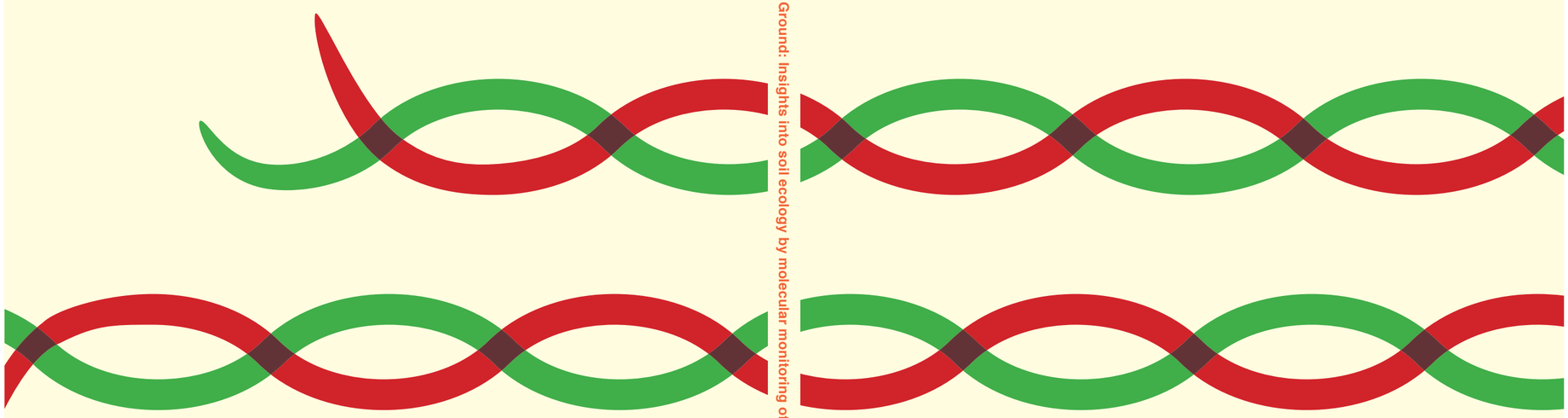
Mariëtte T.W. Vervoort

Covering ground:

Insights into soil ecology by molecular monitoring of nematode assemblages



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INVITATION

You are kindly invited to attend the public defense of my PhD thesis entitled:

COVERING GROUND:

Insights into soil ecology by molecular monitoring of nematode assemblages

On Friday, October 11th 2013
at 13:30 in the aula of
Wageningen University,
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INVITATION

Covering Ground: Insights into Soil Ecology by Molecular Monitoring of Nematode Assemblages

Mariëtte T.W. Vervoort

Covering Ground: Insights into Soil Ecology by Molecular Monitoring of Nematode Assemblages

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Covering Ground: Insights into Soil Ecology by Molecular Monitoring of Nematode Assemblages

Mariëtte T.W. Vervoort

Thesis
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There's a big dark town
it's a place I've found
there's a world going on
underground.

- Tom Waits, Underground, 1983

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Chapter 1

General introduction

Mariëtte T.W. Vervoort

Introduction

Soil biota: diverse and important

Soil is of vital importance for our existence. It is essential for, among others, food production, water purification and carbon cycling. Less known is the fact that an immense diversity of soil organisms plays a key role in allowing soil to perform these functions. It takes just a stroll through a nature reserve and a cornfield to get an idea of the differences in biodiversity of plants and animals aboveground, but how would you assess the diversity of the invisible life below your feet?

Simply taking a scoop of soil in your hands will allow you to feel its structure, moistness and estimate its organic matter content, but little divulges the immense biodiversity of organisms residing in it. In fact, in most terrestrial systems, the largest fraction of the diversity of organisms can be found belowground (Wardle and Giller, 1996). Soil biota not only consist of the generally known earthworms and ants, but also for instance tardigrades, collembolans, mites, protozoa, nematodes, fungi, as well as thousands of bacteria species (Brussaard et al., 1997). This diversity is unsurprising considering the high heterogeneity of niches, provided in soil by factors such as soil structure, moisture, organic matter content and composition, pH, and the presence of plant roots, and which may vary on both field- and micro scale (Killham, 1994).

The basis for soil ecological research dates back to the 19th century, in which the first textbooks on different groups of soil biota were published (Whalen and Sampedro, 2010). These first studies were mainly of a descriptive nature and only focused on one specific species or group:

"…if a fallen bough be examined, a heap of moss shaken over a pocket-handkerchief, or any long herbage swept with a hand-net, the naturalist will not fail to find, together with numerous beetles, flies, and other insects, certain delicate, hexapod, active little creatures…"

(Fragment from Collembola and Thysanura by Sir John Lubbock, 1873)

"*Even on the same field, worms are much more frequent in some places than in others without any visible difference in the nature of the soil*."

(Fragment from: The Formation of Vegetable Mould: Through the Action of Worms by Charles Darwin, 1881)

Subsequently, most studies involving soil biodiversity were conducted from a plant-pathological perspective. However, from the 1970s onwards, the important role of soil biota in vital ecosystem processes (*e.g.* decomposition, nutrient cycling, water remediation etc.) became more and more apparent (Wall and Moore, 1999, Powell, 2007, Brussaard et al., 1997). Before the discovery of DNA and the development of molecular techniques, the inability to culture and identify a large part of all soil organisms restricted the knowledge of the actual diversity in soil (Fitter et al., 2005). By having these tools at our disposal, we are able to make better estimations of the virtually inconceivable diversity of soil life.

Although the diversity, abundance and importance of soil biota have become clearer over time, the exact roles of most species in soil functioning have not (Fitter et al., 2005). Soil is still often referred to as the black box, not only because of the diversity of its inhabitants, but also

due to the complexity of the interacting processes that take place in it (Cortois and de Deyn, 2012, Andrén et al., 2008). As soil functioning lies at the base of our daily needs, we require a better understanding of the dynamics and functional diversity of key players in soil processes. This will allow us to better assess the consequences of human disturbance (*e.g.* intense agriculture, forest disturbance), invasive species, climate change, and will help shape future management (Wall and Moore, 1999).

The soil food web and environmental indicators

Similar as for aboveground ecosystems, the soil ecosystem runs on nutrients provided by primary producers, *i.e.* plants. Nutrients can enter the soil system directly via plant roots (living plant material) but also, for the largest part, as detritus *i.e.* dead organic material (Cyr and Pace, 1993). In turn, nutrients are made available again for plant uptake through a cascade of feeding relationships between different soil biota, which is as a whole referred to as the soil food web (Fig 1.). In figure 1, an example is given of a soil food web, which is divided into four trophic levels. A trophic level can be defined as a group of organisms that obtain the majority of their energy by feeding upon the adjacent trophic level, which is nearer to the energy source (Hairston Jr and Hairston Sr, 1993). The first trophic level (TL1) of the soil food web contains organisms feeding on living plant material (*e.g.* plant parasitic nematodes), as well as saprotrophic fungi and bacteria, the so-called primary decomposers, which are able to break down detritus. The second level (TL2) consists of the secondary decomposers (*e.g.* nematodes, collembolans, amoebae, flagellates and mites), which feed upon fungi and bacteria and are themselves in turn eaten by predaceous soil organisms (*e.g.* predaceous nematodes, mites or collembolans), which are at the highest trophic level of the food web (TL3). The presence of higher trophic levels in the soil food web is necessary for nutrient cycling, since fungi and bacteria, in the first layer, partially retain nutrients for their own metabolism and growth. For instance, soil biota from higher trophic levels generally contribute around 30-40% of total nitrogen mineralization in soil (Verhoef and Brussaard, 1990).

Because of the soil food web's mayor role in soil functioning, it can provide us with information on soil functions; such as decomposition, nutrient cycling, primary production or disease suppression and on the status of the soil; such as the effects of agricultural practices, or its state of succession (Wurst et al., 2012, Holtkamp et al., 2008, De Deyn et al., 2003, van Capelle et al., 2012, Mulder et al., 2003). To obtain information, it would be ideal if we could measure all the different components of a soil food web. However, as illustrated in the last paragraph, the diversity of organisms in soil is immense and their interactions, with each other and with abiotic factors, introduce a high degree of complexity. To acquire data on the soil food web and soil processes in a more feasible way, indicator groups are often selected. Soil faunal communities (from TL2 and TL3) are frequently used as indicators for soil status. Not only because they reflect the state of their food sources (TL1 and or TL2), but also because they have longer generation times (days to years) than soil microbes (hours to days), making them a more stable group to monitor (Neher, 2001). So far, regularly used indicators include collembolans (Kopeszki, 1997), arthropods (Van Straalen, 1998), mites (Gormsen et al., 2006) and nematodes (Neher, 2001).

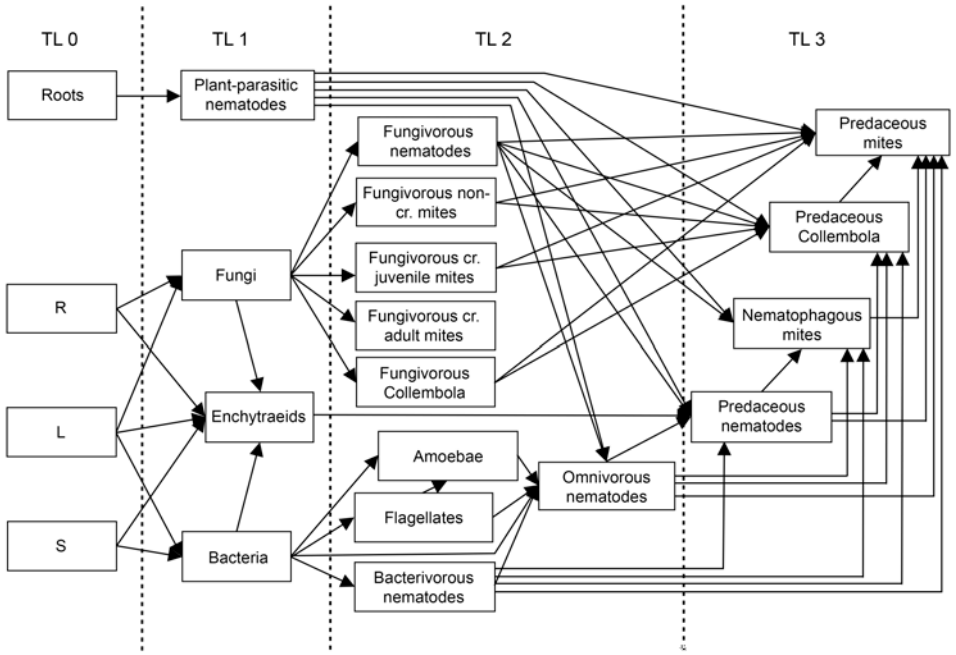

Figure 1. An example of a soil food web presented as a diagram (Holtkamp et al., 2008) in which trophic connections are indicated by the arrows (arrows are pointed towards the consumers). TL: trophic layer, R: recalcitrant organic matter, L: Labile organic matter and S: soluble sugars, cr. cryptostigmatic (now known as orbatid).

Nematodes as environmental indicators

Nematodes are small (mostly between 0.2-2.5 mm in length), worm-shaped animals that taken together constitute the phylum Nematoda. This phylum, which is thought to have arisen during the Cambrian explosion (600-550 million years ago), belongs to the superphylum Ecdysozoa that encompasses all moulting animals (Aguinaldo et al., 1997). Nematodes are present in terrestrial systems, but also in freshwater and marine habitats (Bongers and Ferris, 1999). Next to their ubiquity, nematodes are abundant and can reach densities of up to millions per square meter in soil, residing in plant roots or the water films attached to soil particles (Platt, 1994). Due to their abundance and size, nematodes are easily extractable from soil, when compared to for instance fungi and bacteria. The phylum Nematoda encompasses a high trophic diversity; nematodes may feed upon bacteria, fungi, protozoa, algae, other nematodes or in the case of omnivores on a combination of the aforementioned, or they may be facultative or obligate plant or animal parasites (Yeates, 1993). Due to this diversity in feeding habits, nematodes can be found at all three levels of the soil food web (Ferris et al., 2001).

Next to their central role in the soil food web and easy extractability, the differential responsiveness amongst nematode taxa to disturbances makes them a suitable indicator group for the status of soil (Neher, 2001). Multiple studies have shown that nematode taxa are differ-

entially sensitive to stressors such as desiccation, toxic compounds, heavy metals (Nagy, 2009) and agricultural practices such as tillage or the application of fertilisers (*e.g*. Fiscus and Neher, 2002, Ferris and Bongers, 2006). In short, it is the combination of the functional and taxonomic diversity within this phylum that makes it so suitable as an indicator (Ferris and Bongers, 2009). Based on this, several nematode-specific community indices have been developed over time. The use of community indices can facilitate the interpretation of elaborate data sets, by condensing information into a single or several variables.

An important step towards the development of indices specific for nematode communities has been the assignment of a so-called colonizer-persister value (*cp*-value) to different nematode families (Bongers and Ferris, 1999). In this classification, ranking from 1-5, families within the same *cp*-class share similar life strategy characteristics such as life cycle length, the number and size of their eggs and the size of their gonads. Families that are the least sensitive to disturbance are assigned the lowest values (1-2) and are defined as colonizers (r-strategists). Typically, these are small bacterivorous nematodes with a short generation time and the ability to produce large numbers of offspring. At the other end of the scale (4-5) are the persisters (K-strategistst); sensitive to disturbance, usually large and often predators or omnivores with a long generation time and low numbers of offspring. Based upon this *cp*-classification, the so-called Maturity Index (MI), one of the most frequently used nematode indices, can be calculated (Bongers, 1990). The MI is expressed as the mean frequency of the *cp*-classes found in a sample and in reflects in this way the degree of disturbance of the community or, in other words, the state of ecological succession.

Over time, several nematode indices were developed for different purposes and the existing indices refined (Ferris and Bongers, 2009). Similar to the maturity index, these indexes focus on the inferred ecosystems functions portrayed by the community rather than its structure. Based on this principle, nematode taxa were categorized into so-called functional guilds. In these guilds, both the life strategy (*cp* classification) as well as the feeding habit (also referred to as trophic group) of a taxon is incorporated (Bongers and Bongers, 1998). Examples nematode-specific indices based on this functional guild division are the Enrichment Index (EI) and the Structure index (SI), which include a weighting of functional guilds based on their indicative value for enrichment or the food web structure, and the Channel Index (CI), which distinguishes between the energy flow through the bacterial and the fungal decomposition channels based on the relative abundance of fungivorous and bacterivorous nematodes (Ferris and Bongers, 2009).

Exploitation of a SSU rDNA framework for nematode identification

Nematodes are morphologically highly conserved. From an evolutionary perspective one might say that the saying 'never change a winning team' is applicable for this phylum. Therefore, as the number of informative morphological characters is limited, identification of nematodes is challenging and requires a considerable amount of experience and expertise. For an untrained eye, it is very difficult to distinguish species in an environmental sample (Fig 2). Nevertheless, the soil from one site can contain over 200 species (Boag and Yeates, 1998). Even for experts, their ability to identify certain species depends on the life stage or sex of the present individuals (Floyd et al., 2002).

Traditionally, nematode identification of environmental samples is performed using a light microscope. In general, a subsample of around 150 individuals (of typically around 2,000 individuals in a sample) is identified which can take an expert up to several hours (*e.g.* Sánchez-Moreno et al., 2006, Ferris et al., 1996, Yeates et al., 2000, Boutsis et al., 2011). In the meantime, the number of experts with the ability to perform the identification is declining rapidly over the years (Coomans, 2002). In a way, this phylum's major asset as an environmental indicator, *i.e.* its diversity, is at the same time its pitfall; making nematode community analysis time-consuming and dependent on the availability of the necessary expertise. It is therefore not surprising to see that the use of molecular techniques for the identification and quantification of nematodes has gained interest over the last years (*e.g.* Powers, 2004, Floyd et al., 2002, Jones et al., 2006, Griffiths et al., 2006).

The DNA sequence of a single gene (typically containing hundreds or thousands of nucleotides) can deliver a multitude of objective characters. Different purposes, *e.g.* phylogenetics (deep or shallow) or molecular identification, require different types of information in the form of resolution and variability. Because the type of information a gene provides is related to the rate at which a gene evolves, not every gene is equally suitable for a certain purpose. For nematodes, the small subunit ribosomal DNA (SSU rDNA) gene has been often used in the past for resolving both deep as well as shallow phylogenetic relationships (Blaxter et al., 1998, Aleshin et al., 1998, Holterman et al., 2006, Van Megen et al., 2009). The SSU rDNA gene codes for SSU rRNA (18S), a part of the ribosomal RNA, and is essential for protein synthesis. Because of its vital importance, the SSU rDNA gene is highly conserved in most animals. Nevertheless, in nematodes, the substitution rate of the SSU rDNA is relatively variable and can therefore be informative for deep phylogenetics, shallow phylogenetics as well as molecular

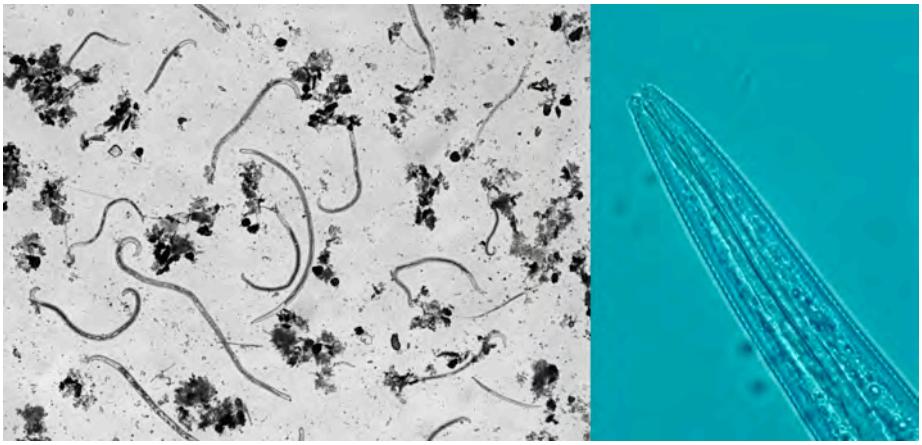

Figure 2. On the left: A nematode suspension, extracted from soil, under a low magnification microscope (400x magnification). Photo credits: Paul Mooijman, Laboratory of Nematology, Wageningen University. On the right: Picture of the head region of a bacterivorous nematode species, Eucephalobus striatus (picture taken at 1,000x magnification). Photo credits: Hanny van Megen, Laboratory of Nematology, Wageningen University.

identification at different taxonomic resolutions (*e.g.* Floyd et al., 2002, Holterman et al., 2008, Van Megen et al., 2009).

Currently, there is a full-length SSU rDNA sequence framework consisting of over 2,800 sequences (and growing). Based on this framework, taxon-specific primers (on species, genus or family-level) have been developed for the qualitative and quantitative monitoring of nematodes. This development has the potential to lift the practical constraints of nematode identification and allows for the large-scale exploitation of the nematode community as an environmental indicator.

Outline of this thesis

The aim of the research described in this thesis was to assess the suitability of molecular taxon-specific assays for the monitoring of nematode assemblages in field experiments. The method was applied to determine the effects of different types of disturbances on the soil food web, *i.e.* agricultural practices, invasive plant species and the effects of a genetically modified potato variety.

In **Chapter 2**, the background of the molecular method and the results of its first field application are presented. It describes the results of an extensive field study in which the seasonal fluctuations of 15 nematode taxa were monitored at 18 times during 10 months in two different habitats; an ex-arable field and its adjacent beech forest. The results show different temporal fluctuations of nematode taxa belonging to the same functional guild. The study demonstrates that the production of elaborate datasets, facilitated by a molecular approach, can lead to new insights in nematode ecology and evolution.

In **Chapter 3**, the effect of an agricultural management practice referred to as biofumigation was determined for the nematode community. Biofumigation is a pest control management measure, which entails the incorporation of brassicaceous plant material resulting in the release of natural, general biocidal compounds into soil. Although strong effects were observed after incorporation of different *Brassica juncea* cultivars, no relationship was observed between the concentrations of the compounds incorporated into the soil and the response of different nematode taxa, including the plant parasites. The results suggest that, at least for the cultivars used in this study, observed effects on the nematode community were related to a combination of tillage and green manuring.

In **Chapter 4**, the results of a study concerning the belowground impact of an invasive plant species, *Solidago gigantea* are presented. Impact was assessed by comparing the vegetation characteristics and soil characteristics of invaded plots (*i.e.* pH, soil moisture content, fungal biomass and the densities of several nematode taxa) to those of uninvaded plots in two contrasting habitat types, riparian and semi-natural grasslands. A habitat-independent significant increase of fungal biomass was observed as well as a systematic increase of one out of the three monitored fungivorous nematode taxa. The results of this study indicate that an asymmetrical boost of the fungal community by *S. gigantea* could explain the observed shift in the fungivorous nematode distribution.

In **Chapter 5,** the effect of a genetically modified potato variety on the nematode community is compared to that of its parental conventional isoline and four other conventional

potato cultivars. The experiment was performed on two locations, differing in soil type, and samples were taken on two occasions during the growing season of the plants. In this study, no differences were observed between the nematode community of soil planted with the GM potato and its parental cultivar or overall between cultivars. However, the results showed a significant effect of location (or soil type) and sampling time.

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Chapter 2

SSU ribosomal DNA-based monitoring of nematode assemblages reveals distinct seasonal fluctuations within evolutionary heterogeneous feeding guilds

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Abstract

Soils are among the most complex, diverse and competitive habitats on Earth and soil biota are responsible for ecosystem services such as nutrient cycling, carbon sequestration and remediation of freshwater. The extreme biodiversity prohibits the making of a full inventory of soil life. Hence, an appropriate indicator group should be selected to determine the biological condition of soil systems. Due to their ubiquity and the diverse responses to abiotic and biotic changes, nematodes are suitable indicators for environmental monitoring. However, the time-consuming microscopic analysis of nematode communities has limited the scale at which this indicator group is used. In an attempt to circumvent this problem, a quantitative PCR-based tool for the detection of a consistent part of the soil nematofauna was developed based on a phylum-wide molecular framework consisting of 2,400 full-length SSU rDNA sequences. Taxon-specific primers were designed and tested for specificity. Furthermore, relationships were determined between the quantitative PCR output and numbers of target nematodes. As a first field test for this DNA sequence signature-based approach, seasonal fluctuations of nematode assemblages under open canopy (one field) and closed canopy (one forest) were monitored. Fifteen taxa from four feeding guilds (covering ~ 65 % of the free-living nematode biodiversity at higher taxonomical level) were detected at two trophic levels. These four feeding guilds are composed of taxa that developed independently by parallel evolution and we detected ecologically interpretable patterns for free-living nematodes belonging to the lower trophic level of soil food webs. Our results show temporal fluctuations, which can be even opposite within taxa belonging to the same guild. This research on nematode assemblages revealed ecological information about the soil food web that had been partly overlooked.

Introduction

The biotic soil fraction is the source of major ecosystem services like water holding, nutrient cycling, and carbon sequestration (Petersen and Luxton, 1982, Myers, 1996, Wardle et al., 2004, Fitter et al., 2005, Postma-Blaauw et al., 2005) and is the bottom-up driving force of the ecosystem (Hunt and Wall 2002, Scheu and Setälä 2002). Therefore, due to their differences in habitat-responses and multitrophic interactions (Hunt and Wall 2002, Scheu and Setälä 2002, Wardle et al., 2004, De Mesel et al., 2006, Mulder et al., 2012), many terrestrial invertebrates are valuable ecological indicators (Birkhofer et al., 2012). However, irrespectively of the environmental characteristics we wish to highlight, the high biodiversity in soils and sediments (Mulder et al., 2005, Höss et al., 2011) forces us to choose a subset that is representative for biological soil quality. Ecological criteria to select indicator groups should include a) distribution across multiple trophic levels, b) methodological interpretability of qualitative and/or quantitative changes, and c) ease of sampling standardization. Soil nematodes meet these criteria.

These vermiform invertebrates, mostly with body lengths ranging between 0.2 and 2.5 mm (Mulder and Vonk 2011), are present in densities up to several millions individuals per square meter, and are easily extractable from the topsoil. Their trophic diversity encompasses all the three energy channels distinguishable within the soil food web: the plant-feeding, the bacterial-feeding, and the fungal-feeding pathway (*e.g.* Ferris et al., 2001). Because of their highly interconnected positions in the detrital soil food web, nematode communities reflect microbial resources, especially the bacterial and the fungal communities (Wardle et al., 2004, Van Eekeren et al., 2010, Christensen et al., 2012), soil fertility and management (Ferris et al., 2001, Yeates 2007, Reuman et al., 2009). Simple food webs with few trophic levels, as those in our study, show more specialization and less omnivory because occurring species (here, nematodes) have a much higher probability of consuming at one single trophic level (Polis et al., 1997, Thompson et al., 2007). Hence, nematodes are a natural avenue to examine the spatial and temporal variance of such food web configurations.

Moreover, the nature and rate by which nematodes respond to changes in the (a)biotical soil condition varies amongst different families and genera. At community level, this variation in responsiveness reflects itself changes in the numerical abundance, species composition, feeding traits and trophic distribution. The interconnected positions in the soil food web, in combination with taxon-specific responsiveness towards environmental stressors, make these invertebrates suitable as indicators.

Over the last decade, substantial progress has been made in collecting phylum-wide genetic information of nematodes (Blaxter et al., 1998, Holterman et al., 2006, Van Megen et al., 2009). This resulted in a small subunit ribosomal DNA-based (SSU-rDNA) framework covering a substantial part of the biodiversity for terrestrial nematode communities in temperate climate zones. Other studies independently introduced molecular tools to analyse nematode communities using qualitative or semi-quantitative techniques such as direct sequencing (Floyd et al., 2002), PCR DGGE (Okada and Oba 2008), and T-RFLP (Donn et al., 2012).

For DNA-based quantitative community analysis, the effect of the (unknown) life-stage distribution within individual taxa should be considered. In the past, nematodes were thought to exhibit cell constancy; all individuals of a given species have the same number of cells. However, at least for one organ, the epidermis, this was shown to be incorrect (Cunha et al.,

1999). During their development from first or second juvenile stage to adult stage, the number of somatic cells appears to show less than a two-fold increase. In case of *Caenorhabditis elegans* (Rhabditidae) the number of non-gonadal cells increase from ≈ 550 (first stage juveniles) to ≈ 810 (mature hermaphrodite) (De Cuyper and Vanfleteren 1982), while *Panagrellus silusiae* (Panagrolaimidae) the number of somatic nuclei was shown to increase from ≈ 410 (second stage juvenile) to ≈ 590 (adult) (Sin and Pasternak 1971). Although data on this issue are scarce (but see Derycke et al., 2012 for cryptic Rhabditidae), we hypothesize here that it is possible to relate quantitative PCR data to the number of individuals of a given family at a logarithmic scale without knowing their exact distribution over the life stages. If this is true, a SSU-rDNA quantitative community analysis will define emergent characteristics of the nematofauna and can provide one common currency to assess the soil food web by comparing the quantitative PCR output of differently-sized nematodes.

According to Neher (2010), more research is needed on nematodes in natural and agricultural soils to test ecological hypotheses. Hence, we chose two adjacent ecosystems to investigate the extent to which the SSU-rDNA tool allows monitoring of soil nematode assemblages in the South of the Veluwe region (central Netherlands). In such a way, we were able to establish the degree of convergence of the soil ecological condition 30 years after ending the agricultural practices by a direct comparison between the compositions of the nematode community in the previously disturbed area ('former arable field') and the adjacent undisturbed control ('mature beech forest'), as recommended in Neher 2010. During 10 months, nematodes were sampled 18 times with intervals of 2-4 weeks. 15 families or genera were detected (monophyletic groups in a phylum-wide SSU rDNA framework that includes ≈ 2,400 taxa as described in Van Megen et al., 2009), within them most feeding guilds were represented. Occurrence of specialized nematodes parasitizing vascular plants greatly depends on the structure of the rhizosphere, hence on the composition of the vegetation and as for the Maturity Index (Bongers 1990) they were not taken into consideration in this nematological research. Shortly, recurring DNA patterns (motifs) were identified for family or genus-specific groups and PCR primers with identical annealing temperatures were developed. We show that nematode assemblages can be monitored frequently using standard molecular laboratory equipment and that this method has the potential to contribute to the full exploitation of this abundant and diverse group of metazoans.

Materials and methods

Study area

Seasonal fluctuations of non-parasitic nematode assemblages were studied in De Planken Wambuis, a nature reserve located on the Veluwe, the largest moraine complex in The Netherlands. Due to the absence of endangered and/or protected species, this investigated area of approximately 100 m length is not protected by law and no specific permits were required. Sandy soil samples were taken from two sites: a 30-year-ago abandoned arable field, known as Dennenkamp (52° 03′ N, 5° 80′ E), and an adjacent more than 100-year-old *Fagus sylvatica* forest.

The former arable field (sampling area 2.5 ha; further referred to as 'field') is a relatively open area with a *Plantagini-Festucion* association (*sensu* Weeda et al., 1996) growing on a soil with pH of 5.7; more characteristics of this site have been published in Holtkamp et al., 2008. The pristine beech forest (further referred to as 'forest') with typical medium humified humus,

hereafter moder soil (pH ≈ 3.7), is characterized by a scarce understory (sampling area 1.5 ha). The precipitation and temperature data were registered by a weather station by the Royal Dutch Meteorological Institute (KNMI Station 06275, 45 m a.s.l., about 7 km from Dennenkamp).

Sampling and nematode extraction

Nematode assemblages were monitored throughout 2009 in an abandoned field and an adjacent pristine beech forest. On these sites, the upper 25 cm of the soil were sampled 18 times from March 17 (week 1) until December 18 (week 40). The humus fraction was still observable as a stratified layer in the forest moder (partly decayed, to some extent mixed with the mineral horizon). At eighteen time points (every 2-4 weeks), we randomly took four composite soil samples from the field, and two composite samples from the adjacent forest. Each sample consisted of 8-10 cores (Ø 1.5 cm, depth 25 cm) taken from a surface of ~ 0.25 m2 and thoroughly mixed. Nematodes were extracted from 100 ml of soil using an elutriator (Oostenbrink, 1960). Nematode density was estimated by counting two subsamples per sample at low magnification (classical analysis); after counting, these subsamples were poured back into the original suspension.

Selection of nematode taxa

To make a selection of monitored taxa for this study, suspensions from both sites were analysed microscopically twice (week 1 and week 39, 2009). In total, 38 genera *sensu* Bongers (1994) were identified in the field and 25 in the forest (Table 1). Fifteen nematode taxa (families and genera; Table 2) were selected based on molecular resolution, trophic ecologies, and sensitivities towards environmental disturbances. This taxonomic selection at genus level covers 59 % of the field and 72 % of the forest nematode biodiversity (excluding the obligate plant parasitic genera; Table 1).

DNA extraction and purification

Nematode suspensions (100 ml) were concentrated by centrifugation at 4,000 rpm, supernatant was removed until an end-volume of approximately 1.5 ml. This volume was further concentrated in a small vial at 14,000 rpm. The supernatant was removed until the final volume of 140 µl was reached. Subsequently, like in Holterman et al. (2006), an equal volume of nematode lysis buffer was added. As an internal standard, 20 µl of mammalian DNA (20 ng/µl) was included. Lysis took place in an oven at 65 °C for two hours. Lysates were purified using a glass fiber-based DNA extraction procedure (essentially according to Ivanova et al., 2006). Purified nematode community DNA was eluted from the filter with T10E1 (10:1, 1 M Tris and 0.5 M EDTA) and immediately used or stored at -20 ˚C. These purified lysates were used for quantitative PCR analysis. We kept this DNA extraction procedure consistent for all samples in our study to ensure full comparability of results (Thonar et al., 2012, note to their S3).

Design and testing of family- and genus-specific primers

For the development of taxon specific PCR primers, a molecular framework consisting of ≈ 2,400 (nearly) full-length SSU rDNA sequences representing all major groups of terrestrial nematodes was used. ARB, a LINUX-based software package (Ludwig et al., 2004), was used to design family and/or genus-specific primers. Most nematode families appeared as monophyletic

groups in a SSU rDNA based phylogenetic tree (Van Megen et al., 2009), and PCR primers were developed on the basis of taxon-specific motifs. In contrast, for some polyphyletic taxa – *e.g.*, fungivorous Diphtherophoridae – separate specific primer combinations were developed for each of the constituting genera. In the case of the poly- and paraphyletic Rhabditidae, embracing 27 genera according to the Fauna Europaea [http://www.faunaeur.org (Accessed 2012 June 6)], no comprehensive DNA barcodes could be generated at such a large family level, albeit for some monophyletic genera, specific primers can still be developed.

When designing the primer combinations, the annealing temperature of the oligonucleotides was assessed *in silico* using the program MELTING (Le Novere 2001). For each nematode taxon (family or genus), the specificity of multiple (up to 5) primer combinations was checked with recombinant SSU rDNA fragments from target(s) and close non-target(s) as identified by ARB (details in Neilson et al., 2009, Rybarczyk-Mydlowska 2012). Apart from the specificity requirements, primer combinations were designed to have an optimal annealing temperature (Ta) of 63˚C. Based on an experimental temperature range test, only target-specific primer combinations with a sharp optimum were selected. This approach allows for a quantitative detection of combinations of taxa with the same PCR temperature profile.

Primer combinations were tested in 25 µl containing 3 µl of 1,000 times diluted template (final concentration: 10 ng/µl), 1 µl of each of the taxon-specific primers (final concentration for each primer: 200 µg/µl), 7.5 µl Milli-Q water and 12.5 µl Absolute SYBR Green Fluorescein Mix (Thermo Fisher). For amplification on a thermal cycler (Bio-Rad iQ5), the following quantitative PCR temperature profile was used: 95 ˚C, 15 min followed by 60× (95 ˚C, 30 sec; 63 ˚C, 1 min; 72 ˚C, 30 sec) followed by a melting curve program 47× (15 sec from 72 to 95 ˚C with steps of 0.5 ˚C).

For each taxon, one primer combination was selected on the basis of optimal specificity (*i.e.*, largest DCt) between target(s) and close non-target(s); assays with a DCt lower than 12 were discarded. Here, Ct value is defined as the number of PCR cycles ('C') at which the reporter dye emission intensity exceeds a predetermined threshold ('t'). In case of similar specificities, primer combinations with lowest Ct value per unit of template were preferred.

Quantitative PCR on total nematode community DNA

Each purified lysate (DNA extract from 100 ml elutriated soil) was used as template with 15 primer combinations on a thermal cycler (Bio-Rad iQ5). A separate primer combination was used to quantify the internal standard (mammalian DNA) in each sample to estimate the efficiency of the lysis and purification procedure. Reaction volume of the quantitative PCR was 25 µl containing 3 µl of 50 times diluted template, 2 µl taxon-specific primers (end concentrations 200 µg/µl), 4.5 µl PVP40, 12.5 µl Absolute SYBR Green Fluorescein Mix (Thermo Fisher). The following quantitative PCR protocol was used: 95 ˚C, 1 min followed (as before) by 60× (95 ˚C, 30 sec; 63 ˚C, 1 min; 72 ˚C, 30 sec) followed by a melting curve program 47× (15 sec from 72 to 95 ˚C with steps of 0.5 ˚C).

Relationships between Ct values and numbers of target nematodes

The quantitative PCR output is expressed in Ct units. The copy number and quantities of the target template are inversely proportional to Ct and can be calculated by direct comparison

with Ct values for known standards (Brunborg et al., 2004, Atkins et al., 2005). In order to get

Table 1. Overview of nematode diversity at genus level (microscopic analysis) in the topsoil (depth 0–25 cm) of the former arable field and the adjacent pristine beech forest. Obligate plant parasitic nematodes are given in green and are not included in the molecular part of this research. Only the genera marked by 'q' are included in the quantitative PCR analysis and for most of these genera quantitative ranges ('r') are available (see Fig. 4 and text for more details). For the taxonomy of the families we adhered to De Ley et al. 2006.

Genus	Family	qPCR analysis	Range	Field	Forest
Achromadora	Achromadoridae			+	
Acrobeles	Cephalobidae	q	r	+	
Acrobeloides	Cephalobidae	q		+	+
Aglenchus	Tylenchidae				+
Alaimus	Alaimidae	q	r	+	+
Anaplectus	Plectidae	q	r	+	
Aphelenchoides	Aphelenchoididae	q	r	+	+
Aphelenchus	Aphelenchidae	q	r	+	
Aporcelaimellus	Aporcelaimidae			+	
Bunonema	Bunonematidae				+
Cephalenchus	Tylodorida				+
Cephalobus	Cephalobidae	q		+	
Cervidellus	Cephalobidae	q	r	+	+
Clarkus	Mononchidae	q	r	+	
Coomansus	Mononchidae	q		+	
Coslenchus	Tylenchidae				+
Cylindrolaimus	Diplopeltidae			+	
Diphtherophora	Diphtherophoridae	q	r	+	
Ditylenchus	Anguinidae			+	+
Eucephalobus	Cephalobidae	q	r	+	+
Eudorylaimus	Qudsianematidae				+
Eumonhystera	Monhysteridae	q	r	+	+
Filenchus	Tylenchidae			+	+
Geomonhystera	Monhysteridae			+	
Helicotylenchus	Hoplolaimidae			+	
Malenchus	Tylenchidae				+
Meloidogyne	Meloidogynidae			+	
Mesorhabditis	Mesorhabditidae			+	
Metateratocephalus	Metateratocephalidae	q	r	+	+
Microdorylaimus	Qudsianematidae			+	
Nygolaimus	Nygolaimidae			+	
Panagrolaimus	Panagrolaimidae			+	
Plectus	Plectidae	q	r	+	+
Pratylenchus	Pratylenchidae			+	
Prismatolaimus	Prismatolaimidae	q	r	+	+
Pungentus	Nordiidae			+	
Rhabditis	Rhabditidae			+	+
Steinernema	Steinernematidae				+
Teratocephalus	Teratocephalidae	q	r	+	+
Thonus	Dorylaimidae	q	r	+	+
Tylencholaimus	Tylencholaimidae			+	+
Tylenchorhynchus	Belonolaimidae			+	
Tylenchus	Tylenchidae			+	+
Tylolaimophorus	Diphtherophoridae	q	r	+	+
Wilsonema	Plectidae	q		+	+
Total # of genera	**45**	**20**	**16**	**38**	**25**

these standards, quantitative series of microscopically identified nematodes (mostly to genus level) were sampled. Vials containing 25 µl sterile water with 1, 5, 10, 50, or 100 hand-picked nematodes were supplemented with an equal volume of lysis buffer (0.2 M NaCl, 0.2 M Tris-HCl [pH 8.0], 1 % (v/v) β-mercaptoethanol) and 800 µg/ml proteinase-K. Lysis took place in a Thermomixer (Eppendorf) at 65 °C as described by Holterman et al. (2006). Quantitative PCR reactions were performed as described above: 3 µl of 1000x diluted lysate, 1 µl of each taxon-specific primer (end concentrations of both primers 200 µg/µl), 7.5 µl Milli-Q water, and 12.5 µl Absolute SYBR Green Fluorescein Mix (Thermo Fisher). In the case of family-specific primers, calibration curves were generated for the major genera within each family.

Data analysis

The total numbers of nematodes were log transformed and an overall comparison (including all sampling times) was made between the two ecosystem types ('forest' and 'field') using *t*-test (equal variances not assumed, a = 0.05). To visualize seasonal patterns and site-dependent differences, trend lines are shown for each family or genus per location. Inter-site comparisons were made for each of the detected families and genera and for the two basal trophic guilds (here as summed 'bacterivores' and summed 'fungivores') using independent Mann Whitney-U test (a = 0.05). To get the temporal variation of the nematode community between our two habitats, a partial Mantel analysis was performed.

Results

Seasonal dynamics and site-specific differences in nematode communities

While the air temperature fluctuated between 20.4 °C (week 14) and -3.5 °C (week 39) and the cumulative rainfall of the latest 21 days before sampling (*sensu* Mulder et al., 2005) fluctuated between 11 and 95 mm (Fig. 1, upper panel), variation of the total nematode density (Fig. 1, bottom panel) was rather low (Coefficient of Variation equals 48.1 % in the field and 63.1 % in the forest). The field had a lower density of nematodes in comparison to the forest (averages per 100 ml elutriated soil were 2,392 ± 1,151 SD *versus* 3,222 ± 2,033 SD individuals; unweighted *t*-test $P = 0.023$).

Composition of the soil nematode assemblages was determined microscopically from two composite suspensions (Table 1). Among these genera and families, taxa that appeared as monophyletic groups in a SSU rDNA-based molecular framework (Van Megen et al., 2009) were chosen for further investigation. From the basal level of the soil food web, 12 taxa were selected to be addressed in the next part, namely 8 bacterivores (7 families and 1 genus) and 4 fungivores (2 families and 2 genera), and from the trophically higher level, 3 taxa were selected, *i.e.* 2 predatory families (here: Mylonchulidae and Mononchidae M3) and one omnivore family (here: Dorylaimidae D3), for monitoring using real time PCR (Table 2). Primary data about densities of individual taxa at each of the time points (average and standard error) are given in the Supplementary Table S1.

In contrast to the total nematode densities, individual taxa show distinct temporal and site-specific patterns. The seasonal fluctuations for 7 bacterivorous families are shown in Fig. 2 (*colonizer-persister cp* ranking as in Bongers 1990, Bongers and Bongers 1998): Teratocephalidae

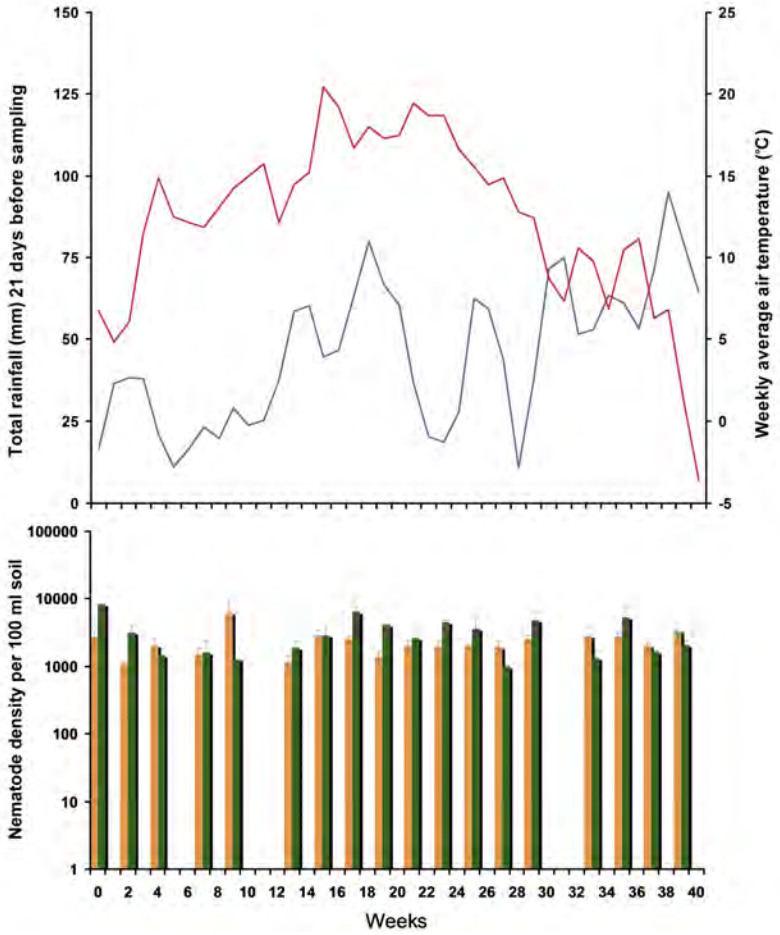

Figure 1. Precipitation and temperature in relation to total nematode densities in open (field) and closed (forest) canopies. Weekly averages of daily temperature (red) and total rainfall over 21 days before sampling (blue) as measured by the Royal Dutch Meteorological Institute (KNMI) are shown above. At the bottom, average nematode densities per 100 ml of soil from a since 25 years abandoned arable field (open canopy, yellow bars) and adjacent pristine beech forest (close canopy, green bars) are given. Sites sampled in 2009 at regular intervals between March 17 (week 1) and December 18 (week 39).

