen as potential leads for more detailed studies on the effect soil management regimes on the activity levels of nematophagous bacteria and/or fungi.

Concluding remarks

The development of a time-efficient and affordable protocol to extract total DNA and RNA from soil (Harkes et al. 2019) allowed us to monitor the effect of a legume, pea, on both resident and active communities of primary decomposers as well as primary consumers of bacterial and fungal assemblages. Pea was shown to exert a large effect on the rhizobiome, and this was not only true for the primary decomposers but also for the protist and metazoan community. We investigated possible biological explanations for the remarkably low levels of the root-knot nematode species *M. chitwoodi*. The fungal family Orbiliaceae, a family that harbor numerous nematophagous fungi, was shown to be present and active in the pea rhizobiome but was unlikely to be involved in the observe *M. chitwoodi* decline. More likely the Pseudomonadaceae – here members of the genus *Pseudomonas* – could have played a role in the biological suppression of this notorious root-knot nematode species. It should be underlined that biological associations have been studied in this research, and it was by no means proven that one or more *Pseudomonas* species that might play a role in plant-parasitic nematode suppression were actually responsible for the observed decline in fields under organic soil management.

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Supplementary information

Table S1: Converted qPCR data (#nematodes/100 g soil) on different nematode taxa for the three different soil management types

Nematode taxon	ConMin	ConSlu	Org
Dorylaimida	11.55	28.52	8.08
Heterorhabditidae	0.01	0.01	0.37
Tylenchorhychus	28.18	11.61	1.77
Coslenchus	0.00	0.00	1.09
Pratylenchus penetrans	0.00	0.00	1.81
Meloidogyne incognita	0.00	0.00	0.00
Meloidogyne chitwoodi	57.70	64.76	1.96
Meloidogyne fallax	0.47	0.00	0.00
Meloidogyne minor	0.00	0.00	0.00
Meloidogyne Naasi	0.15	3.12	0.55
Meloidogyne Hapla	0.00	0.00	0.00
Total nematode abundance	1551	1622	1440

Table S2: Summary of the PERMANOVA based on Bray-Curtis and UniFrac (Weighed and Unweighted) distances for DNA and RNA seperately. Sample type (Bulk/Rhizosphere), Treatment (ConSlu, ConMin, Org) and Time point (Vegetative/Generative) as factors. Differences are considered significant if P< 0.01.

	DNA						RNA					
	Bray Curtis		Unifrac (U)		Unifrac (W)		Bray Curtis		Unifrac (U)		Unifrac (W)	
Source	**R^2**	**P**	**R^2**	**P**	**R^2**	**P**	**R^2**	**P**	**R^2**	**P**	**R^2**	**P**
Bacteria												
Treatment	0.101	0.001	0.097	0.001	0.061	0.001	0.103	0.001	0.125	0.001	0.055	0.001
Sample Type	0.378	0.001	0.185	0.001	0.672	0.001	0.428	0.001	0.215	0.001	0.740	0.001
Time Point	0.048	0.001	0.032	0.001	0.029	0.003	0.063	0.001	0.037	0.001	0.030	0.002
Residuals	0.472		0.686		0.238		0.406		0.622		0.174	
Fungi												
Treatment	0.154	0.001	0.116	0.001	0.110	0.001	0.165	0.001	0.154	0.001	0.137	0.001
Sample Type	0.218	0.001	0.074	0.001	0.295	0.001	0.208	0.001	0.117	0.001	0.236	0.001
Time Point	0.047	0.001	0.039	0.001	0.045	0.001	0.063	0.001	0.048	0.001	0.070	0.001
Residuals	0.581		0.771		0.550		0.565		0.682		0.557	
Protozoa												
Treatment	0.139	0.001	0.205	0.001	0.258	0.001	0.132	0.001	0.156	0.001	0.149	0.001
Sample Type	0.241	0.001	0.182	0.001	0.310	0.001	0.159	0.001	0.151	0.001	0.130	0.001
Time Point	0.136	0.001	0.086	0.002	0.149	0.001	0.120	0.001	0.060	0.001	0.145	0.001
Residuals	0.484		0.527		0.282		0.589		0.633		0.577	
Metazoa												
Treatment	0.138	0.001	0.125	0.001	0.065	0.007	0.101	0.001	0.141	0.001	0.057	0.004
Sample Type	0.168	0.001	0.091	0.001	0.225	0.001	0.200	0.001	0.172	0.001	0.302	0.001
Time Point	0.064	0.001	0.030	0.006	0.076	0.001	0.067	0.001	0.048	0.002	0.059	0.001
Residuals	0.630		0.754		0.634		0.632		0.639		0.582	

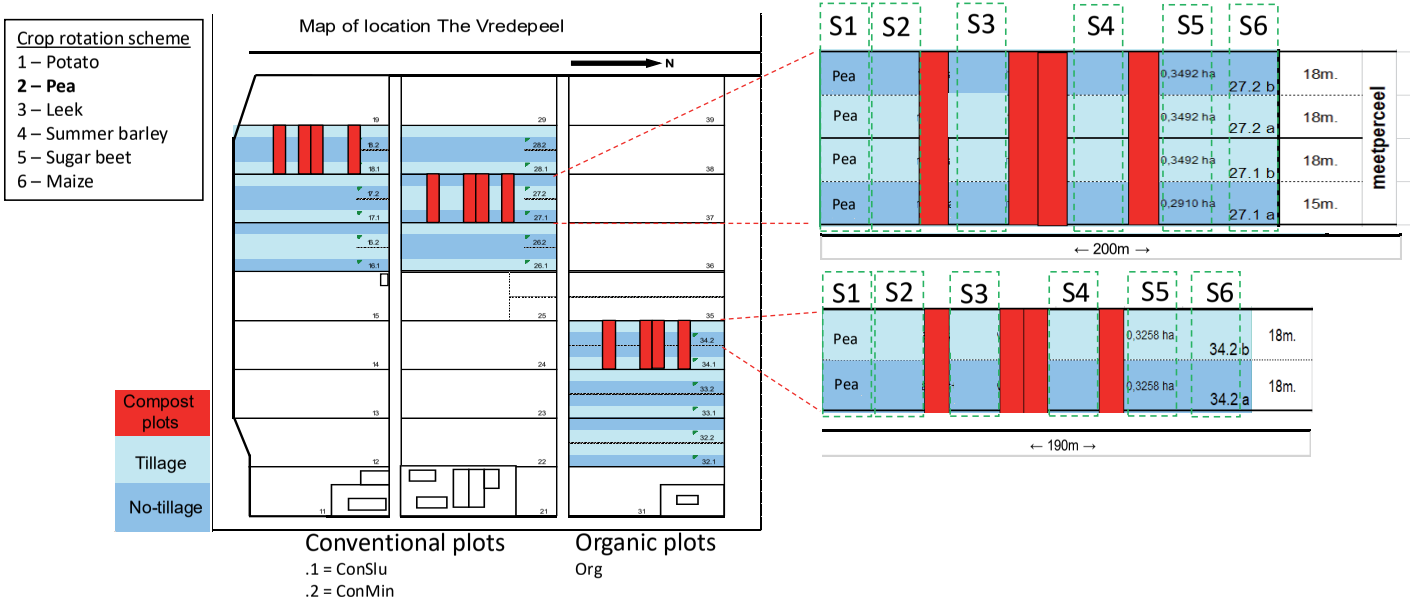

Figure S1: Field set up location Vredepeel (sandy soils). Samples were taken from field 27.2b, 27.1a and 34.2a. The six sampled sub-plots are indicated (S1-S6) with green dotted lines.