(*cp*-3), Prismatolaimidae (*cp*-3), Cephalobidae (*cp*-2), all in the left panel; Plectidae (*cp*-2) and the genus *Anaplectus* (*cp*-2), both in the red box; Alaimidae (*cp*-4), Metateratocephalidae (*cp*-3), and Monhysteridae (*cp*-2), all in the right panel. In particular, bacterivores show distinct temporal patterns in abundances in the two habitats, but also a taxon dependency was observed (Fig. 2). For instance, comparable trends are detectable for all Teratocephalidae (*i.e.*, *Teratocephalus*, being Teratocephalidae monogenic).

Over the entire season, no significant differences between the two habitats were observable in the case of *Teratocephalus* and Monhysteridae (a = 0.05). Two families, Prismatolaimidae and Metateratocephalidae, were consistently more abundant in the forest, whereas Alaimidae, Cephalobidae, members of Plectidae and the genus *Anaplectus* were present in significantly higher densities in the field (Fig. 2). However, if the densities of these bacterivorous taxa are taken together into a single feeding guild (*Bax*, bacterivores with *cp* value *x sensu* Ferris et al., 2001) no significant difference was detectable between the two sites (a = 0.05), despite the remarkable functional differences within bacterial-feeding nematodes known from literature (Postma-Blaauw et al., 2005, De Mesel et al., 2006).

In parallel, three fungivorous families were monitored as well; Aphelenchidae (*cp*-2), Aphelenchoididae (*cp*-2), and Diphtherophoridae (*cp*-3). Ribosomal DNA sequences suggest that Diphtherophoridae are not monophyletic (Van Megen et al., 2009). Hence, representatives of the two constituting genera, *Tylolaimophorus* and *Diphtherophora*, were detected separately. The composition of this guild is site-specific: whereas the forest was dominated by *Tylolaimophorus,* the fungal pathway of the nematofauna in the field was more diverse (Fig. 3), although *Tylolaimophorus* remained predominant. Their densities showed strong temporal fluctuations: from week 20 onwards, all these fungivorous families were present in the field, although at low levels.

SSU rDNA-based assays for the detection of nematode taxa – qualitative aspects

Soil samples typically contain 30-60 nematodes species, and the composition of nematode assemblages is highly dependent on soil conditions (Mulder and Elser 2009, Mulder and Vonk 2011). Keeping this degree of complexity in mind, the development of such a molecular community analysis tool requires a comprehensive SSU rDNA database (Van Megen et al., 2009). A selection of 15 taxa was made with representatives of four major guilds: i– bacterivores, ii– fungivores, iii– omnivores, and iv– carnivores. The strategy followed for the development of specific PCR primers is exemplified here by the Metateratocephalidae, a bacterivorous family harbouring two genera, *Metateratocephalus* and *Euteratocephalus* (Fig. 4). SSU rDNA sequence motifs were used to design primers with an annealing temperature (Ta) of 63 ˚C. To optimize the foreseeable specificity, selected primer combinations showed a sharp increase in Ct (threshold cycle) upon further Ta increase (Fig. 4A). ARB software (Ludwig et al., 2004) was employed to identify potential false positives, and plasmids harbouring relevant SSU rDNA fragments were used for testing PCR primer combinations. Taxonomically, it must be mentioned that potential false positives are not *per se* related to targets, underlining the plea for a phylum-wide database. In case of the most optimal Metateratocephalidae primer combination, the smallest gap between the target and the non-target smallest (ΔCt) measured 26 cycles (Fig. 4B). This value was determined for all primer combinations (Table 2).

SSU rDNA-based assays for the detection of nematode taxa – quantitative aspects

To establish the relationship between a Ct value (the primary output of a quantitative PCR reaction) and the corresponding number of target nematodes (here, members of the Metateratocephalidae), two series of handpicked individuals were generated. The resulting dataset, five Ct values for each *Metateratocephalus* and *Euteratocephalus* (Fig. 4C), was used to define the slope and the *y*-intercept of the regression line describing the linear relationship between log

(# nematodes) and the corresponding Ct values (Fig. 4D). As an assessment of the goodness of fit, *R*2 values are given for each taxon. Although families may harbor more genera than the number given in Table 2, the values presented here only aim to indicate the number of genera that were observed at this particular study area. Considering the life-stage distribution for each taxon (with differences in DNA contents for individual life stages), a taxon-specific degree of uncertainty regarding the exact densities might occur. However, seen the *R*2 values for each taxon (Table 2), we might assume that the SSU rDNA-based densities reflect the actual densities assessed by classical nematological analysis.

Table 2. Molecular overview of the nematode families and genera monitored in our study. Specificity of primer combinations is expressed as the gap between the Ct value of the latest target and the Ct value of the earliest non-target (ΔCt expressed in number of PCR cycles). For relationship between Ct value and number of target nematodes see Fig. 4, and Materials and Methods in '*Relationships between Ct values and numbers of target nematodes*'.

Nematode family / genus	ΔC_t	Relationship between C_t value and log (# target nematodes):			# genera*
		C_t = **a** ×$\log_{10}$ [# nematodes] + **b**			
		a	**b**	R^2	
Alaimidae (B)	N/A	-3.31	25.47	0.996	1
Aphelenchidae (F, FP)	42	-4.31	17.53	0.995	1
Aphelenchoididae (F, FP)	20	-3.06	24.09	0.992	1
Cephalobidae (B)	17	-4.21	21.95	0.855	3
Diphtherophoridae (F) :					
Diphtherophora	18	-3.22	19.18	0.926	
Tylolaimophorus	N/A	-3.02	22.36	0.984	
Dorylaimidae (O)	N/A	-5.90	17.30	0.859	1
Metateratocephalidae (B)	26	-5.09	24.40	0.954	2
Monhysteridae (B)	23	-4.25	21.06	0.954	1
Mononchidae (P)	18	-2.94	15.19	0.990	1
Mylonchulidae (P) **	N/A	-4.02	12.03	0.977	1
Plectidae (B) :					
Plectidae excl. *Anaplectus*	34	-1.93	26.82	0.989	1
Anaplectus	27	-3.33	21.03	0.949	
Prismatolaimidae (B)	13	-5.13	21.64	0.999	1
Teratocephalidae (B)	N/A	-4.41	25.13	0.999	1

B: bacterivore, F: fungivore, FP: facultative plant parasite (only for nematodes where this guild occurred in combination with fungivory), O: omnivore, P: predator; N/A: no quantitative PCR signal produced by non-target(s); *: number of genera within one family assessed by qPCR (families as in De Ley et al., 2006); **: as *Mylonchulus* is expected to occur in this area, its family has been included as additional taxon.

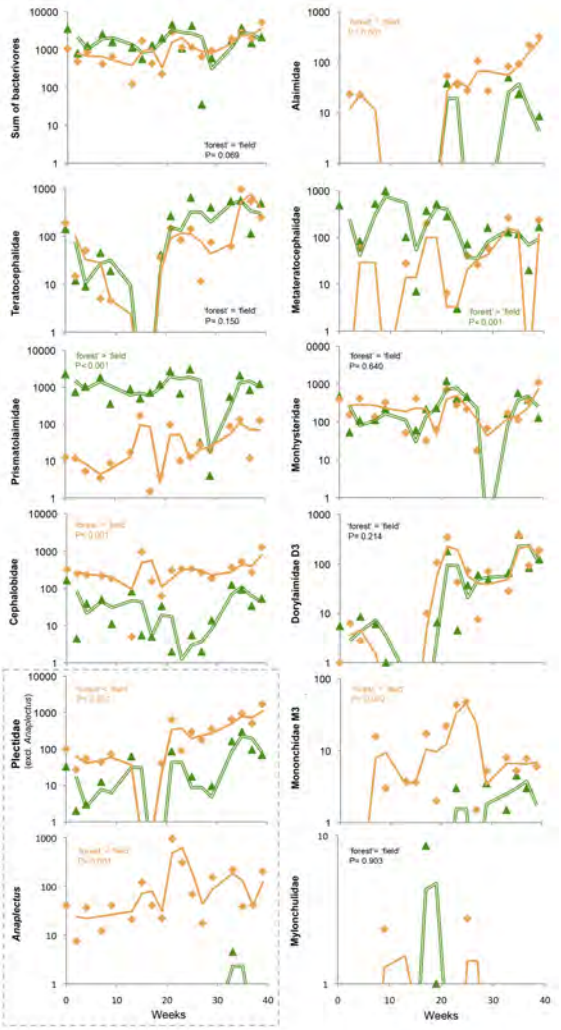

Figure 2. Temporal patterns of bacterivorous, omnivorous and predatory nematode families. We determined DNA-based variation in the nematode densities per 100 ml soil (note differences in y-axes) of representatives from seven bacterivorous families: Teratocephalidae, Prismatolaimidae, Cephalobidae, Plectidae (i.e., all Plectidae excl. *Anaplectus* and '*Anaplectus*', both in a dashed gray box), Alaimidae, Metateratocephalidae, Monhysteridae; the omnivorous family Dorylaimidae (D3 region sensu Holterman et al., 2008); and the predatory families Mononchidae (M3, Holterman et al., 2008) and Mylonchulidae. Sampling weeks as x-axes (constant scales); samples from the field are represented by orange triangles and samples from the forest by green diamonds. Trends are given as two-period moving averages: the averaged 2nd and 3rd data points are portrayed by the 1st data point and so forth.

Quantitative coverage of environmental samples by a 15-taxa nematological analysis

If all taxa present in the samples were covered by this novel quantitative PCR-based community analysis tool, the sum of the densities should equal the total nematode numbers as given in Fig. 1. To check the quantitative coverage of the 15-taxon analysis tool, the total number of nematodes as determined microscopically was compared with the total numbers as estimated by quantitative PCR. We constructed a red dotted line to show virtual data at which the total number of nematodes is equal irrespective whether determined by microscopy or by quantitative PCR (Fig. 5) and one solid line to connect all the points. Ideally, a dataset should not exceed 0.5 log value from the latter solid line, allowing a precision of ± 0.5 order of magnitude. To show the data-range borders, dashed lines have been plotted above and below the solid trend-line. Of all nematode assemblages analysed, 78 % were found within this range. When it is assumed that Fig. 5 provides a summary overview of the relationship between classical and molecular nematological analyses, it is notable, that the slope of the linear log-log regression across all our samples analysed in both ways (solid trend) is allometrically undistinguishable from unity (the slope 0.927 ± 0.192 SE overlaps 1 ± 0 SE; $P < 10\text{-}5$). Discrepancies between counts and qPCR in one fifth of our samples are either due to underestimation or to overestimation of the nematode biomasses. On one hand, lacking appropriate molecular assays are a caveat that

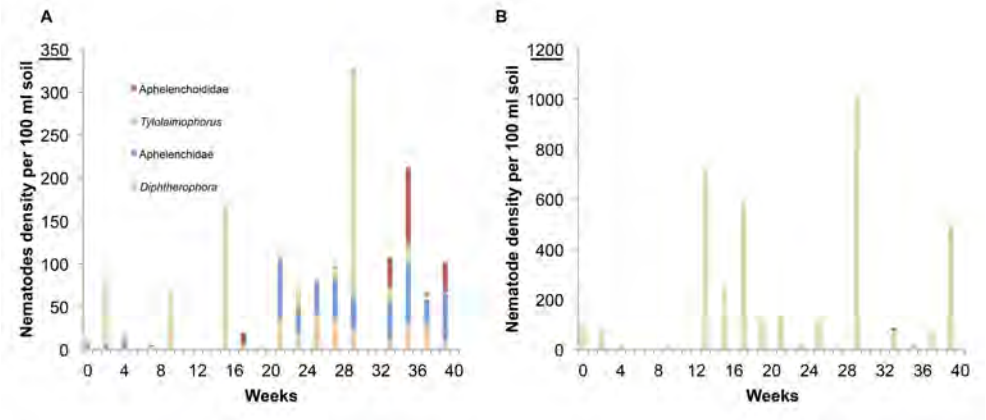

Figure 3. Temporal patterns of fungivorous nematode families. Seasonal variation in densities for fungivores in the field (A) and in the forest (B). Please note that the y-axis scales differ. Aphelenchidae (Aphe, blue), Aphelenchoididae (Acho, red), and two genera belonging to Diphtherophoridae –viz. Tylolaimophorus (Tylo, green) and Diphtherophora (Diph, yellow)– show different patterns over the seasons between open and close canopies. As these taxa represent all observed fungivores, a partial Mantel analysis performed in a matrix describing the community structure in the field (open canopies, matrix Y) and in the forest (close canopies, matrix X) using the squared Euclidean distance was performed using the total entries and the same set of entities. A positive association between the matrices is indicated over the seasons by observed Z greater than average Z from randomized runs (P = 0.0297).

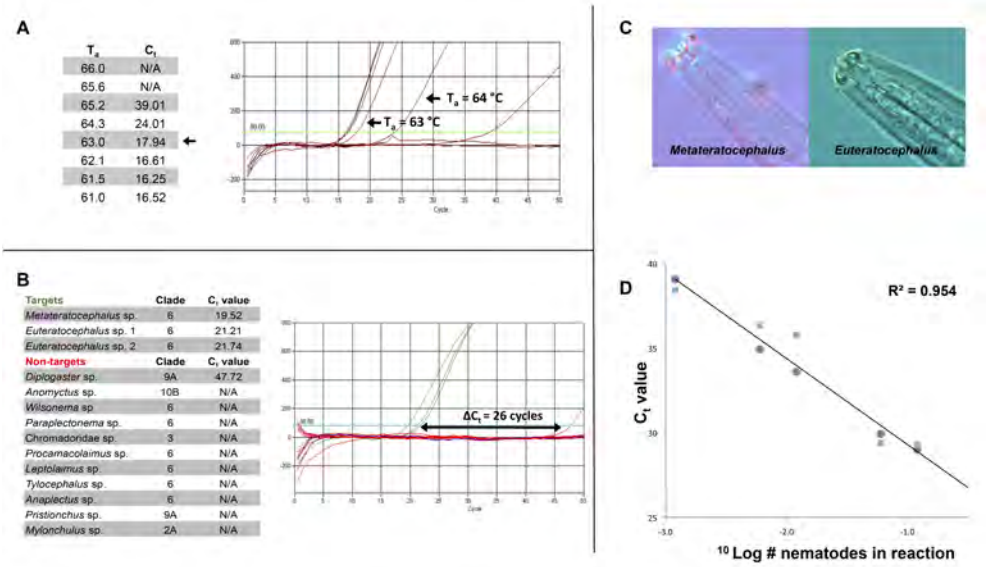

Figure 4. Development and testing of a nematode family-specific primer combination. Here we use the Metateratocephalidae (one bacterivorous family harboring the Metateratocephalus and Euteratocephalus genera) as an example of primer development. (A) All primers were designed to have optimal annealing temperature (Ta) of 63uC, with Ct values varying at temperatures above and below the target Ta. (B) Specificity test of a Metateratocephalidae primer combination with plasmid DNAs from three target species, SSU rDNA fragments from 11 potential false positives (as selected by ARB, Ludwig et al., 2004) and a negative water control. Clade numbers are according to Van Megen et al. (2009). In the quantitative PCR graph the gap between the target and the non-target signal (DCt) is shown. (C) Pictures of the head region of a representative of both genera. (D) The relationship between Ct values and numbers of nematodes for quantification of densities. A linear relationship between Ct values and numbers of nematodes till 1/1,000 part of a single nematode is shown (equivalent to a single nematode cell harbouring 50 copies of the ribosomal DNA cistron). Handpicked individuals of Metateratocephalus (purple circles) and Euteratocephalus (blue squares) were used to quantify the Metateratocephalidae- specific primers.

explains underestimation. For a number of non-monophyletic taxa such as the Rhabditidae, in fact, no molecular assays could be designed. Members of this family (*cp*-1) can respond very quickly to both local environmental changes (*e.g.* eutrification) as to microbial pulses. If such a family would be abundant in a given sample, this would automatically result in a drop of the coverage. On the other hand, though unusual, averages of body-mass values at genus level can be very different within a single family. This phenomenon can be illustrated by the "Plectidae minus *Anaplectus*". In this group, the fresh weight per individual (and – most likely – the individual DNA content) varies substantially between genera (compare *Plectus* with *Wilsonema*, the latter being on average more than 7 times smaller than *Plectus*, Mulder and Vonk 2011). In our paper, the calibration curves were produced at genus level, and the quantification at family

level was based on a qualitative check for Plectidae genera present in a given set of samples.

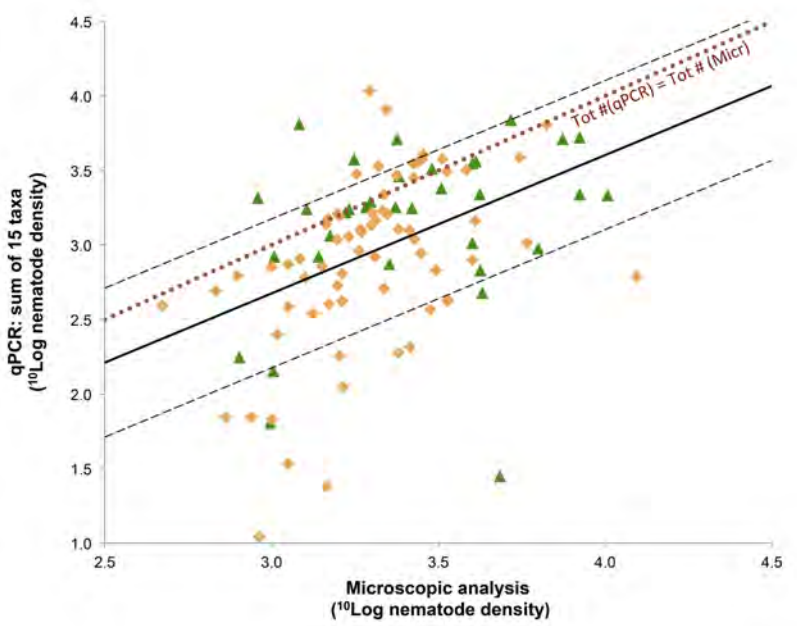

Figure 5. Quantitative coverage of the DNA-based tool using environmental samples. Logarithm of the total of individuals as detected by optical microscopy (x-axis) plotted against the logarithm of the total of individuals as estimated by quantitative PCR (y-axis). The correlations of quantitative PCR with classical analyses seem to be accurate, with no Studentized residuals higher than | 2 |. The solid line shows the trend of all data and the two dashed lines show the boundaries of one-order-of-magnitude precision. The dotted line represents an equal amount of nematodes for both methods. Such a coverage is expected to be lower than 100% as obligate plant parasites were not included, although the fungivorous Aphelenchidae and Aphelenchoididae may harbor facultative plant parasites as shown in Table 2. Given that taxa like Rhabditidae, Qudsianematidae or Nordiidae appear to be both poly- and paraphyletic (Holterman et al., 2008, Van Megen et al., 2009), no rDNA-based detection assay on family level could be developed for those nematodes.

Discussion

Comparison of the abundances of eight bacterivorous taxa during the entire experiment resulted in a diverse picture: for two taxa no difference was detected between the habitats, whereas six differed (four taxa were consistently more abundant in the field and two were present in significantly higher densities in the forest). Lumping the estimated nematode abundances into the feeding guild 'bacterivores' masks the taxon- and site-specific differences. One of those differences is the high density of Prismatolaimidae in the acidic moder. A factor that often contributes to this asymmetric distribution is pH, as some *Prismatolaimus* species prefer acidic conditions (Hirschmann 1952) and our forest moder might constitute an optimal environment for acidophilic bacterivores. Another of those differences was the distinct distribution of *Anaplectus* (Plectidae, Anaplectinae): throughout the seasons, this genus occurred at relatively constant density in the field, whereas it was virtually absent in the forest. This result confirms the outcome of a qualitative study of nematode communities in moder and mull (no stratification in humus-containing layer, organic matter and well-mixed mineral soil, pH 6.2–6.8) beech forest soils, where, *Anaplectus granulosus* was shown to be exclusively present in mull soils (Alphei 1998).

Regarding seasonal changes in bacterivorous nematodes, a decreasing trend was observed for Teratocephalidae and Plectidae in June and July (week 13 to 17 in Fig. 2), a period characterized by intense rainfall. Although precipitation data was recorded at a nearby weather station and not on the site itself, it would be tempting to attribute their absences to their movement to deeper soil layers. For several plant parasitic species, it has been shown that simulating intense rainfall hardly results in leaching of nematodes (Chabrier et al., 2008, Fujimoto et al., 2009). As this ability to withstand leaching is unlikely to be specific for plant parasites alone, we see the absence of Teratocephalidae and Plectidae not as the (passive) result of any leaching below the sampling depth. However, members of these families could *actively* migrate downwards because of the leaching of certain groups of bacteria, their main resource. The passive transport of bacterial cells as a result of rainfall is a well-documented phenomenon (Gagliardi and Karns 2000, Aislabie et al., 2011).

Intra-feeding guild heterogeneity is further illustrated by the fungivorous nematodes. The family Diphtherophoridae harbors two genera, *Diphtherophora* and *Tylolaimophorus*. Whereas representatives of the genus *Diphtherophora* were constantly present in the field from week 15 onwards (though in low densities), they were lacking in the adjacent forest. *Tylolaimophorus*, on the other hand, was the dominant fungivorous nematode in the forest (albeit in fluctuating densities). If soil acidity is so important for belowground Operational Taxonomic Units (OTUs) as suggested by Mulder et al. (2005) and Wu et al. (2011), than the difference in soil pH of our sites might (co)explain the observed patterns: in general, *Tylolaimophorus* spp. is known to be acidophilic (Brzeski 1994), and its high abundance in the beech forest can be attributed to the acidic moder (pH = 3.7). The genus encompasses thirteen species (Brzeski 1994), and we hypothesize that other, non-acidophilic *Tylolaimophorus* species might occur in the abandoned field (pH = 5.7).

A more pronounced, reverse response is observed for *Diphtherophora* (Fig. 3). Either the low pH of the forest soil was directly inhibiting the occurrence of *Diphtherophora* spp., or it is negatively affecting a part of the fungal community that provides an essential food resource for

these nematodes. Members of other fungivorous families (Aphelenchidae and Aphelenchoididae) were respectively non-detectable or present in low amounts (late Autumn) in the acidic moder (Fig. 3B), whereas they occurred in the field at densities up to one individual per ml of soil, especially in the second half of the season. Hence, we hypothesize that (1) fungivorous nematodes are not as polyphagous as suggested (Freckman and Caswell 1985) and that (2) Aphelenchidae and Aphelenchoididae feed on a part of the fungal community different from the segments used as a resource by *Tylolaimophorus*.

Regarding functional changes in nematodes, applied soil ecologists tend to use indices based on so-called guilds, a trait assemblage of nematode taxa sharing the same feeding habits and inferred function in the soil food web (Ferris et al., 2001). The current study did not aim to investigate effects of changes in the nutritional status of a soil food web, nor any other kind of environmental disturbance. Rather it shows that it is possible to monitor communities based on molecular methodology and in this way reveals numerous changes within feeding guilds that might give us more insight in the ecological functioning of soil biota.

In the last decades there have been several extensive studies on both spatial (*e.g.* Neher et al., 1995, Ettema and Yeates 2003) and temporal (*e.g.* Yeates et al., 1999, Sohlenius and Boström 2001) variation within nematode communities. Yeates et al. (1999) monitored nematodes communities on 23 occasions over seven years under an annual and a perennial crop (3-4 sampling occasions per year). Their findings underlined the necessity of long-term monitoring (at least three years) to observe the effects of agricultural practices, and did not pay attention to seasonal fluctuations. Sohlenius and Boström (2001) showed with a study of two annual time series (sampling monthly and every other month) and one long-term time series (10 sampling events over 25 years) in a Swedish pine forest soil, that variations within groups of nematode taxa of the same feeding type were larger compared to variations of the feeding groups as a whole. We did not observe major seasonal fluctuation in total numbers of nematodes as they did (Sohlenius and Boström 2001), but our data are in line with their conclusion stating that precipitation is one of the major drivers of changes in the nematode community composition.

So far, a number of practical obstacles like the time required for microscopic analysis, the limited number of informative morphological characters for some taxa, and the scarcity of people that can analyse nematode assemblages has restricted the number of intensive monitoring studies. DNA-based community analysis can lift such obstacles, and facilitate a wider use of nematodes as indicator for the biological soil quality. In essence, four different molecular approaches are currently used: direct sequencing, PCR DGGE, PCR T-RFLP and real time PCR (Chen et al., 2010).

Direct sequencing shares with PCR DGGE its qualitative rather than quantitative properties. In a careful comparison between microscopic and DGGE-based community analysis, Okada and Oba (2008) found a reasonable match between the two methods. T-RFLP is a semi-quantitative PCR-based technique as well, and for the analysis of nematodes communities, the generation of a molecular framework is required. Recently, Donn et al. (2012) reported on the effects of tillage on nematode communities, and as a start, a database with 516 partial SSU rDNA sequences from the sites under investigation was generated. The requirement of such a location-specific database and its semi-quantitative nature currently makes T-RFLP not an attractive method for routine analyses of nematodes.

Real time PCR is designed for quantification, and Jones et al. (2006) were among the first to

use this method for nematode community analysis. A local and small (74 SSU rDNA sequences) framework was made, and in a next step, community analysis procedure was tested based on the combined use of microscopy (for pre-selection) followed by real time PCR. The molecular procedure presented here allows for the analysis of nematode communities without any microscopic pre-selection because it is based on a considerably broader (2,400 taxa) full length SSU rDNA database that covers all major terrestrial and freshwater nematode taxa. It is noticed that marine nematode are greatly underrepresented in our framework, and consequently it cannot be used for marine nematode assemblages yet. Still, a basic advantage of the detection framework illustrated here is its simplicity, as it only requires standard laboratory equipment.

In most soil nematological studies, data are presented at family or feeding guild level. To allow for a straightforward connection between the large body of ecological data on terrestrial nematode communities and the currently present molecular detection framework, it was decided to preferably develop assays at family level. Families often harbor multiple genera, and regression lines for family-specific primer combinations are based on one or more calibration curves produced on genus-level (*e.g.*, Fig. 4D). In case the relationship between real time PCR output (Ct value) is similar for the constituting genera, the ratio between these genera does not affect the accuracy of the results. However, some families such as Cephalobidae include genera with considerable body-size differences (Mulder and Vonk 2011, Mulder et al., 2011) and, most likely, DNA contents. Therefore, only those genera that were present in the microscopic samples were included in the computation of the regression. As a consequence, the $R2$ for Cephalobidae was slightly lower in comparison to most other primer combinations (Table 2). Hence, the accuracy of density levels of individual families is variable and depends on the variation in average DNA contents of the constituting genera.

On one hand, DNA-based research spans an enormous array of ecological disciplines and we believe that this study demonstrates – among others by showing for two adjacent, undisturbed areas that even 30 years after ending the agricultural practices the soil nematofauna barely seem to converge to the same assemblage – the ecological suitability of a quantitative PCR-based method for nematological and environmental purposes. On the other hand, our results also aim to contribute to the increase of the current knowledge of this phylum, given that the taxonomy of nematodes is still far from complete (Fontaine et al., 2012). It has been shown that the analysis of datasets at genus level can provide more information when comparing analyses at family or order level. Using this DNA barcode-based tool, we have the possibility to work towards a complete view on time trends and soil patterns, enabling the nematode community to become unravelled.

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Chapter 3

Selective alteration of soil food web components by invasive Giant goldenrod (*Solidago gigantea*) in two distinct habitat types

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Abstract

Interactions of plants with soil biota have been suggested to determine the invasive success of exotic plant species. So far, most research has been focussing on primary decomposers and nutrient cycling, and studies on the impact on higher trophic levels of the soil food web are relatively scarce. Here we investigated the aboveground (plant community) and the belowground impact (at multiple trophic levels of the soil food web) of an invasive plant species; Giant goldenrod (*Solidago gigantea*). In order to evaluate the habitat dependency of Giant goldenrod impact, we compared two contrasting environments: riparian zones and semi-natural grasslands. In total, we selected 30 pairs of plots invaded by *S. gigantea* and an un-invaded neighbouring plot. For all plots (n=60), floristic composition, pH, fungal biomass, and the densities of 11 nematode taxa (using a quantitative PCR-based method) were determined. In plots invaded by *S. gigantea*, plant-species richness was reduced by 42% and 55% in riparian and semi-natural grassland habitats respectively. The invader outcompeted both rare and dominant plant species. Belowground, in both habitats, *S. gigantea* invasion reduced pH, increased fungal biomass as well as densities of members of the fungivorous nematode family Aphelenchoididae. However, as two other lineages of fungivorous nematodes, Aphelenchidae and Diphtherophoridae, did not benefit from the increase in fungal biomass, effects on higher trophic level organisms turned out to be selective. Invasive plant species can cause significant alterations of biological conditions in soil. Here, we show that invasive *S. gigantea* can affect the soil food web in a selective way. Fungal biomass was enhanced, but only one fungivorous nematode lineage benefitted from this increase, whereas two other lineages were not affected at all, suggesting that *S. gigantea* stimulates only part of the fungal community. With regard to bacterivorous nematode taxa, no generic effect of Giant goldenrod invasion was observed.

Introduction

The successful establishment of exotic species is considered as one of the major driving forces of changes in biodiversity (Sala et al., 2000). Most naturalised exotic plants behave ecologically comparable to resident species, but a small proportion ('invasive plants') can reach exceptionally high densities in their new environments (for terminology see Pyšek et al., 2004). In various cases, invasive plant species have transformed native plant communities, and locally this has resulted in near monocultures. Hejda and co-workers (2009) studied the main factors determining the impact of invasive plant species on the native plant community. According to them species identity and habitus characteristics such as stand height and cover are major determinants for invasiveness. The number of studies focussing on belowground effects of invasive plant species has grown substantially over the last decade (Vilà et al., 2011). Most of these studies concentrate on the impact on microbial communities (for review see Van Der Putten et al., 2007) and nutrient cycling (for review see Ehrenfeld, 2003). From these studies it has become clear that interactions between plants and soil biota can play a decisive role with regard to the invasive success of exotic plant species. The invasiveness of naturalised plant species has been shown to be promoted by their ability to stimulate generalist soil pathogenic fungi (Mangla et al., 2008) or by the local presence of compatible mycorrhizal fungi (Nuñez et al., 2009). Selective changes in the microbial community can lead to alterations at multiple levels of the food web, and may thereby affect its stability (Dunne et al., 2002). This notion could contribute to our understanding of the ecological impact of exotic plant species. However, little attention has been paid to invader-induced changes on higher trophic levels in the soil food web so far (Belnap et al., 2005, Chen et al., 2007).

Due to the enormous biodiversity of soil biota and the high number of trophic relationships, there are myriad interactions between plants and soil microbial communities (Porazinska et al., 2003). Due to their omnipresence in pores between soil aggregates, their trophic diversity, and their high degree of interconnectedness within the soil food web, nematodes constitute an informative bio-indicator group for soil food web functioning (Neher et al., 2005). A range of studies has focused on interactions between plant community composition and nematode assemblages (De Deyn et al., 2004, Viketoft et al., 2005, Bezemer et al., 2010, Viketoft and Sohlenius, 2011). So far, the impact of exotic plants on nematode communities has received little attention (Van Der Putten et al., 2005, Morriën et al., 2011). Studying changes in nematode communities at high taxonomic resolution can deliver valuable information about shifts in microbial soil communities (Porazinska et al., 1999, Neher et al., 2005, Viketoft and Sohlenius, 2011). However, for experiments with intense sampling designs, microscopy-based community analyses are (too) laborious and time-consuming. A recently developed set of quantitative PCR (qPCR)-based molecular assays (Vervoort et al., 2012) allows for the analysis of nematode assemblages at or below family level in a relatively short time frame.

In the present study, Giant goldenrod (*Solidago gigantea*), a common invasive plant species in most European countries, was selected as a model to examine belowground effects of successful invaders. This plant species has the ability to form near monoculture stands in a broad range of habitats (Weber and Jakobs, 2005). In recent years, several studies revealed properties of *S. gigantea* which possibly contribute to its invasiveness, *e.g.*, high biomass production, high nutrient efficiency, alteration of nutrient turnover (Vanderhoeven et al., 2006, Scharfy et al.,

2009) and the excretion of allelochemicals (Abhilasha et al., 2008). In 2010, Scharfy et al. studied the effect of *S. gigantea* on soil biota in typical wetland soils (gleysols and a gleyic cambisol) under controlled mesocosm conditions. They observed a significant decrease in bacterial and an increase in fungal biomass in soil below a *S. gigantea*-dominated vegetation. However, it is hard to predict whether these are specific or more widespread consequences of invasion by Giant goldenrod, and little is known about possible follow-up changes at higher trophic levels in the soil food web.

The aim of our study is to elucidate the belowground impact of *S. gigantea* on multiple trophic levels in the soil food web. To allow the identification of generic effects of local Giant goldenrod invasions, two contrasting invaded environments were taken into consideration: riparian zones and semi-natural grasslands (characterised by river clay and sandy soils respectively). This approach enabled us to test the following hypotheses: 1) the local increase of the fungal biomass as a result of invasion by Giant goldenrod is a widespread phenomenon. 2) if this invasive plant species provokes a general stimulation of the fungal community, increased densities should be observed for all lineages of fungal feeding nematodes in both habitats. However, in case of an asymmetric boost of the fungal community (*i.e.* only a subset of fungal taxa are benefitting), an asymmetric stimulation of fungivorous lineages can be expected, given that lineages of fungivorous nematode indeed differ in their food preferences as suggested by Vervoort et al., 2012. 3) if the local decrease of the bacterial biomass caused by Giant goldenrod invasion as observed by Scharfy et al. (2010), is a general, invader-driven effect, this should result in a decrease in density of at least a part of the bacterivorous nematode communities, in both habitats.

Materials and methods

Sites of study

Ten *S. gigantea*-invaded sites were selected in two semi-natural habitats: riverbanks of the Rhine and the Walloon and grasslands on Pleistocene sandy soils. Sites were selected within an area of approx. 200 km2 in the central part of The Netherlands (Suppl. Table S1). In this area, naturalised *S. gigantea* plants are present since 1912 (Te Linde and Van den Berg, 2003). In riparian habitats, *S. gigantea* is mainly introduced by surface waters, which carry (fragments of) plants that can sprout under favourable conditions elsewhere (Weber and Jakobs, 2005). The semi-natural grasslands studied here were located relatively close to inhabited areas. Beekeepers and gardeners introduced *S. gigantea* to these areas. All selected sites met the following criteria: 1) *Solidago gigantea* occurred in well-defined patches in the native vegetation, 2) soil and vegetation showed no signs of disturbances caused by *e.g.* foraging wildlife or mowing, 3) soils from sites of the same habitat type were comparable in plant community, pH and humidity.

Sampling

For both invaded habitats, five sites were investigated. For each site, three separate plot-pairs were defined, consisting of two directly neighbouring 4 m2 (2.0 x 2.0 m) plots; one plot in a by *S. gigantea*-dominated patch ('invaded') and one un-invaded plot, just outside this patch. Thus, in total 60 plots were studied. For each plot, the floristic composition was determined, and a composite soil sample was collected. Each composite soil sample consisted of a mixture

of 20 randomly taken soil cores (Æ 1.5 cm, depth: 25 cm) that were homogenised thoroughly, immediately thereafter this mixture was stored at 4°C. Sampling took place during the week of September 12th 2011, when the plant community was at peak standing biomass. One month before, nematode diversity of all 10 sites of this study was assessed microscopically (for details see Suppl. Table S2).

Vegetation analysis

In each plot (n=60), all species of higher vascular plants were recorded and valued on an ordinal scale, based on abundance or coverage (Suppl. Table S3 and S4). Community characteristics were determined by calculating the species richness (S) and the Shannon diversity (H') as described in Hejda et al., 2009.

Soil acidity and humidity

A subsample (20 g) of each composite soil sample was used to determine the moisture content and pH-H2O. Soil moisture content was determined by weight loss after 72 h incubation at 40°C. The dried soil was sieved with a 2 mm mesh; thereupon soil pH was measured in demineralised water using a gel-electrolyte electrode (Sentix 21, WTW, Weilheim, Germany).

Nematode extraction and community analysis

For each of the composite samples, a 100 g subsample was taken, and nematodes were extracted using an elutriator (Oostenbrink, 1960). Nematode suspensions were analysed microscopically, or by a qPCR-based method (Vervoort et al., 2012).

Microscopic analysis (of samples collected in August 2011) was used to assess the nematode community composition for invaded and native plots in each of the habitats. Communities were characterised by the morphological identification (till genus level) of 100 individuals per sample (soil from under invaded vegetation and native vegetation was analysed separately for each site (n=20; for details see Suppl. Tables S1 and S4). On the basis of this nematode biodiversity inventory, sets of taxon-specific PCR primer combinations were selected, hereby optimizing the coverage of the molecular assays.

For the samples collected in September 2011, overall nematode densities were determined by counting two subsamples of each of the nematode suspensions (n=60). DNA extraction from nematode suspensions and subsequent lysate purification were performed as described by Vervoort et al., 2012. DNA extracts were used as a template in qPCR using 11 nematode taxon-specific primer combinations (for details see Vervoort et al., 2012).

Fungal biomass

Fungal biomass was determined by measuring the ergosterol content in soil samples. Ergosterol is a sterol that is present in fungal cell membranes, and which does not occur in plant or animal cells (Gessner and Schmitt, 1996, Van Der Wal et al., 2006, Stahl and Parkin, 1996). This approach largely excludes arbuscular mycorrhizal fungi which are known to contain relatively low amounts of ergosterol (Olsson et al., 2003). Ergosterol was extracted from 1 g of soil using the alkaline extraction protocol described by de Ridder-Duine et al., 2006. Subsequently, high-performance liquid chromatography was used to determine the ergosterol contents of

the samples (de Ridder-Duine et al., 2006).

Data analysis

Soil properties, vegetation, and nematode densities were analysed using mixed linear models (using PROC MIXED of the SAS software system version 9.2, see Littell, 2006). If needed, data were transformed, in order to arrive at approximately normal distributions of residuals as required for valid statistical inference. The variables soil pH, moisture content, plant-species richness, and diversity remained untransformed; nematode densities were square root-transformed; and all other variables (ergosterol and nematode densities) were log-transformed. The log-transformation was applied after addition of a constant (0.05 for ergosterol, and 0.5 for the nematode densities with the exception of Dorylaimidae D3) to push data away from the lower bound zero. Mixed linear models were used, because multiple observations from the same location and/or plot pair within location are not necessarily uncorrelated. The experimental design here is a split-split-plot design with locations as main plots (associated with factor habitat type; see Table 1), plot pairs as split-plots (without an associated treatment factor), and individual plots as split-split-plots (associated with factor invasion; see Table 1). The fixed part of the mixed model contained main effects of habitat and invasion and their interaction. The random part of the model consists of random effects for location, plot pairs, and individual plots, so that total error variance is split into variance components for locations, for plot pairs within locations, and for neighbouring plots within plot pairs. We present the following results from the mixed models: 1) hypothesis tests for interaction and main effects of factors habitat and invasion and 2) back transformed 95% confidence intervals for means per habitat and invasion, and the ratios (impact (%)) of back transformed means for invaded and un-invaded plots per habitat, together with a statement about the significance of the difference between invaded and un-invaded plots.

Results

Changes in native vegetation upon *S. gigantea* invasion

In total, we identified 64 and 78 vascular plant species in riparian vegetation and semi-natural grasslands, respectively. In invaded plots, 35 and 39 vascular plant species were recorded, respectively. For invaded vegetation, plant-species richness (*S*) and diversity (*H'*) were significantly lower compared to native vegetation ($P < 0.001$; Table 1 and Fig. 1). Common native species largely determining the plant community (*e.g. Jacobaea vulgaris, Holcus lanatus, Achillea millefolium*, *Dactylis glomerata,* and *Plantago lanceolata*; Suppl. Tables S2 and S3) were almost absent in invaded vegetation. Relatively rare species such as *Achillea ptarmica, Epipactis sp*. and *Odontites vernus* subsp. *serotinus* (only present in riparian zones), and *Filago vulgaris* (only present in semi-natural grasslands) were completely absent in the plots invaded by *S. gigantea*. On the other hand, Ground ivy, *Glechoma hederacea,* was either unaffected by *S. gigantea* or had more cover in invaded vegetation.

Impact of *S. gigantea* invasion on soil acidity and humidity

Overall, a comparison of pH's of soils from un-invaded *versus* invaded plots revealed slight

Table 1. Summary of ANOVA Fdf and associated P values, testing for differences in the variables soil pH, soil moisture content (%), total nematode density (per 100 g dry soil, analysed by microscope), fungal biomass (expressed as mg ergosterol / kg soil), plant-species richness (S¬¬plant), plant-species diversity (H'plant), and the density of 11 nematode taxa (per 100 g dry soil, analysed by quantitative PCR). These variables were tested for habitat type, invasion (neighboring invaded and un-invaded plots) and their interaction (Habitat type * Invasion), based on mixed models fitted to these variables (see materials and methods). P values < 0.05 are considered significant, and indicated in bold.