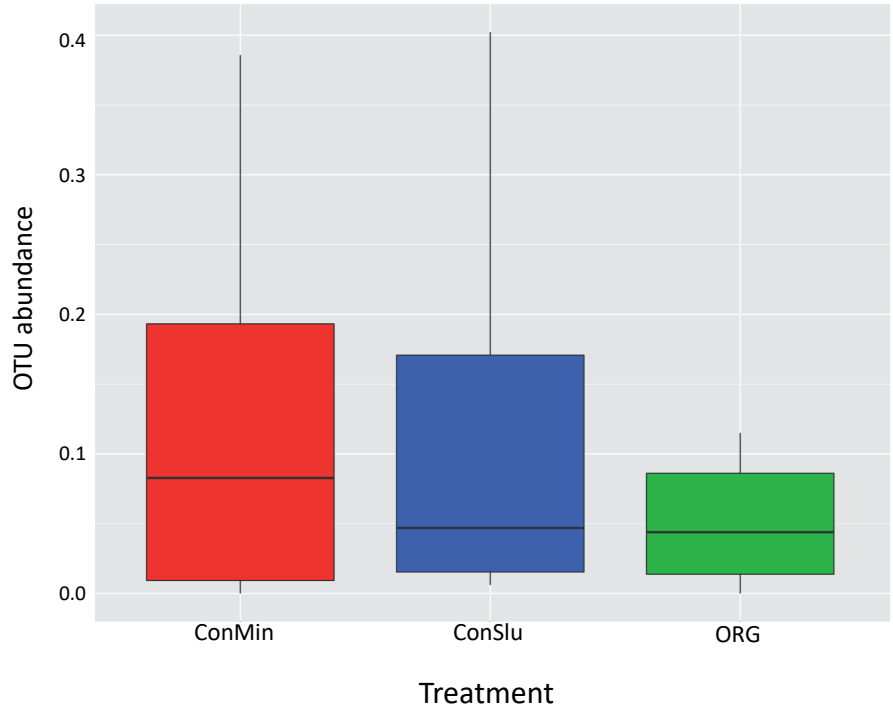

Figure S2: Abundance per treatment for OTU_11 - family *Orbiliaceae*.

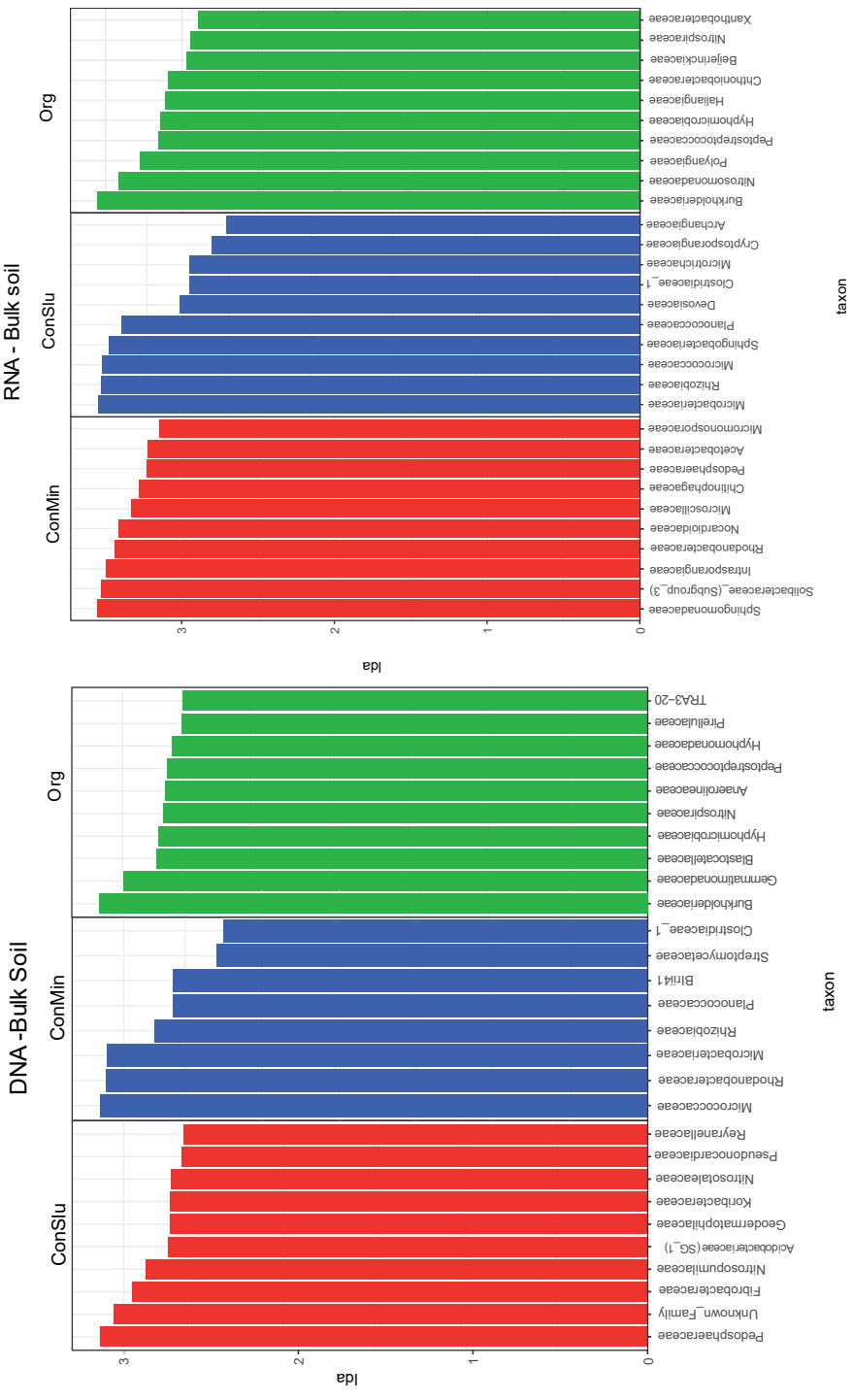

Figure S3: LEfSE per soil management type for DNA-bulk and RNA-bulk.

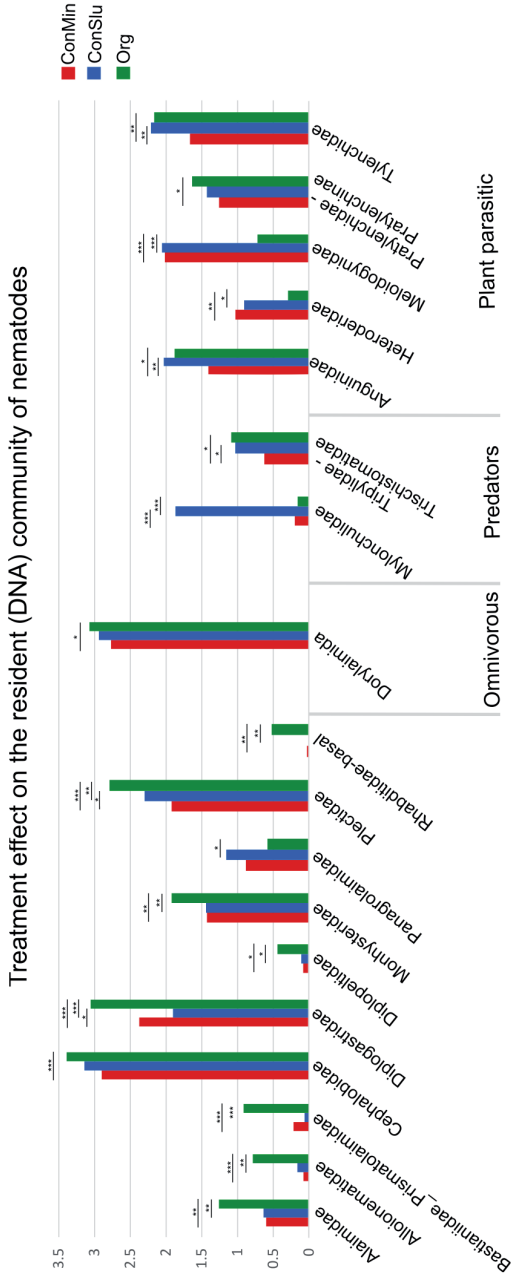

Figure S4: Effect of soil treatment on the total (rDNA) nematode community.

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CHAPTER 7

General discussion

Paula Harkes

> "*Come close... closer... because the more you think you see, the easier it will be to fool you. Because what is seeing? You are looking but what you are really doing is filtering, interpreting, searching for meaning. The closer you look, the less you see.*"
>
> — Daniel Atlas

It is widely acknowledged that soils form a highly complex and dynamic ecosystems in which numerous organisms exist and interact with each other (Bardgett and van der Putten 2014). In this, the soil microbial community acts as a fundamental factor in facilitating ecosystem services and functioning. A growing plant is able to change the chemical, physical and biological properties of the soil in its rhizosphere (Ehrenfeld et al. 2005), adding another dynamic level to the soil microbiome. Mapping the diverse microbial life in soil and, correspondingly, utilizing this information for environmental purposes – such as ecosystem resilience – is a major continuing challenge.

With this thesis I aim to contribute to unravelling soil microbiomes influenced by various plant species and situated in distinct ecosystems. Hereby focusing mainly on the effect of the plant on its rhizosphere community, in conjunction with the effect of various soil management strategies on soil microbiota. The work described in this thesis makes use of several methods and techniques in order to explore the diversity and quantity of organisms participating in plant soil interactions. First, we use (q)PCR on DNA samples at high taxonomic level for the quantification of bacteria, fungi and nematodes. In a next step, we supplemented this type of data with biochemical (ergosterol) measurements and sequencing of fungal DNA. Finally, the development of a low cost and time efficient method to co-extract RNA and DNA from soils allowed us to use high throughput sequencing approaches to identify resident and active soil microbial communities of four major organismal groups.

In this final chapter, I will describe and discuss the different shifts in soil microbiome communities followed by the difficulties of linking soil microbial diversity to ecosystem functioning. In addition, I discuss several issues concerning the use of molecular markers to qualitatively and quantitatively characterize soil microbiomes.

Mapping shifts in the soil microbiome

With the advent of high throughput DNA sequencing techniques, we are now able to take a more detailed look into the soil 'black box'. It is possible to observe and characterize shifts in soil microbial communities. Fig. 1 shows a simplified overview of possible shifts (A-D) that can occur in soil communities. Although portrayed as separate shifts in Fig. 1, it is more likely that these several shifts in soil communities occur simultaneously. For the purpose of interpretability, I will discuss the connection of findings within the chapters of my thesis in light of each of the proposed types of shifts separately in this section.

Selective stimulation

In **Chapter 2**, we investigated the differences in the rhizosphere of two closely related ragwort species. We observed that plants are able to select and boost their microbial community over time (Fig. 1A), demonstrating the importance of plant nature and

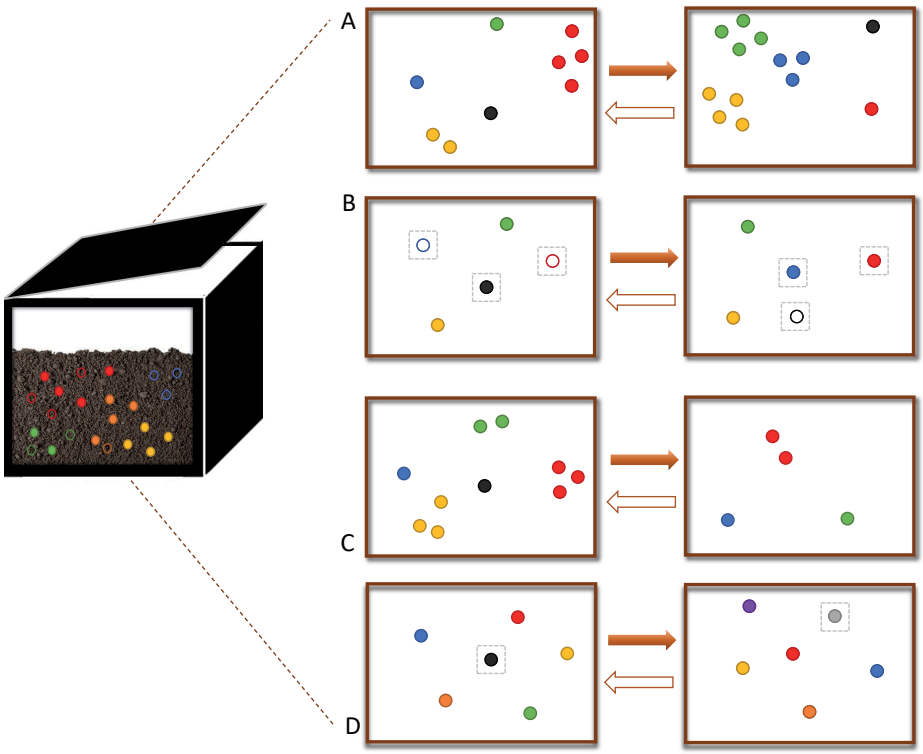

Figure 1: Schematic overview of four possible shifts in the soil microbiome. Each colored circle stands for different microbial life. Open circles indicate dormancy. A) Selective stimulation, B) Activation and de-activation, C) Loss and gain of biodiversity loss, D) Taxon replacement.

developmental stage on the resident microbiome. Especially *Senecio inaequidens* showed an enhanced ability to select its microbial community which strengthened its interconnectedness. Interestingly, during plant development, both ragwort species significantly reduced almost the same subset of phyla. Although the observed shifts in stimulation and selection have been monitored at high taxonomical level, it has been proposed that bacteria within a phylum might share specific ecological traits (Lauber et al. 2009; Philippot et al. 2009; Wessen et al. 2010). Therefore, making it informative to monitor changes in primary decomposers at high taxonomic level (De Gregoris et al. 2011; Philippot et al. 2010; Turner et al. 2013).

As this might be true for bacteria, **Chapter 3** demonstrated that the impact of an invasive plant species on the soil fungal community could not be pinpointed at high taxonomic level. After all, belowground impact of *Solidago gigantea* could not be related to significant quantitative changes at fungal phylum level. Specific differences were only observed when fungal community shifts were determined at family level. Suggesting that phylum level characterisation is probably too rough for fungi, as it only encompasses 5 ecologically high diverse phyla. This contrasts with the domain Bacteria that comprises about 30 phyla (Euzeby 1997).

Activation and de-activation

In **Chapter 4** and **Chapter 6** the resident and active community was examined and showed significant differences between these two communities (Fig. 1B). Whereas DNA indicated almost all taxa were more abundant in organic farming 'only' 1/3 of the 50 bacterial taxa were specifically active under organic soil management conditions (**Chapter 4**). Previous work has shown that up to 80% of the soil living community can be dormant (Lennon and Jones 2011). Additionally, amplifiable extracellular DNA after cell death, can persist in soils for weeks to years (Carini et al. 2017; Pietramellara et al. 2009). As microbial activity – rather than presence – matters in terms of soil food web functioning (Bastida et al. 2018), these results underline the relevance of taking the active communities into account. In fact, it would be advisable to re-evaluate conclusions in previous studies with regard to ecosystem functioning when they are solely based on DNA abundance data. Supporting this view, Gill et al. (2017) found the diversity and community composition patterns based on genomic DNA to be inconsistent predictors of soil differences among active communities. In addition, rare bacterial taxa were found to be disproportionately active, compared to more common bacterial taxa (Gill et al. 2017; Lennon and Jones 2011), leading to an underestimation of the contribution of rare bacteria taxa when only DNA is taken into consideration.

Loss and gain of biodiversity

Intensively managed agricultural systems rely heavily on the use of mineralizers and chemical pesticides, thereby shifting the soil community toward a less biodiverse ecosystem (Figure 1C). Conventionally managed systems typically harbour half the biodiversity as compared to natural ecosystems (Tilman et al. 2002). Organic farming, on the other hand, has been frequently shown to increase soil biodiversity (Francioli et al. 2016; Tuck et al. 2014; Wang et al. 2017). Similarly, **Chapter 4**, **5** and **6**, showed a more diverse and interconnected soil microbial life, compared to more conventional managed systems. Nevertheless, organic harvests on average only 80% of the conventional yield, mainly because of less nitrogen, competition by weeds and damage due to above ground herbivores (Kniss et al. 2016). As a consequence, one can question the importance of soil biodiversity in relation to crop yield. Unfortunately, many studies to biodiversity do not elaborate on crop yield (Kremen et al. 2015). Also in this thesis, only limited attention was paid towards crop yield. In general, crop yield of the organic managed systems in the Vredepeel was lower compared to conventional systems. Only in 2018 (**Chapter 6**), no pea could be harvested in conventional fields because of a severe infestation of *Meloidogyne chitwoodi*, whereas in organic fields low nematode infestation levels resulted in a reasonable pea yield (Harkes et al. 2019). This supports the general thought that organic agriculture, due a higher biodiversity, can be considered more stable. Still, agriculture is in ecological sense an early-successional system which is repeatedly out of balance, making stability difficult to accomplish (Erisman et al. 2016). As soil scientists we should pay attention to the applicability of our research and therefore research might have more practical impact if it would focus on pragmatic intermediate practices rather than seeking the extremes of conventional versus organic.