	Habitat type		**Invasion**		**Habitat type * Invasion**	
	$F_{1,8}$	*P*	$F_{1,28}$	*P*	$F_{1,28}$	*P*
Soil pH	54.64	**<0.001**	5.96	**0.021**	1.22	0.279
Soil moisture content	2.88	0.128	3.36	0.077	0.32	0.579
Nematode density	50.21	**<0.001**	0.43	0.518	0.01	0.924
Fungal biomass	1.89	0.207	20.48	**<0.001**	1.09	0.306
S_{plant}	4.45	0.068	82.02	**<0.001**	7.09	**0.013**
H'_{plant}	3.36	0.104	81.44	**<0.001**	2.16	0.153
Aphelenchidae	12.31	**0.008**	0.00	0.946	1.09	0.306
Aphelenchoididae	13.51	**0.006**	5.58	**0.025**	0.86	0.363
Diphtherophoridae	0.00	0.949	0.33	0.571	0.14	0.712
Dorylaimidae D3	6.25	**0.037**	1.89	0.181	2.47	0.127
Mononchidae M3	0.20	0.668	2.35	0.137	5.71	**0.024**
Cephalobidae	7.99	**0.022**	0.29	0.597	9.79	**0.004**
Plectidae (except *Anaplectus*)	0.93	0.362	0.12	0.731	1.94	0.174
Anaplectus	0.46	0.515	2.00	0.168	0.82	0.374
Alaimidae	7.22	**0.028**	2.14	0.155	3.32	0.079
Prismatolaimidae	0.02	0.879	0.11	0.740	0.65	0.426
Panagrolaimidae	6.51	**0.034**	0.51	0.480	0.70	0.411

[a] Nematode taxa defined as by De Ley et al., 2006, except for Dorylaimidae D3 and Mononchidae M3 (see Holterman et al., 2008).

but significantly lower pH's in invaded soils ($P < 0.001$; Table 1). Soil moisture content tended to be lower in invaded plots, but this effect was not significant (P=0.077; Table 1). In general, the soil pH under semi-natural grasslands was ≈ 1.5 units lower (Table 2) and more variable as compared to the riparian plots ($P < 0.001$; Tables 1, 2). The average moisture content of riparian clay soils was higher, although not significantly, as compared to the sandy soils of the semi-natural grasslands (Table 1).

Impact on fungal biomass

Overall, soil from invaded plots contained significantly higher amounts of fungal biomass compared to un-invaded plots ($P < 0.001$; Table 1 and Fig. 1). Fungal biomass was approximately twice as high in soil collected from *S. gigantea* invaded vegetation, compared to plots with native vegetation (Table 2). Comparison of the two habitats suggests that fungal biomass in the un-invaded, semi-natural grasslands is higher than the biomass detected in the riparian sites (ns; Est. mean 0.43 and 0.72 mg ergosterol per kg soil respectively), whereas the fungal biomass-promoting effect of Giant goldenrod was apparently more pronounced in soil collected from the riparian habitat (ns; Tables 1, 2).

Changes in nematode assemblages upon *S. gigantea* invasion

Overall, total nematode densities (determined microscopically) were similar in neighbouring invaded and un-invaded soils. However, when we investigated the impact of Giant goldenrod at nematode taxon level, only one family, *i.e.* Aphelenchoididae, showed overall higher densities in invaded plots, regardless of habitat type (P=0.025; Fig. 1). Apart from fungivores, the family Aphelenchoididae includes a number of (facultative) plant parasites. The primer-combination used in this study excludes all plant parasites from this family, except for *Aphelenchoides fragariae* (Vervoort et al., 2012). The absence of this plant parasitic species was confirmed using an additional, *A. fragariae*-specific molecular assay (Rybarczyk-Mydlowska et al., 2012) (Data not shown). Aphelenchidae and Diphtherophoridae (represented in these two habitats by a single genus, *Diphtherophora*), two other fungivorous taxa, did not show a difference in density between un-invaded and invaded soil (Fig. 1). For the predatory nematode family Mononchidae M3 (see Holterman et al., 2008) and bacterivorous Cephalobidae, a significant interaction was observed between habitat type and invasion of *S. gigantea* (Table 1), showing that the nature of their response to invasion is habitat-type dependent.

When considering the two habitat types separately, differences between un-invaded and adjacent invaded soil were more pronounced in the riparian habitats than in semi-natural grasslands (Fig. 1, Table 2). While in riparian soils the densities of four out of eleven families differed significantly between invaded and un-invaded plots, this was observed for only one taxon in semi-natural grasslands (Table 2). In invaded riparian soils, the density of Aphelenchoididae was significantly higher, as well as the density of two bacteria feeding families, Cephalobidae and Alaimidae (Fig. 1, Table 2). Other bacteria feeders did not show a consistent response. Mononchidae M3, a family of predatory nematodes, was significantly more abundant in invaded riparian plots as well (P=0.010). In semi-natural grasslands, we found significantly higher densities of omnivorous Dorylaimidae D3 in invaded plots; other taxa did not show a significant response (P=0.046).

Table 2. The 95% confidence intervals for the estimated mean response (Est. mean) of soil variables measured in plots invaded or uninvaded by Solidago gigantea in two habitat types, i.e. riparian vegetation and semi-natural grasslands. Values were back transformed to the original scale if needed and are based on mixed models fitted to the (transformed) variables (see materials and methods). Soil variables include; soil pH, soil moisture content (%), total nematode density (per 100 g dry soil, analysed by microscope), fungal biomass (expressed as mg ergosterol / kg soil), plant-species richness (S¬¬plant), plant- species diversity (H'plant), and the density of 11 nematode taxa (per 100 g dry soil, analysed by quantitative PCR). Nematode taxa are defined as by De Ley et al., (2006), except for Dorylaimidae D3 and Mononchidae M3 (see Holterman et al., 2008).

	Riparian vegetation						Semi-natural grasslands					
	Un-invaded			Invaded			Un-invaded			Invaded		
	lower	Est. mean	upper	lower	Est. mean	upper	lower	Est. mean	upper	lower	Est. mean	upper
Soil pH	7.5	7.7	7.8	7.5	7.6	7.8	5.5	6.0	6.4	5.3	5.8	6.3
Soil moisture content	15.0	18.5	21.9	13.4	16.8	20.3	10.8	14.2	17.7	9.9	13.4	16.8
Nematode density	2190	2584	3010	2104	2491	2910	967	1234	1534	891	1148	1438
Fungal biomass	0.28	0.43	0.67	0.52	0.82	1.32	0.46	0.72	1.15	0.68	1.09	1.77
S_{plant}	10.1	12.3	14.6	4.9	7.1	9.4	15.1	17.3	19.6	5.5	7.8	10.1
H'_{plant}	2.1	2.3	2.5	1.5	1.7	1.9	2.4	2.6	2.8	1.6	1.8	2.0
Aphelenchidae	15.9	33.1	69.4	22.0	45.9	96.4	6.1	12.4	25.6	4.3	8.6	17.7
Aphelenchoididae	5.8	11.0	21.6	16.1	31.8	63.2	2.5	4.5	8.5	3.7	6.9	13.3
Diphtherophoridae	1.1	2.8	9.5	1.1	2.7	9.0	1.2	3.3	11.2	1.0	2.6	8.5
Dorylaimidae D3	17.1	32.5	62.4	16.4	31.2	59.9	4.8	8.9	16.7	8.4	15.9	30.2
Mononchidae M3	1.7	3.9	10.3	5.8	15.9	45.4	2.7	6.9	19.2	2.1	5.1	13.9
Cephalobidae	136.2	211.6	330.4	249.3	389.9	611.2	110.9	171.8	267.7	73.2	112.4	174.1
Plectidae (except *Anaplectus*)	87.8	156.0	277.5	115.1	204.7	364.3	168.5	299.7	533.6	107.2	190.6	339.2
Anaplectus	3.6	10.1	29.9	8.2	24.1	73.0	3.3	9.1	27.0	3.9	11.0	32.8
Alaimidae	18.5	69.1	261.2	62.7	237.0	899.7	4.2	14.7	54.5	3.8	12.9	47.6
Prismatolaimidae	1.5	3.5	9.7	1.6	3.9	11.1	1.6	3.9	11.0	1.3	2.9	8.0
Panagrolaimidae	2.0	3.1	5.1	2.8	4.5	7.7	1.4	2.1	3.3	1.4	2.0	3.3

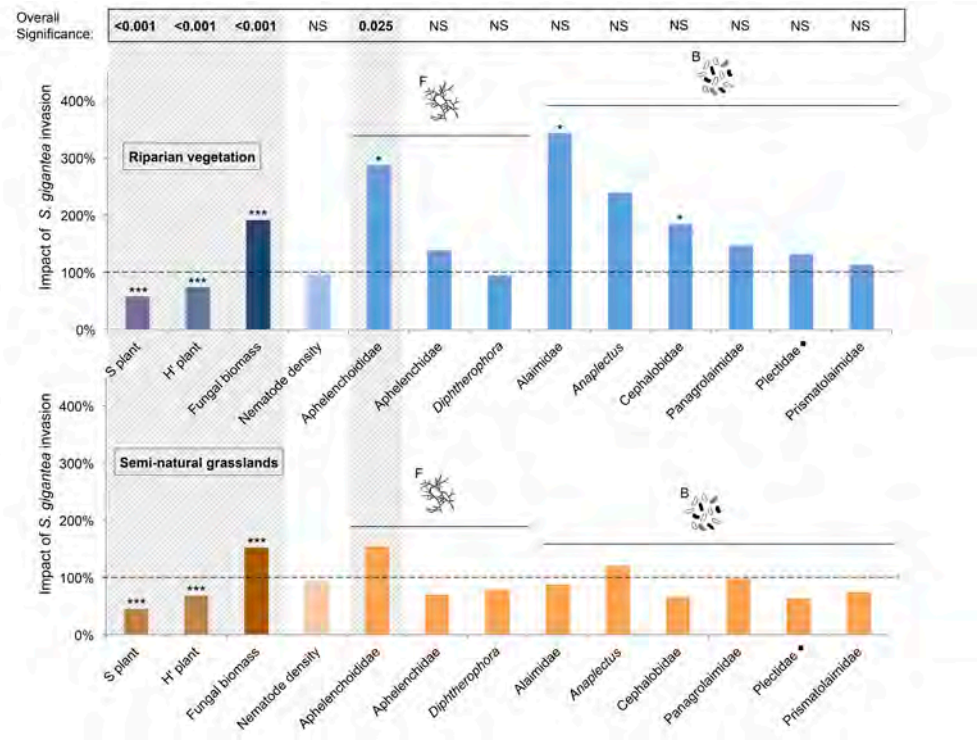

Figure 1. Impact of *Solidago gigantea* invasion in two habitat types, riparian vegetation and semi-natural grasslands, on plant-species richness (S_{plant}), plant-species diversity (H'_{plant}), fungal biomass, total nematode density, and the densities of three fungivorous ('F') and six bacterivorous ('B') nematode taxa. Impacts are expressed as the percentage of the (back transformed) mean values in invaded plots as compared to un-invaded plots (no change = 100%). For each of the two habitats, significant differences between invaded and un-invaded plots are given by asterisks (*P<0.05, **P<0.01 and ***P<0.001; data extracted from the fitted mixed models). Overall significances of the effects of *S. gigantea* invasion (= data from both habitats taken together) are given in top part of this figure (expressed as P values). A shaded background is used to highlight significant variables.

Analysis of samples taken in August 2011 and analysed microscopically, showed that in general nematode diversity was similar for both habitats. Approximately 2,000 nematodes were identified up to genus level. In total, 92 different genera were identified. In riparian soil, 79 genera were found, of which 48 were free-living and 31 plant parasitic. In soil from semi-natural grasslands, we identified 73 genera of which 50 were free-living and 23 plant parasitic. Both habitats shared 60 genera belonging to 40 different families (Suppl. Table S4).

The selection of eleven taxon-specific qPCR assays covered 26 of the 48 free-living genera

shared by both habitat types. For the riparian soil, 46% of the diversity and an estimated average of 86% of the total amount of free-living nematodes were covered by these sets of primer combinations. For soil from the semi-natural grasslands, the molecular assays covered 50% of the free-living nematode diversity and an estimated 80% of the total free-living nematode community (Suppl. Table S4).

Discussion

Investigation of belowground effects of Giant goldenrod (*S. gigantea*) in two (semi) natural habitats – riverbanks and grasslands – revealed a systematic effect of invasion on soil pH, a part of the fungal community, and a single lineage of fungivorous nematodes: invaded soils of two distinct habitats contained more fungal biomass and higher densities of fungivorous Aphelenchoididae than un-invaded soils. Interestingly, the densities of two other lineages of fungivorous nematodes, members of the families Aphelenchidae and Diphtherophoridae, did not change in response to the increased fungal biomass (Fig. 1). No systematic effect was observed on the bacterivorous nematodes community. These results show that – apart from aboveground effects – invasive plant species can cause significant alterations in the nematode community, which appear to be selective for specific taxa within functional groups.

Giant goldenrod effects on soil acidity

The slightly lower pH in invaded plots (Tables 1, 2) may be caused by acidic compounds that are released from *S. gigantea* roots into the rhizosphere (Weber and Jakobs, 2005). Several studies focused on the impact of *S. gigantea* on nutrient pools, and showed a decrease (although site-dependent) in pH in combination with an enhanced P availability (Herr et al., 2007, Chapuis-Lardy et al., 2006). In our study, only small differences in pH were measured, *i.e.* on average 0.1 units, which seem unlikely to play a role in the observed changes in soil biota. It is noted that pH was measured in bulk soil, and more pronounced effect in the rhizosphere cannot be excluded.

Solidago gigantea invaded plant communities

In *S. gigantea*-invaded plant communities, we observed a 42% and 55% reduction of plant-species richness (*S*plant) in the riparian and semi-natural grassland habitats, respectively (Table 2). This impact is relatively high; in a study of (Hejda et al., 2009) an overall reduction of *S*plant of 26% was reported in ruderal plant communities, meadows and along rivers in the Czech Republic. The authors stated that *S. gigantea* had no decisive community-level impact. In addition, *S. gigantea* had limited impact as compared to other invasive plant species such as *Fallopia* spp. (66% - 86% reduction *S*plant) and *Heracleum mantegazzianum* (53% reduction *S*plant). Our results show that the degree of invasiveness of *S. gigantea* in both habitat types is similar to the impact of *H. mantegazzianum* in meadows and forest edges of the Czech Republic (Hejda et al., 2009).

Solidago gigantea renders invaded plots unfit for most resident native plants. At least in part this could be attributed to the high efficiency of *S. gigantea* in the immobilization of minerals such as P and C (Scharfy et al., 2009, Vanderhoeven et al., 2006). For this reason, from June onwards the stems and leaves of *S. gigantea* can become increasingly dense and compete successfully for light. As a result, native plants will invest more in vertical growth, and this might negatively impact seed production. Moreover, Giant goldenrod releases large amounts

of furanoid compounds and acidic compounds in the rhizosphere (Weber and Jakobs, 2005). Rhizodeposition of these toxic compounds could affect resident plant species, as well as belowground communities.

Despite the success of *S. gigantea*, not all plants were negatively affected. We observed a rare and exotic parasitic plant *Cuscata gronovii* (originally from North America), which had strangled and hereby killed *S. gigantea* plants. It is assumed that invasive plants benefit from being released from their natural enemies (Keane and Crawley, 2002). This advantage might not persist (Diez et al., 2010), and *C. gronovii* could become an important factor limiting *S. gigantea* proliferation along rivers.

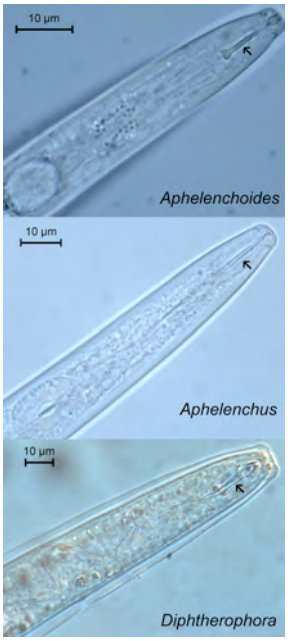

Figure 2. Pictures of the head regions of representatives of the fungivorous nematode genera Aphelenchoides, Aphelenchus and Diphtherophora (pictures taken at 1,000x magnification). To puncture the fungal cell walls, fungivores are equipped with a hardened protrusible piercing device (stylet or spear, indicated by arrows). The protrusibility is facilitated by muscles attached to the knobs or swellings at the basal part of this piercing device. The stylet of Aphelenchoides species is slender with easily observable basal knobs, whereas the stylet of Aphelenchus is characterized by slight basal swellings only. Diphtherophora has a short spear (different ontogeny as compared to a stylet) with a basal swelling of the spear extension.

Effects on soil food web components by *S. gigantea*

In both habitats differing in soil type, floristic composition, and land use history, we found significant belowground effects of *S. gigantea* on soil acidity, fungal biomass, and the density of Aphelenchoididae, a single lineage of fungivorous nematodes. The consistency of these effects suggests that they are general consequences of the dominant presence of *S. gigantea* in its invaded range. Beside overall effects, we observed habitat and site specific responses of other nematode taxa (Fig. 1 and Table 1).

Regarding the increase of fungal biomass and the differential shift observed for fungivorous nematodes, our results suggest that invasion of *S. gigantea* causes an asymmetric boost of the

soil fungal community. We hypothesise that *S. gigantea*'s presence promotes the growth of a subset of taxa within the fungal community, and that only fungivorous Aphelenchoididae were able to benefit from the selective increase in nutritional resources. In Fig. 2, the head regions of the three fungivorous nematode genera are shown. All of them are equipped with a protrusible piercing device that is used to puncture the fungal cell wall. However, the morphologies of these devices (indicated by arrows in Fig. 2) are distinct, and this could point at disparate food preferences. In *in vitro* studies, *Aphelenchoides saprophilus* has been shown to multiply on various mycorrhizal and saprophytic fungal species, whereas *Tylolaimophorus*, a member of the Diphtherophoridae, would not survive on any of these fungi (Ruess and Dighton, 1996). Another *Aphelenchoides* species, *A. hamatus*, could feed and multiply on mycelium from four plant parasitic and a range of edible fungal species (Ruess and Dighton, 1996, Rössner and Nagel, 1984). Among the Aphelenchidae, a family relatively unrelated to the Aphelenchoididae (Van Megen et al., 2009), *Aphelenchus avenae* was reported to prefer plant parasitic fungi to saprophytic species (Okada and Kadota, 2003). This information shows that at least some fungivorous members of the Aphelenchoididae are polyphagous, and our data suggest this could be different for the two other major lineages of fungivorous nematodes, Diphtherophoridae and Aphelenchidae.

Soil born organisms facilitate *S. gigantea* success

The colonisation of Giant goldenrod represents a hazardous factor at the plant community and the landscape scale. After all, we found that next to ruderal communities (Hejda et al., 2009), also relatively biodiverse areas are affected. Compared to most resident plant species, *S. gigantea* has a high nutrient efficiency and biomass production (Vanderhoeven et al., 2006, Scharfy et al., 2009), assumedly because invaders are generally exposed to more favourable plant-soil feedback interactions than their native neighbours (Klironomos, 2002). The results reported here show that the nematode community in *S. gigantea* invaded soil is significantly different from neighbouring soil under the native flora. This is indicative of a shift in their food source, namely the microbial community. We reinforce this hypothesis by the fact that we encountered a two-fold increase of fungal biomass in soil under invaded vegetation. In line with this, considering the impact of plant-soil feedbacks on the invasiveness of *S. gigantea*, the fungal community most probably plays an important role. In order to better understand the invasive success and future perseverance of *S. gigantea* in Europe, we will investigate the nature of the changes in the microbial communities and the way these changes are reflected in the bacterivorous and fungivorous nematode assemblages in more detail.

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Chapter 4

Release of isothiocyanates does not explain the effects of biofumigation with Indian mustard cultivars on nematode assemblages

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Abstract

While the major part of soil biota plays an essential role in ecosystem services, the plant-pathogenic fraction can have a high economic impact on food and feed production. The use of broad-spectrum biocides, so-called fumigants, has been a common practice for controlling soil pathogens, including plant-parasitic nematodes. However, due to their negative environmental impact, many nematicides have been banned or will be banned in the near future. Biofumigation, i.e., the incorporation of mulched (de-compartmentalised) brassicaceous plant material into topsoil, is an example of an alternative management practice. Biofumigation exploits the conversion of glucosinolates (GSLs; secondary metabolites produced by a number of brassicaceous plant species) into nematicidal isothiocyanates (ITCs) upon de-compartmentalisation. Although considerable attention has been paid to effects of biofumigation on plant-parasitic nematodes, the effect on the non-parasitic part of the community has hardly been investigated. Here, we present the results of a field experiment in which we studied the impact of biofumigation with four Indian mustard (Brassica juncea) cultivars on both plant-parasitic and free-living nematodes. Prior to biofumigation, GSL contents of B. juncea plants were determined and expected ITC concentrations in the topsoil were calculated. As positive controls, two concentrations of 2-propenyl ITC were directly applied to wheat plots. Although biofumigation resulted in changes for most nematode taxa, none of these shifts could be attributed to the release of ITCs. Moreover, direct application of a relatively high concentration of ITC did not result in shifts that differed in degree from those observed for the control treatment. We therefore conclude that the observed changes in nematode assemblages are related to intense mechanical disturbance, green manure and the absence of host plants for obligatory plant-parasitic nematode genera, rather than to the release of ITCs.

Introduction

For decades, the use of fumigants in agriculture has been a widespread practice to control soil borne pests (Gamliel et al., 2000). More recently, most synthetic biocides, *e.g.* chloropicrin and methyl bromide, were placed under strict legislation or banned entirely because of their negative impact on the environment (Gamliel et al., 2000, Ruzo, 2006). These restrictions have created a need for alternative management practices. Biofumigation, *i.e.*, the use of *Brassica* green manures for pest control, is considered one of the alternatives (Matthiessen and Kirkegaard, 2006). Plants within the family Brassicaceae are known to produce glucosinolates (GSLs). A major group of hydrolysis products of these GSLs, isothiocyanates (ITCs), act as general biocides (Brown and Morra, 1997). Due to the short release time and half-life of GSLs and ITCs in soils (Gimsing and Kirkegaard, 2009), direct toxic effects on soil borne pathogens are expected within hours to days after biofumigation.

Although biofumigation is often viewed to be less harmful for the environment and soil communities as compared to synthetic fumigants (Matthiessen and Kirkegaard, 2006), these natural mixes of GSLs hydrolysis products can be just as, or even more toxic than synthetic pesticides (Gimsing and Kirkegaard, 2009). ITCs can affect a broad spectrum of soil organisms and may leave previously stable soil food webs vulnerable, as shown for the synthetic fumigant metam sodium (sodium N-methyldithiocarbamate; Cao et al., 2004{Cao, 2004 #2150}). Nevertheless, there has been considerable interest in the extent to which naturally produced ITCs can emulate the efficacy of soil pesticides. Several field studies have shown that the amendment of *Brassica* plant material can have a suppressive effect on a broad range of soil pathogens (Mojtahedi et al., 1993, Motisi et al., 2009). Nevertheless, the efficacy of biofumigation to suppress plant-parasitic nematodes in field trials has been variable (Ploeg, 2008). Results range from high levels of suppression (*e.g.* Mojtahedi et al., 1993, Rahman and Somers, 2005) to no suppression (*e.g.* Johnson et al., 1992, Stirling and Stirling, 2003).

Within the soil food web, free-living (*i.e.*, non plant-parasitic) nematodes are represented at three trophic levels, and the impact of biofumigation on these groups is likely to affect soil functioning. Various effects of biofumigation on free-living nematodes have been reported. After biofumigation, Valdes et al. (2012) observed a decrease in plant-parasitic nematodes and an increase of bacterivorous nematodes. Stirling and Stirling (2003) observed only an increase of free-living nematodes, while Gruver et al. (2010) did not observe any effect. It is hard to link these results unequivocally to the GSL release during biofumigation, as these experiments did not include GSL measurements of the incorporated plant material. In the aforementioned studies, effects were assessed weeks after biofumigation, and it is hard to distinguish the direct toxic effects of ITCs from the impact of tillage and or green manure on nematode communities.

The objectives of this study were to assess the direct and subsequent effects of biofumigation on nematode communities. Direct effects included toxicity of ITCs and disturbance due to tillage; subsequent effects of biofumigation were related to plant biomass incorporation and quality of plant material. We monitored plant-parasitic nematodes using classic identification and free-living nematodes using a DNA-based method (Vervoort et al., 2012) at the start of the growing season as well as just before and at several time points after biofumigation of four Indian mustard (*Brassica juncea*) cultivars differing in their GSL content. The biomass and GSL content of the plant material was determined prior to incorporation. This approach allowed for

an assessment of the impact of different biofumigation-related factors on both plant-parasitic as well as free-living nematodes.

Materials and Methods

Study site

The experiment was performed at the experimental field site of the Julius Kühn-Institut in Münster, Germany, in 2010. Soil type was a medium loamy sand consisting of 9.2% clay, 13.6% silt and 77.2% sand with 1.3% organic matter and a pH (CaCl2) of 6.4. Soil nutrient status at time of planting was 32 mg 100 g-1 P2O5 (above optimum), 17 mg 100-1 g K2O (optimum) and 6 mg 100 g-1 Mg (optimum). Total available mineral nitrogen and sulphur were 52 kg N ha-1 and 42 kg S ha-1, respectively. The experimental plot was prepared on July 7th 2010 by ploughing the remaining stubbles of the previous maize (*Zea mays*) crop and applying 292 kg ha-1 hydrosulfan (24% N, 6% S, Yara GmbH, Germany), *i.e.*, 70 kg N ha-1 and 17.5 kg S ha-1, to ensure optimum plant growth.

Experimental design

Four *B. juncea* cultivars were used: Terrafit, Terratop, Terraplus (P. H. Petersen Saatzucht Lundsgaard GmbH) and ISCI-99 (Bluformula, Italy). Cultivar ISCI-99 was selected as a high GSL producer. As a control (a non-GSL crop), wheat (*Triticum aestivum* cv. Hermann) was chosen. Sinigrin (2-propenyl GSL) is the dominant GSL type in Indian mustard. Its concentration in roots and stems decreases gradually during development, whereas it increases in leaves and reproductive organs of *B. juncea* (Bellostas et al., 2007). At the time of incorporation the plant were in or just beyond the flowering stage. As a positive control, the ITC derivative of sinigrin, 2-propenyl ITC, was directly applied in the wheat plots (see section 2.4).

A randomised block design with four replicates was used, and the plot size was 4 x 15 m. Based on known germination rates, plants were sown at densities of 12 kg ha-1 for *B. juncea* cvs. Terrafit, Terratop and Terraplus, 15 kg ha-1 for *B. juncea* cv. ISCI-99, and 176 kg ha-1 for wheat. All plots were drilled on the July 9th, 2010, hereafter referred to as day 0 (Fig. 1).

Plant sampling and analysis

Immediately before biomass incorporation (day 59), plants were sampled from 50 x 50 cm subplots within each plot, and root and shoot fresh weights were determined. Aliquots (each ≈ 150 g fresh weigh) were collected to determine root and shoot dry weight (weight loss after 24 hrs at 70°C). From each *B. juncea* plot, ten plants were randomly collected, divided into roots and shoots, and plant parts were immediately frozen and kept at -80°C. The plant material was freeze-dried, pulverized with an oscillating mill (MM2, Retsch, Germany) and the resulting plant powder was stored till further chemical analysis. The GSLs were extracted from a 200 mg subsample using 3 mL methanol:water (70:30, vol/vol) at 75°C. One ml of GSL extract was loaded on a micro-column filled with DEAE – A25 Sephadex (CAS Number 12609-80-2, Sigma-Aldrich, Germany), The extracted GSLs were then converted into desulfo-GSL's by incubation for 16 hrs at 39°C with sulfatase from *Helix pomatia* Type H-2 (CAS Number 9016-17-5, Sigma-Aldrich, Germany), eluted with H2O and analysed by High-Performance Liquid Chromatography with Diode-Array Detection at a wavelength of 229 nm. GSL quantification

was made internal standard-based (see also Schütze et al., 1999). Finally, GSL yield per hectare was calculated based on GSL concentration of the plant material and plant dry biomass. C and N content of the *B. juncea* plant material was determined using an elemental analyser (Interscience/Carlo Erba, type EA 1108).

Biofumigation

On day 59, *B. juncea* and *T. aestivum* plant material was chopped and incorporated into the soil. For this, a tractor-driven flail mower was used, and plant parts were immediately incorporated into the top 20 cm of soil with a rotary tiller. Afterwards, the soil surface was slightly rolled to close soil pores and thus reduce evaporation of the ITC's. As a positive control, 2-propenyl ITC was applied directly to the soil. For this purpose, two subplots of 4 m2 each were selected in each of the four wheat plots. These subplots were treated, after the plant material was chopped and prior to incorporation, with 10 l m-2 of a low (1.2 mmol l-1) or a high (4.8 mmol l-1) concentration of 2-propenyl ITC (CAS Number 1476-23-9, 95% purity, Aldrich, Germany). The low concentration treatment was selected to mimic an approximate 100% conversion of the total expected GSL yield of the cultivars into 2-propenyl ITC. The high concentration treatment was chosen to demonstrate the effect of an artificially high ITC concentration on the nematode community.

Soil sampling and nematode extraction

Bulk soil samples were taken on day 3, 59 (just before biofumigation), 60 (one day after biofumigation), 81 and 116 (Fig. 1). Each sample consisted of 30 cores (Ø 2 cm) taken from the top 20 cm following a grid pattern of 3 x 10 m per plot. Samples were then mixed thoroughly, sieved over a 5 mm grid to remove stones and large organic debris and stored at 4°C.

Nematodes were extracted from 250 ml aliquots of the bulk soil in an Oostenbrink elutriator (Oostenbrink, 1960). Nematode density was determined by counting two subsamples of 5 ml out of 100 ml at low magnification under an inverted microscope. After counting, the subsamples were poured back. One half of each suspension (50 ml) was used for microscopic analysis of plant-parasitic nematodes. For this purpose, nematodes were fixed by replacing the water with DESS solution (a solution containing dimethyl sulfoxide (DMSO), disodium EDTA and NaCl, see Yoder et al., 2006) to a total volume of 10 ml. The remaining 50 ml of the suspension was further processed for quantitative PCR (qPCR) analysis to quantify free-living nematode taxa.

Nematode community analysis

Nematode suspensions were split, and one part was used for the morphological identification of plant parasites to genus using an inverted microscope (magnification 40x). The remaining part was lysed, purified, and analysed by quantitative PCR as described in Vervoort et al. (2012). Nematode taxon selection was based on two microscopic analyses of the overall nematode community composition: just before the start of the experiment (May 2010) and on day 81 of the experiment (September 2010).

Based on microscopic nematode biodiversity assessment, 15 taxa were selected for qPCR analysis (this selection covered 83% of the identified free-living genera, see supplementary Table S1). For the suborder Dorylaimina and the family Mononchidae, cluster-specific primers

D1, D3 and M3 were used according to Holterman et al. (2008). For the family Plectidae, separate primers were used targeting either *Anaplectus* or *Plectus* (the only other representative of this family in our field). Many nematode families were represented by a single genus (see supplementary Table S1). Within this experiment, two families were represented by multiple genera: Cephalobidae (five genera) and Dorylaimidae D1 (two genera).

Data analysis

To compare the plant GSL, C and N content on the day of biofumigation (day 59) and the concentrations of the GSLs incorporated into the soil between the four *B. juncea* cultivars ($n = 4$), data was subjected to an ANOVA analysis followed by a least significant difference test (LSD). Differences in nematode densities (total or taxon) between the treatments per each sampling time were tested using a Kruskal-Wallis test. The same test was applied to compare relative changes in nematode densities due to incorporation of plant material between the four *B. juncea* cultivars and the wheat control plots (day 59 – 60; total or taxon). With regard to the comparison of densities of free-living nematode taxa between day 59 and 60, 12 samples were analysed (instead of 16) due to the loss of four samples during processing for qPCR analysis. The Kruskal-Wallis test was also used to check for differences in relative change in nematode densities (total or taxon) between the treatments in the wheat plots from day 59 to 60, day 60 to 81, and day 60 to 118. Significant changes in nematode density (total or taxon) between different sampling times were determined by using either a paired *t*-test (data were log (x+1) transformed) or by a Wilcoxon Signed Rank test. The relationship between the quantity of incorporated GSLs, plant C and N, and changes in nematode densities (total or per taxon) in the *B. juncea* plots were analysed using linear regression. For the wheat plots, linear regression was used to determine if there was a relationship between the relative change in nematode densities (between day 59 and 60) and the two directly applied ITC concentrations. For all tests, differences were considered significant when $P < 0.05$. Data were analysed using SPSS 19 (IBM Corporation).

Results

Points of departure: Glucosinolate contents of *Brassica juncea* plant material and characterization of nematode community

Just prior to biofumigation (day 59), the glucosinolate (GSL) contents of the shoots and roots of the individual Indian mustard cultivars were determined (Table 1). Sinigrin (2-propenyl GSL) was the most abundant glucosinolate in the *B. juncea* plants. Between the four cultivars, only minor differences in GSL contents were observed. As compared to two other cultivars, *B. juncea* cultivar Terraplus showed a slightly higher concentration of indole-GSLs in the shoots (Table 1). Among the Indian mustard cultivars no significant differences were observed in sinigrin concentration, the C and N contents of the plant parts, or the total plant biomasses.

With regard to the nematode communities, both the plant-parasitic and the free-living fraction were characterised. In total 33 genera were identified, of which eight were plant parasites (supplementary Table S1). Five of these plant-parasitic nematode genera, known to feed exclusively on higher plants, were included by microscopic analysis; *i.e.* two ectoparasites (*Trichodorus* and *Tylenchorhynchus*), two sedentary endoparasites (*Heterodera* and *Meloidogyne*)

Table 1. Plant biomass and tissue concentrations of relevant elements (mean ± SD, n = 4) from four *Brassica juncea* cultivars just prior to biofumigation. GSL: glucosinolate, dw: dry weight, Mg: megagram (= metric ton), ha: hectare. Sinigrin is the common name for 2-propenyl GSL.

		***B. juncea* cultivar**			
		Terrafit	**Terratop**	**Terraplus**	**ISCI-99**
Shoot	**GSL, total**(μmol g^{-1})	24.2 ± 7.3	16.9 ± 2.9	14.2 ± 2.7	24.8 ± 8.0
	Sinigrin (μmol g^{-1})	22.6 ± 7.2	15.2 ± 2.7	12.3 ± 2.7	22.9 ± 8.1
	Indole GSLs (μmol g^{-1})*	1.5 ± 0.1 ^{a}	1.7 ± 0.3 ^{ab}	1.8 ± 0.2 ^{b}	1.9 ± 0.3 ^{ab}
	N (% dw)	1.5 ± 0.1	1.7 ± 0.4	1.3 ± 0.5	1.8 ± 0.4
	C (% dw)	83.4 ± 2.0	84.3 ± 2.0	82.3 ± 1.3	82.2 ± 2.7
	Biomass (Mg dw ha^{-1})	5.9 ± 2.0	6.9 ± 2.3	6.4 ± 2.3	8.8 ± 1.9
Root	**GSL, total** (μmol g^{-1})	20.2 ± 9.8	16.5 ± 7.6	25.9 ± 15.8	33.2 ± 10.1
	Sinigrin (μmol g^{-1})	15.9 ± 9.5	10.9 ± 6.7	18.5 ± 14.0	27.2 ± 9.5
	Indole GSLs (μmol g^{-1})	4.3 ± 0.4	5.6 ± 1.3	7.5 ± 2.1	6.1 ± 0.8
	N (% dw)	1.9 ± 1.1	1.7 ± 0.6	2.1 ± 1.1	2.2 ± 0.8
	C (% dw)	76.6 ± 1.9	68.8 ± 8.1	75.3 ± 7.0	75.2 ± 5.8
	Biomass (Mg dw ha^{-1})	0.9 ± 0.3	1.5 ± 0.6	1.0 ± 0.4	1.0 ± 0.1
Whole plant	**Biomass** (Mg dw ha^{-1})	6.9 ± 1.8	8.4 ± 2.5	7.5 ± 2.5	9.9 ± 2.0

* $P < 0.05$ (different letters indicate significantly different groups)

and one migratory endoparasite (*Pratylenchus*). Microscopic analysis revealed that *Tylenchorhynchus* was the most dominantly plant-parasitic genus with densities of 590 ± 379 individuals per 100 g dry soil (average ± stdev, *n* =120, present in all samples). The second most dominant plant parasite was *Trichodorus*, present in 97% of all samples with an overall average density of 107 ± 79 individuals per 100 g dry soil. *Heterodera*, *Pratylenchus* and *Meloidogyne* were only incidentally present at low densities of respectively 11 ± 28 (26%), 6 ± 15 (29%) and 3 ± 10 (11%) individuals per 100 g dry soil (average ± stdev; % samples present).

Free-living nematodes were analysed on the basis of unique SSU rDNA motifs using quantitative PCR (Vervoort et al. 2012). Preliminary analyses revealed that some taxa – Alaimidae, Aphelenchoididae, *Cruznema*, Dorylaimidae D3 and Mylonchulidae – were present in less than 50% of all samples. To allow paired comparisons between subsequent sampling times, only taxa present in at least 50% of soil samples were included for further analysis. These target taxa, two plant-parasitic genera and ten free-living nematode taxa (Fig. 2), were taken into consideration for the overall analyses.

Growing season

Both at the start (day 3) and the end (day 59) of the growing season, total nematode densities (Fig. 1) and the densities of individual nematode taxa (qPCR data for free-living nematodes and microscopic counts for plant parasites) were comparable for all cultivars. Although not significantly different, total densities in the wheat plots appeared to be relatively low during the growing season (Fig. 1). As no significant differences were observed in GSL, C and N contents of the *B. juncea* cultivars, and in the absence of clear qualitative or quantitative differences between the nematode assemblages in the individual plots, we decided to combine all *B. juncea*

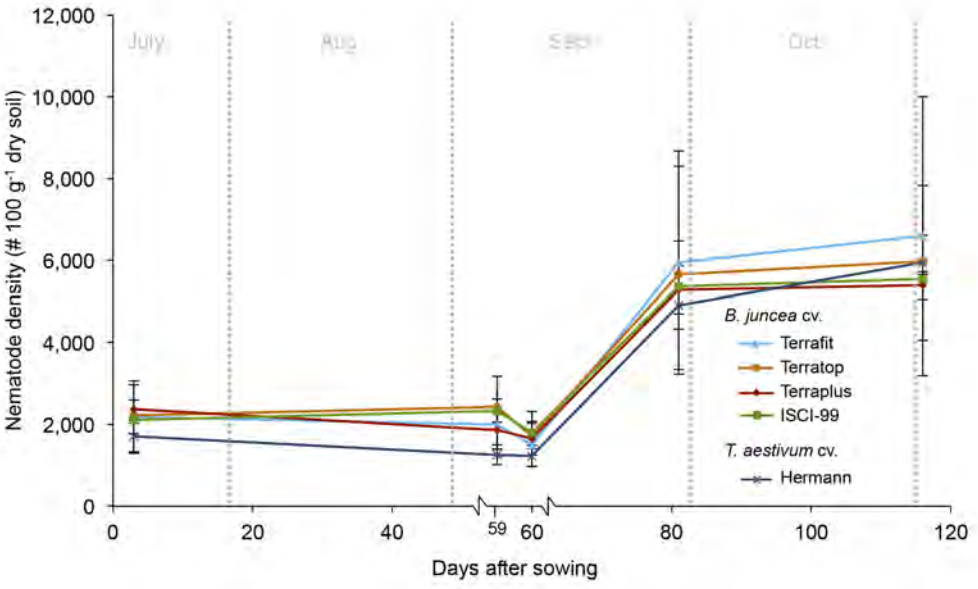

Figure 1. Total nematode density (analysed by microscope) per 100 g dry soil during the growing season (day 3 and 59) and after biofumigation (day 60, 81 and 116) with four *Brassica juncea* cultivars and wheat (Triticum aestivum). Data represents averages ± stdev (n=4).

samples of each sampling time point for the analyses at family and genus level. Although no overall change in nematode densities was observed in between day 3 and 59, significant shifts were detected for individual taxa (Fig. 2). A significant decrease in densities of Monhysteridae (Fig. 2B, bacterivores) and Mononchida M3 (Fig. 2A, carnivores) was paralleled by an increase in densities for Aphelenchidae (Fig. 2A, fungivores) and *Tylenchorhynchus* (Fig. 2A, plant parasites). In wheat plots, the only change between these two sampling times was seen for the Mononchida M3, of which density decreased significantly during the growing season (paired *t*-test, $P = 0.012$).

Biofumigation: direct effects on nematode community

The amount of plant GSLs, C and N incorporated into the soil was calculated for each plot of the *B. juncea* cultivars (Table 2). Overall, only the estimated concentration of sinigrin in the topsoil differed significantly between cultivars; topsoils with chopped ISCI-99 plants were exposed to a two times higher sinigrin concentration. This was due to the combined effect of slightly (but not significantly) higher GSL content in combination with a slightly (but not significantly) higher shoot biomass (Table 1).

Mulching, in fact mechanical de-compartimentalisation of plant tissues, followed by incorporation into the topsoil resulted in a significant decrease in the total nematode density in the *B. juncea* plots (n = 16, paired *t*-test, P=0.005). Apart from Monhysteridae and Mononchida M3

(Holterman et al., 2008), the density of the free-living taxa decreased significantly between day 59 and 60 (Fig. 2). Remarkably, no significant changes were observed for the two plant-parasitic genera (Fig. 2). In the untreated wheat plots, we did not observe a significant change in total nematode density between days 59 and 60 (n = 4, paired *t*-test 0.059). Fig. 1 could suggest for a difference in the degree of change in total nematode densities in the time interval day 59 - day 60 between the wheat plots on the one hand and the *B. juncea* cultivars on the other, but this difference was not significant.

Table 2. Calculated concentrations of relevant plant substances from *Brassica juncea* and *Triticum aestivum* cultivars in the topsoil (upper 20 cm) after biofumigation. For the determination of these concentrations, distinct values given for the plant parts (shoots and roots; Table 1) were transformed into either moles or weight and added together. For each of the cultivars, mean calculated concentrations in top soil ± SD of two types of glucosinolates (GSL), sinigrin (2-propenyl GSL) and indole GSLs, as well as plant C and N and total dry plant biomass are given (n = 4).

		B. juncea cv.				*T. aestivum* cv.
	g^{-1} dry soil	Terrafit	Terratop	Terraplus	ISCI-99	Hermann
Sinigrin**	nmol	55.8 ± 7.9[a]	52.5 ± 12.5[a]	54.5 ± 12.8[a]	91.4 ± 9.1[b]	nd
Indole GSLs	nmol	5.3 ± 1.2	9.3 ± 1.6	8.9 ± 3.5	9.1 ± 3.2	nd
C	µmol	189.6 ± 52.1	231.6 ± 73.6	208.2 ± 68.7	269.4 ± 56.7	nd
N	µmol	2.9 ± 0.8	4.2 ± 1.9	3.1 ± 1.6	5.4 ± 2.1	nd
Biomass	mg	2.8 ± 0.8	3.4 ± 1.1	3.1 ± 1.0	4.0 ± 0.9	3.0 ± 0.9

** $P < 0.01$ (different letters indicate significantly different groups)

Mulching-induced hydrolysis of GSLs should result in a rapid release of ITCs in the topsoil (Gimsing and Kirkegaard, 2006, Gimsing et al., 2009). Hence, we investigated whether the concentrations of GSLs in the topsoil (Table 2) could be related to the degree of change in nematode densities immediately before and directly after biofumigation. The same approach was used to test if there was a relationship between nematode density changes and the ITC concentrations in the soil of the wheat plots. For both *B. juncea* treatments and wheat treatments, no such relationships could be identified (data not shown).

As it is hard to assess the transformation efficiency of GSLs to ITCs in the topsoil *in situ*, the direct effects of 2-propenyl ITC on nematode assemblages were studied in small areas (4 m2) within the wheat plots. 2-propenyl ITC was directly applied to the topsoil in two concentrations. 'Low' treatment plots received a dosis comparable to that produced by cultivars Terrafit, Terratop and Terraplus treatments, whereas the high concentration is equivalent to twice the amount produced by ISCI-99. Both the applications of low and high concentrations of 2-propenyl ITC did not result in any difference in the relative increases or decreases in the total nematode density, or in the densities of individual taxa between day 59 and 60 (Table 3).

Biofumigation: prolonged effects on nematode community

Three weeks after biofumigation (day 81), the total nematode densities had increased significantly as compared to day 60, one day after incorporation of the Indian mustard or wheat plants

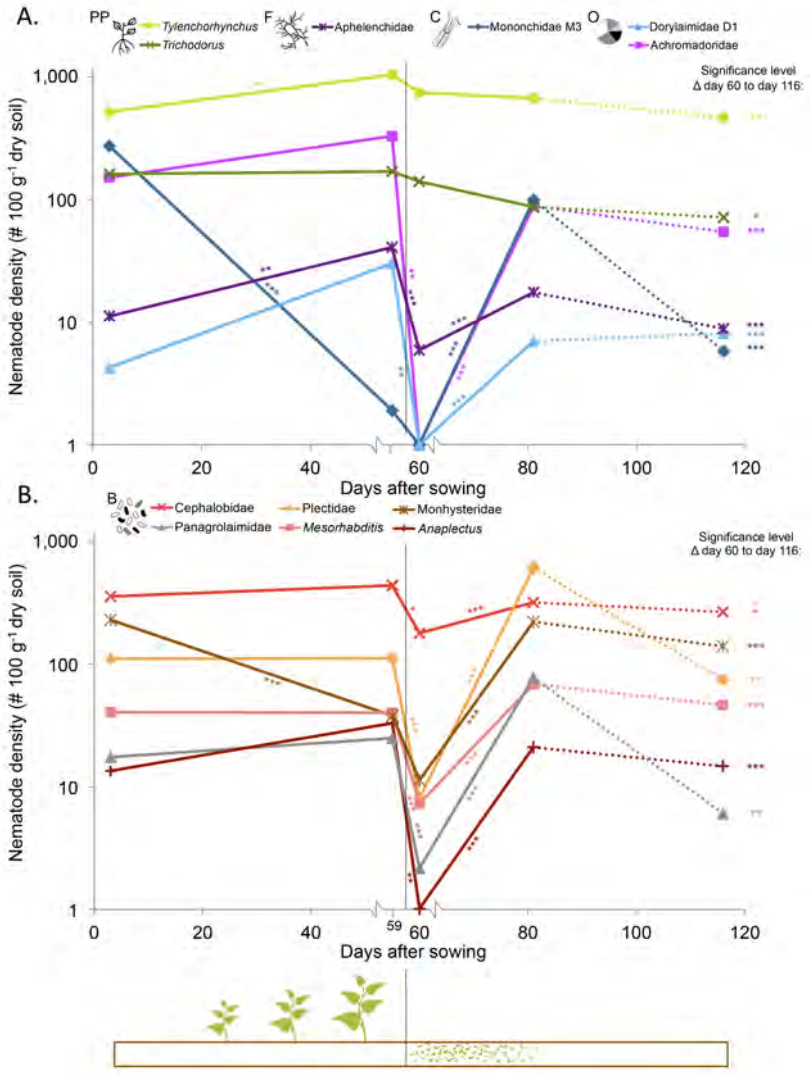

Figure 2. Biofumigation effects on the densities of 12 nematode taxa. PP: plant parasites, F: fungivores, C: carnivores, O: omnivores (panel A), B: bacterivores (panel B) Biofumigation took place on day 59. Significant changes between two connected time points are indicated with asterisks (*P < 0.05, **P < 0.01 and ***P < 0.001). Dashed lines are used to connect data from day 81 and 116. Taxon densities at day 116 were compared to densities one day after biofumigation (60), of which significances are shown by asterisks on the right part of the panels A and B.

(Fig. 1, paired *t*-test, $P < 0.001$). Analyses at the level of individual taxa revealed an increase for all groups of free-living nematodes under investigation (Fig. 2). However, the densities of the plant-parasitic taxa *Trichodorus* and *Tylenchorhynchus* showed no significant change (Fig. 2A). Only for Cephalobidae, we found a significant correlation between the degree of increase and the total quantity of biomass incorporated (R2 = 0.56, $F = 12.49$, $P = 0.005$).

Two months after biofumigation (day 60 compared to day 116), the population increase for free-living taxa as observed three weeks after biofumigation (day 81) was still visible, although less pronounced. In contrast to these taxa, the population densities of the plant-parasitic taxa *Tylenchorhynchus* and *Trichodorus* decreased. The relative increase of Monhysteridae was positively correlated with the total amount of plant biomass that had been incorporated (R2 = 0.46, $F = 8.36$, $P = 0.016$). Apart from short-term direct effects of 2-propenyl ITC (Table 3), we also looked for possible long-term consequences. However, no significant differences in total nematode density or densities of individual nematode taxa between treated and non-treated wheat plots between day 60 and 81 (all $P \geq 0.123$) or day 60 and 116 (all $P \geq 0.077$) were detected.

Table 3. Impact of low and high 2-propenyl isothiocyanate (2-p ITC) concentrations on total nematode abundance and the levels of individual nematode taxa in non-GSL (wheat) control plots expressed as the difference in density between before and after biofumigation (Δ density [day 60 – day 59]; Δ individuals per 100 g dry soil). 'Control' reflects the effect of incorporation fragmented wheat material into the top-soil, whereas 'Low' and 'High' present the combined effects of 2-p ITC and wheat incorporation. Low: 48.8 ± 1.6 nmol 2-p ITC g-1 dry soil; high: 195.1 ± 6.5 nmol 2-p ITC g-1 dry soil.

		Treatment			*P* value[c]
	Trophic group[a]	Control	Low	High	
Nematode density[m]		-27.2 ± 100.1	-185.3 ± 213.6	-107.9 ± 494.9	0.694
Achromadoridae[q]	O	-25.1 ± 87.8	-38.1 ± 75.5	-38.1 ± 75.5	0.938
Anaplectus[q]	B	-2.6 ± 3.8	-2.7 ± 4.3	-3.2 ± 3.7	0.825
Aphelenchidae[q]	F	-11.4 ± 8.2	-9.4 ± 7.5	-12.3 ± 6.8	0.967
Cephalobidae[q]	B	-33.2 ± 89.0	-1.0 ± 122	-55.5 ± 148.2	0.735
Dorylaimidae D1[q]	O	-27.5 ± 34.4	-27.4 ± 34.6	-27.3 ± 34.6	0.980
Monhysteridae[q]	B	-7.2 ± 32.4	-17 ± 19.4	-16.7 ± 19.4	0.944
Mononchidae M3[q]	O	-9.5 ± 17.2	-0.9 ± 9.8	-5 ± 21.3	0.546
Panagrolaimidae[q]	B	-4.2 ± 3.0	-4.7 ± 3.9	-4.5 ± 5.1	0.938
Plectidae[q,b]	B	-82.0 ± 142.5	-80.7 ± 142.9	-82.4 ± 142.7	0.943
Mesorhabditis[q]	B	-26.0 ± 23.2	-24.5 ± 20.5	-29.2 ± 22.6	0.837
Tylenchorhynchus[m]	PP	-129.3 ± 156.8	-31.8 ± 287.8	-301.6 ± 300.9	0.437
Trichodorus[m]	PP	-26.7 ± 92.9	-55 ± 70.2	-39.3 ± 48.7	0.794

[a] B: bacterivorous, F: fungivorous, C: carnivorous, O: omnivorous, PP: plant-parasitic

[b] Plectidae except for the genus Anaplectus.

[c] Kruskall-Wallis test

[m] Analysed microscopically

[q] Analysed by quantitative PCR

Discussion

Soil fumigants, among the most rigorous and non-discriminative measures that can be taken to control soil-borne pathogens including plant-parasitic nematodes, have even been banned in many countries or will be banned in the near future. Hence, there is a strong need for alternative, more sustainable control methods. One of the relatively new approaches exploits the presence of glucosinolates (GSLs) in many Brassicaceae. GSLs are produced by these plants to prevent herbivory by phytophagous insects. Here, we investigate the effects of various Indian mustard cultivars on targeted and non-targeted fractions of the nematode community.

Relationship between GSLs (ITCs) and biofumigation effects on different nematode taxa

Based on the GSL contents of the plants just prior to incorporation, GSL concentrations in the topsoil were calculated. The reportedly high producing cultivar ISCI-99 indeed produced the highest yield of GSLs. Overall, the total incorporated biomass and GSL levels of the four cultivars were comparable with the levels previously reported for Indian mustard cultivars (*e.g.* Hartz et al., 2005, Morra and Kirkegaard, 2002).

One day after biofumigation, no significant differences in densities of individual nematode taxa were detected between the three commercial Indian mustard cultivars on the one hand, and the high producer ISCI-99 on the other. We are aware that the calculated final GSL concentrations in the topsoil are a precursor of the bioactive ITCs, and that several factors co-determine the efficiency of the desired conversion. Upon incorporation of GSL-containing plant material into the topsoil, the degree of cell disruption of the material (Morra and Kirkegaard, 2002) and the activity of myrosinase as influenced by temperature and pH (Van Eylen et al., 2007) can have a considerable effect on the release efficiency of ITCs. However, as mulching and incorporation took place by a standardised procedure and on the same day, a substantial difference in conversion efficiencies between cultivars seems unlikely.

To address the issue of the possible low ITC release, we included treatments in which two concentrations of 2-propenyl ITC were directly applied to wheat plots. However, also in this "positive control" experiment no differential change in nematode taxa between the low and the high 2-propenyl ITC concentration and untreated wheat subplots could be detected (Table 3). If 2-propenyl ITC is released or directly applied to the soil, its volatility is influenced by soil texture and temperature (Price et al., 2005), its degradation rate depends on the activity of the *in situ* soil microbial community (Gimsing et al., 2009, Warton et al., 2003), and its degree of sorption is affected by the organic matter fraction in the soil (Gimsing et al., 2009).

Translation of toxicity levels for pathogens derived from *in vitro* settings to soil systems is not straightforward. In one *in vitro* study with autoclaved silica sand, Zasada and Ferris (2003) showed that the LC50 values of 2-propenyl ITC and of the soil fumigant metam sodium were similar for the plant-parasitic nematodes *Tylenchulus semipenetrans* and *Meloidogyne javanica*. In a follow-up greenhouse study, Zasada and Ferris (2004) amended 0.7 to 2.9% (w/w biomass levels) *B. junce*a into a soil (inoculated with either *T. semipenetrans* or *M. javanica*) theoretically corresponding to the LC50 and LC90 levels determined *in vitro* while correcting for the estimated release and conversion efficiency of GSLs. They showed that the amount of GSLs added to the

soil through the plant material had to be up to 70% higher as compared to the *in vitro* experiment in order to achieve the same results. In our study, the concentration of the highest 2-propenyl ITC treatment added to the soil of the wheat plots was about 2.5 and 12 times higher than the expected LC50 values for the two plant-parasitic nematodes mentioned before. However, we did not observe any effect on nematode densities in our field experiment. An explanation could be that *T. semipenetrans* and *M. javanica* are more susceptible to 2-propenyl ITC than the taxa occurring in our field experiment, but – more likely – other unknown parameters might hamper the translation of results from *in vitro* or greenhouse experiments (*e.g.* Lord et al., 2011, Zasada and Ferris, 2004) into field conditions with biofumigation protocols that will robustly reduce (plant-parasitic) nematode populations.

Non-ITC-related effects of biofumigation on different nematode taxa

If the observed reduction in total nematode density as well as the decreased densities of most free-living nematode taxa (eight out of ten studied taxa) one day after biofumigation are unrelated to GSL or ITC concentrations, other explanatory factors should identified. These decrements may be related to a combination of mechanical stress (tillage) and or the release of compounds other than ITC from the plant material. Little is known about sensitivities of individual nematode genera or families to the mechanical stress of tillage, as most studies focus on the overall effect of tillage (including enrichment) at trophic group levels (*e.g.* Fu et al., 2000, Timper et al., 2012, Treonis et al., 2010). However, Fiscus and Neher (2002) demonstrated distinct sensitivities of nematode genera for the direct and indirect effects of tillage by performing a canonical analysis of data from two field experiments. The categories of sensitivity appointed to different nematode genera in their study only partially explain our results. For instance, the genus *Achromadora* (Achromadoridae) showed a significant decrease on day 60 (Fig. 2A), while *Monhystera* (Monhysteridae), which is placed into the same tillage sensitivity category as *Achromadora*, did not (Fig. 2B). In many studies on the effects of tillage on nematode communities, data are analysed at trophic group level, while the original data, at genus level, are not included (*e.g.* Lenz and Eisenbeis, 2000, Rahman et al., 2007, Timper et al., 2012). Reviewing primary data from tillage studies performed could give insight in the differential effects of tillage on nematode genera.

Next to ITCs, non-glucosinolate derived, sulphur-containing compounds are released during the incorporation of *Brassica* plant material in topsoil. In *B. juncea,* these sulfides are generally less toxic than ITCs but are present in higher concentrations (Bending and Lincoln, 1999). Wang et al. (2009) demonstrated that high concentrations of dimethyl disulfide and methylsulfide could be related to suppression of pathogens. However, as we did not observe any difference in the degree of nematode decrease between *B. juncea* and wheat plots, we cannot confirm this assumption for our data. We conclude that the observed responses of the free-living nematode taxa one day after biofumigation are mainly due to mechanical disturbance.

Subsequent effects of biofumigation on nematode taxa density

Effects on nematode densities three weeks and two months after incorporation of mulched Indian mustard into the topsoil are supposedly unrelated to the release of ITCs. All densities of free-living nematode taxa had increased, while the numbers of plant-parasitic taxa declined.

These results correspond to earlier findings by Valdes and coworkers (2012), who studied the nematode density before biofumigation and 6 weeks after. As also mentioned in their study, the increase in the density of free-living nematode taxa is most likely related to the incorporation of plant biomass, *i.e.* green manuring. Our results suggest that all free-living nematode taxa were able to benefit from this green manure within a timespan of weeks to months. Gruver et al. (2010) revealed that the quality of the plant material was major determining factor for the subsequent (> 6 weeks) effects of biofumigation on the nematode community. In their study, they used various *Brassica* species with distinct C/N ratios. Our experiment concentrated on the effects of various cultivars of a single *Brassica* species with consequently low variation in plant biomass and C/N ratios, and, hence, no differential, green manure-related effects were observed.

We ascribe the decrease of plant-parasitic nematodes after biofumigation (three weeks and two months) to the mere absence of host plants. This option is also considered in the study by Valdes et al. (2012), in which a decrease in potato cyst nematodes (PCNs) was observed 6 weeks after biofumigation with yellow mustard. However, encysted PCNs can survive for months if not years in absence of a host, and – more likely – this decrease was attributed to green manure related biotic suppression. Stirling et al. (2001) showed that the density of *Tylenchorhynchus* was very low in bare fallow soil and when planted with sugarcane could return to very high numbers. This indirectly suggests that a fraction of the population can survive without a host for a long period, and due to its short reproduction cycle it can re-establish high densities whenever a host is available again.

Conclusions

Our results demonstrate that 'biofumigation', the release of isothiocyanates due to incorporation of mulched plant material from four distinct *B. juncea* cultivars, did not directly affect plant-parasitic or free-living nematode populations. The observed short-term (day 1 after biofumigation) reduction in nematode densities was non-distinct from the effects of wheat incorporation, and therefore unrelated to *B. juncea*-derived GSLs. Also, direct application of relatively high concentrations of 2-propenyl ITC (up to around 480 mol ha-1 resulting in 195 nmol g-1 dry soil) did not result in shifts distinct from those observed in untreated wheat plots for the plant-parasitic and free-living nematode taxa under investigation. We therefore conclude that the observed direct and subsequent effects of biofumigation on the nematode community were mainly attributable to a combination of tillage and green manuring, and not to the effects of *B. juncea*-produced ITCs *per se*.

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Chapter 5

In comparison to its parental line and four other conventional cultivars, GM waxy starch potato has no distinct detectable effect on the soil food web as revealed by nematode community analysis

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Abstract

Since the first introduction of genetically modified (GM) crops in 1996, there has been intensive debate about the impact of this new and powerful technique on the environment. Soils constitute highly complex and biodiverse habitats, and – compared to aboveground parts of the ecosystem – little attention has been paid to possible adverse effect of GM crops on its ecological functioning. Here we investigated belowground impact of a marker-free GM potato (Solanum tuberosum, cultivar Modena) blocked in its amylose biosynthesis pathway. In a follow-up on studies concentrating on two organismal groups residing in the first trophic of the soil food web, bacteria and fungi (İnceoğlu et al. 2010, Hannula et al. 2012), we investigated possible side-effects of the waxy starch GM potato 'Modena' on higher trophic levels. For this, we concentrated on the free-living nematode community, as this organismal group is represented in multiple trophic levels. A field experiment was performed at two sites in which nematode communities were analysed from plots grown with the GM variety Modena, its parental conventional isoline, and four other conventional potato cultivars, each at two time points. Although quantitative PCR-based analysis of eight nematode taxa revealed clear location and sampling-time effects, no GM waxy starch potato-related changes were observed in the parts of the nematode community examined here. In combination with results from previous studies on other components of the soil food web, we tentatively conclude that the GM potato cultivar Modena has no observable GM-related effects on the soil food web during its growing season.

Introduction

Since the initial commercialization of genetically modified (GM) crops in 1996, the area planted with these crop varieties has increased from 1.7 million to 160 million hectares in 2011 (Privalle et al., 2012, James, 2011). At a worldwide scale, this acreage is asymmetrically distributed; about 2/3 of this area is located in USA, Brazil and Argentina (James, 2011). As a result of considerable public concern about the safety with of this new technology, the overall GM crop area in Europe is very small.

Before commercialisation, newly developed GM crops are subjected to risk assessment. Although the amplitude and stringency of these testing procedures differ per region and country, most schemes focus on the safety of such GM crops (*i.e.* harvested plant parts, its derivatives, and the remains) for use as food and feed for humans or livestock. In addition, GM crops are often tested for their possible adverse effects on the environment (Privalle et al., 2012). Such an environmental risk assessment may include the effects of GM crops on soil biota (Icoz and Stotzky, 2008).

Depending on the nature of the genetic modification(s), GM plant parts could affect biological soil functioning through changes in the plant's root physiology, root exudates, and litter quality (Powell, 2007). Several studies have focused on the direct effects of GM crops on bacterial and fungal communities, as main organismal groups that use GM crop exudates or remains as primary food source, during the growth season of GM crops (*e.g.* (Gschwendtner et al., 2011, Girlanda et al., 2008, Hannula et al., 2010, Hannula et al., 2012, Weinert et al., 2009). Although it is conceivable that subtle disturbances at the first trophic level of the soil food web might trigger more robust secondary effects at higher trophic levels, only few studies paid attention to effects of GM crops on higher trophic levels of the soil food web (Powell, 2007).

Due to their trophic diversity and differential sensitivities to environmental disturbances, the free-living nematode community constitutes an informative component of soil food webs in arable fields (Neher, 2001, Bongers, 1990). Qualitative and/or quantitative changes in the composition of nematode assemblages can be indicative for the impact of agricultural practices (Mulder et al., 2003), and for the differential effect of plant genotypes on soil biota. Changes in nematode communities are not only induced by crop rotation and/or changes in the composition of the plant community (Wardle, 2002) but can even be visible amongst varieties of a single crop species (Palomares-Rius et al., 2012).

Nematodes have occasionally been used as indicators for the impact of GM crops on belowground systems (*e.g.* Höss et al., 2011, Griffiths et al., 2006, O'Callaghan et al., 2008). The microscopic analysis of nematode assemblages from environmental samples by microscope requires a high level of taxonomic expertise and is invariably time-consuming. The recent development of molecular assays for the quantitative analysis of nematode communities based on taxon-specific sequence motifs in the small subunit of the ribosomal DNA (Vervoort et al., 2012) greatly facilitates the processing of large number of environmental samples in a relatively short time span.

In this study, we concentrated on the possible belowground side effects of a marker-free GM potato (*Solanum tuberosum*) cultivar modified in its starch production. "Marker-free" refers to the absence of a plant-transformation marker. Normally starch in potato consists of amylose and amylopectin in a ratio of about 1:5 (Broothaerts et al., 2007), but tubers of the

cultivar Modena (BASF Science co. GmbH, city, Germany), produce amylose-free starch. This was accomplished by the inhibition of the transcription of the granule-bound starch synthase (GBSS) gene, which is essential for amylose production. The resulting amylose-free starch, so-called waxy starch, is clearer, stickier and retrogradates slowly as compared to normal potato starch. These characteristics make waxy potato starch an attractive ingredient for a range of industrial applications. Because of the nature of this particular trait, the directed inhibition of a pathway in the carbohydrate household and the absence of a plant-transformation marker (De Vetten et al., 2003), its potential effects should be sought in changes in the plant's carbohydrate metabolism. This could have consequences for the composition and/or quantity of exudates released in the rhizosphere.

Our objective was to compare the nematode communities in the close vicinity of roots, stolons and developing tubers from various potato varieties, including the GM Modena and Karnico, the latter being parental line of Modena. In order to frame potential GM trait-related effects in the background of variation due to conventional genotype variation (Griffiths et al., 2007), we included four conventional potato cultivars, two with high and two with low starch content. Belowground effects were studied in two experimental fields at two time points during the growth season. Densities of individual nematode taxa were monitored using a recently developed quantitative PCR-based method (Vervoort et al., 2012).

Materials and Methods

Study sites and experimental design

The experiment was performed at two experimental fields ('VMD' and 'BUI'), located at approximately 10 km distance of each other in the province of Drenthe, the Netherlands. Soil from VMD was characterized as a sandy peat soil (organic matter: 26%, pH (H2O): 5.0) and the soil from field BUI as loamy sand (organic matter: 6%, pH (H2O): 5.0). The water retention ranged from 40-46% at VMD to 25-29% at the BUI location (Hannula et al., 2012). At each field, six different potato (*Solanum tuberosum*) cultivars were grown; two cultivars have relatively high growth rates and a low starch content: 'Premiere' (P) and 'Désirée' (D), four cultivars with a relatively low growth rate and high in starch content: 'Aveka' (A), 'Aventra' (Av), 'Karnico' (K), and an amylose-free potato cultivar named Modena (M). Each field consisted of four replicate plots per cultivar (24 plots in total), which were distributed using a randomized complete block design. Each plot contained 28 plants divided over four ridges.

Soil sampling and nematode extraction

At both experimental fields, soil samples were taken at two time points in the growth season, on July 12th (flowering stage) and August 15th in 2011(senescence stage). Per plot, a composite soil sample was taken, consisting of 16 cores (Ø 2 cm, depth 20 cm) taken from the four potato ridges in each plot, and was stored at 4 °C until further use. Nematodes were extracted from 100 ml bulk soil using an elutriator (Oostenbrink, 1960). Nematode densities were determined in the resulting nematode suspensions (100 ml, in tap water) by counting two subsamples (5 ml each) under a low magnification inverted microscope. Subsamples were poured back after counting and the total nematode suspensions were further processed for quantitative PCR (qPCR) analysis.

Nematode analysis

Nematode suspensions (n = 96) were used for DNA extraction and lysate purification. Purified DNA extracts were used as a template in qPCR using nematode taxon-specific primer combinations (for details see Vervoort et al., 2012). A microscopic analysis of the nematode biodiversity at the two locations (sampling time: July 2010, see Supplementary Table S1) was used to select the most informative taxon-specific PCR primer combinations. In total, 12 primer combinations specific for free-living taxa were selected for qPCR analysis of the samples. Of these 12 analysed taxa, four taxa, Anatonchidae, Dorylaimidae D3, Metateratocephalidae and Mylonchulidae, were present in less than 50 % of all samples and were not included in the full data analysis (Table 1).

Data analysis

Nematode densities were analysed using mixed linear models (using PROC MIXED of the SAS software system version 9.2, see (Littell et al., 2006)). With the exception of the total nematode densities, all data were transformed to obtain approximate normal distributions of residuals as required for valid statistical inference. Cephalobidae densities were square root-transformed; and all other nematode taxon densities were log-transformed. The log-transformation was applied after addition of a constant (0.5 in general, and 5 for Monhysteridae). The fixed part of the mixed model contained main effects of cultivar, location and time and their two-way interactions. The random part of the model consisted of random effects for blocks, individual plots, and residual error. In addition, because of the special interest in the comparison of the starch-modified potato (Modena) with the parental isoline (Karnico), the differences in nematode densities between the two groups were estimated and tested (by approximate t-tests), averaged over time and locations, and location-specific averaging over time alone, using tailor-made ESTIMATE statements of PROC MIXED within the larger mixed model.

Results

In a field experiment designed to detect possible environmental effects of the knock out of a granule-bound starch synthase (GBSS) gene, a gene encoding an essential enzyme in amylose biosynthesis, in potato, no significant differences in nematode densities (both overall and at individual taxon level) between the waxy starch GM Modena and the non-GM parental cultivar Karnico could be detected (t-test statistic for difference Karnico-Modena in total nematode density t30 = -0.28, P = 0.782; for individual taxa t-test statistic t30 ranges from -1.20 to -0.10, all P ≥ 0.238). In addition, no significant cultivar effects were observed for both total and taxon-specific nematode densities (Table 1).

The variables ‘sampling time’ and ‘location’ strongly affected both the overall nematode densities and the levels of several nematode taxa (Table 1). On the first sampling date, total nematode density was similar for both locations (Fig 1. and Table 2). Nematode density at the VMD location remained comparable over time, while at the BUI location total nematode density was lower on the second sampling date compared to the first (Table 2 and Fig. 1). The qPCR-monitored taxa included in this analysis encompassed five bacterivorous (B), one fungivorous (F), one omnivorous (O) and one carnivorous taxon (C). Although present in over

Table 1. Summary of ANOVA (Fdf and associated P values) for differences in total nematode density (100 g-1 dry soil, analysed by microscope) and the density of 8 individual nematode taxa (100 g-1 dry soil, analysed by quantitative PCR) between six different potato (*Solanum tuberosum*) cultivars. The belowground impact of the genetically modified, waxy starch cultivar Modena was compared with its parental line Karnico, and four other cultivars: Aveka, Aventra, Désirée, and Premiere at two locations and at two time points. Both main and interaction effects were determined based on mixed models fitted to the variables (see materials and methods, significant P values (< 0.05) in bold).

		Main effects						**Interaction effects**					
		Cultivar		**Location**		**Time**		**Cultivar*Location**		**Cultivar*Time**		**Location*Time**	
	Trophic[a]	$F_{5,30}$	*P*	$F_{1,30}$	*P*	$F_{1,41}$	*P*	$F_{5,30}$	*P*	$F_{5,41}$	*P*	$F_{1,41}$	*P*
Nematode density [M]		1.25	0.313	15.33	**0.008**	11.73	**0.001**	2.58	**0.047**	0.56	0.726	9.41	**0.004**
Achromadoridae	O	0.16	0.976	8.96	**0.024**	39.20	**<0.001**	0.89	0.499	0.09	0.993	1.17	0.286
Aphelenchidae	F	1.79	0.145	51.73	**<0.001**	7.48	**0.009**	1.01	0.427	1.80	0.134	15.51	**<0.001**
Cephalobidae	B	1.73	0.158	10.28	**0.019**	1.73	0.158	0.69	0.632	0.79	0.566	46.15	**<0.001**
Monhysteridae	B	1.51	0.218	0.46	0.524	1.58	0.216	0.34	0.884	1.09	0.382	1.34	0.253
Mononchidae M3	C	1.20	0.333	172.66	**<0.001**	1.05	0.310	0.95	0.463	0.47	0.798	0.67	0.418
Panagrolaimidae	B	0.31	0.905	0.16	0.704	1.42	0.240	2.39	0.061	1.89	0.116	0.09	0.764
Plectidae*	B	1.28	0.297	3.23	0.122	22.49	**<0.001**	0.15	0.978	1.26	0.302	27.72	**<0.001**
Prismatolaimidae	B	0.95	0.465	20.59	**0.004**	2.31	0.136	0.38	0.862	2.75	**0.031**	0.07	0.799

[a] Main trophic groups assigned primarily as in Yeates et al., 1993; B: bacterivorous, F: fungivorous, C: carnivorous, O: omnivorous.

[M] Total nematode density was determined by microscope prior to DNA extraction for qPCR analyses.

* Plectidae except the genus *Anaplectus*.

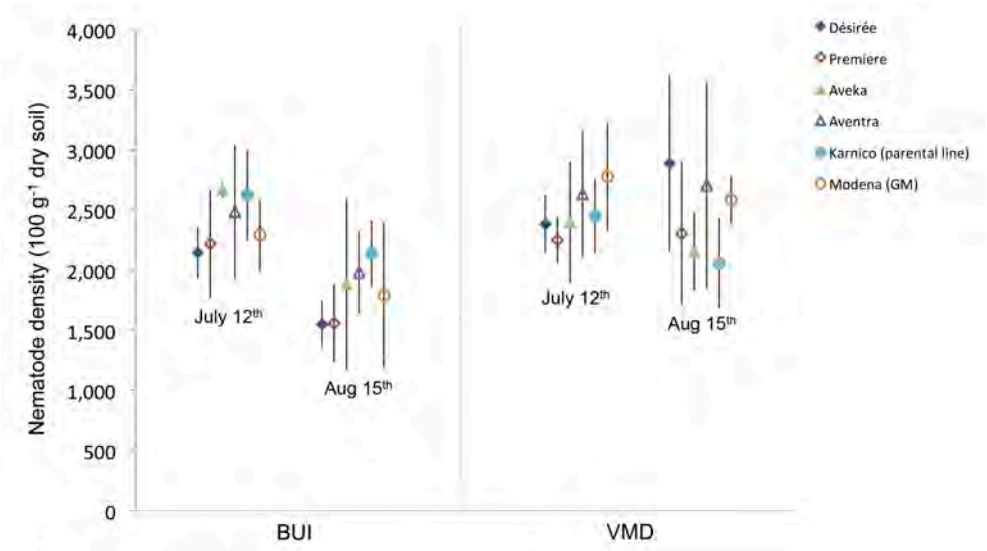

Figure 1. Nematode density (100 g-1 dry soil) in soil planted with six different potato (*Solanum tuberosum*) cultivars, including the genetically modified waxy starch variety Modena of one of the other five included conventional cultivars (i.e. Karnico), measured at two time points during the growing season (the 12th of July and the 15th of August) at two experimental fields (BUI and VMD).

50% of all samples (n = 96, both locations combined), the densities of the taxa Aphelenchidae (fungivore), Panagrolaimidae (bacterivore), Mononchidae M3 (predator) and Dorylaimidae D3 (omnivore) were relatively low as compared to the other taxa (on average below 10 individuals per 100 g dry soil)

both locations, the bacterivorous families Plectidae and Cephalobidae were the most abundantly and consistently present. 'Location' had a significant effect on densities of five out of the eight taxa covering all occurring feeding habits (Table 1): the densities of Achromadoridae (O), Aphelenchidae (F), Cephalobidae (B) were higher at the BUI location (Table 1 and 2, *P*-values: 0.024, <0.001 and 0.019, respectively), while the overall difference in (both time points combined) the density of the taxa Mononchidae M3 (C) and Prismatolaimidae (B) was higher at the VMD location (Table 1 and 2, *P*-values: <0.001 and 0.004, respectively).

Time of sampling had a significant effect on the densities of the taxa Achromadoridae, Aphelenchidae and Plectidae (Table 1). For the families Aphelenchidae, Cephalobidae and Plectidae, there was an interaction of time and location (Table 1). At both locations, Achromadoridae increased both in abundance (Table 2) and occurrence (BUI: from 76% to 97%. VMD: from 50% to 88%). As mentioned before, the predominantly fungivorous family Aphelenchidae was overall present in relatively low numbers and showed a decrease in abundance (Table 2) as well as occurrence (100% to 58%) at the BUI location and an increase in abundance (Table 2) and slightly in occurrence (38% to 50) at the VMD location. For the bacterivorous family Cephalobidae the average density decreased over time for Location BUI, but increased for

VMD (table 2), resulting in a significant interaction (P=<0.0001, Table 1). Another relatively abundant bacterivorous family, the Plectidae, showed a strong effect of time, and its density was higher at the second sampling time at both locations. This increase was much larger in location VMD than in BUI, resulting in a significant location*time interaction.

Table 2. Descriptive statistics (overall average ± standard deviation) of untransformed total nematode density (100 g-1 dry soil, analysed by microscope), and density of eight nematode taxa (100 g-1 dry soil, analysed by quantitative PCR) in two experimental fields (BUI and VMD) at which the belowground impact of the GM waxy starch cultivar Modena was compared to five other potato cultivars at two time points during the growth season. In addition, the percentage of samples in which the taxon was detected is given per location. Nematode taxa are defined as by (De Ley et al., 2006), except for Mononchidae M3 (see (Holterman et al., 2008)).

	BUI			VMD		
	July 12th	Aug 15th	%	July 12th	Aug 15th	%
Nematode density	2406 ± 380	1818 ± 453	100	2483 ± 391	2451 ± 586	100
Achromadoridae	41.3 ± 101.1	348.1 ± 683.2	87.5	4.2 ± 9.8	40.6 ± 56.2	68.8
Aphelenchidae	5.9 ± 4.3	2.8 ± 4.4	79.2	0.5 ± 0.7	0.9 ± 1.2	43.8
Cephalobidae	441 ± 150.7	150.4 ± 146.9	100.0	143.4 ± 123.2	232.5 ± 140.5	100.0
Monhysteridae	35.5 ± 50.4	33.9 ± 38.9	70.8	21.9 ± 17.8	68.6 ± 76.1	75.0
Mononchidae M3	0.5 ± 1	0.5 ± 0.9	29.2	6.1 ± 5.2	7.3 ± 4.6	97.9
Panagrolaimidae	0.8 ± 0.6	1.7 ± 2.8	75.0	0.8 ± 0.6	1.4 ± 2	62.5
Plectidae*	44.5 ± 40.8	111.7 ± 192.6	95.8	76 ± 150.4	1594.4 ± 2091.1	100.0
Prismatolaimidae	1.4 ± 2.9	3.1 ± 6.5	68.8	14.5 ± 22.5	58.9 ± 104.1	93.8

* Plectidae except for the genus *Anaplectus*.

Discussion

Introduction of genetically modified (GM) crop varieties is invariably preceded by an extensive risk assessment. Apart from testing the safety of resulting products on the end-consumers, humans or livestock, this risk assessment also includes the testing for possible adverse effects on the environment. In this study, we measured the belowground effect of a GM waxy starch potato cultivar, as compared to its untransformed parental line and four other conventional potato cultivars with different starch contents during their growing season. Nematodes are trophically diverse, and changes in the bacterial or fungal community will be observable as corresponding shifts in a subset of the bacterivorous and fungivorous nematode taxa. In this field experiment, densities of different nematode taxa were determined at two locations and two time points. Overall, neither a cultivar effect (taking all six cultivars into consideration), nor a genetic modification related shift (direct comparison between Karnico and the derived waxy starch cultivar Modena) was detected in the total nematode density or in the densities of the individual taxa. However, clear effects of sampling time and of location on densities of nematode taxa were observed.

We did not observe any effect of the plant genotype and genetic modification on the nematode taxa included in this study. This result corresponds to the outcomes of studies that focussed on

the effects on the microbial community in the same experimental fields (Hannula et al., 2010, Hannula et al., 2012b, İnceoğlu et al., 2010, İnceoğlu et al., 2011). In these studies, differences in the fungal or bacterial community between Karnico and Modena were either not significant or transient and fell within the range of variation observed for the other potato cultivars. For both, the major determining factors were growth stage of the plant and soil type, and not the genotypic constitution of the potato cultivars included in this research (Hannula et al., 2012, İnceoğlu et al., 2010). Hence, the currently presented nematode community composition data point at the same direction as previously presented data on the bacterial and fungal community suggesting that factors such as plant development and soil type affect the composition of the soil food web, whereas – within this particular set of six potato cultivars – the impact of plant genotype is not significant.

Here, two soil types representing the two types of starch potato production areas in The Netherlands were included. Overall, we observed a strong effect of location on the total nematode density and several of the monitored nematode taxa. The overall differences in nematode community composition between the two sites ('location effect') can almost fully be attributed to the difference in soil structure as the two other major determinants, temperature and precipitation / evaporation, hardly differed between the two experimental sites (distance between sites just 10 km, no natural barriers between sites). Although located relatively close to each other, the soils of the two experimental fields differed considerably in their organic matter (OM) content (VMD: 26% OM, BUI: 6% OM). In a survey on the impact of various crops (92 fields in North Carolina, USA) on the nematode community composition, no direct correlation was found between organic matter contents – ranging in these fields from 0.6 to 45.7% – and the relative abundances of trophic groups (Neher and Lee Campbell, 1994). Alternatively, the observed location effects could be attributed to differences in the soil particle sizes and water retention of the two soils, as nematodes are dependent on the water film between soil particles for their movement and reaching their food sources. (Neher et al., 1999) studied the effects of soil moisture content on nematode communities. Their results showed the genus *Prismatolaimus* to be consistently present in high moisture containing soils, which corresponds to our results. In the case of the family Cephalobidae, for which we see a similar pattern in our study, their study also showed a dependency on soil moisture content, however this effect interacted with the month of sampling (Neher et al., 1999).

The results of our field experiment confirm the relevance of the inclusion of multiple soil structures, and showed that neither of the two distinct nematodes assemblages was affected to a detectable degree by the nature of the potato cultivar and or genetic modification. Soil texture is one of the major determinants of the nematode community (*e.g.* Ronn et al., 1995), and possible community-disturbing effects of GM crops should therefore be investigated in the range of soil types relevant for the crop of interest. As we also detected effects of sampling time, it seems favourable to include different sampling times during the growing season when monitoring GM crop effects. In arable fields, seasonal effects such as plant development, rainfall or temperature can have an impact on the soil faunal composition (Wardle, 2002, Sohlenius and Boström, 2001).

The GM potato variety Modena had no detectable effect on the nematode community in our field study. It must be underlined that the results of this study concern the effect of a specific GM-trait, actually the knock down of a specific carbohydrate metabolism pathway,

and are not translatable to other modifications or crops. Within our experimental set up with two experimental fields and two sampling moments, only soil type and sampling time had a significant effect on the native nematode community.

Investigations on possible unwanted or unexpected side effect on soil biota including betaproteobacterial, fungal and nematode communities all indicate that this particular GM waxy starch potato variety has no measurable consistent GM-related effect on one of these organismal groups (İnceoğlu et al., 2010, Hannula et al., 2010, Hannula et al., 2012). Based on the relevance of these groups in terms of biodiversity, biomass and trophic connectedness, we tentatively conclude that the waxy starch GM potato cultivar Modena has no significant effect on the soil food web that could be related to its GM nature.

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Chapter 6

General discussion

Mariëtte T.W. Vervoort

General discussion

The use of indicators for assessing the biological functioning of ecosystems is common practice. Nematodes meet several requirements that make them suitable as a bio-indicator group of the soil ecosystem. In comparison to other major soil inhabitants such as bacteria and fungi, they are easily extractable from soil, occupy central positions in the soil food web and have differential sensitivities to both natural and anthropogenic disturbances. However, the microscopic identification of nematodes demands taxonomic expertise and a considerable investment of time. This thesis presents the results of the first field applications of a molecular tool for the quantitative and qualitative monitoring of nematode assemblages. It shows the impact on the nematode community of different types of disturbances, either directly or indirectly related to anthropogenic practices. In this chapter, several aspects concerning the molecular method, the use of nematode taxa as indicators and the challenges and future opportunities for this group of organisms in soil ecological research will be discussed.

A molecular approach to nematode community analysis

As described in Chapter 1, multiple molecular methods have been developed over time for the analysis of nematode communities (Chen et al. 2010). Even without the sequence technology available to us today, Van Der Knaap et al. (1993) demonstrated the value of molecular methods in nematode identification by showing the ability to distinguish species of handpicked individuals based on their produced band patterns using random amplification of polymorphic DNA (RAPD-PCR). Twenty years later, we have arrived at the point of being able to perform directed metagenomic studies using high-throughput sequencing (Porazinska et al. 2009). Nevertheless, data processing and analysis of these large (around 10,000 reads per sample) high-resolution datasets, takes a considerable amount of time. Although high-throughput sequencing delivers a vast amount of information, this type of data may not be required for monitoring studies and its laborious nature may at this stage form a practical limitation for the experimental set up. For monitoring studies, methods such as terminal restriction fragment length polymorphism (T-RFLP) (Donn et al. 2012) or a quantitative PCR (qPCR) based approach (Vervoort et al. 2012) may be more practical and will allow for more intense sampling schemes. In other words, the method of choice for nematode community analysis will very much depend on the underlying research question(s). Directed (T-RFLP and qPCR) and undirected (high throughput rDNA sequencing) have their inherent pros and cons and suitability will depend on the precision, resolution and scale required to comply with the aims of the study in which it will be applied.