Taxon replacement

Although not observed in the studies presented in this thesis, another shift in the soil microbiome could be the replacement of certain community members by other community members (Fig. 1D). This is most likely when soil transplantation or soil inoculation is carried out (Wubs et al. 2016). However, in a natural ecosystem, it is unlikely that soil organisms will be replaced permanently, since the rhizosphere effect exceeds the effect of soil inoculation (Yergeau et al. 2015). Nevertheless, soil transplantation is considered a tool to restore disturbed terrestrial ecosystems, especially when top soils are removed (Wubs et al. 2016). This offers an interesting perspective once we have developed parameters for plant growth promoting soil food webs.

Linking diversity to function

As also demonstrated by the studies in this thesis, great strides have been made in our ability to the identify microbial shifts the soil food web. At the same time, relating specific shifts to soil ecosystem functioning is at an initial stage. There are still only a few studies linking ecosystem functioning to the soil food web (de Vries et al. 2013; Wang et al. 2019) and most studies in which shifts are observed conclude that it is challenging to link the interpretation of their results to ecosystem functioning. A factor that contributes to this challenge is the fact that all shift in soil communities are shaped by multiple environmental factors (*e.g.* climate, land use) and therefore remain highly context dependent (Bender et al. 2016). The taxonomic level at which we can analyse our samples might form a limiting factor in our attempt to link community composition information to soil functioning. However, high resolution is not always required. In **Chapter 2**, we demonstrated that the difference in ecological impact between two related plant species (native and invasive) could be observed at (bacterial) phylum level. On the other hand, mapping the condition of the nematophagous microbial community required information at species level (**Chapter 6**). In other words, some soil ecological questions with regard to the microbiome can be addressed at a remarkable high taxonomic level, whereas for other phenomena we need to have resolution at species level, or even below.

At this point in time, most high throughput studies of soil biota reach can identification at genus level. Current read length are too short to provide a higher resolution as typical Illumina sequencers produce read lengths in the range of 50–300 bp, which makes it hard to reliable map beyond genus level (Hermes 2016). In **Chapter 3**, we experienced that, due to the quality of the reads, the family Glomeraceae could not even be determined to genus level. This limitation in resolution hampered our ability to compare our results with related, published data. Even when genus level can be reached and assuming that a genus is heterogenous for relevant ecological traits a plethora of species can be hold accountable for the observed shifts. On the bright side, sequencing techniques are continually developing and techniques creating longer read lengths will soon be widely available (Gupta and Verma 2019).

Once a specific indicator species is determined by means of sequencing, it is hard to validate its function, as most soil organisms remain unculturable. Nevertheless, we can and should, expand our knowledge on organisms that we are able to grow in cultures. After all, such cultures are pivotal in studying direct trophic interactions or the influence of soil microbes on plant growth (Gao et al. 2019). In **Chapter 6** it was demonstrate that increased activity of the bacterial genus *Pseudomonas* was associated with lower abundances of the parasitic root-knot nematode *Meloidogyne*

chitwoodi. Members of the genus *Pseudomonas* might have acted as an antagonist for *M. chitwoodi*, a phenomenon worth studying in a more controlled setting. Similarly, **Chapter 3** showed the genus *Cladosporium* was abundantly present in plots that were invaded with *Solidago gigantea*. Therefore, *Cladosporium* spp. abundance could potentially relate to the invasive success of giant goldenrod.

The preceding paragraphs show that a higher taxonomic resolution of the soil microbiome is required for a number of questions. On the other hand, when we would be able to zoom into species level and know far more about the ecologies of specific organisms, the danger lurks that we forget about the bigger picture. In search for ecological meaning, we start to filter certain species. Thereby, unintentionally eliminate possible relevant interactions in the soil food web that will influence the outcome. We should not forget, that every approach that we use to map soil biodiversity has its own limitations. As Geisen et al. (2019) clearly pointed out, a combination of reductionist and holistic approaches will be essential to understand and discover soil microbial functioning, that can be exploited for agriculture or nature conservation.

The challenges of high-throughput sequencing

The use of high-throughput sequencing techniques for the analysis of complex soil microbial communities has increased tremendously in recent years. Caused by a continual reduction of costs and the improved ability to analyze large data sets, next generation sequencing is exceedingly preferred over traditional culture-based methods as standard method to map and monitor soil biodiversity (Hayden 2014). At this moment sequencing is the only method known that allows a direct assessment of the microbial community composition at multiple trophic levels (Vestergaard et al. 2017). This rather holistic analysis approach might enhance our understanding of soil food web. Even though, the broad use of high-throughput sequencing in soil sciences provides us with new opportunities, we should, at the same time, be careful not to fall for the law of the instrument; "*If the only tool you have is a hammer, you treat everything as if it were a nail*". In other words, an over-reliance on a familiar tool or method and lack of reflection, could lead to a cognitive bias. In the case of high-throughput sequencing it includes DNA/RNA isolation techniques, bioinformatics pipelines and statistical tests.

Isolation and amplification techniques

As soil is an extremely heterogeneous matrix, not only from a physiological but also from a biological point of view, each choice in the process – from soil sample

to sequence – bring its own bias (*e.g.* soil sampling, isolation, purification) (Hata et al. 2011; Krsek and Wellington 1999). It has been frequently shown that methodology, rather than biology, is the largest driver of variation in microbiome studies (Clooney et al. 2016; Inceoglu et al. 2010 ; Ramirez et al. 2018; Soliman et al. 2017). For example, the choice of a lysing method could can have dramatic effects on the obtained results, as each organism has its own lysing optimum (Kirk et al. 2004). Furthermore, isolation methods seldom access more than 60% of the nucleic acids present in a sample (Robe et al. 2003). This inefficiency in isolation creates the possibility that isolating DNA from the same sample twice results in differences in obtained microbial patterns (Luna et al. 2006). Delmont et al. (2013) showed that using two different DNA extraction methods for the same soil resulted in a difference of ~40% of the microbial distribution in the results, which points out that each extraction method strongly affects the apparent soil diversity and community structure.

Another factor causing potential bias in soil microbiome studies, is the amplification of the obtained DNA or cDNA in order to prepare a sequence library. Soil contains many PCR-inhibiting substances, making amplification success dependent on dilutions, thereby introducing bias towards less abundant species (Gonzalez et al. 2012). Moreover, the choice of primer region as well as the length of the obtained amplicon may causes significant differences in community structure (Engelbrektson et al. 2010; Morales et al. 2009; Wu et al. 2010). All aforementioned issues cause that everyone that wishes to compare different soil metagenome studies one-on-one will enter dangerous grounds.

Bioinformatic pipelines

After sequencing the amplicon library, the generated dataset will be analyzed by using a pipeline (a combination of diverse bioinformatics tools). In this pipeline, sequence reads undergo quality trimming, filtering and finally reads will be clustered into OTUs. A recent study by Ramiro-Garcia et al. (2016) compared a newly developed pipeline (NG-tax) with QIIME – a pipeline originally developed to analyze the bacterial community of the gut but also widely used to determine soil microbiota (Caporaso et al. 2010). In their study, they sequenced different mock communities – synthetic microbial communities of varying complexity – and compared the results of each pipeline. The results showed that by using NG-tax more than 90% of the reads could be assigned to genus level whereas with QIMME only 20% of the OTU's could be assigned beyond class level. As each bioinformatic pipeline applies its own filtering and clustering it raises the question: "What is more accurate and what comes closer to the truth?" With the fast development of new scripts and improvements of pipelines, we need to increase the frequency at

which we question, discuss and critically review the different variants of methods that we use to process sequence data. Especially when data handling is 'hidden' in pipelines, it is tempting to use such pipelines without fully understanding what it encompasses. This makes it imaginable that the most habituated method for data analyses is not necessarily the most suitable for a given data set.