Approaches based on quantitative PCR (qPCR) and Terminal-Restriction Fragment Length Polymophism (T-RFLP)

In this thesis, we monitored the nematode community using a qPCR-based approach. So far, most nematological studies using qPCR have designed probes for a specific species or a particular group and or required pre-selection and prescreening of environmental nematode samples (Jones et al. 2006, Toyota et al. 2008, Derycke et al. 2012, Green et al. 2012). Thereby these studies still relied, to a certain degree, on microscopic analysis. A distinctive property of the assays used in the studies presented in this thesis, is their development based on a relatively

versatile, phylum-wide SSU ribosomal DNA framework (Holterman et al. 2006, Van Megen et al. 2009), currently covering over 2,800 sequences. Both the availability of this sequence framework as well as the accompanying rDNA clone library (thousands of *Escherichia coli* clones harbouring nematode rDNA fragments) permits for the development and testing of taxon-specific qPCR assays. The rDNA framework is mined to define *in silico* taxon-specific DNA motifs and to identify close non-targets. It is noted that potential false positives are not necessarily taxonomically related to the target (see Chapter 1). Subsequently, relevant recombinant rDNA fragments are used to test the specificity of assays for which – in a first selection round – cloned rDNA fragments are selected (Holterman et al., 2008). In a second selection round, the most promising primer combination were tested on real nematodes. Primers developed and tested in this way are applicable for use in complex DNA backgrounds without the necessity of pre-selection or pre-screening. Although it is not a necessity, for the studies in this thesis we decided to determine the nematode diversity by microscope, as well. In this way we were able to select a set of assays covering the majority of the free-living nematode diversity at each location. In the meantime, we have developed approx. 70 taxon-specific (family, genus or species level) assays. In future studies, we can now use this set to perform a molecular pre-screening of nematode diversity and composition.

Next to qPCR-based assays, T-RFLP, a molecular fingerprinting method, has been successfully applied for the monitoring of nematode communities in field studies (Donn et al. 2012, Griffiths et al. 2012). For this method, DNA is extracted from soil samples and SSU rDNA is amplified using nematode-specific primers. This complex amplicon is then digested by taxon-specific restriction enzymes, resulting in fragments of different lengths. The relative abundance of each fragment size detected, portrays the abundance of a specific group of nematodes present in the initial sample. The identification of the peaks measured can be accomplished by the beforehand production of a location-specific sequence database; a strategy termed 'directed T-RFLP' (Donn et al. 2012). The necessity of producing location-specific sequence databases combined with the relatively complex sampling handling – amplified of nematode DNA is separated on a capillary sequencer upon restriction by a range of enzymes – could be considered as limitations of this method. Nevertheless, (Donn et al. 2012) showed that this method can reveal changes in the community related to agricultural practice.

The link between microscopic and molecular-based analyses

Our knowledge of nematode ecology is largely based on microscopically acquired data. It is therefore important to find a way to link results produced by a molecular method to those produced by microscope (Chen et al. 2010). However, every molecular method has its own efficiency and precision, dependent on factors such as the extraction efficiency, the occurrence of PCR bias or the precision of the calibration curves used. For microscopic analysis the composition of a sample is generally determined by the analysis of a subsample (often the first 150 adults in a mass slide) of the total community and consequential extrapolation. For a number of species, the scarcity of informative morphological characters for non-adult life stages, often these individuals are discarded from further analysis, introduces an error margin of its own. The direct comparison of data produced by different methods is therefore not as straightforward as it may seem. Nevertheless, it may be possible to qualitatively compare the results

obtained by different methods, for instance, in terms of their ability or sensitivity to detect certain changes or differences in a community. While determining which method is overall the best to use is difficult, at least in this way it will become clear what type of information each method can provide.

Nematode taxa as indicators for human-related disturbances

Especially since the start of soil cultivation, anthropogenic activities have had an impact on the aboveground and the belowground biodiversity and functioning of ecosystems (Bardgett and Hefin Jones 2012). Soil ecological research has in different ways contributed to our understanding of the consequences of these disturbances. For the soil ecosystem, the nematode community has been used as an environmental indicator of the impact of different types of disturbances directly or indirectly related to human practices such as tillage, pollution, climate change, invasive plant species, GM crops (Powell 2007). To a certain degree, all chapters in this thesis concern a different type of disturbance directly or indirectly related to human practices; recovery after prolonged use for arable farming (Chapter 2), the impact of agricultural management practices (Chapter 4), the effect of an introduced invasive plant species (Chapter 3), and the possible unintended effects of a GM crop (Chapter 5). These field studies were performed in both semi-natural (Chapter 2 and 3) and agricultural (Chapter 3 and 4) systems. Inherently, an agricultural system is exposed to disturbances of a different character and at a different frequency compared to a natural system. However, it is not only the nature and frequency of a disturbance that determines its effect, but also the pre-disturbance state or history of the soil in which it occurs (Grandy et al., 2012). In this section, I will first discuss the relative degree of impact by different types of disturbances on the nematode community and the soil food web. Finally, I will discuss the potential of individual nematode taxa as indicators for specific types of disturbances.

The impact of land-use change on the soil food web

In Chapter 2 of this thesis, we monitored the nematode community of an ex-arable field and its adjacent beech forest and showed that these differed considerably both spatially and throughout time. Apart from the differences in vegetation between the two areas, the soil pH as well as soil structure were distinct. The ex-arable field studied in Chapter 2 was previously included in a study focused on the succession and development of above and belowground communities after abandonment (*e.g.* Kardol et al. 2005, Van Der Wal et al. 2006, Holtkamp et al. 2008). For this purpose, the soil food web was monitored for a chronosequence of abandoned agricultural fields sharing the same soil type and located within a region in the Netherlands. The results of these studies showed that when a field is abandoned after a period of cultivation, there is an initial short period of change in the nematode as well as soil community. This short period of change is most likely related to the release of physical stress from agricultural practices, such as tillage (Kardol et al. 2005), which for instance in Chapter 4 but also in other literature (*e.g.* Fu et al. 2000) has been shown to have a strong effect on the nematode community. However, the chronosequence studies showed that in the decades following this response there is a difference in the speed of aboveground and belowground succession, with the development of the nematode community as well as the overall structure of the soil food web lagging behind

when compared to that of the plant community. Other studies concerning belowground recovery after cultivation have shown similar results (Buckley and Schmidt 2001). Overall, this demonstrates that effects of land use, the combination of *e.g.* liming, fumigating, fertilizing, tilling the soil, can have a considerably effect on an ecosystem that lingers over a period of decades. Hence, for the assessment of ecosystem recovery, the monitoring of aboveground succession only doesn't provide us with the full picture with regard to ecosystem recovery; inclusion of the belowground part – the soil ecosystem – is essential (Young et al., 2005).

Plant identity effects belowground

Plant species can differ in their effects on the soil ecosystem and its inhabitants. These differences cannot only be ascribed to variation between species in their nutrient uptake or their quantity of input into soil, but also to differences in the quality of the resource input, *e.g.* carbon to nitrogen ratio, lignin or cellulose content or due to differences in the production of different secondary metabolites (Wardle 2002). Next to the quantity and character of resource input and output, the presence of a plant species can also partially determine microclimate conditions, affecting *e.g.* soil moisture retention or soil temperature through their height and density (Ehrenfeld et al., 2001). For the nematode community, De Deyn et al. (2004) showed that plant identity is a determining factor; in this relationship resource quality has shown to be more influential than resource quantity.

Ehrenfeld et al. (2001) showed that invasive plants species can change soil properties and that their effects on the soil ecosystem differ from those caused by native plant species. Moreover, effects may persist after the invasive plants have been removed and these areas can thereafter remain more sensitive to reinvasion (Eviner et al., 2010). This ability of invasive plant species may contribute to their invasiveness and demonstrates the closeness of the relationship between above- and belowground communities. In Chapter 3, we studied the local effect of invasion by the plant species *Solidago gigantea* (Giant goldenrod, native in North America) above and belowground and observed habitat-independent effects on different groups of soil organisms. The observation that *Solidago gigantea* is able to locally alter nutrient cycling (Chapuis-Lardy et al. 2006, Vanderhoeven et al. 2006, Herr et al. 2007) could be related to the shift in the soil community that we observed. Apparently *S. gigantea* is to a certain degree able to change the soil food web in such a way that it benefits its own growth, potentially through the quality of its litter or root exudates and or through the exudation of secondary metabolites. The latter can be via *S. gigantea's* release of furanoid and acidic compounds into its rhizosphere (Weber and Jakobs 2005), which are potentially toxic for both native plants as well as present soil organisms. There are strong indications that allelochemicals can play a large role in the invasiveness of plant species. Studies in which activated coal was added to the soil to bind toxic compounds from an invasive plant species resulted in cover reduction of the invader (Kulmatiski and Beard 2006, Eviner et al., 2010). This exemplifies how recent insights in the process by which different invasive species can affect the soil ecosystem may lead to the development of strategies for limiting invasion (Baer et al., 2012). However, at this moment our general knowledge of plant-specific effects belowground is too limited for this purpose. Studies concerning invasive plant species may be a way to serve both the need for nature conservation strategies concerning invasive plant species as well as our need to learn more about plant species-specific effects on the soil ecosystem.

While in Chapter 3 we focused on the effect of an exotic plant species and compared it to native vegetation, in Chapter 5 the effects of different varieties of another exotic plant species in Europe were assessed, namely potato. Moreover, we included what may be considered as an exotic version of this exotic plant species, namely a genetically modified (GM) variety of a conventional potato cultivar, altered in its starch production. The variation of nematode communities under these varieties was monitored to see if the effects of the GM variety on the soil ecosystem differed from those of conventional varieties. In our study, no differences in nematode assemblages under all the included varieties of potato were observed, whether it being a GM, a low producing-, or a high starch-producing conventional cultivar. Naturally, these results cannot be generalized to other GM crops as the character of GM crop traits can be very distinct and should therefore be assessed for each modification individually. Moreover, our study was limited to a single growing season and the results can therefore not account for possible long-term effects.

Individual nematode taxa as indicators for specific disturbances

The nematode community has often been used to study the effects of different measures or disturbances in agricultural systems, such as fertilisation (Ferris and Bongers 2006), the effects of tillage (Fiscus and Neher 2002) or nematicides (Timper et al. 2012) etc. Nematode community indices are widely applied in most of these studies. The most commonly used nematode community indices are based on the so called *cp*-scaling (colonizer – persister), which distinguishes nematode families based on their life strategies, and – related to this – their sensitivity to stress (see Chapter 1). In this thesis, community indices were not applied and we focused mainly on taxon-specific responses to disturbances. This choice has two underlying reasons: firstly a number of families distinguished in classical nematological community analyses such as Rhabditidae, Nordiidae or the Qudsianematidae appeared to by para- and/or polyphyletic (*e.g.* Van Megen et al. 2009). The second reason is that the use of nematode indices typically involves the lumping of data, and generalizations concerning nematode life strategies. Already in 1996, Yeates and Van Der Meulen suggested that the elucidation of so-called "key populations", individual taxa for which the population size can be linked to specific soil conditions or processes, could be more valuable than the use of general nematode indexes. Several recent studies have started to work towards this elucidation by combining datasets and the application of statistical tools (Fiscus and Neher 2002, Zhao and Neher 2013, Zhao et al. 2013).

Zhao and Neher (2013) demonstrated the potential value of meta-analysis of existing data to elucidate consistently responding nematode genera to different types of disturbances. For example, their results (based on data from 7 studies) showed that the genera *Diphtherophora* (Clade 1, fungivore), *Prismatolaimus* (Clade 1, bacterivore) and *Tylenchorhynchus* (Clade 12, a plant parasite) were present in lower densities in standard cultivation fields compared to fields managed by using conservation tillage, suggesting these taxa to be more sensitive to physical disturbance. A high sensitivity of *Prismatolaimus* to physical disturbance in combination with a legacy of tillage may partially explain the contrast in the number of *Prismatolaimus* between ex-arable field and beech forest that we observed in Chapter 2. However, for *Tylenchorhynchus* we monitored short-term sensitivity to tillage and we did not see a significant decrease in abundance between right before and after the soil was tilled while most of the other taxa did

respond (Chapter 4). Zhao and Neher (2013) mention several discrepancies between their overall findings and the results of certain studies. These differences may originate from the variation in soil characteristics, crop types and weather conditions of the individual experiments that were grouped in order to have enough data to perform the analyses.

While Zhao and Neher (2013) show the potential of using meta-analysis studies to elucidate individual taxon indicators, their study also reveals its current limitations. Data of suitable studies is often analysed and presented in papers on the level of trophic groups, although the microscopic analyses often resulted in community characterisation at genus level. In addition, requesting raw data from authors may not always be efficient. The latter is illustrated by the study of Zhao and Neher (2013) in which they received the primary community composition data underlying 21 out of the 48 requested papers. Another factor that complicates data comparison is the fact that research groups often use different nematode extraction methods. Extraction methods can differ in their efficiency of extracting nematodes of different sizes and mobility and can also vary in their suitability for different soil types (Nagy 1996, Yeates and Bongers 1999). The variation introduced by different extraction methodologies may therefore complicate comparison of results from different studies. In the same way, future use of different molecular methods may also complicate the performance of meta-analysis studies and the identification of single taxon indicators for specific disturbances. To achieve this identification, standardised sampling, extraction and analysis methodologies within the nematological research community may increase the use and acceptance of nematodes as bio-indicators.

Challenges in nematode ecology: Scaling up and zooming in

By lifting the practical limitations of microscopic analysis, it is now possible to analyse the nematode communities of large numbers of soil samples at an unprecedented rate and, eventually, at a resolution of our choice. Producing elaborate datasets can reveal new information about nematode ecology as well as soil ecology in general. Nevertheless, our limited knowledge of for instance nematode feeding habits and spatial and temporal variation at high resolutions may hamper progress. It is therefore not only important to gain the possibility to scale up sample sizes (*e.g.* by using a molecular method) but also to zoom in on nematode ecology. Therefore, in this section, I will first discuss the necessity of high-resolution information on nematode feeding habits and the possibilities of molecular tools to contribute to this. Secondly, I will discuss the relevance of gaining more insight into the spatial and temporal patterns of different nematode taxa.

The assignment of nematode feeding types and differential intra-guild feeding preferences

Throughout this thesis, the occurrence or density changes of different nematode taxa are discussed on the basis of their assigned feeding habit or the results of observational studies. However, most feeding types assigned to nematode taxa are inferred from their morphology supplemented with fragmented experimental data and/or anecdotal observations introducing a degree of uncertainty with regard to their real food preferences (Yeates 2003). To illustrate this point the family Tylenchidae can be taken as an example. In soil samples members of this family harbouring five subfamilies and over 50 genera often constitute more than 30% of the

total nematode community (Ferris and Bongers 2006). A genus level their feeding habits are far from clear, and they are generally either assigned to be herbivores (algae, mosses, lichen or higher plants) or fungivores or both (Yeates 2003).

Next to the uncertainty that resides in the assignment of feeding groups, differential feeding preferences within a trophic group, *e.g.* the preference of nematode taxa for specific groups of bacteria or fungi as a food source, are often not defined. In this thesis, both the results from Chapter 2 and 4 show variation in the response of nematodes assigned to the same feeding guild, which is most likely indicative for differences in their food preference. So far, most studies concerning food preference of nematodes have been performed on medium plates in the lab (*e.g.* Bilgrami 1993, Ruess and Dighton 1996, Okada and Kadota 2003, Hasna et al. 2007). However, it has been shown that the results from feeding preference studies performed on agar plates can be different to those performed in soil (Okada and Kadota 2003).

Ideally, as was already stated by Yeates et al. in their overview of feeding habits in 1993, feeding habits and preferences of nematode taxa should be assessed per ecological setting. Even though today this task is still daunting, the application of molecular methods may be able to contribute to this goal. This can be done by a direct approach which is demonstrated by (Read et al. 2006), who studied the feeding behaviour of soil micro-arthropods by sequencing and performing PCRs on the gut content of individuals taken directly from field samples. For this approach, the use of PCR and next generation sequencing allow for identification of feeding associations with an unparalleled precision level and can thereby reveal trophic links which would have been impossible to uncover without molecular methods (Pompanon et al. 2012). Another indirect approach would be the associational divergence of information on feeding relationships from large datasets encompassing high-resolution measurements of groups of soil organism from multiple trophic levels.

Spatial and temporal variation of nematode taxa

Although spatial and temporal variation of aboveground populations has been elaborately studied, this is not the case for belowground communities (Ettema and Wardle 2002). For belowground organisms, progression in this field has been hampered by the inability to process large numbers of samples and distinguish communities of soil biota at high resolutions. Therefore, we are often unable to interpret this type of variation in data and are limited in our ability to extract the information it contains (Ettema and Wardle 2002). Nevertheless, spatial and temporal variation patterns could provide us with information on species coexistence, their relation to abiotic factors and differential sensitivities to disturbances (Levin and Paine 1974). In addition, the fundamental knowledge of the spatial and temporal heterogeneity of nematode taxa in different soils and systems will also improve sampling schemes of future studies. It may also reveal that, depending on the research question, certain nematode taxa are more suitable for use as indicators compared to others. Extensive sampling combined with molecular analyses of the nematode community has the potential to provide us with more insight into spatial as well as temporal variation. At this moment, our group is producing a dataset consisting of the output of molecular analyses of nematode taxa for over 1000 soil samples. With this dataset the patchiness (horizontal spatial variation) of free-living nematode taxa in arable fields will be determined for different soil types.

Conclusions

The results presented in this thesis demonstrate that the use of taxon-specific quantitative PCR-based assays is a suitable method for the monitoring of nematode assemblages in field experiments. This method has a number of advantages: no subsampling is required, analysis is life stage independent, and it does not require microscopic pre-screening or pre-characterisation of samples or sampling sites. The results of these field experiments show that even though changes in the plant community, such as the invasion by an exotic plant species, can have distinct effects on the soil food web, these changes, however, are small compared to the impact of land use practices. Furthermore, the results indicate that nematode genera belonging to the same trophic guild can respond differentially to natural and anthropogenic disturbances, and, moreover, this refined community analysis provides us with new ecological insights. The implementation of molecular methods for the monitoring of nematode communities allows the formation of large, high-resolution datasets, which will contribute to an improved understanding about nematode ecology and, thereby, about soil ecology in general.

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Supplementary data

Chapter 2 - Supplementary Table S1. Nematode densities (average ± standard error) in numbers of individuals per 100 ml elutriated soil at different times in a former arable field and its adjacent pristine beech forest. Feeding guilds are given in capitals: B: bacterivore, F: fungivore, FP: facultative plant parasite, O: omnivore, P: predator. The weeks are defined as number of weeks after March 17.

i	Teratocephalidae		Prismatolaimidae		Plectidae		Cephalobidae		*Anaplectus*	
	B		B		B		B		B	
Wk	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'
0	190±121	144±79	13±7	2245±1159	100±27	33±5	328±79	166±62	40±16	0±0
2	15±12	12±12	12±7	733±74	27±11	2±2	248±100	4±0	8±5	0±0
4	52±29	9±8	5±4	1031±302	55±31	3±1	232±35	39±35	37±11	0±0
7	5±5	45±33	3±2	1833±1833	44±41	13±12	222±172	51±30	12±10	0±0
9	35±30	13±6	11±6	2949±2598	55±54	0±0	139±49	93±82	49±22	0±0
13	0±0	0±0	17±16	881±311	0±0	63±63	5±1	83±67	21±19	0±0
15	0±0	0±0	128±98	494±375	0±0	0±0	889±610	6±5	261±141	0±0
17	0±0	0±0	2±1	710±169	0±0	0±0	157±35	5±1	40±25	0±0
19	36±13	1±0	4±2	1107±1107	41±22	0±0	62±33	10±8	22±22	0±0
21	148±83	270±267	96±28	2735±601	645±412	86±85	306±148	2±2	935±730	0±0
23	84±79	0±0	10±8	669±669	91±59	0±0	337±220	0±0	308±188	0±0
25	145±50	652±340	13±5	3039±1907	294±98	18±14	338±162	5±3	69±23	0±0
27	12±12	0±0	28±16	33±32	180±87	0±0	271±99	2±2	18±11	0±0
29	76±76	404±209	20±7	4±4	354±157	10±6	189±85	14±2	154±120	0±0
33	62±41	550±223	87±53	542±73	662±370	163±66	370±126	125±63	218±185	5±5
35	978±459	568±204	130±76	2077±861	942±270	298±118	524±157	94±30	38±24	0±0
37	552±459	115	12±8	845	506±263	96	279±122	34	42±16	0
39	252±180	496±195	126±53	1217±1123	1714±870	68±31	1298±342	53±36	203±126	0±0

ii	Alaimidae		Metateratocephalidae		Monhysteridae		Aphelenchoididae		Aphelenchidae	
	B		B		B		F/FP		F/FP	
Wk	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'
0	0±0	0±0	0±0	550±410	393±94	468±185	0±0	0±0	4±4	0±0
2	24±23	0±0	0±0	0±0	156±36	52±28	0±0	0±0	4±3	0±0
4	22±19	0±0	67±67	94±11	414±160	108±108	0±0	0±0	11±4	0±0
7	0±0	0±0	0±0	591±591	133±125	112±37	0±0	0±0	2±2	0±0
9	0±0	0±0	0±0	1121	436±125	2900±2677	0±0	0±0	0±0	0±0
13	0±0	0±0	32±32	115±83	51±49	0±0	0±0	0±0	0±0	0±0
15	0±0	0±0	0±0	8±8	2139±1736	60±40	0±0	0±0	18±18	0±0
17	0±0	0±0	226±226	423±26	32±14	218±218	13±13	0±0	0±0	0±0
19	0±0	0±0	0±0	587±384	62±41	289±99	0±0	0±0	4±4	0±0
21	53±17	38±38	7±7	322±321	724±400	1196±172	0±0	0±0	70±25	0±0
23	35±24	0±0	0±0	3±3	279±88	409±409	0±0	0±0	30±4	0±0
25	27±9	0±0	47±47	82±69	221±64	459±169	2±2	0±0	36±10	0±0
27	106±52	0±0	30±30	1±1	18±11	0±0	2±2	0±0	45±16	0±0
29	27±13	0±0	68±68	182±85	69±12	0±0	2±1	0±0	39±23	0±0
33	83±60	50±29	295±295	145±78	174±162	164±59	35±35	14±9	45±13	0±0
35	92±45	23±23	0±0	134±56	114±38	580±521	90±41	1±1	71±26	0±0
37	219±103	0	0±0	22	362±128	384	3±2	0	29±8	0
39	316±166	9±7	267±166	189±35	1114±445	128±128	35±19	8±6	55±15	1±1

iii	*Tylolaimophorus*		*Diphtherophora*		Dorylaimidae		Mononchidae		Mylonchulidae	
	F		F		O		P		P	
Wk	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'
0	8±8	101±87	1±1	0±0	1±1	6±5	0±0	0±0	0±0	0±0
2	81±78	82±82	1±1	0±0	6±6	0±0	0±0	0±0	0±0	0±0
4	8±7	19±18	1±0	0±0	3±2	8±8	0±0	0±0	0±0	0±0
7	0±0	5±2	9±6	0±0	0±0	6±6	16±12	0±0	0±0	0±0
9	40±40	12±6	26±15	0±0	0±0	4±3	9±7	2±2	2±1	4±4
13	0±0	721±669	0±0	0±0	0±0	0±0	4±2	0±0	1±1	1±1
15	189±189	250±7	8±6	0±0	0±0	0±0	3±3	0±0	1±1	0±0
17	0±0	590±554	6±6	0±0	10±10	0±0	17±12	0±0	1±0	9±3
19	3±3	119±87	2±2	0±0	108±85	0±0	2±1	0±0	0±0	1±1
21	4±4	157±149	36±21	0±0	349±141	182±182	22±14	0±0	0±0	0±0
23	22±21	21±2	19±5	0±0	43±43	5±5	43±37	3±3	0±0	0±0
25	0±0	114±13	43±30	0±0	74±74	37±28	48±44	0±0	3±3	0±0
27	15±11	8±8	36±21	0±0	8±8	60±60	1±1	0±0	0±0	0±0
29	261±150	1007±598	23±13	0±0	71±71	48±2	5±3	4±4	0±0	0±0
33	16±9	74±16	11±5	0±0	28±28	66±39	8±8	2±2	0±0	0±0
35	22±15	11±3	30±13	0±0	379±41	396±107	5±1	5±3	0±0	0±0
37	5±3	70	29±20	0	93±57	83	8±8	3	0±0	0
39	2±2	472±61	10±5	0±0	189±114	125±16	6±2	1±0	0±0	0±0

Chapter 3 - Supplementary Table S1. Study site locations and descriptions.

	No.	Site	Soil type	Coordinates	Year of introduction
Semi-natural grassland sites	1	Planken Wambuis	Pleistocene sandy soil	52^0 01' 45.64" N 5^0 47' 53.50" E	1982
	2	Reinaerde Den Dolder	Pleistocene sandy soil	52^0 08' 59.53" N 5^0 14' 35.14" E	2000
	3	Plantage Willem III	Pleistocene sandy soil	51^0 58' 48.62" N 5^0 31' 08.47" E	1995
	4	Hollandseweg Wageningen	Pleistocene sandy soil	51^0 58' 49.89" N 5^0 40' 59.84" E	before 2005
	5	Reijerscamp	Pleistocene sandy soil	52^0 00' 47.49" N 5^{0}46 '08.64" E	2006
Riparian vegetation sites	6	Duffeltdijk (Walloon)	Riverclay	51^0 51' 58.11" N 6^0 00' 35.47" E	~ 1950
	7	Center of Millingerwaard (Walloon)	Riverclay	51^0 52' 26.39" N 6^0 00' 31.46" E	~ 1950
	8	Ewijkse plaat (Walloon)	Riverclay	51^0 52' 47.36" N 5^0 44' 52.17" E	~ 1950
	9	Blauwe kamer West (Rhine)	Riverclay	51^0 56' 40.22" N 5^0 36' 19.90" E	after 1950
	10	Blauwe kamer East (Rhine)	Riverclay and sand	51^0 56' 32.56" N 5^0 37' 09.54" E	after 1950

Chapter 3 - Supplementary Table S2. Floristic composition of semi-natural grasslands at five sites, all with three plot-pairs composed of an invaded *Solidago gigantea* patch (I) and a corresponding adjacent vegetation (U) (see Table S1). All vascular plant species were recorded and valued on an ordinal scale, based on abundance or coverage (numerical transformation of Braun Blanquets' scale).

Site number	1						2						3						4						5					
Plot-pair number	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15	
State	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I
Plantspecies																														
Achillea millefolium	3		3		2		4		7		6	2	2				3													
Aegopodium podagraria							2	5		3		3																		
Agrostis capillaris	7		8		9		4		3		3		9	3	8	4	5		9	8	8		4	3	2		2			
Arrhenatherum elatius																					2									
Artemisia vulgaris							2	2		2		2	2		2		3	2												
Bromus hordeaceus															2															
Bryophyta spp.	7	9	3	2		4	9		9		9	9		8	7	7		7	4					5	8	9	9	9		
Calystegia sepium																					3	3	2	2						
Calluna vulgaris					3																									
Cardamine sp.				1	1	3							4	3		3		3									3			
Centaurea jacea							2		1			1																		
Cerastium fontanum												3			2										4					
Chenopodium album															1															
Cirsium arvense																									2	3	3	2	5	2
Cirsium vulgare																											1			
Conyza canadensis			2																								3			
Crataegus monogyna			1														1													
Crepis capillaris	3		3		2								2		5		2										2			
Cytisus scoparius													2		3		3													
Dactylis glomerata							7	4	1	2									3	2	6		2	1	2		1		2	
Daucus carota	2	2	2					2			1																			
Deschampsia flexuosa	3		3		4																									
Elytrigia repens	4				2					3	2								5	8	9	5	9	5		1	3		4	4
Epilobium sp.																											2			
Equisetum arvense									3	2	2	4																		
Fallopia convolvulus													2				2													
Festuca rubra					3	2			3			3							7						6		6		4	3
Filago vulgaris																											1			
Fragaria sp.																			3											
Galium aparine																						5								
Galium mollugo							4	2	2	3	1	3																		
Geranium molle	3		3		3						2		4		3		3	3												
Geranium pusillum													1				1												1	
Glechoma hederacea								3		3							3		5	8	3	2	2	2						
Gnaphalium sylvaticum																									2	2				
Hieracium aurantiacum													2																	
Hieracium sabaudum	1				2																									
Holcus lanatus	6		3		4	3							5	5	3	3	9	3	2		3						3		4	

Holcus mollis									2		3																					
Hypericum perforatum	2		2	2	3		1					2	3		2																
Hypochaeris radicata									1		2		5		2		2		2								5		3		2	
Jacobeae vulgaris	3				4				2		1		2				3										5	3	7	3	3	3
Leontodon autumnale													1				1															
Leucanthemum vulgare							2		2																						5	2
Lotus corniculatus					2																											
Lolium perenne																											5					
Luzula campestris													2																			
Malva alcea																											1					
Medicago lupulina									2																							
Myosotis arvensis	3				2																											
Oenothera sp.									3	1	3				5																	
Plantago lanceolata	7		2				4		4	2	5	2					1		1	1							2					
Phleum pratense subsp. *pratense*																													1		7	
Poa sp.								3							3	6	3					4		3								
Poa annua	3	3	3		4	2					2								4		3				4	2					4
Pteropsida spp.																		1												2		
Ranunculus repens							2																									
Rubus caesius			2	1			1		1			1																				
Rumex sp.																			3													
Rumex acetosa			2		2		3	2																								
Rumex acetosella	1						2		2		3		3		2		2															
Saponaria officinalis									1			2																				
Scrophularia nodosa	1		2	1																												
Sonchus arvensis																													2			
Solidago gigantea	2	9	2	9		9	2	9	2	9	1	9		9		9		9		9		9		9		9	2	9	2	9		9
Stellaria media									3	2			2				3												2			
Taraxacum officinale	2		2		3	2							1														3		3		2	2
Tragopogon pratensis															2																	
Trifolium arvense							1		2		6				2																	
Trifolium pretense									3																							
Trifolium repens	3				2						3	1															5		1			
Urtica dioica						1																2	1	3								
Verbascum nigrum												2			2	2																
Veronica arvensis	2		3														3												2		3	
Veronica officinalis	2		3										2																			
Vicia hirsuta	2		2		1	1									2	1					1											
Vicia sativa															2																	
Viola arvensis			1																										2			

Chapter 3 - Supplementary Table S3. Floristic composition of riparian vegetation at five sites, all with three plot-pairs composed of an invaded Solidago gigantea patch (I) and a corresponding adjacent vegetation (U) (see Table S1). All vascular plant species were recorded and valued on an ordinal scale, based on abundance or coverage (numerical transformation of Braun Blanquets' scale).

Site number	6						7						8						9						10					
Plot-pair number	16		17		18		19		20		21		22		23		24		25		26		27		28		29		30	
State	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I
Plantspecies																														
Achillea millefolium							2						2		2		2													
Achillea ptarmica					2																									
Agrostis stolonifera	3																													
Arctium lappa															1											1		7		
Arrhenatherum elatius					2																									
Artemisia vulgaris							2						2	2	4	2					2	2								
Brassica nigra			1	1												2	2		2	2	5		2	1	9		8		2	
Calamagrostis sp.							6	3	9	3	9	3													2					
Calystegia sepium			2	2	2																		3		2		9	3	2	2
Carex sp.	5	2	2		3	2																				1				
Cerastium fontanum			1														2													
Cirsium arvense	2	2	3		2						1				2	2		2	6		5	2	6	2	2	2	6	2	7	5
Cirsium vulgare																			2	2		1	1							
Conyza canadensis							1																							
Crataegus monogyna									2																					
Crepis capillaris							2																							
Cuscata gronovii																				2		1				1				1
Dactylis glomerata	2								3				7	3	8		7		2		5		3		5					
Daucus carota							2										2													
Elytrigia repens	5	4	9		9		7								6	5	5	3	8	6	7	5	9	5	8	5			7	
Epipactis sp.											1																			
Equisetum arvense			2			2																				2				
Eryngium campestre													8		5	2	2	2												
Euphorbia esula			1	2																										
Festuca rubra							7		2		2		9	2	8		8	2												
Galeopsis tetrahit																									3					
Galium aparine					2												1													1
Galium verum																3	4													
Glechoma hederacea	4	4	4	4	4		5	3	6		3	2	3	2	4	8	4	7	3	2			4	3	4	4	3	5	3	2
Heracleum sphondylium			2	1	1	1										2			2				2		1					
Holcus lanatus	3																													
Hypericum perforatum							1		1																					
Jacobeae vulgaris			2				3		2		2				4										1					
Lamium album																			3	2	6	2								
Lamium purpureum																					2	2								
Lycopus europaeus									1		2																			
Lysimachia nummularia	5	6																												
Lythrum salicaria	2				2	1																								

Matricaria recutita	1																															
Mentha aquatica	2		2		1		2		2																							
Odontites vernus subsp. *serotinus*	3		2																													
Pastinaca sativa											2																					
Persicaria amphibia	3		2		3																						1					
Plantago lanceolata	8		5				2		2						3		4															
Poa sp.																													3			4
Poa annua											3								8	4	4											
Potentilla sp.							2																2	2								
Ranunculus acris																										2	2					2
Ranunculus repens	2		2				3		2																							
Rubus sp.								1																								
Rubus caesius									2		6	3																				
Rumex sp.										3																	2		2			
Rumex crispus															1																	
Rumex obtusifolius																						2										
Senecio inaequidens																			2													
Solidago gigantea		9		9		9		9		9		9		9		9		9		9		9		9		9		1	9			9
Stachys palustris										1																		3		7		1
Stellaria media													2																			
Tanacetum vulgare													2				2	2													1	2
Taraxacum officinale	2	2																														
Trifolium pratense	3								1																							
Trifolium repens																			2													
Urtica dioica		2	4	2	3	3		3	2	2			2	3		2	4	4	3	5	3	2	3	3	7		6	2	5	3		
Vicia sativa									1								1															

Chapter 3 - Supplementary Table S4. Overall nematode diversity up to genus level of soil (depth: 25 cm) from five riparian sites ('Riparian') and five semi-natural grasslands ('Grassland') invaded by Solidago gigantea (microscopic analysis). The taxonomy of the nematode families is presented as according to (De Ley, Decraemer & Abebe 2006) and trophic groups are assigned as in (Yeates 1993). Plant parasitic nematodes are given in green. Genera marked by a q in the qPCR column were included in quantitative PCR analysis and for genera marked with both a q and r, a quantitative range was available (see Vervoort et al. under review for more details).

Genus	Family	Trophic	qPCR	Range	Riparian	Grassland
Achromadora	Achromadoridae	U			+	+
Acrobeles	Cephalobidae	B	q	r	+	+
Acrobeloides	Cephalobidae	B	q		+	+
Aglenchus	Tylenchidae	PP			+	+
Alaimus	Alaimidae	B	q	r	+	+
Amphidelus	Alaimidae	B	q		+	+
Amplimerlinius	Dolichodoridae	PP				+
Anaplectus	Plectidae	B	q	r	+	+
Anatonchus	Anatonchidae	P				+
Aphelenchoides	Aphelenchoididae	F	q	r	+	+
Aphelenchus	Aphelenchidae	F	q	r	+	+
Aporcelaimellus	Aporcelaimidae	P, O	q	r	+	+
Basiria	Tylenchidae	PP			+	+
Bastiania	Bastianiidae	B			+	+
Boleodorus	Tylenchidae	PP or F			+	+
Cephalobus	Cephalobidae	B	q		+	+
Cervidellus	Cephalobidae	B	q	r	+	+
Chiloplacus	Cephalobidae	B	q	r	+	+
Clarkus	Mononchidae	P	q	r	+	+
Coomansus	Mononchidae	P	q		+	
Coslenchus	Tylenchidae	PP			+	+
Criconematidae (Other genera)	Criconematidae	PP			+	+
Criconemoides	Criconematidae	PP				+
Cylindrolaimus	Diplopeltidae	B			+	+
Diphtherophora	Diphtherophoridae	F	q	r	+	+
Ditylenchus	Anguinidae	F or PP			+	+
Dorylaimoides	Mydonomidae	O				+
Dorylaimus	Dorylaimidae	O				+
Drilocephalobus	Osstellidae	B			+	+
Epidorylaimus	Qudsianematidae	O	q	r		+
Eucephalobus	Cephalobidae	B	q	r	+	+
Eudorylaimus	Qudsianematidae	P, O	q		+	+
Eumonhystera	Monhysteridae	B, S			+	+
Euteratocephalus	Metateratocephalidae	B			+	+
Filenchus	Tylenchidae	PP			+	+
Geomonhystera	Monhysteridae	B, S			+	
Globodera/Heterodera	Heteroderidae	PP				+
Helicotylenchus	Hoplolaimidae	PP			+	+
Hemicycliophora	Hemicycliophoridae	PP			+	+
Heterocephalobus	Cephalobidae	B	q	r	+	+
Laimydorus	Dorylaimidae	O				+
Lelenchus	Tylenchidae	PP			+	
Leptonchus	Leptonchidae	F				+
Longidorella	Nordiidae	PP				+
Longidorus	Longidoridae	PP			+	+
Malenchus	Tylenchidae	PP				+
Meloidogyne	Meloidogynidae	PP			+	+
Merlinius	Dolichodoridae	PP			+	+
Mesocriconema	Criconematidae	PP				+
Mesodorylaimus	Dorylaimidae	O			+	+
Mesorhabditis	Mesorhabditidae	B			+	+
Metateratocephalus	Metateratocephalidae	B			+	+
Microdorylaimus	Qudsianematidae	O			+	+
Mononchus	Mononchidae	P				+
Mylonchulus	Mylonchulidae	P			+	+
Nagelus	Belonolaimidae	PP				+
Nygolaimus	Nygolaimidae	P			+	+
Oxydirus	Belondiridae	PP, O				+
Panagrolaimus	Panagrolaimidae	B	q	r	+	+
Paramphidelus	Alaimidae	B	q	r	+	
Pararotylenchus	Hoplolaimidae	PP			+	+
Paratrichodorus	Trichodoridae	PP			+	
Paratylenchus	Paratylenchidae	PP			+	+
Paraxonchium	Aporcelaimidae	P, O			+	
Plectus	Plectidae	B	q	r	+	+
Pratylenchus	Pratylenchidae	PP			+	+
Prionchulus	Mononchidae	P	q	r	+	
Prismatolaimus	Prismatolaimidae	B	q	r	+	+

Genus	Family	Trophic	qPCR	Range	Riparian	Grassland
Pro/Mesodorylaimus	Dorylaimidae	O			+	+
Pseudhalenchus	Anguinidae	F				+
Psilenchus	Tylenchidae	PP			+	+
Pungentus	Nordiidae	PP, P, O			+	
Rhabditis	Rhabditidae	B			+	+
Rhabditophanes	Alloionematidae	B			+	+
Rhabdolaimus	Rhabdolaimidae	B			+	
Rotylenchulus	Rotylenchulidae	PP				+
Rotylenchus	Hoplolaimidae	PP			+	+
Scutylenchus	Telotylenchidae	PP			+	+
Sectonema	Aporcelaimidae	P/O				+
Seinura	Seinuridae	P			+	+
Steinernema	Steinernematidae	E			+	+
Teratocephalus	Teratocephalidae	B			+	
Theristus	Xyalidae	B, S or U			+	
Thonus	Qudsianematidae	P/O	q		+	+
Trichodorus	Trichodoridae	PP			+	+
Tylencholaimellus	Tylencholaimellidae	F			+	+
Tylencholaimus	Tylencholaimidae	F			+	+
Tylenchorhynchus	Belonolaimidae	PP			+	+
Tylenchus	Tylenchidae	PP/F				+
Tylocephalus	Plectidae	B	q	r	+	+
Tylolaimophorus	Diphtherophoridae	F			+	
Wilsonema	Plectidae	B	q	r	+	
Total # of genera					**79**	**73**

Chapter 4 - Supplementary Table S1. Estimation of the nematode diversity at genus level in the soil (upper 20 cm) of the experimental field based on two microscopic analyses of a mixed sample taken 44 days before sowing and on day 81 of the experiment. Genera further analyzed by microscope (m) or by quantitative PCR analysis (q) are marked. For genera marked with in the last column (r), also a quantitative range was available (for more details see Vervoort et al. 2012).

Family	Genus	Trophic group[a]	qPCR/Microscope	Range
Achromadoridae	*Achromadora*	O	q	r
Alaimidae	*Alaimus*	B	q	r
Aphelenchidae	*Aphelenchus*	F	q	r
Aphelenchoididae	*Aphelenchoides*	F	q	r
Aporcelaimidae	*Aporcelaimellus**	C		
Cephalobidae	*Acrobeles*	B	q	r
Cephalobidae	*Acrobeloides*	B	q	
Cephalobidae	*Cephalobus**	B	q	
Cephalobidae	*Eucephalobus*	B	q	r
Cephalobidae	*Heterocephalobus**	B	q	r
Diplogastridae	*Diplogaster**	B		
Discolaimidae	*Discolaimus*	C		
Dorylaimidae D1	*Mesodorylaimus*	O	q	r
Dorylaimidae D1	*Ecumenicus*	O	q	r
Dorylaimidae D3	*Thonus*	O	q	r
Mesorhabditidae	*Mesorhabditis*	B	q	r
Monhysteridae	*Eumonhystera*	B	q	r
Mononchidae M3	*Clarkus*	C	q	
Mylonchulidae	*Mylonchulus*	C	q	r
Neodiplogastridae	*Pristionchus*	B		
Panagrolaimidae	*Panagrolaimus*	B	q	r
Plectidae	*Anaplectus*	B	q	r
Plectidae	*Plectus*	B	q	r
Rhabditidae	*Cruznema*	B	q	r
Rhabditidae	Rhabditidae (s.l.)[‡]	B		
Belonolaimidae	*Tylenchorhynchus*	PP1	m	
Heteroderidae	*Heterodera*	PP4	m	
Meloidogynidae	*Meloidogyne**	PP4	m	
Pratylenchidae	*Pratylenchus*	PP3	m	
Trichodoridae	*Trichodorus*	PP1	m	
Tylenchidae	*Basiria*	PP2		
Tylenchidae	*Coslenchus*	PP2		
Tylenchidae	*Tylenchus*	PP5		

[a] B: bacterivorous, F: fungivorous, C: carnivorous, O: omnivorous, PP: plant-parasitic (1: ectoparasite, 2: epidermal cell and root hair feeder, 3: migratory endoparasite, 4: sedentary parasite, 5: algal, lichen or moss feeder).

*Genera only observed on day 81.

[‡] Family, not identified to genus.

Chapter 5 - Supplementary Table S1. Estimation of the overall nematode diversity at genus level in the soil (upper 20 cm depth) of the experimental fields BUI and VMD based on a microscopic analysis of two mixed samples (consisting of 20 randomly taken cores from each field) taken in July 2010 (one year before the experiment). Plant parasitic nematodes are shown in green. Genera further analysed by quantitative PCR analysis (q) are marked. For genera marked with in the last column (r), a quantitative range was available (for more details see Vervoort et al., 2012).

Family[a]	Genus	Trophic group[b]	qPCR	Range
Achromadoridae	Achromadora	O	q	r
Anatonchidae	Anatonchus	C	q	r
Anguinidae	Ditylenchus	PP		
Aphelenchidae	Aphelenchus	F	q	r
Aphelenchoididae	Aphelenchoides	F	q	r
Belonolaimidae	Tylenchorhynchus	PP		
Cephalobidae	Acrobeles	B	q	r
Cephalobidae	Acrobeloides	B	q	
Cephalobidae	Chiloplacus	B	q	r
Cephalobidae	Eucephalobus	B	q	r
Dorylaimidae D3	Thonus	O	q	
Hoplolaimidae	Helicotylenchus	PP		
Metateratocephalidae	Metateratocephalus	B	q	r
Monhysteridae	Eumonhystera	B	q	r
Monhysteridae	Monhystera	B	q	r
Mononchidae M3	Prionchulus	C	q	r
Mylonchulidae	Mylonchulus	C	q	r
Panagrolaimidae	Panagrolaimus	B	q	r
Paratylenchidae	Paratylenchus	PP		
Plectidae	Plectus	B	q	r
Plectidae	Wilsonema	B	q	r
Pratylenchidae	Pratylenchus	PP		
Prismatolaimidae	Prismatolaimus	B	q	r
Rhabditidae	Rhabditis	B		
Tylenchidae	Filenchus	PP		
Tylenchidae	Psilenchus	PP		
Tylenchidae	Tylenchus	PP		

[a] Taxonomy as according to De Ley et al., 2006 with exception of Mononchidae M3 and Dorylaimidae D3 (see Holterman et al., 2008).

[b] Main trophic groups assigned primarily as in Yeates et al., 1993; B:bacterivorous, F: fungivorous, C: carnivorous, O: omnivorous, PP: plant parasitic.

Summary

Soil performs numerous functions, which allow us to produce food and feed and provide us with clean freshwater. These functions rely on the high diversity of organisms residing in soils. Within the high complexity of the soil food web, nematodes, worm-shaped animals belonging to the phylum Nematoda, are an informative group for assessing the status of a soil-dwelling community due to their ubiquity, abundance and trophic diversity. Although nematodes also possess several other assets favourable for a biological indicator of soil ecosystems (*e.g.* easy extractability, differential sensitivities to disturbances, ecological interpretability), their microscopic identification demands a considerable amount of expertise and time because of their relatively conserved morphology. Hence, the use of a molecular method for the identification and quantification of nematode assemblages has the potential to lift practical limitations and allows for more intensive sampling schemes. The aim of the research described in this thesis was to assess the suitability of molecular taxon-specific assays, developed on the basis of a phylum-wide molecular framework of 2,400 full-length small subunit ribosomal DNA sequences, for the monitoring of nematode assemblages in field experiments. The method was applied to monitor the impact of different types of disturbances on the soil food web, *i.e.* agricultural practices, invasive plant species and the effects of genetically modified crop (potato).

The second chapter of this thesis presents the background of the molecular method and the results of its first field application. It demonstrates the suitability of this method for use in extensive field experiments and the results of this study reveal distinct seasonal fluctuations between nematode genera classified to belong to the same feeding type group. A distinct response of nematode genera within trophic groups – taxonomically diverse groups of nematodes having the nature of their main food source as a common denominator – was also observed in the study described in Chapter 3. In this chapter, a study was conducted to investigate the impact of an invasive plant species called Giant goldenrod (*Solidago gigantea*, native to North America) on the plant community as well as on different trophic levels of the soil food web. In addition to monitoring the nematode community, pH and fungal biomass were measured in plots invaded or uninvaded by Giant goldenrod in two contrasting habitats. The results revealed that, in addition to outcompeting native plant species, this invader also reduced pH and increased fungal biomass in the soil of both habitats. Based on the results concerning the nematode community, the impact on the soil food web seemed to be selective since the local increase of fungal biomass appeared to benefit only one fungivorous nematode lineage of the three present in the field. This suggests that invasion by Giant goldenrod only stimulates one part of the fungal community.

Contrary to the preceding chapters, the effects of different disturbances on the nematode community were studied in an arable setting in chapters 4 and 5. Chapter 4 describes a field experiment in which the impact of biofumigation, a pest control measure, on the nematode community was assessed. Biofumigation is considered as an alternative for the use of synthetic fumigants and entails the incorporation of mulched brassicaceous plant material, which, upon de-compartmentalisation, releases general biocides called isothiocyanates. In our experiment, these compounds as well as their precursors could not be related to the effects observed for the nematode community. Therefore, changes in nematode assemblages are more likely to be related to the intense mechanical disturbance and green manure – the addition of a large

quantity of fresh plant material to the topsoil – rather than the release of isothiocyanates from the plant material

Chapter 5 presents a field experiment in which the possible belowground side effects of a waxy starch GM potato, a genetically modified plant blocked in its amylose biosynthesis, were investigated. The nematode community was monitored during the growing season of this GM variety, its parental line and four other conventional potato cultivars in two experimental fields. Although we observed clear effects of location and time, no GM-related effects were observed on the nematode community. Our results, in line with previous studies concerning the microbial community, indicate there are no observable, non-transient effects related to this particular GM trait on the soil food web during the growing season.

Overall, the results presented in this thesis demonstrate that, first of all, the developed molecular approach is suitable as a tool for the quantitative monitoring of nematode assemblages in field experiments, and, secondly, how a molecular monitoring method based on nematode taxon-specific DNA motifs can be exploited to get new insights into the ecology of terrestrial nematodes and – more in general – into the ecological functioning of this obscure, highly biodiverse and poorly understood habitat below our feet.

Samenvatting

De bodem vervult talrijke functies die ons onder andere de mogelijkheid geven om voedsel te produceren en die ons toegang bieden tot schoon drinkwater. Deze functies zijn afhankelijk van de hoge diversiteit van organismen die in de bodem leven. Binnen de complexiteit van het bodemvoedselweb zijn nematoden, wormvormige dieren uit het fylum Nematoda, een informatieve groep voor het bepalen van de status van het bodemleven dankzij hun alomtegenwoordigheid, talrijkheid en trofische diversiteit. Hoewel nematoden nog meer eigenschappen bezitten die wenselijk zijn voor een biologische indicator voor bodemsystemen (bijvoorbeeld: gemakkelijke extractie uit grond, variatie in gevoeligheid voor verstoringen en ecologische interpreteerbaarheid van veranderingen), vereist het identificeren van nematoden met behulp van een microscoop een aanzienlijke hoeveelheid expertise en tijd. Dit laatste heeft alles te maken met de geconserveerde morfologie binnen dit fylum (*i.e.* ze lijken op elkaar). Om deze reden kan het gebruik van een moleculaire methode voor de identificatie en kwantificering van nematodengemeenschappen praktische beperkingen opheffen en het mogelijk maken om experimenten uit te voeren met intensievere monstername. Het doel van het onderzoek dat beschreven staat in dit proefschrift was om de geschiktheid van moleculaire taxon-specifieke assays, ontworpen op basis van een fylum-breed raamwerk bestaande uit 2.400 full-length small subunit ribosomal DNA sequenties, voor het monitoren van nematodengemeenschappen in veldexperimenten. De methode was toegepast om de impact op het bodemvoedselweb te bepalen van verschillende type verstoringen, namelijk agrarische toepassingen, invasieve plantensoorten en effecten van genetische gemodificeerde gewassen (aardappel).

In hoofdstuk 2 van dit proefschrift presenteer ik de achtergrond van de moleculaire methode en de resultaten van de eerste toepassing hiervan in veldcondities. Dit hoofdstuk demonstreert de geschiktheid van deze methode voor gebruik in extensieve veldexperimenten, ook laten de resultaten verschillen zien tussen de fluctuaties door het seizoen van nematoden genera behorende bij dezelfde trofische groep (gekenmerkt door hetzelfde type voedselbron). Een verschil in respons tussen genera van dezelfde trofische groep werd ook geobserveerd in het experiment beschreven in hoofdstuk 3. Dit hoofdstuk betreft een studie naar de impact van een invasieve plantensoort genaamd Late guldenroede (*Solidago gigantea*, afkomstig uit Noord-Amerika) op zowel de plantengemeenschap als op verschillende trofische niveaus van het bodemvoedselweb. Naast het monitoren van de nematodengemeenschap, werden pH en schimmelbiomassa gemeten in plots met en zonder invasie door Late guldenroede in twee verschillende habitats. De resultaten lieten zien dat deze invasieve soort, naast het verdringen van inheemse plantensoorten, lokaal ook leidt tot een lagere pH en hogere schimmelbiomassa in de bodem van beide habitat types. Gebaseerd op de resultaten van de nematodengemeenschap, lijkt het effect op het bodemvoedselweb selectief, omdat de toename van schimmelbiomassa enkel benut leek te worden door slechts een van de drie aanwezige fungivore nematode genera in deze locaties. Dit suggereert dat invasie door Late guldenroede slechts een specifiek gedeelte van de schimmelgemeenschap stimuleert.

In tegenstelling tot de voorgaande hoofdstukken, betreffen hoofdstuk 4 en 5 de effecten van verschillende verstoringen op de nematodengemeenschap in een agrarische setting. Hoofdstuk 4 beschrijft een veldexperiment waarin het effect van biofumigatie, een grondontsmettings-

methode, op de nematodengemeenschap werd bestudeerd. Biofumigatie wordt beschouwd als een alternatief voor het gebruik van synthetische fumigantia en betreft het incorporeren van fijngehakseld plantenmateriaal afkomstig van koolachtige gewassen (Brassicaceae) in de bodem. Bij dit proces komen stoffen vrij, isothiocyanaten, die een breed toxisch effect hebben.

In dit experiment gepresenteerd in hoofdstuk 4 werd geen relatie gevonden tussen de hoeveelheid isothiocyanaten of de uitgangsstoffen hiervan en de geobserveerde effecten van biofumigatie op de nematodengemeenschap. Deze effecten zijn waarschijnlijk gerelateerd aan de intense mechanische verstoring en de toevoeging van grote hoeveelheden plantenbiomassa aan de bodem, in plaats van het vrijkomen van isothiocyanaten van het plantenmateriaal.

In hoofdstuk 5 worden de resultaten gepresenteerd van een veldexperiment waarin gekeken is naar de ondergrondse effecten van een aardappelras dat genetisch gemodificeerd is om enkel zetmeel te produceren in de vorm van amylopectine (middels de blokkering van de synthese van amylose). In een veldexperiment werd de nematodengemeenschap gemonitord gedurende het groeiseizoen van deze GM variant, de uitgangs-cultivar en van vier andere conventionele aardappelcultivars in twee verschillende proefvelden. Hoewel we sterke effecten zagen van locatie en tijd, werden er geen observeerbare, consistente effecten waargenomen in relatie met dit specifieke GM kenmerk gedurende het groeiseizoen.

De resultaten gepresenteerd in deze dissertatie tonen aan dat de ontwikkelde moleculaire methode geschikt is voor het kwantitatief monitoren van nematodengemeenschappen in veldexperimenten. Daarnaast toont dit onderzoek dat de toepassing van moleculair monitoren gebruikt kan worden voor het vergaren van nieuwe inzichten wat betreft de ecologie van terrestrische nematoden en – in het algemeen – in het ecologische functioneren van de obscure, moeilijk te doorgronden, hoog biodivers habitat dat zich onder onze voeten bevindt.

Curriculum vitae

Mariëtte Theodora Wilhelmina Vervoort was born on the 23rd of May 1986 in Nijmegen, The Netherlands. She holds a MSc. in Plant Biology from Utrecht University. During her studies at Utrecht, she specialized in plant pathology and microbiology. In August 2008 she started her PhD at the Laboratory of Nematology, Plant Science group at Wageningen University. In May 2013 she started teaching at this department.

Acknowledgements

A PhD programme is often described as a road. In my case, I would like to take that comparison a step further and compare my PhD to the A12 between Utrecht and Wageningen. My relationship with the A12 is quite intense; I work next to it, I drive it every day and I live only 500 m away from it. Both cars I have driven since I started working in Wageningen (magnificent Toyota Starlets) probably know every bump and corner of the A12 by heart now. From the start to the end of my PhD, there has been a lot of construction work on this road, leading to traffic jams and stricter speed limits. Nevertheless, this work has finally resulted in an extra lane for the whole distance and, in the end, a smoother ride. So, yes, although kind of a cliché, I do believe I can compare my PhD to a freeway. Fortunately, there is one big difference between the two, because my metaphorical PhD car was always filled with all these people, without whom, I would never have made it to the end.

First of all, I would like to thank the people that contributed to my thesis. I would like to thank my supervisors, Hans and Jaap. Hans, my daily supervisor, thank you for your help with bending my often-chaotic thoughts into straight lines and for always remaining optimistic and calm. Jaap, thank you for our talks and the trust you have bestowed upon me in my new job. Sven and Paul, my two paranymphs, I am really grateful for your help and advice over the last years and I couldn't imagine doing this thesis without the two of you. Also, equally important, we had a lot of fun and I can't even start to count the times you guys made me laugh to tears. I would also like to give a special thanks to the most modest person I know, Hanny. You are at the basis of everything we do in our subgroup at Nematology. I am very happy to have had the opportunity to work with you just before you retired and that I still get to visit you afterwards. Casper, my former masterstudent and current colleague, your high energy level and ever-present optimism have been infectious and made working on our 'top-project' really enjoyable. Even after having listened to the trololo-man for more than three hours straight! I am also incredibly grateful to Arie and Christian. A memorable moment was when I got stuck with my boots in our completely rain-drenched experimental field in Münster while Arie was laughing in another plot as I almost fell facedown in the mud. Arie, apart from that single occasion, you have been a rock to me, always willing to take a look at my work and asking me how things were going. I can't say it enough; thank you! The same holds true for Christian; you helped me through the most difficult part of my PhD. I have really enjoyed the days at the RIVM, drinking liters of coffee and puzzling with the data. Another person I would like to thank is Karst: thank you for all the fun and good times while preparing and working on our field experiments together. Those long days of taking soil samples for hours on end have left me with really nice memories. In addition, I would like to thank Ellis and Ron for their advice and contributions to the work presented in this thesis. I would also like to thank Johannes and his colleagues at the Julius Kühn Institute, who made it possible for us to perform the experiment in Münster. Johannes; thank you for your help with the manuscript and for always answering my question-filled emails. Gerrit Gort, I really appreciate your help during the last year. Wim van der Putten, thank you for your advice and support throughout my PhD.

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PE&RC PhD Training Certificate

With the educational activities listed below the PhD candidate has complied with the educational 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 (5.6 ECTS)
- Glucosinolate profiles in different Brassica species (2008)
- Nematode community shifts (2008)
- GM crop risk assessment studies for soil ecosystems (2010)

Post-graduate courses (3 ECTS)
- Soil ecology: linking theory to practice: PE&RC (2010)
- Sampling in space and time for survey and monitoring of natural resources: PE&RC (2012)
- Introduction to R for statistical analysis: PE&RC (2012)

Laboratory training and working visits (4.5 ECTS)
- Barcode-based monitoring of nematode communities; BLGG (2008, 2009)
- Field experiment; Julius Kühn Institut, Münster (2010)
- Statistical data analysis; RIVM (2009-2012)
- Sample analysis; NIOO (2011)
- Nematode behavioural models; AMOLF (2012)

Invited review of (unpublished) journal manuscript (1 ECTS)
- Plant and Soil: hyperaccumlators and their associated nematodes (2012)

Deficiency, refresh, brush-up courses (3 ECTS)
- Nematodes in environmental studies/identification course (2009)
- Food web ecology; lectures

Competence strengthening / skills courses (4.5 ECTS)
- PhD competence assessment: PE&RC (2009)
- Afstudeervak organiseren en begeleiden; DO, WUR (2009)
- Career perspectives ; WGS, WUR (2011)
- Coaching of ACT groups; DO, WUR (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
- PE&RC Weekend (2008)
- PE&RC Day: expect the unexpected (2008)
- PE&RC Day: intelligent nature (2009)
- PE&RC Day: selling science (2010)
- PE&RC Day: innovation for sustainability (2011)

Discussion groups / local seminars / other scientific meetings (5 ECTS)
- Current themes in ecology: Plants-Insects-Microbes: an ecological dance for three (2008)
- Discussion group meetings: Experimental Evolution; Ecogenomics; Plant-Soil interactions (2008-2012)
- EPOS Meeting, interactions between plants and biotic agents; with presentation (2009)
- National Ecogenomics Day; with presentation (2009, 2010)
- Presentation at the Julius Kühn Institut Münster (2009, 2012)
- NOW ERGO Meetings; with presentation (2009, 2010, 2012)
- Congres Biodiversiteit: kansen voor een nieuwe economie (2010)
- Presentation at the RIVM, LER meeting (2010)

International symposia, workshops and conferences (5.1 ECTS)
- Society of Nematologists annual meeting; Portland, Oregon (2011)
- Nematodes as bio indicators; presentation: impact of four Indian mustard cultivars with distinct glucosinolate levels on free living and plant parasitic nematodes (2012)

Lecturing / supervision of practical's/ tutorials (3 ECTS)
- Introduction environmental studies (2008- 2011)
- Biology and management of plant pathogens insects and weeds I (2009)
- Food web ecology (2009)
- Nematology (2010)
- Ecological aspects of biological interactions (2010, 2011)

Supervision of a MSc student (3 ECTS)
- Belowground impact of invasive plant species *Solidago gigantea*

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Covering Ground: Insights into Soil Ecology by Molecular Monitoring of Nematode Assemblages

Mariëtte T.W. Vervoort

Thesis
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In the presence of the
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There's a big dark town
it's a place I've found
there's a world going on
underground.

- Tom Waits, Underground, 1983

Contents

Chapter 1

General introduction

Mariëtte T.W. Vervoort

Introduction

Soil biota: diverse and important

Soil is of vital importance for our existence. It is essential for, among others, food production, water purification and carbon cycling. Less known is the fact that an immense diversity of soil organisms plays a key role in allowing soil to perform these functions. It takes just a stroll through a nature reserve and a cornfield to get an idea of the differences in biodiversity of plants and animals aboveground, but how would you assess the diversity of the invisible life below your feet?

Simply taking a scoop of soil in your hands will allow you to feel its structure, moistness and estimate its organic matter content, but little divulges the immense biodiversity of organisms residing in it. In fact, in most terrestrial systems, the largest fraction of the diversity of organisms can be found belowground (Wardle and Giller, 1996). Soil biota not only consist of the generally known earthworms and ants, but also for instance tardigrades, collembolans, mites, protozoa, nematodes, fungi, as well as thousands of bacteria species (Brussaard et al., 1997). This diversity is unsurprising considering the high heterogeneity of niches, provided in soil by factors such as soil structure, moisture, organic matter content and composition, pH, and the presence of plant roots, and which may vary on both field- and micro scale (Killham, 1994).

The basis for soil ecological research dates back to the 19th century, in which the first textbooks on different groups of soil biota were published (Whalen and Sampedro, 2010). These first studies were mainly of a descriptive nature and only focused on one specific species or group:

"…if a fallen bough be examined, a heap of moss shaken over a pocket-handkerchief, or any long herbage swept with a hand-net, the naturalist will not fail to find, together with numerous beetles, flies, and other insects, certain delicate, hexapod, active little creatures…"

(Fragment from Collembola and Thysanura by Sir John Lubbock, 1873)

"*Even on the same field, worms are much more frequent in some places than in others without any visible difference in the nature of the soil*."

(Fragment from: The Formation of Vegetable Mould: Through the Action of Worms by Charles Darwin, 1881)

Subsequently, most studies involving soil biodiversity were conducted from a plant-pathological perspective. However, from the 1970s onwards, the important role of soil biota in vital ecosystem processes (*e.g.* decomposition, nutrient cycling, water remediation etc.) became more and more apparent (Wall and Moore, 1999, Powell, 2007, Brussaard et al., 1997). Before the discovery of DNA and the development of molecular techniques, the inability to culture and identify a large part of all soil organisms restricted the knowledge of the actual diversity in soil (Fitter et al., 2005). By having these tools at our disposal, we are able to make better estimations of the virtually inconceivable diversity of soil life.

Although the diversity, abundance and importance of soil biota have become clearer over time, the exact roles of most species in soil functioning have not (Fitter et al., 2005). Soil is still often referred to as the black box, not only because of the diversity of its inhabitants, but also

due to the complexity of the interacting processes that take place in it (Cortois and de Deyn, 2012, Andrén et al., 2008). As soil functioning lies at the base of our daily needs, we require a better understanding of the dynamics and functional diversity of key players in soil processes. This will allow us to better assess the consequences of human disturbance (*e.g.* intense agriculture, forest disturbance), invasive species, climate change, and will help shape future management (Wall and Moore, 1999).

The soil food web and environmental indicators

Similar as for aboveground ecosystems, the soil ecosystem runs on nutrients provided by primary producers, *i.e.* plants. Nutrients can enter the soil system directly via plant roots (living plant material) but also, for the largest part, as detritus *i.e.* dead organic material (Cyr and Pace, 1993). In turn, nutrients are made available again for plant uptake through a cascade of feeding relationships between different soil biota, which is as a whole referred to as the soil food web (Fig 1.). In figure 1, an example is given of a soil food web, which is divided into four trophic levels. A trophic level can be defined as a group of organisms that obtain the majority of their energy by feeding upon the adjacent trophic level, which is nearer to the energy source (Hairston Jr and Hairston Sr, 1993). The first trophic level (TL1) of the soil food web contains organisms feeding on living plant material (*e.g.* plant parasitic nematodes), as well as saprotrophic fungi and bacteria, the so-called primary decomposers, which are able to break down detritus. The second level (TL2) consists of the secondary decomposers (*e.g.* nematodes, collembolans, amoebae, flagellates and mites), which feed upon fungi and bacteria and are themselves in turn eaten by predaceous soil organisms (*e.g.* predaceous nematodes, mites or collembolans), which are at the highest trophic level of the food web (TL3). The presence of higher trophic levels in the soil food web is necessary for nutrient cycling, since fungi and bacteria, in the first layer, partially retain nutrients for their own metabolism and growth. For instance, soil biota from higher trophic levels generally contribute around 30-40% of total nitrogen mineralization in soil (Verhoef and Brussaard, 1990).

Because of the soil food web's mayor role in soil functioning, it can provide us with information on soil functions; such as decomposition, nutrient cycling, primary production or disease suppression and on the status of the soil; such as the effects of agricultural practices, or its state of succession (Wurst et al., 2012, Holtkamp et al., 2008, De Deyn et al., 2003, van Capelle et al., 2012, Mulder et al., 2003). To obtain information, it would be ideal if we could measure all the different components of a soil food web. However, as illustrated in the last paragraph, the diversity of organisms in soil is immense and their interactions, with each other and with abiotic factors, introduce a high degree of complexity. To acquire data on the soil food web and soil processes in a more feasible way, indicator groups are often selected. Soil faunal communities (from TL2 and TL3) are frequently used as indicators for soil status. Not only because they reflect the state of their food sources (TL1 and or TL2), but also because they have longer generation times (days to years) than soil microbes (hours to days), making them a more stable group to monitor (Neher, 2001). So far, regularly used indicators include collembolans (Kopeszki, 1997), arthropods (Van Straalen, 1998), mites (Gormsen et al., 2006) and nematodes (Neher, 2001).

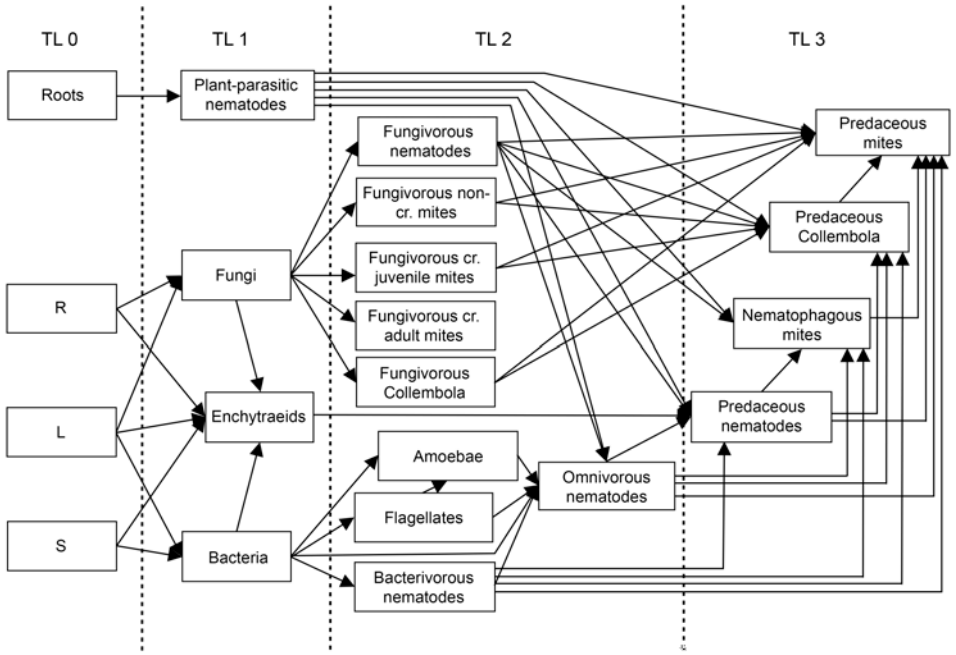

Figure 1. An example of a soil food web presented as a diagram (Holtkamp et al., 2008) in which trophic connections are indicated by the arrows (arrows are pointed towards the consumers). TL: trophic layer, R: recalcitrant organic matter, L: Labile organic matter and S: soluble sugars, cr. cryptostigmatic (now known as orbatid).

Nematodes as environmental indicators

Nematodes are small (mostly between 0.2-2.5 mm in length), worm-shaped animals that taken together constitute the phylum Nematoda. This phylum, which is thought to have arisen during the Cambrian explosion (600-550 million years ago), belongs to the superphylum Ecdysozoa that encompasses all moulting animals (Aguinaldo et al., 1997). Nematodes are present in terrestrial systems, but also in freshwater and marine habitats (Bongers and Ferris, 1999). Next to their ubiquity, nematodes are abundant and can reach densities of up to millions per square meter in soil, residing in plant roots or the water films attached to soil particles (Platt, 1994). Due to their abundance and size, nematodes are easily extractable from soil, when compared to for instance fungi and bacteria. The phylum Nematoda encompasses a high trophic diversity; nematodes may feed upon bacteria, fungi, protozoa, algae, other nematodes or in the case of omnivores on a combination of the aforementioned, or they may be facultative or obligate plant or animal parasites (Yeates, 1993). Due to this diversity in feeding habits, nematodes can be found at all three levels of the soil food web (Ferris et al., 2001).

Next to their central role in the soil food web and easy extractability, the differential responsiveness amongst nematode taxa to disturbances makes them a suitable indicator group for the status of soil (Neher, 2001). Multiple studies have shown that nematode taxa are differ-

entially sensitive to stressors such as desiccation, toxic compounds, heavy metals (Nagy, 2009) and agricultural practices such as tillage or the application of fertilisers (*e.g*. Fiscus and Neher, 2002, Ferris and Bongers, 2006). In short, it is the combination of the functional and taxonomic diversity within this phylum that makes it so suitable as an indicator (Ferris and Bongers, 2009). Based on this, several nematode-specific community indices have been developed over time. The use of community indices can facilitate the interpretation of elaborate data sets, by condensing information into a single or several variables.

An important step towards the development of indices specific for nematode communities has been the assignment of a so-called colonizer-persister value (*cp*-value) to different nematode families (Bongers and Ferris, 1999). In this classification, ranking from 1-5, families within the same *cp*-class share similar life strategy characteristics such as life cycle length, the number and size of their eggs and the size of their gonads. Families that are the least sensitive to disturbance are assigned the lowest values (1-2) and are defined as colonizers (r-strategists). Typically, these are small bacterivorous nematodes with a short generation time and the ability to produce large numbers of offspring. At the other end of the scale (4-5) are the persisters (K-strategistst); sensitive to disturbance, usually large and often predators or omnivores with a long generation time and low numbers of offspring. Based upon this *cp*-classification, the so-called Maturity Index (MI), one of the most frequently used nematode indices, can be calculated (Bongers, 1990). The MI is expressed as the mean frequency of the *cp*-classes found in a sample and in reflects in this way the degree of disturbance of the community or, in other words, the state of ecological succession.

Over time, several nematode indices were developed for different purposes and the existing indices refined (Ferris and Bongers, 2009). Similar to the maturity index, these indexes focus on the inferred ecosystems functions portrayed by the community rather than its structure. Based on this principle, nematode taxa were categorized into so-called functional guilds. In these guilds, both the life strategy (*cp* classification) as well as the feeding habit (also referred to as trophic group) of a taxon is incorporated (Bongers and Bongers, 1998). Examples nematode-specific indices based on this functional guild division are the Enrichment Index (EI) and the Structure index (SI), which include a weighting of functional guilds based on their indicative value for enrichment or the food web structure, and the Channel Index (CI), which distinguishes between the energy flow through the bacterial and the fungal decomposition channels based on the relative abundance of fungivorous and bacterivorous nematodes (Ferris and Bongers, 2009).

Exploitation of a SSU rDNA framework for nematode identification

Nematodes are morphologically highly conserved. From an evolutionary perspective one might say that the saying 'never change a winning team' is applicable for this phylum. Therefore, as the number of informative morphological characters is limited, identification of nematodes is challenging and requires a considerable amount of experience and expertise. For an untrained eye, it is very difficult to distinguish species in an environmental sample (Fig 2). Nevertheless, the soil from one site can contain over 200 species (Boag and Yeates, 1998). Even for experts, their ability to identify certain species depends on the life stage or sex of the present individuals (Floyd et al., 2002).

Traditionally, nematode identification of environmental samples is performed using a light microscope. In general, a subsample of around 150 individuals (of typically around 2,000 individuals in a sample) is identified which can take an expert up to several hours (*e.g.* Sánchez-Moreno et al., 2006, Ferris et al., 1996, Yeates et al., 2000, Boutsis et al., 2011). In the meantime, the number of experts with the ability to perform the identification is declining rapidly over the years (Coomans, 2002). In a way, this phylum's major asset as an environmental indicator, *i.e.* its diversity, is at the same time its pitfall; making nematode community analysis time-consuming and dependent on the availability of the necessary expertise. It is therefore not surprising to see that the use of molecular techniques for the identification and quantification of nematodes has gained interest over the last years (*e.g.* Powers, 2004, Floyd et al., 2002, Jones et al., 2006, Griffiths et al., 2006).

The DNA sequence of a single gene (typically containing hundreds or thousands of nucleotides) can deliver a multitude of objective characters. Different purposes, *e.g.* phylogenetics (deep or shallow) or molecular identification, require different types of information in the form of resolution and variability. Because the type of information a gene provides is related to the rate at which a gene evolves, not every gene is equally suitable for a certain purpose. For nematodes, the small subunit ribosomal DNA (SSU rDNA) gene has been often used in the past for resolving both deep as well as shallow phylogenetic relationships (Blaxter et al., 1998, Aleshin et al., 1998, Holterman et al., 2006, Van Megen et al., 2009). The SSU rDNA gene codes for SSU rRNA (18S), a part of the ribosomal RNA, and is essential for protein synthesis. Because of its vital importance, the SSU rDNA gene is highly conserved in most animals. Nevertheless, in nematodes, the substitution rate of the SSU rDNA is relatively variable and can therefore be informative for deep phylogenetics, shallow phylogenetics as well as molecular

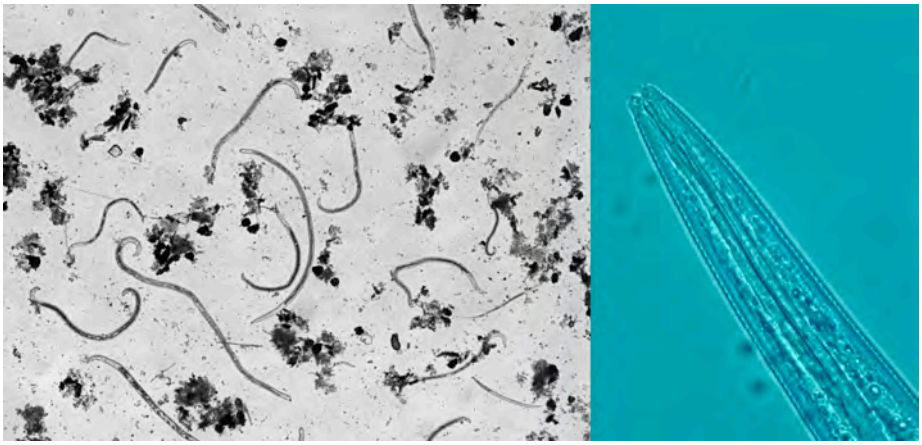

Figure 2. On the left: A nematode suspension, extracted from soil, under a low magnification microscope (400x magnification). Photo credits: Paul Mooijman, Laboratory of Nematology, Wageningen University. On the right: Picture of the head region of a bacterivorous nematode species, Eucephalobus striatus (picture taken at 1,000x magnification). Photo credits: Hanny van Megen, Laboratory of Nematology, Wageningen University.

identification at different taxonomic resolutions (*e.g.* Floyd et al., 2002, Holterman et al., 2008, Van Megen et al., 2009).

Currently, there is a full-length SSU rDNA sequence framework consisting of over 2,800 sequences (and growing). Based on this framework, taxon-specific primers (on species, genus or family-level) have been developed for the qualitative and quantitative monitoring of nematodes. This development has the potential to lift the practical constraints of nematode identification and allows for the large-scale exploitation of the nematode community as an environmental indicator.

Outline of this thesis

The aim of the research described in this thesis was to assess the suitability of molecular taxon-specific assays for the monitoring of nematode assemblages in field experiments. The method was applied to determine the effects of different types of disturbances on the soil food web, *i.e.* agricultural practices, invasive plant species and the effects of a genetically modified potato variety.

In **Chapter 2**, the background of the molecular method and the results of its first field application are presented. It describes the results of an extensive field study in which the seasonal fluctuations of 15 nematode taxa were monitored at 18 times during 10 months in two different habitats; an ex-arable field and its adjacent beech forest. The results show different temporal fluctuations of nematode taxa belonging to the same functional guild. The study demonstrates that the production of elaborate datasets, facilitated by a molecular approach, can lead to new insights in nematode ecology and evolution.

In **Chapter 3**, the effect of an agricultural management practice referred to as biofumigation was determined for the nematode community. Biofumigation is a pest control management measure, which entails the incorporation of brassicaceous plant material resulting in the release of natural, general biocidal compounds into soil. Although strong effects were observed after incorporation of different *Brassica juncea* cultivars, no relationship was observed between the concentrations of the compounds incorporated into the soil and the response of different nematode taxa, including the plant parasites. The results suggest that, at least for the cultivars used in this study, observed effects on the nematode community were related to a combination of tillage and green manuring.

In **Chapter 4**, the results of a study concerning the belowground impact of an invasive plant species, *Solidago gigantea* are presented. Impact was assessed by comparing the vegetation characteristics and soil characteristics of invaded plots (*i.e.* pH, soil moisture content, fungal biomass and the densities of several nematode taxa) to those of uninvaded plots in two contrasting habitat types, riparian and semi-natural grasslands. A habitat-independent significant increase of fungal biomass was observed as well as a systematic increase of one out of the three monitored fungivorous nematode taxa. The results of this study indicate that an asymmetrical boost of the fungal community by *S. gigantea* could explain the observed shift in the fungivorous nematode distribution.

In **Chapter 5,** the effect of a genetically modified potato variety on the nematode community is compared to that of its parental conventional isoline and four other conventional

potato cultivars. The experiment was performed on two locations, differing in soil type, and samples were taken on two occasions during the growing season of the plants. In this study, no differences were observed between the nematode community of soil planted with the GM potato and its parental cultivar or overall between cultivars. However, the results showed a significant effect of location (or soil type) and sampling time.

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Chapter 2

SSU ribosomal DNA-based monitoring of nematode assemblages reveals distinct seasonal fluctuations within evolutionary heterogeneous feeding guilds

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Abstract

Soils are among the most complex, diverse and competitive habitats on Earth and soil biota are responsible for ecosystem services such as nutrient cycling, carbon sequestration and remediation of freshwater. The extreme biodiversity prohibits the making of a full inventory of soil life. Hence, an appropriate indicator group should be selected to determine the biological condition of soil systems. Due to their ubiquity and the diverse responses to abiotic and biotic changes, nematodes are suitable indicators for environmental monitoring. However, the time-consuming microscopic analysis of nematode communities has limited the scale at which this indicator group is used. In an attempt to circumvent this problem, a quantitative PCR-based tool for the detection of a consistent part of the soil nematofauna was developed based on a phylum-wide molecular framework consisting of 2,400 full-length SSU rDNA sequences. Taxon-specific primers were designed and tested for specificity. Furthermore, relationships were determined between the quantitative PCR output and numbers of target nematodes. As a first field test for this DNA sequence signature-based approach, seasonal fluctuations of nematode assemblages under open canopy (one field) and closed canopy (one forest) were monitored. Fifteen taxa from four feeding guilds (covering ~ 65 % of the free-living nematode biodiversity at higher taxonomical level) were detected at two trophic levels. These four feeding guilds are composed of taxa that developed independently by parallel evolution and we detected ecologically interpretable patterns for free-living nematodes belonging to the lower trophic level of soil food webs. Our results show temporal fluctuations, which can be even opposite within taxa belonging to the same guild. This research on nematode assemblages revealed ecological information about the soil food web that had been partly overlooked.

Introduction

The biotic soil fraction is the source of major ecosystem services like water holding, nutrient cycling, and carbon sequestration (Petersen and Luxton, 1982, Myers, 1996, Wardle et al., 2004, Fitter et al., 2005, Postma-Blaauw et al., 2005) and is the bottom-up driving force of the ecosystem (Hunt and Wall 2002, Scheu and Setälä 2002). Therefore, due to their differences in habitat-responses and multitrophic interactions (Hunt and Wall 2002, Scheu and Setälä 2002, Wardle et al., 2004, De Mesel et al., 2006, Mulder et al., 2012), many terrestrial invertebrates are valuable ecological indicators (Birkhofer et al., 2012). However, irrespectively of the environmental characteristics we wish to highlight, the high biodiversity in soils and sediments (Mulder et al., 2005, Höss et al., 2011) forces us to choose a subset that is representative for biological soil quality. Ecological criteria to select indicator groups should include a) distribution across multiple trophic levels, b) methodological interpretability of qualitative and/or quantitative changes, and c) ease of sampling standardization. Soil nematodes meet these criteria.

These vermiform invertebrates, mostly with body lengths ranging between 0.2 and 2.5 mm (Mulder and Vonk 2011), are present in densities up to several millions individuals per square meter, and are easily extractable from the topsoil. Their trophic diversity encompasses all the three energy channels distinguishable within the soil food web: the plant-feeding, the bacterial-feeding, and the fungal-feeding pathway (*e.g.* Ferris et al., 2001). Because of their highly interconnected positions in the detrital soil food web, nematode communities reflect microbial resources, especially the bacterial and the fungal communities (Wardle et al., 2004, Van Eekeren et al., 2010, Christensen et al., 2012), soil fertility and management (Ferris et al., 2001, Yeates 2007, Reuman et al., 2009). Simple food webs with few trophic levels, as those in our study, show more specialization and less omnivory because occurring species (here, nematodes) have a much higher probability of consuming at one single trophic level (Polis et al., 1997, Thompson et al., 2007). Hence, nematodes are a natural avenue to examine the spatial and temporal variance of such food web configurations.

Moreover, the nature and rate by which nematodes respond to changes in the (a)biotical soil condition varies amongst different families and genera. At community level, this variation in responsiveness reflects itself changes in the numerical abundance, species composition, feeding traits and trophic distribution. The interconnected positions in the soil food web, in combination with taxon-specific responsiveness towards environmental stressors, make these invertebrates suitable as indicators.

Over the last decade, substantial progress has been made in collecting phylum-wide genetic information of nematodes (Blaxter et al., 1998, Holterman et al., 2006, Van Megen et al., 2009). This resulted in a small subunit ribosomal DNA-based (SSU-rDNA) framework covering a substantial part of the biodiversity for terrestrial nematode communities in temperate climate zones. Other studies independently introduced molecular tools to analyse nematode communities using qualitative or semi-quantitative techniques such as direct sequencing (Floyd et al., 2002), PCR DGGE (Okada and Oba 2008), and T-RFLP (Donn et al., 2012).

For DNA-based quantitative community analysis, the effect of the (unknown) life-stage distribution within individual taxa should be considered. In the past, nematodes were thought to exhibit cell constancy; all individuals of a given species have the same number of cells. However, at least for one organ, the epidermis, this was shown to be incorrect (Cunha et al.,

1999). During their development from first or second juvenile stage to adult stage, the number of somatic cells appears to show less than a two-fold increase. In case of *Caenorhabditis elegans* (Rhabditidae) the number of non-gonadal cells increase from ≈ 550 (first stage juveniles) to ≈ 810 (mature hermaphrodite) (De Cuyper and Vanfleteren 1982), while *Panagrellus silusiae* (Panagrolaimidae) the number of somatic nuclei was shown to increase from ≈ 410 (second stage juvenile) to ≈ 590 (adult) (Sin and Pasternak 1971). Although data on this issue are scarce (but see Derycke et al., 2012 for cryptic Rhabditidae), we hypothesize here that it is possible to relate quantitative PCR data to the number of individuals of a given family at a logarithmic scale without knowing their exact distribution over the life stages. If this is true, a SSU-rDNA quantitative community analysis will define emergent characteristics of the nematofauna and can provide one common currency to assess the soil food web by comparing the quantitative PCR output of differently-sized nematodes.

According to Neher (2010), more research is needed on nematodes in natural and agricultural soils to test ecological hypotheses. Hence, we chose two adjacent ecosystems to investigate the extent to which the SSU-rDNA tool allows monitoring of soil nematode assemblages in the South of the Veluwe region (central Netherlands). In such a way, we were able to establish the degree of convergence of the soil ecological condition 30 years after ending the agricultural practices by a direct comparison between the compositions of the nematode community in the previously disturbed area ('former arable field') and the adjacent undisturbed control ('mature beech forest'), as recommended in Neher 2010. During 10 months, nematodes were sampled 18 times with intervals of 2-4 weeks. 15 families or genera were detected (monophyletic groups in a phylum-wide SSU rDNA framework that includes ≈ 2,400 taxa as described in Van Megen et al., 2009), within them most feeding guilds were represented. Occurrence of specialized nematodes parasitizing vascular plants greatly depends on the structure of the rhizosphere, hence on the composition of the vegetation and as for the Maturity Index (Bongers 1990) they were not taken into consideration in this nematological research. Shortly, recurring DNA patterns (motifs) were identified for family or genus-specific groups and PCR primers with identical annealing temperatures were developed. We show that nematode assemblages can be monitored frequently using standard molecular laboratory equipment and that this method has the potential to contribute to the full exploitation of this abundant and diverse group of metazoans.

Materials and methods

Study area

Seasonal fluctuations of non-parasitic nematode assemblages were studied in De Planken Wambuis, a nature reserve located on the Veluwe, the largest moraine complex in The Netherlands. Due to the absence of endangered and/or protected species, this investigated area of approximately 100 m length is not protected by law and no specific permits were required. Sandy soil samples were taken from two sites: a 30-year-ago abandoned arable field, known as Dennenkamp (52° 03′ N, 5° 80′ E), and an adjacent more than 100-year-old *Fagus sylvatica* forest.