Statistical tests

The need for more frequent reflection on methodology also applies to choices that are made in statistical tests and in approaches we use to depict soil microbial community data. Many dissimilarity measures that (soil)ecologists use, relativize absolute differences in abundance to display community structures. For instance, if two communities have the same proportion in two samples they may be considered similar (Bray-Curtis, Hellinger, chi-squared) (Yoshioka 2008). These standardizations do not only affect patterns of dispersion but can also lead to misinterpretation. An important ecological issue is to understand how much of the dissimilarity is driven by compositional differences and how much is driven by differences in relative abundance (Anderson et al. 2006). In **Chapter 4** and **Chapter 6** we supplemented the Bray-Curtis dissimilarity-based method with UniFrac, a method that uses phylogenetic distances as a measure for the comparison of microbial communities. Although the main factors (nucleic acid type and soil type) were still significantly different, there were changes in relation to the amount of variance explained by the individual factors. This difference in variance became even more clear by visualization of the different measures, as the PCoA plots displayed very different patterns. A similar observation was made by Anderson et al. (2011) when they examined β diversity variation with different measures and saw contrasting results depending on the measure they implemented. This raises the question about the most reliable way of displaying the reality.

Outlook

High throughput sequencing has brought us a lot of knowledge about the diversity of the soil microbiome. As this field progresses with high speed, it does not only lead us to more data, but also to new insights in in how to gain, process and analyze this data. In order to examine the soil microbiome, it would be beneficial to generate data in such a way that they allow for more robust comparison between studies. Therefore, it could be helpful to create and share a methodology selection protocol for soil microbiome studies in order to prevent as much technical bias as possible. Such a protocol could encompass, methods for soil sampling, DNA and RNA isolation techniques, bio-informatic pipelines and the choice of statistical

testing and should be subjected to frequent evaluation and discussions worldwide. Eventually, with a more uniform approach in how methods are selected, obtained data should be shared on a global platform as proposed by Ramirez et al. (2015) to identify previously overlooked taxa and factors that are relevant for structuring soil communities. At this point, just sequencing more will not be the answer towards unravelling the soil microbiome. Sequencing more accurate with a diverse set of proper tools, making it possible to combine data universally, could lead us to unravel the complex world of soil.

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Summary

As early as 1937, Franklin D. Roosevelt raised the importance of soil with his statement: "*A nation that destroys its soil, destroys itself*". Soil is the groundwork of ecosystem functioning as it filters and stores freshwater, provides essential nutrients for plant growth, and regulates the earth's temperature. Essential for this functioning is the abundance and diversity of the microbial life living in soil. However, as the majority of soil organisms are not culturable, for a long time it was impossible to identify all biota present in soil. Nowadays, using molecular markers (both DNA and RNA) as a proxy, it is possible to map soil communities much faster and in greater detail. Therefore, we have new opportunities to deepen our understanding of the soil microbiome in different contexts. One area of specific interest is the rhizosphere, soil in the direct vicinity of plant roots. Plants are able to influence the rhizosphere by releasing a broad range of carbon-containing substances in the soil, resulting in selection and boosting a subset of the soil living community. In this thesis the central aim was to explore to what extent plants are able to affect the rhizosphere food web to their own benefit under different circumstances.

Chapter 2 describes the differences in rhizosphere food web composition of the invasive narrow-leaved ragwort (*Senecio inaequidens*) and the native tansy ragwort (*Jacobaea vulgaris*) co-occurring in three semi-natural habitats. For both species, two life stages were examined (1 year and ≥ 2 years). Bacteria, fungi, and nematode abundances were analysed using phylum (bacteria, fungi) and family (nematodes) specific quantitative (q)PCR primers. The results show subtle, yet significant differences in bacterial- and nematode communities between the native and the invasive species. On the other hand, the impact of plant life stage on rhizosphere biota was larger than the impact of plant species. The overall bacterial and fungal biomasses, as well as a remarkably consistent set of constituents (Actinobacteria, α- and β-Proteobacteria, and Bacteroidetes) were negatively affected by plant stage for both ragwort species. Most of the included nematode families also decreased in abundance in older life stages of the plant. However, densities of the fungivorous genera *Diphtherophora* and *Tylolaimophorus* remained constant. This suggests that a growing plant is well capable of altering the soil rhizosphere food web which differentially affects the major consumers of the primary decomposer community.

Chapter 3 also examines the effect of an invasive species on the rhizosphere food web. Here, the impact of the invasive giant goldenrod (*Solidago gigantea*) on microbial communities in two soil types was examined. Both biochemical and DNA-based markers were employed to investigate quantitative effects of giant goldenrod on the fungal community and on fungivorous nematodes. In invaded plots, elevated levels of fungal biomass were accompanied by the selective upregulation

of two out of three fungivorous nematode lineages present in these ecosystems (Aphelenchidae and Aphelenchoididae). With the use of DNA sequencing, we identified the fungal family Cladosporiaceae as indicative for invasion. Following the same line of reasoning as in Chapter 2, this indicates that the fungivorous nematode families Aphelenchidae and Aphelenchoididae may be characterized by a distinct food preference towards members of the Cladosporiaceae. As earlier research already showed an upregulation of the fungal biomass, we can now conclude that *S. gigantea* has a structural impact on the belowground soil community by increasing the fungal biomass independent of sampling moment, sampling year, or habitat.

Where natural ecosystems were studied in experimental Chapters 2 and 3, **Chapter 4** focusses on the effects of agricultural management practices – organic vs conventional, and compost vs no-compost – on major soil food web constituents. The rhizosphere of barley (*Hordeum vulgare*) was compared with bulk soil at two different growth stages (vegetative and generative). Here, we took a novel approach by mapping active (rRNA) and the resident (rDNA) communities of bacteria, fungi, protozoa, and metazoa with the use of high throughput sequencing. This was possible due to the development of a fast and affordable in-house RNA/DNA extraction protocol that was also applied in Chapters 5 and 6. Chapter 4 demonstrates the relevance of discriminating between the resident- and active communities, which explained 22%, 14%, 21%, and 25% of the variance within the bacterial, fungal, protozoan, and metazoan communities respectively. In addition, soil management (organic *versus* conventional) affected the four organismal groups significantly and organic management generally increased the presence and activity of soil organisms. Hence, prolonged (> 15 consecutive years) exposure to distinct soil management regimes causes significant shifts in soil communities.

Chapter 5 presents the analysis of a specific part of the Chapter 4 dataset. Here, we focus solely on the active (rRNA) fraction of bacteria, fungi, protozoa, and metazoa, during the generative stage of barley in organic and conventional fields. We studied interkingdom relationships, focusing on the interactions between bacteria and the three eukaryotic groups. First, for bacteria-protozoa, only positive interactions were found in conventional management. Organic management showed a more complex environment, with 7 positive and 12 negative interactions in the rhizosphere and 14 positive and 24 negative interactions in the bulk compartment. The same trend was observed for bacteria-fungi interactions. This suggests that organic fertilizer increases the complexity of the soil food web. Therefore, the application of organic fertilizers combined with abstinence of pesticides selects orders with different associations. These associations are most likely related to survival strategies and predation for protozoans and metazoans, while fungal-bacterial associations are most likely facilitated by support in decomposition.

Chapter 6 continues the research into organic and conventional farming practices. The same soil management types as in chapter 4 and 5 were assessed but now we investigated the impact of a legume, green pea (*Pisum sativum*), on the active and resident soil communities. Additionally, the active rhizosphere biota in association with different densities of the root-knot nematode *Meloidogyne chitwoodi* was evaluated. As was previously found in Chapter 4, large differences in resident and active communities were demonstrated. Novel was the high level of variance that could be explained by the variable sample type (bulk soil *versus* rhizosphere) for the two groups of primary decomposers. This indicates that green pea exerts a large effect on the bacterial and fungal communities in its rhizosphere, to the extent that it exceeds soil management effects. With regards to *M. chitwoodi* densities, prolonged organic soil management was accompanied by significantly lower numbers of the root-knot nematodes as compared to the conventionally managed soil systems. The increased abundance of the bacterial genus *Pseudomonas* offers as a possible explanation for the observed low pathogen abundance. This study proposes potential leads for more detailed studies on the effect soil management regimes on the activity levels of nematophagous bacteria and/or fungi.