The former arable field (sampling area 2.5 ha; further referred to as 'field') is a relatively open area with a *Plantagini-Festucion* association (*sensu* Weeda et al., 1996) growing on a soil with pH of 5.7; more characteristics of this site have been published in Holtkamp et al., 2008. The pristine beech forest (further referred to as 'forest') with typical medium humified humus,

hereafter moder soil (pH ≈ 3.7), is characterized by a scarce understory (sampling area 1.5 ha). The precipitation and temperature data were registered by a weather station by the Royal Dutch Meteorological Institute (KNMI Station 06275, 45 m a.s.l., about 7 km from Dennenkamp).

Sampling and nematode extraction

Nematode assemblages were monitored throughout 2009 in an abandoned field and an adjacent pristine beech forest. On these sites, the upper 25 cm of the soil were sampled 18 times from March 17 (week 1) until December 18 (week 40). The humus fraction was still observable as a stratified layer in the forest moder (partly decayed, to some extent mixed with the mineral horizon). At eighteen time points (every 2-4 weeks), we randomly took four composite soil samples from the field, and two composite samples from the adjacent forest. Each sample consisted of 8-10 cores (Ø 1.5 cm, depth 25 cm) taken from a surface of ~ 0.25 m2 and thoroughly mixed. Nematodes were extracted from 100 ml of soil using an elutriator (Oostenbrink, 1960). Nematode density was estimated by counting two subsamples per sample at low magnification (classical analysis); after counting, these subsamples were poured back into the original suspension.

Selection of nematode taxa

To make a selection of monitored taxa for this study, suspensions from both sites were analysed microscopically twice (week 1 and week 39, 2009). In total, 38 genera *sensu* Bongers (1994) were identified in the field and 25 in the forest (Table 1). Fifteen nematode taxa (families and genera; Table 2) were selected based on molecular resolution, trophic ecologies, and sensitivities towards environmental disturbances. This taxonomic selection at genus level covers 59 % of the field and 72 % of the forest nematode biodiversity (excluding the obligate plant parasitic genera; Table 1).

DNA extraction and purification

Nematode suspensions (100 ml) were concentrated by centrifugation at 4,000 rpm, supernatant was removed until an end-volume of approximately 1.5 ml. This volume was further concentrated in a small vial at 14,000 rpm. The supernatant was removed until the final volume of 140 µl was reached. Subsequently, like in Holterman et al. (2006), an equal volume of nematode lysis buffer was added. As an internal standard, 20 µl of mammalian DNA (20 ng/µl) was included. Lysis took place in an oven at 65 °C for two hours. Lysates were purified using a glass fiber-based DNA extraction procedure (essentially according to Ivanova et al., 2006). Purified nematode community DNA was eluted from the filter with T10E1 (10:1, 1 M Tris and 0.5 M EDTA) and immediately used or stored at -20 ˚C. These purified lysates were used for quantitative PCR analysis. We kept this DNA extraction procedure consistent for all samples in our study to ensure full comparability of results (Thonar et al., 2012, note to their S3).

Design and testing of family- and genus-specific primers

For the development of taxon specific PCR primers, a molecular framework consisting of ≈ 2,400 (nearly) full-length SSU rDNA sequences representing all major groups of terrestrial nematodes was used. ARB, a LINUX-based software package (Ludwig et al., 2004), was used to design family and/or genus-specific primers. Most nematode families appeared as monophyletic

groups in a SSU rDNA based phylogenetic tree (Van Megen et al., 2009), and PCR primers were developed on the basis of taxon-specific motifs. In contrast, for some polyphyletic taxa – *e.g.*, fungivorous Diphtherophoridae – separate specific primer combinations were developed for each of the constituting genera. In the case of the poly- and paraphyletic Rhabditidae, embracing 27 genera according to the Fauna Europaea [http://www.faunaeur.org (Accessed 2012 June 6)], no comprehensive DNA barcodes could be generated at such a large family level, albeit for some monophyletic genera, specific primers can still be developed.

When designing the primer combinations, the annealing temperature of the oligonucleotides was assessed *in silico* using the program MELTING (Le Novere 2001). For each nematode taxon (family or genus), the specificity of multiple (up to 5) primer combinations was checked with recombinant SSU rDNA fragments from target(s) and close non-target(s) as identified by ARB (details in Neilson et al., 2009, Rybarczyk-Mydlowska 2012). Apart from the specificity requirements, primer combinations were designed to have an optimal annealing temperature (Ta) of 63˚C. Based on an experimental temperature range test, only target-specific primer combinations with a sharp optimum were selected. This approach allows for a quantitative detection of combinations of taxa with the same PCR temperature profile.

Primer combinations were tested in 25 µl containing 3 µl of 1,000 times diluted template (final concentration: 10 ng/µl), 1 µl of each of the taxon-specific primers (final concentration for each primer: 200 µg/µl), 7.5 µl Milli-Q water and 12.5 µl Absolute SYBR Green Fluorescein Mix (Thermo Fisher). For amplification on a thermal cycler (Bio-Rad iQ5), the following quantitative PCR temperature profile was used: 95 ˚C, 15 min followed by 60× (95 ˚C, 30 sec; 63 ˚C, 1 min; 72 ˚C, 30 sec) followed by a melting curve program 47× (15 sec from 72 to 95 ˚C with steps of 0.5 ˚C).

For each taxon, one primer combination was selected on the basis of optimal specificity (*i.e.*, largest DCt) between target(s) and close non-target(s); assays with a DCt lower than 12 were discarded. Here, Ct value is defined as the number of PCR cycles ('C') at which the reporter dye emission intensity exceeds a predetermined threshold ('t'). In case of similar specificities, primer combinations with lowest Ct value per unit of template were preferred.

Quantitative PCR on total nematode community DNA

Each purified lysate (DNA extract from 100 ml elutriated soil) was used as template with 15 primer combinations on a thermal cycler (Bio-Rad iQ5). A separate primer combination was used to quantify the internal standard (mammalian DNA) in each sample to estimate the efficiency of the lysis and purification procedure. Reaction volume of the quantitative PCR was 25 µl containing 3 µl of 50 times diluted template, 2 µl taxon-specific primers (end concentrations 200 µg/µl), 4.5 µl PVP40, 12.5 µl Absolute SYBR Green Fluorescein Mix (Thermo Fisher). The following quantitative PCR protocol was used: 95 ˚C, 1 min followed (as before) by 60× (95 ˚C, 30 sec; 63 ˚C, 1 min; 72 ˚C, 30 sec) followed by a melting curve program 47× (15 sec from 72 to 95 ˚C with steps of 0.5 ˚C).

Relationships between Ct values and numbers of target nematodes

The quantitative PCR output is expressed in Ct units. The copy number and quantities of the target template are inversely proportional to Ct and can be calculated by direct comparison

with Ct values for known standards (Brunborg et al., 2004, Atkins et al., 2005). In order to get

Table 1. Overview of nematode diversity at genus level (microscopic analysis) in the topsoil (depth 0–25 cm) of the former arable field and the adjacent pristine beech forest. Obligate plant parasitic nematodes are given in green and are not included in the molecular part of this research. Only the genera marked by 'q' are included in the quantitative PCR analysis and for most of these genera quantitative ranges ('r') are available (see Fig. 4 and text for more details). For the taxonomy of the families we adhered to De Ley et al. 2006.

Genus	Family	qPCR analysis	Range	Field	Forest
Achromadora	Achromadoridae			+	
Acrobeles	Cephalobidae	q	r	+	
Acrobeloides	Cephalobidae	q		+	+
Aglenchus	Tylenchidae				+
Alaimus	Alaimidae	q	r	+	+
Anaplectus	Plectidae	q	r	+	
Aphelenchoides	Aphelenchoididae	q	r	+	+
Aphelenchus	Aphelenchidae	q	r	+	
Aporcelaimellus	Aporcelaimidae			+	
Bunonema	Bunonematidae				+
Cephalenchus	Tylodorida				+
Cephalobus	Cephalobidae	q		+	
Cervidellus	Cephalobidae	q	r	+	+
Clarkus	Mononchidae	q	r	+	
Coomansus	Mononchidae	q		+	
Coslenchus	Tylenchidae				+
Cylindrolaimus	Diplopeltidae			+	
Diphtherophora	Diphtherophoridae	q	r	+	
Ditylenchus	Anguinidae			+	+
Eucephalobus	Cephalobidae	q	r	+	+
Eudorylaimus	Qudsianematidae				+
Eumonhystera	Monhysteridae	q	r	+	+
Filenchus	Tylenchidae			+	+
Geomonhystera	Monhysteridae			+	
Helicotylenchus	Hoplolaimidae			+	
Malenchus	Tylenchidae				+
Meloidogyne	Meloidogynidae			+	
Mesorhabditis	Mesorhabditidae			+	
Metateratocephalus	Metateratocephalidae	q	r	+	+
Microdorylaimus	Qudsianematidae			+	
Nygolaimus	Nygolaimidae			+	
Panagrolaimus	Panagrolaimidae			+	
Plectus	Plectidae	q	r	+	+
Pratylenchus	Pratylenchidae			+	
Prismatolaimus	Prismatolaimidae	q	r	+	+
Pungentus	Nordiidae			+	
Rhabditis	Rhabditidae			+	+
Steinernema	Steinernematidae				+
Teratocephalus	Teratocephalidae	q	r	+	+
Thonus	Dorylaimidae	q	r	+	+
Tylencholaimus	Tylencholaimidae			+	+
Tylenchorhynchus	Belonolaimidae			+	
Tylenchus	Tylenchidae			+	+
Tylolaimophorus	Diphtherophoridae	q	r	+	+
Wilsonema	Plectidae	q		+	+
Total # of genera	**45**	**20**	**16**	**38**	**25**

these standards, quantitative series of microscopically identified nematodes (mostly to genus level) were sampled. Vials containing 25 µl sterile water with 1, 5, 10, 50, or 100 hand-picked nematodes were supplemented with an equal volume of lysis buffer (0.2 M NaCl, 0.2 M Tris-HCl [pH 8.0], 1 % (v/v) β-mercaptoethanol) and 800 µg/ml proteinase-K. Lysis took place in a Thermomixer (Eppendorf) at 65 °C as described by Holterman et al. (2006). Quantitative PCR reactions were performed as described above: 3 µl of 1000x diluted lysate, 1 µl of each taxon-specific primer (end concentrations of both primers 200 µg/µl), 7.5 µl Milli-Q water, and 12.5 µl Absolute SYBR Green Fluorescein Mix (Thermo Fisher). In the case of family-specific primers, calibration curves were generated for the major genera within each family.

Data analysis

The total numbers of nematodes were log transformed and an overall comparison (including all sampling times) was made between the two ecosystem types ('forest' and 'field') using *t*-test (equal variances not assumed, a = 0.05). To visualize seasonal patterns and site-dependent differences, trend lines are shown for each family or genus per location. Inter-site comparisons were made for each of the detected families and genera and for the two basal trophic guilds (here as summed 'bacterivores' and summed 'fungivores') using independent Mann Whitney-U test (a = 0.05). To get the temporal variation of the nematode community between our two habitats, a partial Mantel analysis was performed.

Results

Seasonal dynamics and site-specific differences in nematode communities

While the air temperature fluctuated between 20.4 °C (week 14) and -3.5 °C (week 39) and the cumulative rainfall of the latest 21 days before sampling (*sensu* Mulder et al., 2005) fluctuated between 11 and 95 mm (Fig. 1, upper panel), variation of the total nematode density (Fig. 1, bottom panel) was rather low (Coefficient of Variation equals 48.1 % in the field and 63.1 % in the forest). The field had a lower density of nematodes in comparison to the forest (averages per 100 ml elutriated soil were 2,392 ± 1,151 SD *versus* 3,222 ± 2,033 SD individuals; unweighted *t*-test $P = 0.023$).

Composition of the soil nematode assemblages was determined microscopically from two composite suspensions (Table 1). Among these genera and families, taxa that appeared as monophyletic groups in a SSU rDNA-based molecular framework (Van Megen et al., 2009) were chosen for further investigation. From the basal level of the soil food web, 12 taxa were selected to be addressed in the next part, namely 8 bacterivores (7 families and 1 genus) and 4 fungivores (2 families and 2 genera), and from the trophically higher level, 3 taxa were selected, *i.e.* 2 predatory families (here: Mylonchulidae and Mononchidae M3) and one omnivore family (here: Dorylaimidae D3), for monitoring using real time PCR (Table 2). Primary data about densities of individual taxa at each of the time points (average and standard error) are given in the Supplementary Table S1.

In contrast to the total nematode densities, individual taxa show distinct temporal and site-specific patterns. The seasonal fluctuations for 7 bacterivorous families are shown in Fig. 2 (*colonizer-persister cp* ranking as in Bongers 1990, Bongers and Bongers 1998): Teratocephalidae

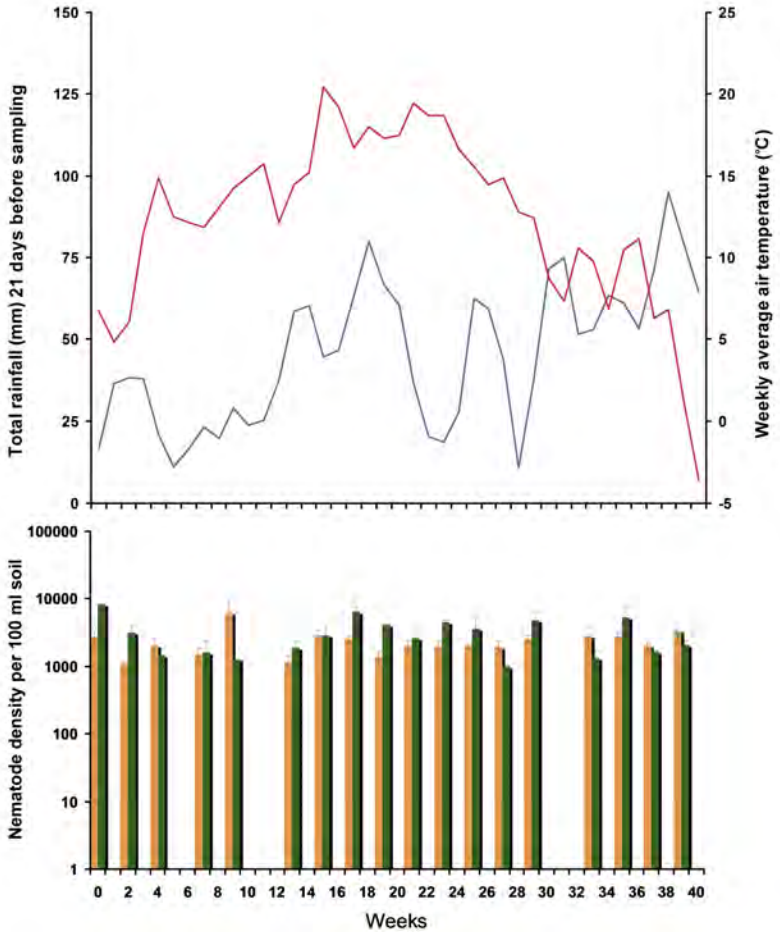

Figure 1. Precipitation and temperature in relation to total nematode densities in open (field) and closed (forest) canopies. Weekly averages of daily temperature (red) and total rainfall over 21 days before sampling (blue) as measured by the Royal Dutch Meteorological Institute (KNMI) are shown above. At the bottom, average nematode densities per 100 ml of soil from a since 25 years abandoned arable field (open canopy, yellow bars) and adjacent pristine beech forest (close canopy, green bars) are given. Sites sampled in 2009 at regular intervals between March 17 (week 1) and December 18 (week 39).

(*cp*-3), Prismatolaimidae (*cp*-3), Cephalobidae (*cp*-2), all in the left panel; Plectidae (*cp*-2) and the genus *Anaplectus* (*cp*-2), both in the red box; Alaimidae (*cp*-4), Metateratocephalidae (*cp*-3), and Monhysteridae (*cp*-2), all in the right panel. In particular, bacterivores show distinct temporal patterns in abundances in the two habitats, but also a taxon dependency was observed (Fig. 2). For instance, comparable trends are detectable for all Teratocephalidae (*i.e.*, *Teratocephalus*, being Teratocephalidae monogenic).

Over the entire season, no significant differences between the two habitats were observable in the case of *Teratocephalus* and Monhysteridae (a = 0.05). Two families, Prismatolaimidae and Metateratocephalidae, were consistently more abundant in the forest, whereas Alaimidae, Cephalobidae, members of Plectidae and the genus *Anaplectus* were present in significantly higher densities in the field (Fig. 2). However, if the densities of these bacterivorous taxa are taken together into a single feeding guild (*Bax*, bacterivores with *cp* value *x sensu* Ferris et al., 2001) no significant difference was detectable between the two sites (a = 0.05), despite the remarkable functional differences within bacterial-feeding nematodes known from literature (Postma-Blaauw et al., 2005, De Mesel et al., 2006).

In parallel, three fungivorous families were monitored as well; Aphelenchidae (*cp*-2), Aphelenchoididae (*cp*-2), and Diphtherophoridae (*cp*-3). Ribosomal DNA sequences suggest that Diphtherophoridae are not monophyletic (Van Megen et al., 2009). Hence, representatives of the two constituting genera, *Tylolaimophorus* and *Diphtherophora*, were detected separately. The composition of this guild is site-specific: whereas the forest was dominated by *Tylolaimophorus,* the fungal pathway of the nematofauna in the field was more diverse (Fig. 3), although *Tylolaimophorus* remained predominant. Their densities showed strong temporal fluctuations: from week 20 onwards, all these fungivorous families were present in the field, although at low levels.

SSU rDNA-based assays for the detection of nematode taxa – qualitative aspects

Soil samples typically contain 30-60 nematodes species, and the composition of nematode assemblages is highly dependent on soil conditions (Mulder and Elser 2009, Mulder and Vonk 2011). Keeping this degree of complexity in mind, the development of such a molecular community analysis tool requires a comprehensive SSU rDNA database (Van Megen et al., 2009). A selection of 15 taxa was made with representatives of four major guilds: i– bacterivores, ii– fungivores, iii– omnivores, and iv– carnivores. The strategy followed for the development of specific PCR primers is exemplified here by the Metateratocephalidae, a bacterivorous family harbouring two genera, *Metateratocephalus* and *Euteratocephalus* (Fig. 4). SSU rDNA sequence motifs were used to design primers with an annealing temperature (Ta) of 63 ˚C. To optimize the foreseeable specificity, selected primer combinations showed a sharp increase in Ct (threshold cycle) upon further Ta increase (Fig. 4A). ARB software (Ludwig et al., 2004) was employed to identify potential false positives, and plasmids harbouring relevant SSU rDNA fragments were used for testing PCR primer combinations. Taxonomically, it must be mentioned that potential false positives are not *per se* related to targets, underlining the plea for a phylum-wide database. In case of the most optimal Metateratocephalidae primer combination, the smallest gap between the target and the non-target smallest (ΔCt) measured 26 cycles (Fig. 4B). This value was determined for all primer combinations (Table 2).

SSU rDNA-based assays for the detection of nematode taxa – quantitative aspects

To establish the relationship between a Ct value (the primary output of a quantitative PCR reaction) and the corresponding number of target nematodes (here, members of the Metateratocephalidae), two series of handpicked individuals were generated. The resulting dataset, five Ct values for each *Metateratocephalus* and *Euteratocephalus* (Fig. 4C), was used to define the slope and the *y*-intercept of the regression line describing the linear relationship between log

(# nematodes) and the corresponding Ct values (Fig. 4D). As an assessment of the goodness of fit, *R*2 values are given for each taxon. Although families may harbor more genera than the number given in Table 2, the values presented here only aim to indicate the number of genera that were observed at this particular study area. Considering the life-stage distribution for each taxon (with differences in DNA contents for individual life stages), a taxon-specific degree of uncertainty regarding the exact densities might occur. However, seen the *R*2 values for each taxon (Table 2), we might assume that the SSU rDNA-based densities reflect the actual densities assessed by classical nematological analysis.

Table 2. Molecular overview of the nematode families and genera monitored in our study. Specificity of primer combinations is expressed as the gap between the Ct value of the latest target and the Ct value of the earliest non-target (ΔCt expressed in number of PCR cycles). For relationship between Ct value and number of target nematodes see Fig. 4, and Materials and Methods in '*Relationships between Ct values and numbers of target nematodes*'.

Nematode family / genus	ΔC_t	Relationship between C_t value and log (# target nematodes):			# genera*
		C_t = **a** ×$\log_{10}$ [# nematodes] + **b**			
		a	**b**	R^2	
Alaimidae (B)	N/A	-3.31	25.47	0.996	1
Aphelenchidae (F, FP)	42	-4.31	17.53	0.995	1
Aphelenchoididae (F, FP)	20	-3.06	24.09	0.992	1
Cephalobidae (B)	17	-4.21	21.95	0.855	3
Diphtherophoridae (F) :					
Diphtherophora	18	-3.22	19.18	0.926	
Tylolaimophorus	N/A	-3.02	22.36	0.984	
Dorylaimidae (O)	N/A	-5.90	17.30	0.859	1
Metateratocephalidae (B)	26	-5.09	24.40	0.954	2
Monhysteridae (B)	23	-4.25	21.06	0.954	1
Mononchidae (P)	18	-2.94	15.19	0.990	1
Mylonchulidae (P) **	N/A	-4.02	12.03	0.977	1
Plectidae (B) :					
Plectidae excl. *Anaplectus*	34	-1.93	26.82	0.989	1
Anaplectus	27	-3.33	21.03	0.949	
Prismatolaimidae (B)	13	-5.13	21.64	0.999	1
Teratocephalidae (B)	N/A	-4.41	25.13	0.999	1

B: bacterivore, F: fungivore, FP: facultative plant parasite (only for nematodes where this guild occurred in combination with fungivory), O: omnivore, P: predator; N/A: no quantitative PCR signal produced by non-target(s); *: number of genera within one family assessed by qPCR (families as in De Ley et al., 2006); **: as *Mylonchulus* is expected to occur in this area, its family has been included as additional taxon.

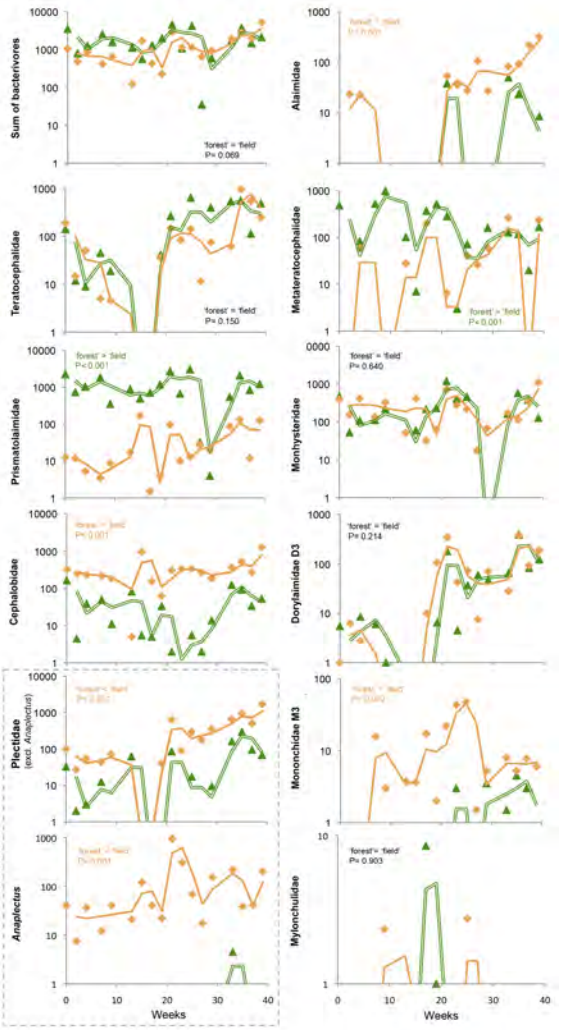

Figure 2. Temporal patterns of bacterivorous, omnivorous and predatory nematode families. We determined DNA-based variation in the nematode densities per 100 ml soil (note differences in y-axes) of representatives from seven bacterivorous families: Teratocephalidae, Prismatolaimidae, Cephalobidae, Plectidae (i.e., all Plectidae excl. *Anaplectus* and '*Anaplectus*', both in a dashed gray box), Alaimidae, Metateratocephalidae, Monhysteridae; the omnivorous family Dorylaimidae (D3 region sensu Holterman et al., 2008); and the predatory families Mononchidae (M3, Holterman et al., 2008) and Mylonchulidae. Sampling weeks as x-axes (constant scales); samples from the field are represented by orange triangles and samples from the forest by green diamonds. Trends are given as two-period moving averages: the averaged 2nd and 3rd data points are portrayed by the 1st data point and so forth.

Quantitative coverage of environmental samples by a 15-taxa nematological analysis

If all taxa present in the samples were covered by this novel quantitative PCR-based community analysis tool, the sum of the densities should equal the total nematode numbers as given in Fig. 1. To check the quantitative coverage of the 15-taxon analysis tool, the total number of nematodes as determined microscopically was compared with the total numbers as estimated by quantitative PCR. We constructed a red dotted line to show virtual data at which the total number of nematodes is equal irrespective whether determined by microscopy or by quantitative PCR (Fig. 5) and one solid line to connect all the points. Ideally, a dataset should not exceed 0.5 log value from the latter solid line, allowing a precision of ± 0.5 order of magnitude. To show the data-range borders, dashed lines have been plotted above and below the solid trend-line. Of all nematode assemblages analysed, 78 % were found within this range. When it is assumed that Fig. 5 provides a summary overview of the relationship between classical and molecular nematological analyses, it is notable, that the slope of the linear log-log regression across all our samples analysed in both ways (solid trend) is allometrically undistinguishable from unity (the slope 0.927 ± 0.192 SE overlaps 1 ± 0 SE; $P < 10\text{-}5$). Discrepancies between counts and qPCR in one fifth of our samples are either due to underestimation or to overestimation of the nematode biomasses. On one hand, lacking appropriate molecular assays are a caveat that

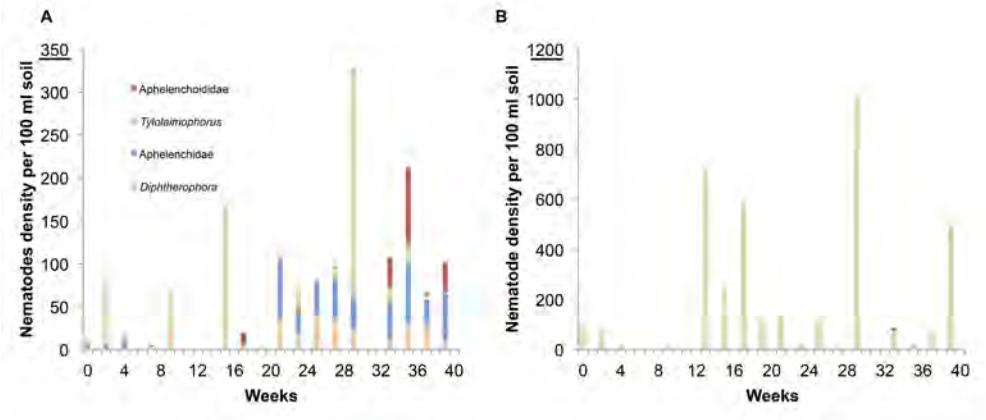

Figure 3. Temporal patterns of fungivorous nematode families. Seasonal variation in densities for fungivores in the field (A) and in the forest (B). Please note that the y-axis scales differ. Aphelenchidae (Aphe, blue), Aphelenchoididae (Acho, red), and two genera belonging to Diphtherophoridae –viz. Tylolaimophorus (Tylo, green) and Diphtherophora (Diph, yellow)– show different patterns over the seasons between open and close canopies. As these taxa represent all observed fungivores, a partial Mantel analysis performed in a matrix describing the community structure in the field (open canopies, matrix Y) and in the forest (close canopies, matrix X) using the squared Euclidean distance was performed using the total entries and the same set of entities. A positive association between the matrices is indicated over the seasons by observed Z greater than average Z from randomized runs (P = 0.0297).

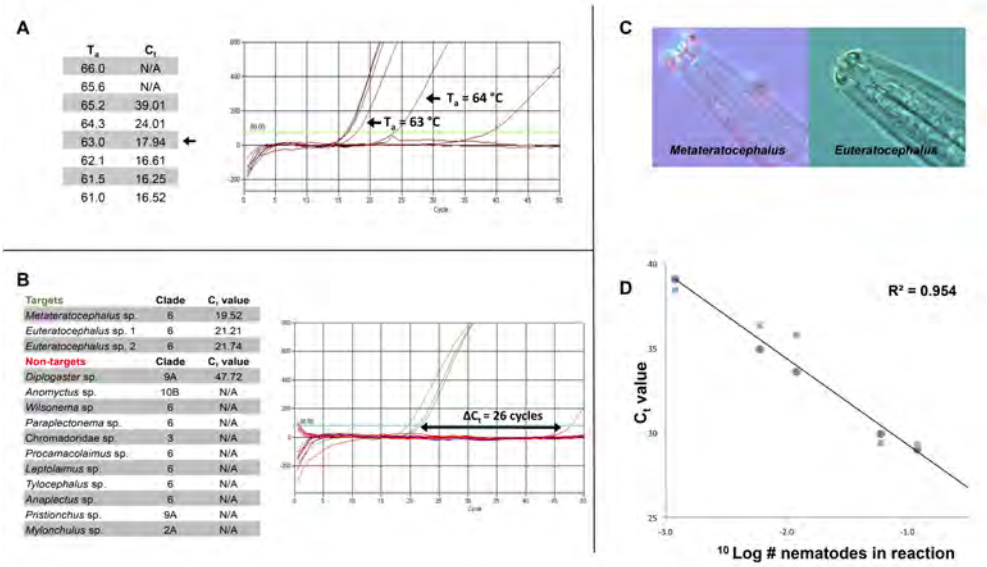

Figure 4. Development and testing of a nematode family-specific primer combination. Here we use the Metateratocephalidae (one bacterivorous family harboring the Metateratocephalus and Euteratocephalus genera) as an example of primer development. (A) All primers were designed to have optimal annealing temperature (Ta) of 63uC, with Ct values varying at temperatures above and below the target Ta. (B) Specificity test of a Metateratocephalidae primer combination with plasmid DNAs from three target species, SSU rDNA fragments from 11 potential false positives (as selected by ARB, Ludwig et al., 2004) and a negative water control. Clade numbers are according to Van Megen et al. (2009). In the quantitative PCR graph the gap between the target and the non-target signal (DCt) is shown. (C) Pictures of the head region of a representative of both genera. (D) The relationship between Ct values and numbers of nematodes for quantification of densities. A linear relationship between Ct values and numbers of nematodes till 1/1,000 part of a single nematode is shown (equivalent to a single nematode cell harbouring 50 copies of the ribosomal DNA cistron). Handpicked individuals of Metateratocephalus (purple circles) and Euteratocephalus (blue squares) were used to quantify the Metateratocephalidae- specific primers.

explains underestimation. For a number of non-monophyletic taxa such as the Rhabditidae, in fact, no molecular assays could be designed. Members of this family (*cp*-1) can respond very quickly to both local environmental changes (*e.g.* eutrification) as to microbial pulses. If such a family would be abundant in a given sample, this would automatically result in a drop of the coverage. On the other hand, though unusual, averages of body-mass values at genus level can be very different within a single family. This phenomenon can be illustrated by the "Plectidae minus *Anaplectus*". In this group, the fresh weight per individual (and – most likely – the individual DNA content) varies substantially between genera (compare *Plectus* with *Wilsonema*, the latter being on average more than 7 times smaller than *Plectus*, Mulder and Vonk 2011). In our paper, the calibration curves were produced at genus level, and the quantification at family

level was based on a qualitative check for Plectidae genera present in a given set of samples.

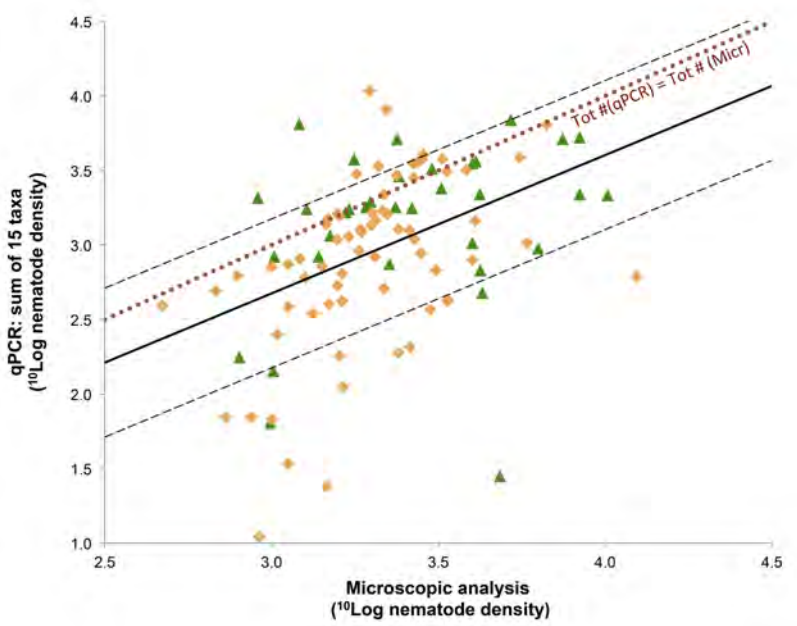

Figure 5. Quantitative coverage of the DNA-based tool using environmental samples. Logarithm of the total of individuals as detected by optical microscopy (x-axis) plotted against the logarithm of the total of individuals as estimated by quantitative PCR (y-axis). The correlations of quantitative PCR with classical analyses seem to be accurate, with no Studentized residuals higher than | 2 |. The solid line shows the trend of all data and the two dashed lines show the boundaries of one-order-of-magnitude precision. The dotted line represents an equal amount of nematodes for both methods. Such a coverage is expected to be lower than 100% as obligate plant parasites were not included, although the fungivorous Aphelenchidae and Aphelenchoididae may harbor facultative plant parasites as shown in Table 2. Given that taxa like Rhabditidae, Qudsianematidae or Nordiidae appear to be both poly- and paraphyletic (Holterman et al., 2008, Van Megen et al., 2009), no rDNA-based detection assay on family level could be developed for those nematodes.

Discussion

Comparison of the abundances of eight bacterivorous taxa during the entire experiment resulted in a diverse picture: for two taxa no difference was detected between the habitats, whereas six differed (four taxa were consistently more abundant in the field and two were present in significantly higher densities in the forest). Lumping the estimated nematode abundances into the feeding guild 'bacterivores' masks the taxon- and site-specific differences. One of those differences is the high density of Prismatolaimidae in the acidic moder. A factor that often contributes to this asymmetric distribution is pH, as some *Prismatolaimus* species prefer acidic conditions (Hirschmann 1952) and our forest moder might constitute an optimal environment for acidophilic bacterivores. Another of those differences was the distinct distribution of *Anaplectus* (Plectidae, Anaplectinae): throughout the seasons, this genus occurred at relatively constant density in the field, whereas it was virtually absent in the forest. This result confirms the outcome of a qualitative study of nematode communities in moder and mull (no stratification in humus-containing layer, organic matter and well-mixed mineral soil, pH 6.2–6.8) beech forest soils, where, *Anaplectus granulosus* was shown to be exclusively present in mull soils (Alphei 1998).

Regarding seasonal changes in bacterivorous nematodes, a decreasing trend was observed for Teratocephalidae and Plectidae in June and July (week 13 to 17 in Fig. 2), a period characterized by intense rainfall. Although precipitation data was recorded at a nearby weather station and not on the site itself, it would be tempting to attribute their absences to their movement to deeper soil layers. For several plant parasitic species, it has been shown that simulating intense rainfall hardly results in leaching of nematodes (Chabrier et al., 2008, Fujimoto et al., 2009). As this ability to withstand leaching is unlikely to be specific for plant parasites alone, we see the absence of Teratocephalidae and Plectidae not as the (passive) result of any leaching below the sampling depth. However, members of these families could *actively* migrate downwards because of the leaching of certain groups of bacteria, their main resource. The passive transport of bacterial cells as a result of rainfall is a well-documented phenomenon (Gagliardi and Karns 2000, Aislabie et al., 2011).

Intra-feeding guild heterogeneity is further illustrated by the fungivorous nematodes. The family Diphtherophoridae harbors two genera, *Diphtherophora* and *Tylolaimophorus*. Whereas representatives of the genus *Diphtherophora* were constantly present in the field from week 15 onwards (though in low densities), they were lacking in the adjacent forest. *Tylolaimophorus*, on the other hand, was the dominant fungivorous nematode in the forest (albeit in fluctuating densities). If soil acidity is so important for belowground Operational Taxonomic Units (OTUs) as suggested by Mulder et al. (2005) and Wu et al. (2011), than the difference in soil pH of our sites might (co)explain the observed patterns: in general, *Tylolaimophorus* spp. is known to be acidophilic (Brzeski 1994), and its high abundance in the beech forest can be attributed to the acidic moder (pH = 3.7). The genus encompasses thirteen species (Brzeski 1994), and we hypothesize that other, non-acidophilic *Tylolaimophorus* species might occur in the abandoned field (pH = 5.7).

A more pronounced, reverse response is observed for *Diphtherophora* (Fig. 3). Either the low pH of the forest soil was directly inhibiting the occurrence of *Diphtherophora* spp., or it is negatively affecting a part of the fungal community that provides an essential food resource for

these nematodes. Members of other fungivorous families (Aphelenchidae and Aphelenchoididae) were respectively non-detectable or present in low amounts (late Autumn) in the acidic moder (Fig. 3B), whereas they occurred in the field at densities up to one individual per ml of soil, especially in the second half of the season. Hence, we hypothesize that (1) fungivorous nematodes are not as polyphagous as suggested (Freckman and Caswell 1985) and that (2) Aphelenchidae and Aphelenchoididae feed on a part of the fungal community different from the segments used as a resource by *Tylolaimophorus*.

Regarding functional changes in nematodes, applied soil ecologists tend to use indices based on so-called guilds, a trait assemblage of nematode taxa sharing the same feeding habits and inferred function in the soil food web (Ferris et al., 2001). The current study did not aim to investigate effects of changes in the nutritional status of a soil food web, nor any other kind of environmental disturbance. Rather it shows that it is possible to monitor communities based on molecular methodology and in this way reveals numerous changes within feeding guilds that might give us more insight in the ecological functioning of soil biota.

In the last decades there have been several extensive studies on both spatial (*e.g.* Neher et al., 1995, Ettema and Yeates 2003) and temporal (*e.g.* Yeates et al., 1999, Sohlenius and Boström 2001) variation within nematode communities. Yeates et al. (1999) monitored nematodes communities on 23 occasions over seven years under an annual and a perennial crop (3-4 sampling occasions per year). Their findings underlined the necessity of long-term monitoring (at least three years) to observe the effects of agricultural practices, and did not pay attention to seasonal fluctuations. Sohlenius and Boström (2001) showed with a study of two annual time series (sampling monthly and every other month) and one long-term time series (10 sampling events over 25 years) in a Swedish pine forest soil, that variations within groups of nematode taxa of the same feeding type were larger compared to variations of the feeding groups as a whole. We did not observe major seasonal fluctuation in total numbers of nematodes as they did (Sohlenius and Boström 2001), but our data are in line with their conclusion stating that precipitation is one of the major drivers of changes in the nematode community composition.

So far, a number of practical obstacles like the time required for microscopic analysis, the limited number of informative morphological characters for some taxa, and the scarcity of people that can analyse nematode assemblages has restricted the number of intensive monitoring studies. DNA-based community analysis can lift such obstacles, and facilitate a wider use of nematodes as indicator for the biological soil quality. In essence, four different molecular approaches are currently used: direct sequencing, PCR DGGE, PCR T-RFLP and real time PCR (Chen et al., 2010).

Direct sequencing shares with PCR DGGE its qualitative rather than quantitative properties. In a careful comparison between microscopic and DGGE-based community analysis, Okada and Oba (2008) found a reasonable match between the two methods. T-RFLP is a semi-quantitative PCR-based technique as well, and for the analysis of nematodes communities, the generation of a molecular framework is required. Recently, Donn et al. (2012) reported on the effects of tillage on nematode communities, and as a start, a database with 516 partial SSU rDNA sequences from the sites under investigation was generated. The requirement of such a location-specific database and its semi-quantitative nature currently makes T-RFLP not an attractive method for routine analyses of nematodes.

Real time PCR is designed for quantification, and Jones et al. (2006) were among the first to

use this method for nematode community analysis. A local and small (74 SSU rDNA sequences) framework was made, and in a next step, community analysis procedure was tested based on the combined use of microscopy (for pre-selection) followed by real time PCR. The molecular procedure presented here allows for the analysis of nematode communities without any microscopic pre-selection because it is based on a considerably broader (2,400 taxa) full length SSU rDNA database that covers all major terrestrial and freshwater nematode taxa. It is noticed that marine nematode are greatly underrepresented in our framework, and consequently it cannot be used for marine nematode assemblages yet. Still, a basic advantage of the detection framework illustrated here is its simplicity, as it only requires standard laboratory equipment.

In most soil nematological studies, data are presented at family or feeding guild level. To allow for a straightforward connection between the large body of ecological data on terrestrial nematode communities and the currently present molecular detection framework, it was decided to preferably develop assays at family level. Families often harbor multiple genera, and regression lines for family-specific primer combinations are based on one or more calibration curves produced on genus-level (*e.g.*, Fig. 4D). In case the relationship between real time PCR output (Ct value) is similar for the constituting genera, the ratio between these genera does not affect the accuracy of the results. However, some families such as Cephalobidae include genera with considerable body-size differences (Mulder and Vonk 2011, Mulder et al., 2011) and, most likely, DNA contents. Therefore, only those genera that were present in the microscopic samples were included in the computation of the regression. As a consequence, the $R2$ for Cephalobidae was slightly lower in comparison to most other primer combinations (Table 2). Hence, the accuracy of density levels of individual families is variable and depends on the variation in average DNA contents of the constituting genera.

On one hand, DNA-based research spans an enormous array of ecological disciplines and we believe that this study demonstrates – among others by showing for two adjacent, undisturbed areas that even 30 years after ending the agricultural practices the soil nematofauna barely seem to converge to the same assemblage – the ecological suitability of a quantitative PCR-based method for nematological and environmental purposes. On the other hand, our results also aim to contribute to the increase of the current knowledge of this phylum, given that the taxonomy of nematodes is still far from complete (Fontaine et al., 2012). It has been shown that the analysis of datasets at genus level can provide more information when comparing analyses at family or order level. Using this DNA barcode-based tool, we have the possibility to work towards a complete view on time trends and soil patterns, enabling the nematode community to become unravelled.