The research presented in this thesis contributes to the identification of factors affecting the soil microbiome in different ecosystems. This thesis shows shifts in the soil microbiome induced by different plants and management systems. Providing evidence that long term soil management practices are able to structurally change the soil microbiome. Moreover, plants are well capable of changing the local microbiome, to the extent that it exceeds the effect of management practices. Next, this thesis provides insights into the distinction between active and resident microbial communities, which became possible by developing a fast and affordable DNA/RNA isolation technique as presented. As a consequence, it revealed the importance of discriminating between 'being present' and 'being present and active' in soil ecology. With the fast developments in this field it is important to remain critical of the tools we use to estimate soil biodiversity. Because currently high methodical variation hampers robust comparison of sequencing data between studies. Ahead of us lies the challenge to link soil biodiversity to soil function.

Samenvatting

Al in 1939 stipte Franklin D. Roosevelt het belang van de bodem aan met zijn statement: "*Een land dat zijn bodem vernietigt, vernietigt zichzelf*". De bodem ligt ten grondslag aan het functioneren van het ecosysteem: het filtert zoet water en slaat dat op, het voorziet in essentiële nutriënten die nodig zijn voor planten groei en het reguleert de temperatuur van de aarde. Essentieel voor dit functioneren is de diversiteit en de hoeveelheid van het microbiële leven in de bodem. Een lange tijd was het niet mogelijk om al het leven in de bodem te identificeren omdat het overgrote deel van het bodemleven niet in een laboratorium te kweken is. Tegenwoordig, doormiddel van het gebruiken van moleculaire merkers (zowel DNA als RNA) als graadmeter, is het wel mogelijk om de bodem gemeenschappen nauwkeurig en snel in kaart te brengen. Dit creëert de mogelijkheid om meer kennis te vergaren over het microbiële bodemleven in verschillende omstandigheden. In dit proefschrift wordt voornamelijk onderzocht in hoeverre planten in staat zijn om het voedsel web rondom hun wortels (rhizosfeer) in verschillende situaties te veranderen ten behoeve van zichzelf.

Hoofdstuk 2 beschrijft de verschillen in de samenstelling van het rhizosfeer voedsel web tussen het invasieve Bezemkruiskruid (*Senecio inaequidens*) en het inheemse Jakobskruiskruid (*Jacobaea vulgaris*) die samen voorkomen in drie verschillende natuurgebieden. Voor beide soorten werden twee verschillende groeistadia gebruikt (1 jaar en ≥ 2 jaar). De hoeveelheden bacteriën, schimmels en nematoden werden geanalyseerd doormiddel van fylum (bacteriën, schimmels) en familie (nematoden) specifieke (q)PCR primers. De resultaten waren subtiel maar er werden wel significante verschillen in bacterie en nematoden gemeenschap gevonden tussen de invasieve en de inheemse plantensoort. De impact die het groeistadium had op het bodemleven was groter dan de impact van de plantensoort. Over het algemeen werd de biomassa van bacteriën en schimmels negatief beïnvloed als een plant ouder werd, eveneens het geval voor een aantal specifieke fyla van bacteriën (Actinobacteria, α- en β-Proteobacteria en Bacteroidetes). De meerderheid van de geteste nematode families daalden ook in aantal met een toenemende leeftijd van de plant. Echter, aantallen van de schimmel etende genera *Diphtherophora* en *Tylolaimophorus* bleven constant. Dit suggereert dat een groeiende plant in staat is om het bodem voedsel web rondom zijn wortels te veranderen, gevolgd door een specifieke verandering in de consumenten (nematoden) van de reducenten (bacteriën en schimmels).

Hoofdstuk 3 onderzoekt ook het effect van een invasieve planten soort op het bodem voedsel web. In dit hoofdstuk wordt de impact van de late guldenroede (*Solidago gigantea*) op de microbiële gemeenschap, in twee verschillende

bodemtypes onderzocht. Zowel biochemische als DNA merkers werden gebruikt om kwantitatieve effecten van de late guldenroede op de schimmel gemeenschap en op schimmel etende nematoden te onderzoeken. In plots met de invasieve plant werd een verhoogde concentratie aan schimmels gevonden gepaard met een verhoogde concentratie aan schimmel etende nematoden (Aphelenchidae en Aphelenchoididae). Doormiddel van het sequensen van DNA konden we de schimmelfamilie Cladosporiaceae identificeren als indicatief voor invasieve plots. Wanneer we dezelfde redernatie gebruiken als in Hoofdstuk 2, geven deze bevindingen aan dat de nematoden families Aphelenchidae en Aphelenchoididae een voedselvoorkeur kunnen hebben voor leden van de Cladosporiaceae. Omdat eerder onderzoek al een toename van de schimmelbiomassa aantoonde in plots met *S. gigantea*, kunnen we nu concluderen dat *S. gigantea* een structurele impact heeft op de ondergrondse bodemgemeenschap, door de schimmelbiomassa te vergroten onafhankelijk van het bemonsteringsmoment, het bemonsteringsjaar of de habitat.

Natuurlijke ecosystemen werden bestudeerd in de experimentele hoofdstukken 1 en 2, maar in **Hoofdstuk 4** ligt de focus op het effect van verschillende landbouw management types – biologisch *versus* conventioneel en compost *versus* geen compost – op belangrijke organismen van het bodem voedsel web. De rhizosfeer van gerst (*Hordeum vulgare*) werd vergeleken met naastgelegen bulk grond gedurende twee verschillende groei stadia (vegetatief en generatief). We maken gebruik van een nieuwe aanpak door zowel de actieve (rRNA) als de totale (rDNA) gemeenschappen van bacteriën, schimmels, protozoa en metazoa in kaart te brengen doormiddel van sequensen(MiSeq). Dit was mogelijk door de ontwikkeling van een snel en betaalbaar RNA/DNA extractie protocol en dit protocol wordt vervolgens ook toegepast in de hoofdstukken 5 en 6. Hoofdstuk 4 laat zien dat het belangrijk is om een onderscheid te maken tussen de totale en de actieve gemeenschappen, aangezien dit 22%, 14%, 21%, en 25% van de variantie verklaard in de respectievelijke bacteriën, schimmels, protozoa en metazoa gemeenschappen. Bovendien beïnvloedde bodembeheer (organisch *versus* conventioneel) de vier groepen organismen aanzienlijk en verhoogde organisch beheer in het algemeen de aanwezigheid en activiteit van bodemorganismen. Langdurige (> 15 opeenvolgende jaren) blootstelling aan verschillende bodembeheersregimes veroorzaakt dan ook aanzienlijke verschuivingen in bodem-gemeenschappen.

Hoofdstuk 5 presenteert de analyse van een specifiek deel van de dataset uit Hoofdstuk 4. In dit hoofdstuk focussen we alleen op de actieve (rRNA) fractie van bacteriën, schimmels, protozoa en metazoa gedurende de generatieve fase van gerst in biologische en conventionele velden. We bestuderen interacties tussen verschillende rijken, waarbij de focus voornamelijk ligt op de interacties tussen bacteriën en de drie groepen eukaryoten. Er werden alleen positieve interacties gevonden in

conventionele velden tussen bacteriën en protozoa. Biologische velden lieten een meer complexte omgeving zien met 7 positieven en 12 negatieve interacties in de rhizosfeer en 14 positief en 24 negatieve interacties in de bulk grond. Dezelfde trend – meer complexe interacties in biologische velden – werd gevonden wanneer er gekeken werd naar interacties tussen bacteriën en schimmels. Dit suggereert dat biologische landbouw de complexiteit van het bodem voedsel web verhoogt. Ergo, de toevoeging van organische bemesting en de afwezigheid van pesticiden selecteert verschillende orders met andere functies. Deze functies zijn waarschijnlijk gerelateerd aan overlevingsstrategieën en predatie voor protozoa en metazoa, terwijl de interacties tussen schimmels en bacteriën waarschijnlijk worden gefaciliteerd door hun ondersteuning in decompositie

Hoofdstuk 6 vervolgt het onderzoek naar biologische en conventionele landbouw. Dezelfde management types als in Hoofstuk 4 en 5 werden bestudeerd echter nu werd het effect van een vlinderbloemige, erwt (*Pisum sativum*), op de actieve en totale bodemgemeenschap in kaart gebracht. Daarnaast werd de actieve rhizosfeer gemeenschap onderzocht in samenhang met verschillende dichtheden van de wortelknobbelaaltje *Meloidogyne chitwoodi*. Zoals eerder in hoofdstuk 4 werd gevonden, werden ook nu grote verschillen in actieve en totale gemeenschappen aangetoond. Nieuw was dat er een groot deel van de variantie kon worden verklaard door het type monster (bulk grond of rhizosfeer) in het geval van de twee reducenten (bacteriën en schimmels). Dit geeft aan dat erwt een groot effect heeft op de bacterie- en schimmelgemeenschappen in zijn rhizosfeer, dusdanig dat het de effecten van bodembeheer overschrijdt. Wanneer er gekeken werd naar *M. chitwoodi* dichtheden ging langdurig organisch bodembeheer gepaard met aanzienlijk lagere aantallen wortelknobbelaaltjes in vergelijking met de conventioneel beheerde bodem. Een verhoogde aanwezigheid van het bacterie genus *Pseudomonas* in de biologische velden kan een mogelijke verklaring zijn voor de vertegenwoordiging van lage pathogenen. Deze studie geeft verschillende aanknopings-mogelijkheden om meer gedetailleerd onderzoek te doen naar het effect van bodem management op de mate van activiteit van verschillende nematofage bacteriën en/of schimmels.

Het onderzoek dat wordt gepresenteerd in dit proefschrift draagt bij aan de identificatie van factoren die het bodem leven beïnvloed in verschillende ecosystemen. Dit proefschrift laat zien dat verschuivingen in bodemleven worden geïnduceerd door de plant en verschillende landbouw systemen. Het voorziet in bewijs dat langdurig bodembeheer het bodemleven structureel kan veranderen. Bovendien zijn planten goed in staat om hun lokale bodemleven te veranderen, zelfs dusdanig dat dit het effect van bodembeheer overstijgt. Daarnaast biedt dit proefzicht inzicht het verschil tussen actief en totale microbiële gemeenschappen. Dit werd mogelijk gemaakt door de ontwikkeling van een snel en betaalbaar DNA/

RNA isolatie protocol, zoals te lezen is in dit proefschrift. Hierdoor werd duidelijk dat het belangrijk is om onderscheid te kunnen maken tussen 'aanwezig zijn' en 'actief aanwezig zijn' in bodem. Met de snelle ontwikkelingen in het veld van sequensen blijft het belangrijk om kritisch te zijn op de instrumenten en methodes die we gebruiken om bodem diversiteit in kaart te brengen. Op dit moment belemmert grote methodologische variatie om verschillende sequentie data tussen studies te vergelijken. Voor ons ligt de interessante en grote uitdaging om bodem diversiteit te kunnen koppelen aan bodem functies.

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Basten every time we went for a beer it was great fun! I regret not doing that more often. Thank you for your help creating the charming networks reviewers were so 'horny' on back then. Rikus, I miss your laughter and your grapefruit at the coffee table for a long time already but I want to thank you for all your help in the lab. Whenever I needed something, no matter how small or old fashioned it was, you knew where to find it. Debbie, I remember you said to me you were so happy that a matching minded girl had entered Nematology, unfortunately, it was soon after that I had to miss your risqué jokes but I am thankful for the time we spend together at Nema. Lotte, the trip to Gent was brilliant (B*tches!!!) and I look forward to the next party with you. Paul, thank you for your help with my second chapter and your humour. Erik thank you very much for your help and expertise in the field practical's and that you accommodated Ava in a proper house without fungi.

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Being able to teach is something I highly value. Although it is somewhat less comfortable when you realize you are at the same (student)party. My dear students: Daniel, Linda, Hilde, Brent, and Koen. It was nice to see growing interest in soil biodiversity over the years and I am glad I was able to work with you on this topic. Thank you for the enjoyable distraction.

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K(T)ikkie Poels, thanks for being there with B&J every time my life felt apart. I will never forget Ibiza and our frozen yogurt breakfast at 4 pm. I hope your PhD journey will be as hospitable as mine (pun intended). Nichterige Niels, Awesome Anne, Mobiele Marianne, and Korrie thank you for your friendship, although we don't see each other that much it was always nice to reunite. Thanks to all other members of Gymnast association Split, although I stopped (twice) with gymnastics I want to thank you for the Monday training and activities we did. Marcel Wubs a special thanks to you, for not only being my trainer but also a friend during the 12 years of gymnastics at the Bongerd and always referring to my PhD as "the worm thing"

Bart, Freek, Pompe, Renée, Knillis, Sam (WC de Koffiemolen), thank you for all the rides we made on our carbon stallions. The trip to La Marmotte was something I will never forget. The amount of suffering to get to the top of the mountain, but also the feeling of awesomeness when you reach it, is something which would be the overdramatic analogy of my PhD, if I had to pick one. Thank you for supporting me and for the weirdly developed friendship that we have nowadays. This also includes Pien and Ellen ofcourse, as they give the best sticky bottles. All members of cycling association Hellingproof, thank you for the introduction to road racing and the nice activities. Whenever I was stressed out, this was the perfect relieve for me.

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these years. Although you baby-boomed the hell out of Alexandra and me, it was never an excuse to cancel our dates. Thank you for the nice outing and baking adventures during my PhD. I am happy I escaped this 'room' in time.

Marielle, Eeltje, Anne, (Onno), our "Vreetzeugen" dates were hilarious all-you-can-eat feastings. I think we ate a lot of restaurants bankrupt, but more importantly you made me laugh so hard by being so honest about everything, that I forgot the things I was stressed about.

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Henrieke (Henk), you are my pen-pal and we have written many e-mail to each other throughout my PhD. It was useful to put on paper what was troubling me, and you always replied. Every year we made resolutions, which made me think of goals beside my PhD. I really liked that and I hope we will continue to do this. Pieter(tje), I want to thank you for your serenity and your friendship during the past years. Because of you, I joined one of the most diverse and approachable Bible study groups, which was a valuable addition in my somewhat dissolute life. Thanks to all members of Kring, for exploring the deeper meaning of our existence together.

Onno (Onjo), I still don't know exactly why, but you decided 4 years ago (out of nowhere) that we should look for a house together. Which, by some means, turned out great! Although you started looking for a new house already a year later, you still have a bed in my house and a place in my heart. Thank you for all the relaxing coach potato evenings and for eating every single candy in the house, so I would not get fat.

"A moment of love is better than a lifetime alone": André, Gerben, Danny, and Aram thank you for stepping along for a while in my PhD life. Gerben a special word of thanks to you, for my very first road-bike experience, and for your willingness to share your micro-expertise with me in the final stage of my PhD (although we both know that nematodes rule the earth).

Anne (Plofje), you feel a little like my baby sister and I am thankful that we ran into each other. We are quite alike yet also very different. The friendship we have is like the journey of a fart; developed out of nowhere, slowly grew, swelled up, released the sweet scent of ~~compost~~ roses and is now giving way to a relaxed comfort. I want to thank you for the countless drives to McDonalds and your support in my PhD and with everything else I do.

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everything I do and I know you will be there for me whatever path I will choose. Thank you for your never-ending interest and faith in me.

"Be nice to your siblings, they're your best link to your past and the people most likely to stick with you in the future." Rolf, my big brother and also a great example (google R. Harkes and you find interesting stuff that no-one will understand but sounds incredibly cool). Sometimes it is hard to be a second child and you feel you'll have to live up to the expectations the firstborn created. Thank you for pretending to fail the first three years of your PhD in order to lower the performance bar for me. It was always a pleasure to be in Leiden amongst Froukje, Lorijn, and Renske. I am very proud to be your sister and happy you are my paranymph during the day of my defence.

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"If I have the gift of prophecy, and know all mysteries and all knowledge; and if I have all faith, so as to remove mountains, but do not have love, I am nothing". Mom and Dad, you are love in its truest form to me and the most inspiring people in my life. Thank you for learning me how to be critical and discuss issues that can be uncomfortable, yet important. Thank you for the wisdom of self-mockery and perspective. Thank you for raising and teaching me the way you did. The unconditional love and the absolute faith you have in me was my motivation to finish my PhD, therefore I dedicate this thesis to you both. I love you.

Paula Harkes

About the author

Paula Harkes was born on the 28th of July 1988 in Hengelo, Overijssel, the Netherlands. She grew up in Deventer and always had a fascination for animals and their wellbeing. After finishing high school (Etty Hillesum Lyceum) she was rejected for veterinarian medicine and decided to start a BSc Animal Science at the Wageningen University. When she was concluding her BSc with a minor in animal health and disease, she decided to switch to the MSc Biology in order to broaden her view. Within her MSc she did a thesis at the laboratory of Nematology to investigate the effect of anthelmintic on the microbiome of sheep. Furthermore she spend 5 months at the Universidad Autónoma de Yucatán (Media, Mexico), researching the effect of a TGF-β blocker against Chagas disease in mice. In 2013 she graduated as a biologist from the Wageningen University with a specialization in health and disease.

At the beginning of 2014 she was appointed as a research assistant at the department of Nematology to develop a molecular methodology to characterize bacterial and fungal communities in soil samples. After one year she got a contract as a junior researcher at the department in order to work on the ecological food web in the rhizosphere between plants, nematodes and bacteria. In summer 2015 a NWO grant was appointed and she started her PhD project at the laboratory of Nematology under supervision of Dr. ir. Hans Helder.

During her PhD she focused on soil microbiomes influenced by various plant species and situated in distinct ecosystems, links between primary decomposers and specific changes in the next trophic level. Hereby focusing mainly on the effect of the plant on its rhizosphere community, in conjunction with the effect of various soil management strategies on soil microbiota. She enjoyed supervising several BSc and MSc thesis students and organizing different practical courses. Furthermore she was involved in hosting plant soil interaction seminars for two years. Her firmly guarded free time was filled with cycling, running, making music and go out for dinner with friends.

List of publications

Harkes P, Verhoeven A, Sterken MG, Snoek LB, van den Elsen SJJ, Mooijman PJW, Quist CW, Vervoort MTW, Helder J (2017) The differential impact of a native and a non-native ragwort species (Senecioneae) on the first and second trophic level of the rhizosphere food web. Oikos 126: 1790-1803.

Wilschut RA, van der Putten WH, Garbeva P, **Harkes P**, Konings W, Kulkarni P, Martens H, Geisen S (2019) Root traits and belowground herbivores relate to plant-soil feedback variation among congeners. Nature Communications 10: 1564.

Suleiman AKA*, **Harkes P***, van den Elsen S, Holterman MHM, Korthals GW, Helder J, Kuramae EE (2019) Organic amendment strengthens interkingdom associations in the soil and rhizosphere of barley (Hordeum vulgare). Science of The Total Environment 695: 133885.

Harkes P, Suleiman AKA, van den Elsen SJJ, de Haan JJ, Holterman MHM, Kuramae EE, Helder J (2019) Conventional and organic soil management as divergent drivers of resident and active fractions of major soil food web constituents. Scientific Reports 9: 13521.

Di Lonardo P, van der Wal A, **Harkes P**, de Boer W (2019) Effect of nitrogen on fungal growth efficiency. Di Lonardo DP. The microbial side of soil priming effects. Wageningen University. https://doi.org/10.18174/470122

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 of a minimum total of 32 ECTS (= 22 weeks of activities).

Review of literature (4.5 ECTS)
- The effect of alien plant species on the rhizosphere food web

Post-graduate courses (4.1 ECTS)
- Next generation sequencing data analysis; Erasmus Rotterdam (2016)
- Introduction to R for statistical analysis; PE&RC (2016)
- R programming; Coursera (2017)
- Soil ecology; PE&RC (2019)

Laboratory training and working visits (1.5 ECTS)
- Data analysis (MiSeq); NIOO (2017)
- Ergosterol measurement techniques; NIOO (2016)

Invited review of (unpublished) journal manuscript (2 ECTS)
- Environmental Microbiology & Environmental Microbiology reports: cropping history shapes fungal, oomycetes and nematode communities in arable soil (2017)
- Microbial Ecology: the response of the soil microbiota to long-term mineral and organic nitrogen fertilization is stronger in the bulk soil than in the rhizosphere (2019)

Competence strengthening / skills courses (5 ECTS)
- Ethics, philosophy and history of the life sciences (MSc); RSO (2011)
- PhD competence assessment; WGS (2016)
- Efficient writing strategies; WGS (2016)
- Teaching and supervising thesis students; WGS (2016)
- Career assessment; WGS (2018)
- Ethics in plant science; WGS (2018)
- Start to teach; WGS
- Lecturing; WGS (2018)
- Brain based teaching; Educational Staff Development (2018)
- Last stretch of your PhD & writing propositions; WGS (2018)
- Ethics in plant science; WGS (2018)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.3 ECTS)
- PE&RC Day: one's waste... another's treasure? (2015)
- PE&RC First years weekend (2016)
- PE&RC Workshop: plant-soil-microbe interactions for crop and pest management (2016)
- PE&RC Day: PE&RC's got talent (2016)
- PE&RC Course carrousel (2016, 2017)
- Frontiers in ecology: the soil plant interface (2017)
- PE&RC Day: preventing the end of the year (2017)
- Plant-soil feedback: linkages between root traits and soil biota (2019)

Discussion groups / local seminars / other scientific meetings (9.1 ECTS)
- Rhizosphere congress: stretching the interface of life; Maastricht (2015)
- Soil science in a changing world, 2nd Wageningen conference on soil science; Wageningen (2015)
- Working group soilborn pathogens and soil microbiology; Wageningen (2015, 2017)
- Discussion group PE&RC: plant soil interactions; Wageningen (2016–2018)
- NWO Green rhizosphere fine-mapping project meeting; Wageningen (2016–2019)
- Farewell symposium Ton Bisseling EPS: underground labyrinth; Wageningen (2017)
- Plant-soil feedback: linkages between root traits and soil biota; Wageningen (2019)
- Wageningen PhD symposium: science: from Local to Global; Wageningen (2017)

International symposia, workshops and conferences (9.5 ECTS)
- ESN; oral presentation; Braga, Portugal (2016)
- NEAM Meeting; poster presentation; Lunteren, the Netherlands (2018)
- 2nd Plant microbiome symposium; poster presentation; Amsterdam, the Netherlands (2018)
- ESN; oral presentation; Gent, Belgium (2018)
- NEAM; oral presentation (3rd presentation award); Lunteren, the Netherlands (2019)

Societally relevant exposure (0.9 ECTS)
- Online video: interview/performance; Worm&Co, Wageningen (2017)
- Guest lecture: weekend van de wetenschap; Wonderryck, Twente (2019)
- Guest lecture: organic vs conventional agricultural soils; HAS, Den Bosch (2019)

Lecturing / supervision of practicals / tutorials (25.9 ECTS)

- Webs of Terrestrial Diversity (2014–2019)
- Functional Morphology, Biosystematics and DNA-based identification (2014–2016)
- Introduction Environmental studies (2014–2017)
- Ecological aspects of bio-interactions (2015–2018)
- Host Parasite Interactions (2018)

Supervision of MSc students (12 ECTS)

- Parasitic nematodes species biodiversity betweensSheep farming systems: ribosomal DNA sequence approach
- The effect of breeding for tolerance or resistance on beet cyst nematodes, and the impact of these plant genetic constitutions on the rhizosphere microbial community
- The bacterial and nematode rhizosphere composition and its change with compost addition
- Differences in rhizosphere community between organic and conventional farming of *Pisum sativum*
- Rhizosphere community composition changes under influence of plant developmental stage in *Pisum sativum*

We still do not know the magnitude
of what we do not know.

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