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Chapter 3

Selective alteration of soil food web components by invasive Giant goldenrod (*Solidago gigantea*) in two distinct habitat types

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Abstract

Interactions of plants with soil biota have been suggested to determine the invasive success of exotic plant species. So far, most research has been focussing on primary decomposers and nutrient cycling, and studies on the impact on higher trophic levels of the soil food web are relatively scarce. Here we investigated the aboveground (plant community) and the belowground impact (at multiple trophic levels of the soil food web) of an invasive plant species; Giant goldenrod (*Solidago gigantea*). In order to evaluate the habitat dependency of Giant goldenrod impact, we compared two contrasting environments: riparian zones and semi-natural grasslands. In total, we selected 30 pairs of plots invaded by *S. gigantea* and an un-invaded neighbouring plot. For all plots (n=60), floristic composition, pH, fungal biomass, and the densities of 11 nematode taxa (using a quantitative PCR-based method) were determined. In plots invaded by *S. gigantea*, plant-species richness was reduced by 42% and 55% in riparian and semi-natural grassland habitats respectively. The invader outcompeted both rare and dominant plant species. Belowground, in both habitats, *S. gigantea* invasion reduced pH, increased fungal biomass as well as densities of members of the fungivorous nematode family Aphelenchoididae. However, as two other lineages of fungivorous nematodes, Aphelenchidae and Diphtherophoridae, did not benefit from the increase in fungal biomass, effects on higher trophic level organisms turned out to be selective. Invasive plant species can cause significant alterations of biological conditions in soil. Here, we show that invasive *S. gigantea* can affect the soil food web in a selective way. Fungal biomass was enhanced, but only one fungivorous nematode lineage benefitted from this increase, whereas two other lineages were not affected at all, suggesting that *S. gigantea* stimulates only part of the fungal community. With regard to bacterivorous nematode taxa, no generic effect of Giant goldenrod invasion was observed.

Introduction

The successful establishment of exotic species is considered as one of the major driving forces of changes in biodiversity (Sala et al., 2000). Most naturalised exotic plants behave ecologically comparable to resident species, but a small proportion ('invasive plants') can reach exceptionally high densities in their new environments (for terminology see Pyšek et al., 2004). In various cases, invasive plant species have transformed native plant communities, and locally this has resulted in near monocultures. Hejda and co-workers (2009) studied the main factors determining the impact of invasive plant species on the native plant community. According to them species identity and habitus characteristics such as stand height and cover are major determinants for invasiveness. The number of studies focussing on belowground effects of invasive plant species has grown substantially over the last decade (Vilà et al., 2011). Most of these studies concentrate on the impact on microbial communities (for review see Van Der Putten et al., 2007) and nutrient cycling (for review see Ehrenfeld, 2003). From these studies it has become clear that interactions between plants and soil biota can play a decisive role with regard to the invasive success of exotic plant species. The invasiveness of naturalised plant species has been shown to be promoted by their ability to stimulate generalist soil pathogenic fungi (Mangla et al., 2008) or by the local presence of compatible mycorrhizal fungi (Nuñez et al., 2009). Selective changes in the microbial community can lead to alterations at multiple levels of the food web, and may thereby affect its stability (Dunne et al., 2002). This notion could contribute to our understanding of the ecological impact of exotic plant species. However, little attention has been paid to invader-induced changes on higher trophic levels in the soil food web so far (Belnap et al., 2005, Chen et al., 2007).

Due to the enormous biodiversity of soil biota and the high number of trophic relationships, there are myriad interactions between plants and soil microbial communities (Porazinska et al., 2003). Due to their omnipresence in pores between soil aggregates, their trophic diversity, and their high degree of interconnectedness within the soil food web, nematodes constitute an informative bio-indicator group for soil food web functioning (Neher et al., 2005). A range of studies has focused on interactions between plant community composition and nematode assemblages (De Deyn et al., 2004, Viketoft et al., 2005, Bezemer et al., 2010, Viketoft and Sohlenius, 2011). So far, the impact of exotic plants on nematode communities has received little attention (Van Der Putten et al., 2005, Morriën et al., 2011). Studying changes in nematode communities at high taxonomic resolution can deliver valuable information about shifts in microbial soil communities (Porazinska et al., 1999, Neher et al., 2005, Viketoft and Sohlenius, 2011). However, for experiments with intense sampling designs, microscopy-based community analyses are (too) laborious and time-consuming. A recently developed set of quantitative PCR (qPCR)-based molecular assays (Vervoort et al., 2012) allows for the analysis of nematode assemblages at or below family level in a relatively short time frame.

In the present study, Giant goldenrod (*Solidago gigantea*), a common invasive plant species in most European countries, was selected as a model to examine belowground effects of successful invaders. This plant species has the ability to form near monoculture stands in a broad range of habitats (Weber and Jakobs, 2005). In recent years, several studies revealed properties of *S. gigantea* which possibly contribute to its invasiveness, *e.g.*, high biomass production, high nutrient efficiency, alteration of nutrient turnover (Vanderhoeven et al., 2006, Scharfy et al.,

2009) and the excretion of allelochemicals (Abhilasha et al., 2008). In 2010, Scharfy et al. studied the effect of *S. gigantea* on soil biota in typical wetland soils (gleysols and a gleyic cambisol) under controlled mesocosm conditions. They observed a significant decrease in bacterial and an increase in fungal biomass in soil below a *S. gigantea*-dominated vegetation. However, it is hard to predict whether these are specific or more widespread consequences of invasion by Giant goldenrod, and little is known about possible follow-up changes at higher trophic levels in the soil food web.

The aim of our study is to elucidate the belowground impact of *S. gigantea* on multiple trophic levels in the soil food web. To allow the identification of generic effects of local Giant goldenrod invasions, two contrasting invaded environments were taken into consideration: riparian zones and semi-natural grasslands (characterised by river clay and sandy soils respectively). This approach enabled us to test the following hypotheses: 1) the local increase of the fungal biomass as a result of invasion by Giant goldenrod is a widespread phenomenon. 2) if this invasive plant species provokes a general stimulation of the fungal community, increased densities should be observed for all lineages of fungal feeding nematodes in both habitats. However, in case of an asymmetric boost of the fungal community (*i.e.* only a subset of fungal taxa are benefitting), an asymmetric stimulation of fungivorous lineages can be expected, given that lineages of fungivorous nematode indeed differ in their food preferences as suggested by Vervoort et al., 2012. 3) if the local decrease of the bacterial biomass caused by Giant goldenrod invasion as observed by Scharfy et al. (2010), is a general, invader-driven effect, this should result in a decrease in density of at least a part of the bacterivorous nematode communities, in both habitats.

Materials and methods

Sites of study

Ten *S. gigantea*-invaded sites were selected in two semi-natural habitats: riverbanks of the Rhine and the Walloon and grasslands on Pleistocene sandy soils. Sites were selected within an area of approx. 200 km2 in the central part of The Netherlands (Suppl. Table S1). In this area, naturalised *S. gigantea* plants are present since 1912 (Te Linde and Van den Berg, 2003). In riparian habitats, *S. gigantea* is mainly introduced by surface waters, which carry (fragments of) plants that can sprout under favourable conditions elsewhere (Weber and Jakobs, 2005). The semi-natural grasslands studied here were located relatively close to inhabited areas. Beekeepers and gardeners introduced *S. gigantea* to these areas. All selected sites met the following criteria: 1) *Solidago gigantea* occurred in well-defined patches in the native vegetation, 2) soil and vegetation showed no signs of disturbances caused by *e.g.* foraging wildlife or mowing, 3) soils from sites of the same habitat type were comparable in plant community, pH and humidity.

Sampling

For both invaded habitats, five sites were investigated. For each site, three separate plot-pairs were defined, consisting of two directly neighbouring 4 m2 (2.0 x 2.0 m) plots; one plot in a by *S. gigantea*-dominated patch ('invaded') and one un-invaded plot, just outside this patch. Thus, in total 60 plots were studied. For each plot, the floristic composition was determined, and a composite soil sample was collected. Each composite soil sample consisted of a mixture

of 20 randomly taken soil cores (Æ 1.5 cm, depth: 25 cm) that were homogenised thoroughly, immediately thereafter this mixture was stored at 4°C. Sampling took place during the week of September 12th 2011, when the plant community was at peak standing biomass. One month before, nematode diversity of all 10 sites of this study was assessed microscopically (for details see Suppl. Table S2).

Vegetation analysis

In each plot (n=60), all species of higher vascular plants were recorded and valued on an ordinal scale, based on abundance or coverage (Suppl. Table S3 and S4). Community characteristics were determined by calculating the species richness (S) and the Shannon diversity (H') as described in Hejda et al., 2009.

Soil acidity and humidity

A subsample (20 g) of each composite soil sample was used to determine the moisture content and pH-H2O. Soil moisture content was determined by weight loss after 72 h incubation at 40°C. The dried soil was sieved with a 2 mm mesh; thereupon soil pH was measured in demineralised water using a gel-electrolyte electrode (Sentix 21, WTW, Weilheim, Germany).

Nematode extraction and community analysis

For each of the composite samples, a 100 g subsample was taken, and nematodes were extracted using an elutriator (Oostenbrink, 1960). Nematode suspensions were analysed microscopically, or by a qPCR-based method (Vervoort et al., 2012).

Microscopic analysis (of samples collected in August 2011) was used to assess the nematode community composition for invaded and native plots in each of the habitats. Communities were characterised by the morphological identification (till genus level) of 100 individuals per sample (soil from under invaded vegetation and native vegetation was analysed separately for each site (n=20; for details see Suppl. Tables S1 and S4). On the basis of this nematode biodiversity inventory, sets of taxon-specific PCR primer combinations were selected, hereby optimizing the coverage of the molecular assays.

For the samples collected in September 2011, overall nematode densities were determined by counting two subsamples of each of the nematode suspensions (n=60). DNA extraction from nematode suspensions and subsequent lysate purification were performed as described by Vervoort et al., 2012. DNA extracts were used as a template in qPCR using 11 nematode taxon-specific primer combinations (for details see Vervoort et al., 2012).

Fungal biomass

Fungal biomass was determined by measuring the ergosterol content in soil samples. Ergosterol is a sterol that is present in fungal cell membranes, and which does not occur in plant or animal cells (Gessner and Schmitt, 1996, Van Der Wal et al., 2006, Stahl and Parkin, 1996). This approach largely excludes arbuscular mycorrhizal fungi which are known to contain relatively low amounts of ergosterol (Olsson et al., 2003). Ergosterol was extracted from 1 g of soil using the alkaline extraction protocol described by de Ridder-Duine et al., 2006. Subsequently, high-performance liquid chromatography was used to determine the ergosterol contents of

the samples (de Ridder-Duine et al., 2006).

Data analysis

Soil properties, vegetation, and nematode densities were analysed using mixed linear models (using PROC MIXED of the SAS software system version 9.2, see Littell, 2006). If needed, data were transformed, in order to arrive at approximately normal distributions of residuals as required for valid statistical inference. The variables soil pH, moisture content, plant-species richness, and diversity remained untransformed; nematode densities were square root-transformed; and all other variables (ergosterol and nematode densities) were log-transformed. The log-transformation was applied after addition of a constant (0.05 for ergosterol, and 0.5 for the nematode densities with the exception of Dorylaimidae D3) to push data away from the lower bound zero. Mixed linear models were used, because multiple observations from the same location and/or plot pair within location are not necessarily uncorrelated. The experimental design here is a split-split-plot design with locations as main plots (associated with factor habitat type; see Table 1), plot pairs as split-plots (without an associated treatment factor), and individual plots as split-split-plots (associated with factor invasion; see Table 1). The fixed part of the mixed model contained main effects of habitat and invasion and their interaction. The random part of the model consists of random effects for location, plot pairs, and individual plots, so that total error variance is split into variance components for locations, for plot pairs within locations, and for neighbouring plots within plot pairs. We present the following results from the mixed models: 1) hypothesis tests for interaction and main effects of factors habitat and invasion and 2) back transformed 95% confidence intervals for means per habitat and invasion, and the ratios (impact (%)) of back transformed means for invaded and un-invaded plots per habitat, together with a statement about the significance of the difference between invaded and un-invaded plots.

Results

Changes in native vegetation upon *S. gigantea* invasion

In total, we identified 64 and 78 vascular plant species in riparian vegetation and semi-natural grasslands, respectively. In invaded plots, 35 and 39 vascular plant species were recorded, respectively. For invaded vegetation, plant-species richness (*S*) and diversity (*H'*) were significantly lower compared to native vegetation ($P < 0.001$; Table 1 and Fig. 1). Common native species largely determining the plant community (*e.g. Jacobaea vulgaris, Holcus lanatus, Achillea millefolium*, *Dactylis glomerata,* and *Plantago lanceolata*; Suppl. Tables S2 and S3) were almost absent in invaded vegetation. Relatively rare species such as *Achillea ptarmica, Epipactis sp.* and *Odontites vernus* subsp. *serotinus* (only present in riparian zones), and *Filago vulgaris* (only present in semi-natural grasslands) were completely absent in the plots invaded by *S. gigantea*. On the other hand, Ground ivy, *Glechoma hederacea,* was either unaffected by *S. gigantea* or had more cover in invaded vegetation.

Impact of *S. gigantea* invasion on soil acidity and humidity

Overall, a comparison of pH's of soils from un-invaded *versus* invaded plots revealed slight

Table 1. Summary of ANOVA Fdf and associated P values, testing for differences in the variables soil pH, soil moisture content (%), total nematode density (per 100 g dry soil, analysed by microscope), fungal biomass (expressed as mg ergosterol / kg soil), plant-species richness (S¬¬plant), plant-species diversity (H'plant), and the density of 11 nematode taxa (per 100 g dry soil, analysed by quantitative PCR). These variables were tested for habitat type, invasion (neighboring invaded and un-invaded plots) and their interaction (Habitat type * Invasion), based on mixed models fitted to these variables (see materials and methods). P values < 0.05 are considered significant, and indicated in bold.

	Habitat type		**Invasion**		**Habitat type * Invasion**	
	$F_{1,8}$	*P*	$F_{1,28}$	*P*	$F_{1,28}$	*P*
Soil pH	54.64	**<0.001**	5.96	**0.021**	1.22	0.279
Soil moisture content	2.88	0.128	3.36	0.077	0.32	0.579
Nematode density	50.21	**<0.001**	0.43	0.518	0.01	0.924
Fungal biomass	1.89	0.207	20.48	**<0.001**	1.09	0.306
S_{plant}	4.45	0.068	82.02	**<0.001**	7.09	**0.013**
H'_{plant}	3.36	0.104	81.44	**<0.001**	2.16	0.153
Aphelenchidae	12.31	**0.008**	0.00	0.946	1.09	0.306
Aphelenchoididae	13.51	**0.006**	5.58	**0.025**	0.86	0.363
Diphtherophoridae	0.00	0.949	0.33	0.571	0.14	0.712
Dorylaimidae D3	6.25	**0.037**	1.89	0.181	2.47	0.127
Mononchidae M3	0.20	0.668	2.35	0.137	5.71	**0.024**
Cephalobidae	7.99	**0.022**	0.29	0.597	9.79	**0.004**
Plectidae (except *Anaplectus*)	0.93	0.362	0.12	0.731	1.94	0.174
Anaplectus	0.46	0.515	2.00	0.168	0.82	0.374
Alaimidae	7.22	**0.028**	2.14	0.155	3.32	0.079
Prismatolaimidae	0.02	0.879	0.11	0.740	0.65	0.426
Panagrolaimidae	6.51	**0.034**	0.51	0.480	0.70	0.411

[a] Nematode taxa defined as by De Ley et al., 2006, except for Dorylaimidae D3 and Mononchidae M3 (see Holterman et al., 2008).

but significantly lower pH's in invaded soils ($P < 0.001$; Table 1). Soil moisture content tended to be lower in invaded plots, but this effect was not significant (P=0.077; Table 1). In general, the soil pH under semi-natural grasslands was ≈ 1.5 units lower (Table 2) and more variable as compared to the riparian plots ($P < 0.001$; Tables 1, 2). The average moisture content of riparian clay soils was higher, although not significantly, as compared to the sandy soils of the semi-natural grasslands (Table 1).

Impact on fungal biomass

Overall, soil from invaded plots contained significantly higher amounts of fungal biomass compared to un-invaded plots ($P < 0.001$; Table 1 and Fig. 1). Fungal biomass was approximately twice as high in soil collected from *S. gigantea* invaded vegetation, compared to plots with native vegetation (Table 2). Comparison of the two habitats suggests that fungal biomass in the un-invaded, semi-natural grasslands is higher than the biomass detected in the riparian sites (ns; Est. mean 0.43 and 0.72 mg ergosterol per kg soil respectively), whereas the fungal biomass-promoting effect of Giant goldenrod was apparently more pronounced in soil collected from the riparian habitat (ns; Tables 1, 2).

Changes in nematode assemblages upon *S. gigantea* invasion

Overall, total nematode densities (determined microscopically) were similar in neighbouring invaded and un-invaded soils. However, when we investigated the impact of Giant goldenrod at nematode taxon level, only one family, *i.e.* Aphelenchoididae, showed overall higher densities in invaded plots, regardless of habitat type (P=0.025; Fig. 1). Apart from fungivores, the family Aphelenchoididae includes a number of (facultative) plant parasites. The primer-combination used in this study excludes all plant parasites from this family, except for *Aphelenchoides fragariae* (Vervoort et al., 2012). The absence of this plant parasitic species was confirmed using an additional, *A. fragariae*-specific molecular assay (Rybarczyk-Mydlowska et al., 2012) (Data not shown). Aphelenchidae and Diphtherophoridae (represented in these two habitats by a single genus, *Diphtherophora*), two other fungivorous taxa, did not show a difference in density between un-invaded and invaded soil (Fig. 1). For the predatory nematode family Mononchidae M3 (see Holterman et al., 2008) and bacterivorous Cephalobidae, a significant interaction was observed between habitat type and invasion of *S. gigantea* (Table 1), showing that the nature of their response to invasion is habitat-type dependent.

When considering the two habitat types separately, differences between un-invaded and adjacent invaded soil were more pronounced in the riparian habitats than in semi-natural grasslands (Fig. 1, Table 2). While in riparian soils the densities of four out of eleven families differed significantly between invaded and un-invaded plots, this was observed for only one taxon in semi-natural grasslands (Table 2). In invaded riparian soils, the density of Aphelenchoididae was significantly higher, as well as the density of two bacteria feeding families, Cephalobidae and Alaimidae (Fig. 1, Table 2). Other bacteria feeders did not show a consistent response. Mononchidae M3, a family of predatory nematodes, was significantly more abundant in invaded riparian plots as well (P=0.010). In semi-natural grasslands, we found significantly higher densities of omnivorous Dorylaimidae D3 in invaded plots; other taxa did not show a significant response (P=0.046).

Table 2. The 95% confidence intervals for the estimated mean response (Est. mean) of soil variables measured in plots invaded or uninvaded by Solidago gigantea in two habitat types, i.e. riparian vegetation and semi-natural grasslands. Values were back transformed to the original scale if needed and are based on mixed models fitted to the (transformed) variables (see materials and methods). Soil variables include; soil pH, soil moisture content (%), total nematode density (per 100 g dry soil, analysed by microscope), fungal biomass (expressed as mg ergosterol / kg soil), plant-species richness (S¬¬plant), plant- species diversity (H'plant), and the density of 11 nematode taxa (per 100 g dry soil, analysed by quantitative PCR). Nematode taxa are defined as by De Ley et al., (2006), except for Dorylaimidae D3 and Mononchidae M3 (see Holterman et al., 2008).

	Riparian vegetation						Semi-natural grasslands					
	Un-invaded			Invaded			Un-invaded			Invaded		
	lower	Est. mean	upper	lower	Est. mean	upper	lower	Est. mean	upper	lower	Est. mean	upper
Soil pH	7.5	7.7	7.8	7.5	7.6	7.8	5.5	6.0	6.4	5.3	5.8	6.3
Soil moisture content	15.0	18.5	21.9	13.4	16.8	20.3	10.8	14.2	17.7	9.9	13.4	16.8
Nematode density	2190	2584	3010	2104	2491	2910	967	1234	1534	891	1148	1438
Fungal biomass	0.28	0.43	0.67	0.52	0.82	1.32	0.46	0.72	1.15	0.68	1.09	1.77
S_{plant}	10.1	12.3	14.6	4.9	7.1	9.4	15.1	17.3	19.6	5.5	7.8	10.1
H'_{plant}	2.1	2.3	2.5	1.5	1.7	1.9	2.4	2.6	2.8	1.6	1.8	2.0
Aphelenchidae	15.9	33.1	69.4	22.0	45.9	96.4	6.1	12.4	25.6	4.3	8.6	17.7
Aphelenchoididae	5.8	11.0	21.6	16.1	31.8	63.2	2.5	4.5	8.5	3.7	6.9	13.3
Diphtherophoridae	1.1	2.8	9.5	1.1	2.7	9.0	1.2	3.3	11.2	1.0	2.6	8.5
Dorylaimidae D3	17.1	32.5	62.4	16.4	31.2	59.9	4.8	8.9	16.7	8.4	15.9	30.2
Mononchidae M3	1.7	3.9	10.3	5.8	15.9	45.4	2.7	6.9	19.2	2.1	5.1	13.9
Cephalobidae	136.2	211.6	330.4	249.3	389.9	611.2	110.9	171.8	267.7	73.2	112.4	174.1
Plectidae (except *Anaplectus*)	87.8	156.0	277.5	115.1	204.7	364.3	168.5	299.7	533.6	107.2	190.6	339.2
Anaplectus	3.6	10.1	29.9	8.2	24.1	73.0	3.3	9.1	27.0	3.9	11.0	32.8
Alaimidae	18.5	69.1	261.2	62.7	237.0	899.7	4.2	14.7	54.5	3.8	12.9	47.6
Prismatolaimidae	1.5	3.5	9.7	1.6	3.9	11.1	1.6	3.9	11.0	1.3	2.9	8.0
Panagrolaimidae	2.0	3.1	5.1	2.8	4.5	7.7	1.4	2.1	3.3	1.4	2.0	3.3

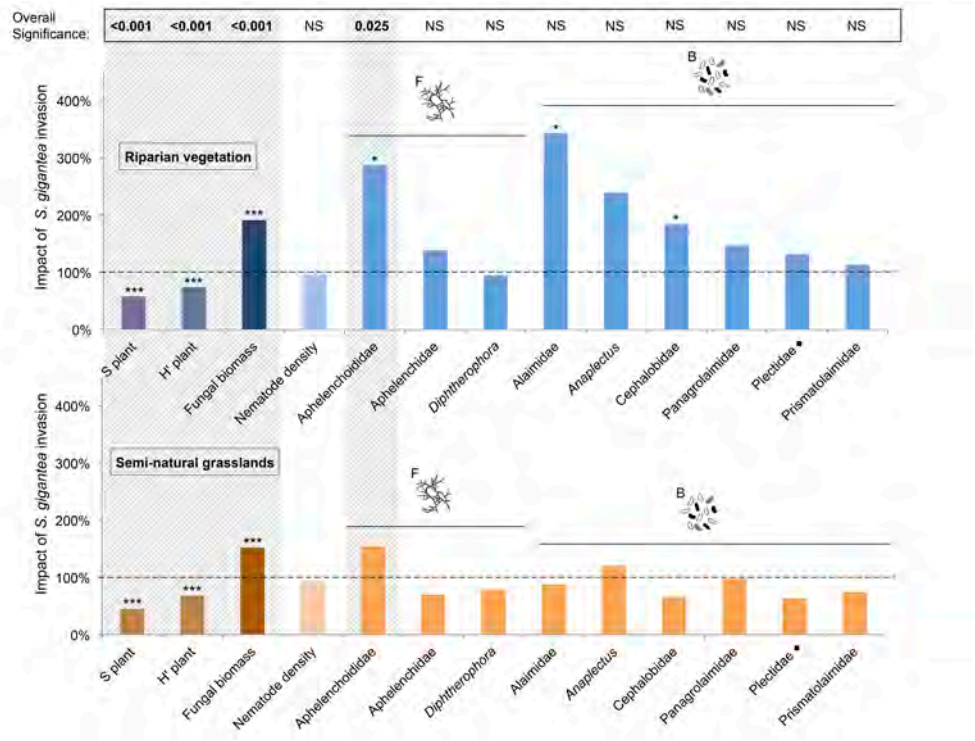

Figure 1. Impact of *Solidago gigantea* invasion in two habitat types, riparian vegetation and semi-natural grasslands, on plant-species richness (S_{plant}), plant-species diversity (H'_{plant}), fungal biomass, total nematode density, and the densities of three fungivorous ('F') and six bacterivorous ('B') nematode taxa. Impacts are expressed as the percentage of the (back transformed) mean values in invaded plots as compared to un-invaded plots (no change = 100%). For each of the two habitats, significant differences between invaded and un-invaded plots are given by asterisks (*P<0.05, **P<0.01 and ***P<0.001; data extracted from the fitted mixed models). Overall significances of the effects of *S. gigantea* invasion (= data from both habitats taken together) are given in top part of this figure (expressed as P values). A shaded background is used to highlight significant variables.

Analysis of samples taken in August 2011 and analysed microscopically, showed that in general nematode diversity was similar for both habitats. Approximately 2,000 nematodes were identified up to genus level. In total, 92 different genera were identified. In riparian soil, 79 genera were found, of which 48 were free-living and 31 plant parasitic. In soil from semi-natural grasslands, we identified 73 genera of which 50 were free-living and 23 plant parasitic. Both habitats shared 60 genera belonging to 40 different families (Suppl. Table S4).

The selection of eleven taxon-specific qPCR assays covered 26 of the 48 free-living genera

shared by both habitat types. For the riparian soil, 46% of the diversity and an estimated average of 86% of the total amount of free-living nematodes were covered by these sets of primer combinations. For soil from the semi-natural grasslands, the molecular assays covered 50% of the free-living nematode diversity and an estimated 80% of the total free-living nematode community (Suppl. Table S4).

Discussion

Investigation of belowground effects of Giant goldenrod (*S. gigantea*) in two (semi) natural habitats – riverbanks and grasslands – revealed a systematic effect of invasion on soil pH, a part of the fungal community, and a single lineage of fungivorous nematodes: invaded soils of two distinct habitats contained more fungal biomass and higher densities of fungivorous Aphelenchoididae than un-invaded soils. Interestingly, the densities of two other lineages of fungivorous nematodes, members of the families Aphelenchidae and Diphtherophoridae, did not change in response to the increased fungal biomass (Fig. 1). No systematic effect was observed on the bacterivorous nematodes community. These results show that – apart from aboveground effects – invasive plant species can cause significant alterations in the nematode community, which appear to be selective for specific taxa within functional groups.

Giant goldenrod effects on soil acidity

The slightly lower pH in invaded plots (Tables 1, 2) may be caused by acidic compounds that are released from *S. gigantea* roots into the rhizosphere (Weber and Jakobs, 2005). Several studies focused on the impact of *S. gigantea* on nutrient pools, and showed a decrease (although site-dependent) in pH in combination with an enhanced P availability (Herr et al., 2007, Chapuis-Lardy et al., 2006). In our study, only small differences in pH were measured, *i.e.* on average 0.1 units, which seem unlikely to play a role in the observed changes in soil biota. It is noted that pH was measured in bulk soil, and more pronounced effect in the rhizosphere cannot be excluded.

Solidago gigantea invaded plant communities

In *S. gigantea*-invaded plant communities, we observed a 42% and 55% reduction of plant-species richness (*S*plant) in the riparian and semi-natural grassland habitats, respectively (Table 2). This impact is relatively high; in a study of (Hejda et al., 2009) an overall reduction of *S*plant of 26% was reported in ruderal plant communities, meadows and along rivers in the Czech Republic. The authors stated that *S. gigantea* had no decisive community-level impact. In addition, *S. gigantea* had limited impact as compared to other invasive plant species such as *Fallopia* spp. (66% - 86% reduction *S*plant) and *Heracleum mantegazzianum* (53% reduction *S*plant). Our results show that the degree of invasiveness of *S. gigantea* in both habitat types is similar to the impact of *H. mantegazzianum* in meadows and forest edges of the Czech Republic (Hejda et al., 2009).

Solidago gigantea renders invaded plots unfit for most resident native plants. At least in part this could be attributed to the high efficiency of *S. gigantea* in the immobilization of minerals such as P and C (Scharfy et al., 2009, Vanderhoeven et al., 2006). For this reason, from June onwards the stems and leaves of *S. gigantea* can become increasingly dense and compete successfully for light. As a result, native plants will invest more in vertical growth, and this might negatively impact seed production. Moreover, Giant goldenrod releases large amounts

of furanoid compounds and acidic compounds in the rhizosphere (Weber and Jakobs, 2005). Rhizodeposition of these toxic compounds could affect resident plant species, as well as belowground communities.

Despite the success of *S. gigantea*, not all plants were negatively affected. We observed a rare and exotic parasitic plant *Cuscata gronovii* (originally from North America), which had strangled and hereby killed *S. gigantea* plants. It is assumed that invasive plants benefit from being released from their natural enemies (Keane and Crawley, 2002). This advantage might not persist (Diez et al., 2010), and *C. gronovii* could become an important factor limiting *S. gigantea* proliferation along rivers.

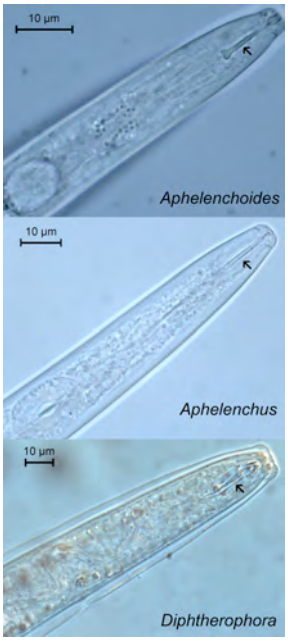

Figure 2. Pictures of the head regions of representatives of the fungivorous nematode genera Aphelenchoides, Aphelenchus and Diphtherophora (pictures taken at 1,000x magnification). To puncture the fungal cell walls, fungivores are equipped with a hardened protrusible piercing device (stylet or spear, indicated by arrows). The protrusibility is facilitated by muscles attached to the knobs or swellings at the basal part of this piercing device. The stylet of Aphelenchoides species is slender with easily observable basal knobs, whereas the stylet of Aphelenchus is characterized by slight basal swellings only. Diphtherophora has a short spear (different ontogeny as compared to a stylet) with a basal swelling of the spear extension.

Effects on soil food web components by *S. gigantea*

In both habitats differing in soil type, floristic composition, and land use history, we found significant belowground effects of *S. gigantea* on soil acidity, fungal biomass, and the density of Aphelenchoididae, a single lineage of fungivorous nematodes. The consistency of these effects suggests that they are general consequences of the dominant presence of *S. gigantea* in its invaded range. Beside overall effects, we observed habitat and site specific responses of other nematode taxa (Fig. 1 and Table 1).

Regarding the increase of fungal biomass and the differential shift observed for fungivorous nematodes, our results suggest that invasion of *S. gigantea* causes an asymmetric boost of the

soil fungal community. We hypothesise that *S. gigantea*'s presence promotes the growth of a subset of taxa within the fungal community, and that only fungivorous Aphelenchoididae were able to benefit from the selective increase in nutritional resources. In Fig. 2, the head regions of the three fungivorous nematode genera are shown. All of them are equipped with a protrusible piercing device that is used to puncture the fungal cell wall. However, the morphologies of these devices (indicated by arrows in Fig. 2) are distinct, and this could point at disparate food preferences. In *in vitro* studies, *Aphelenchoides saprophilus* has been shown to multiply on various mycorrhizal and saprophytic fungal species, whereas *Tylolaimophorus*, a member of the Diphtherophoridae, would not survive on any of these fungi (Ruess and Dighton, 1996). Another *Aphelenchoides* species, *A. hamatus*, could feed and multiply on mycelium from four plant parasitic and a range of edible fungal species (Ruess and Dighton, 1996, Rössner and Nagel, 1984). Among the Aphelenchidae, a family relatively unrelated to the Aphelenchoididae (Van Megen et al., 2009), *Aphelenchus avenae* was reported to prefer plant parasitic fungi to saprophytic species (Okada and Kadota, 2003). This information shows that at least some fungivorous members of the Aphelenchoididae are polyphagous, and our data suggest this could be different for the two other major lineages of fungivorous nematodes, Diphtherophoridae and Aphelenchidae.

Soil born organisms facilitate *S. gigantea* success

The colonisation of Giant goldenrod represents a hazardous factor at the plant community and the landscape scale. After all, we found that next to ruderal communities (Hejda et al., 2009), also relatively biodiverse areas are affected. Compared to most resident plant species, *S. gigantea* has a high nutrient efficiency and biomass production (Vanderhoeven et al., 2006, Scharfy et al., 2009), assumedly because invaders are generally exposed to more favourable plant-soil feedback interactions than their native neighbours (Klironomos, 2002). The results reported here show that the nematode community in *S. gigantea* invaded soil is significantly different from neighbouring soil under the native flora. This is indicative of a shift in their food source, namely the microbial community. We reinforce this hypothesis by the fact that we encountered a two-fold increase of fungal biomass in soil under invaded vegetation. In line with this, considering the impact of plant-soil feedbacks on the invasiveness of *S. gigantea*, the fungal community most probably plays an important role. In order to better understand the invasive success and future perseverance of *S. gigantea* in Europe, we will investigate the nature of the changes in the microbial communities and the way these changes are reflected in the bacterivorous and fungivorous nematode assemblages in more detail.

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Chapter 4

Release of isothiocyanates does not explain the effects of biofumigation with Indian mustard cultivars on nematode assemblages

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Abstract

While the major part of soil biota plays an essential role in ecosystem services, the plant-pathogenic fraction can have a high economic impact on food and feed production. The use of broad-spectrum biocides, so-called fumigants, has been a common practice for controlling soil pathogens, including plant-parasitic nematodes. However, due to their negative environmental impact, many nematicides have been banned or will be banned in the near future. Biofumigation, i.e., the incorporation of mulched (de-compartmentalised) brassicaceous plant material into topsoil, is an example of an alternative management practice. Biofumigation exploits the conversion of glucosinolates (GSLs; secondary metabolites produced by a number of brassicaceous plant species) into nematicidal isothiocyanates (ITCs) upon de-compartmentalisation. Although considerable attention has been paid to effects of biofumigation on plant-parasitic nematodes, the effect on the non-parasitic part of the community has hardly been investigated. Here, we present the results of a field experiment in which we studied the impact of biofumigation with four Indian mustard (Brassica juncea) cultivars on both plant-parasitic and free-living nematodes. Prior to biofumigation, GSL contents of B. juncea plants were determined and expected ITC concentrations in the topsoil were calculated. As positive controls, two concentrations of 2-propenyl ITC were directly applied to wheat plots. Although biofumigation resulted in changes for most nematode taxa, none of these shifts could be attributed to the release of ITCs. Moreover, direct application of a relatively high concentration of ITC did not result in shifts that differed in degree from those observed for the control treatment. We therefore conclude that the observed changes in nematode assemblages are related to intense mechanical disturbance, green manure and the absence of host plants for obligatory plant-parasitic nematode genera, rather than to the release of ITCs.

Introduction

For decades, the use of fumigants in agriculture has been a widespread practice to control soil borne pests (Gamliel et al., 2000). More recently, most synthetic biocides, *e.g.* chloropicrin and methyl bromide, were placed under strict legislation or banned entirely because of their negative impact on the environment (Gamliel et al., 2000, Ruzo, 2006). These restrictions have created a need for alternative management practices. Biofumigation, *i.e.*, the use of *Brassica* green manures for pest control, is considered one of the alternatives (Matthiessen and Kirkegaard, 2006). Plants within the family Brassicaceae are known to produce glucosinolates (GSLs). A major group of hydrolysis products of these GSLs, isothiocyanates (ITCs), act as general biocides (Brown and Morra, 1997). Due to the short release time and half-life of GSLs and ITCs in soils (Gimsing and Kirkegaard, 2009), direct toxic effects on soil borne pathogens are expected within hours to days after biofumigation.

Although biofumigation is often viewed to be less harmful for the environment and soil communities as compared to synthetic fumigants (Matthiessen and Kirkegaard, 2006), these natural mixes of GSLs hydrolysis products can be just as, or even more toxic than synthetic pesticides (Gimsing and Kirkegaard, 2009). ITCs can affect a broad spectrum of soil organisms and may leave previously stable soil food webs vulnerable, as shown for the synthetic fumigant metam sodium (sodium N-methyldithiocarbamate; Cao et al., 2004{Cao, 2004 #2150}). Nevertheless, there has been considerable interest in the extent to which naturally produced ITCs can emulate the efficacy of soil pesticides. Several field studies have shown that the amendment of *Brassica* plant material can have a suppressive effect on a broad range of soil pathogens (Mojtahedi et al., 1993, Motisi et al., 2009). Nevertheless, the efficacy of biofumigation to suppress plant-parasitic nematodes in field trials has been variable (Ploeg, 2008). Results range from high levels of suppression (*e.g.* Mojtahedi et al., 1993, Rahman and Somers, 2005) to no suppression (*e.g.* Johnson et al., 1992, Stirling and Stirling, 2003).

Within the soil food web, free-living (*i.e.*, non plant-parasitic) nematodes are represented at three trophic levels, and the impact of biofumigation on these groups is likely to affect soil functioning. Various effects of biofumigation on free-living nematodes have been reported. After biofumigation, Valdes et al. (2012) observed a decrease in plant-parasitic nematodes and an increase of bacterivorous nematodes. Stirling and Stirling (2003) observed only an increase of free-living nematodes, while Gruver et al. (2010) did not observe any effect. It is hard to link these results unequivocally to the GSL release during biofumigation, as these experiments did not include GSL measurements of the incorporated plant material. In the aforementioned studies, effects were assessed weeks after biofumigation, and it is hard to distinguish the direct toxic effects of ITCs from the impact of tillage and or green manure on nematode communities.

The objectives of this study were to assess the direct and subsequent effects of biofumigation on nematode communities. Direct effects included toxicity of ITCs and disturbance due to tillage; subsequent effects of biofumigation were related to plant biomass incorporation and quality of plant material. We monitored plant-parasitic nematodes using classic identification and free-living nematodes using a DNA-based method (Vervoort et al., 2012) at the start of the growing season as well as just before and at several time points after biofumigation of four Indian mustard (*Brassica juncea*) cultivars differing in their GSL content. The biomass and GSL content of the plant material was determined prior to incorporation. This approach allowed for

an assessment of the impact of different biofumigation-related factors on both plant-parasitic as well as free-living nematodes.

Materials and Methods

Study site

The experiment was performed at the experimental field site of the Julius Kühn-Institut in Münster, Germany, in 2010. Soil type was a medium loamy sand consisting of 9.2% clay, 13.6% silt and 77.2% sand with 1.3% organic matter and a pH (CaCl2) of 6.4. Soil nutrient status at time of planting was 32 mg 100 g-1 P2O5 (above optimum), 17 mg 100-1 g K2O (optimum) and 6 mg 100 g-1 Mg (optimum). Total available mineral nitrogen and sulphur were 52 kg N ha-1 and 42 kg S ha-1, respectively. The experimental plot was prepared on July 7th 2010 by ploughing the remaining stubbles of the previous maize (*Zea mays*) crop and applying 292 kg ha-1 hydrosulfan (24% N, 6% S, Yara GmbH, Germany), *i.e.*, 70 kg N ha-1 and 17.5 kg S ha-1, to ensure optimum plant growth.

Experimental design

Four *B. juncea* cultivars were used: Terrafit, Terratop, Terraplus (P. H. Petersen Saatzucht Lundsgaard GmbH) and ISCI-99 (Bluformula, Italy). Cultivar ISCI-99 was selected as a high GSL producer. As a control (a non-GSL crop), wheat (*Triticum aestivum* cv. Hermann) was chosen. Sinigrin (2-propenyl GSL) is the dominant GSL type in Indian mustard. Its concentration in roots and stems decreases gradually during development, whereas it increases in leaves and reproductive organs of *B. juncea* (Bellostas et al., 2007). At the time of incorporation the plant were in or just beyond the flowering stage. As a positive control, the ITC derivative of sinigrin, 2-propenyl ITC, was directly applied in the wheat plots (see section 2.4).

A randomised block design with four replicates was used, and the plot size was 4 x 15 m. Based on known germination rates, plants were sown at densities of 12 kg ha-1 for *B. juncea* cvs. Terrafit, Terratop and Terraplus, 15 kg ha-1 for *B. juncea* cv. ISCI-99, and 176 kg ha-1 for wheat. All plots were drilled on the July 9th, 2010, hereafter referred to as day 0 (Fig. 1).

Plant sampling and analysis

Immediately before biomass incorporation (day 59), plants were sampled from 50 x 50 cm subplots within each plot, and root and shoot fresh weights were determined. Aliquots (each ≈ 150 g fresh weigh) were collected to determine root and shoot dry weight (weight loss after 24 hrs at 70°C). From each *B. juncea* plot, ten plants were randomly collected, divided into roots and shoots, and plant parts were immediately frozen and kept at -80°C. The plant material was freeze-dried, pulverized with an oscillating mill (MM2, Retsch, Germany) and the resulting plant powder was stored till further chemical analysis. The GSLs were extracted from a 200 mg subsample using 3 mL methanol:water (70:30, vol/vol) at 75°C. One ml of GSL extract was loaded on a micro-column filled with DEAE – A25 Sephadex (CAS Number 12609-80-2, Sigma-Aldrich, Germany), The extracted GSLs were then converted into desulfo-GSL's by incubation for 16 hrs at 39°C with sulfatase from *Helix pomatia* Type H-2 (CAS Number 9016-17-5, Sigma-Aldrich, Germany), eluted with H2O and analysed by High-Performance Liquid Chromatography with Diode-Array Detection at a wavelength of 229 nm. GSL quantification

was made internal standard-based (see also Schütze et al., 1999). Finally, GSL yield per hectare was calculated based on GSL concentration of the plant material and plant dry biomass. C and N content of the *B. juncea* plant material was determined using an elemental analyser (Interscience / Carlo Erba, type EA 1108).

Biofumigation

On day 59, *B. juncea* and *T. aestivum* plant material was chopped and incorporated into the soil. For this, a tractor-driven flail mower was used, and plant parts were immediately incorporated into the top 20 cm of soil with a rotary tiller. Afterwards, the soil surface was slightly rolled to close soil pores and thus reduce evaporation of the ITC's. As a positive control, 2-propenyl ITC was applied directly to the soil. For this purpose, two subplots of 4 m2 each were selected in each of the four wheat plots. These subplots were treated, after the plant material was chopped and prior to incorporation, with 10 l m-2 of a low (1.2 mmol l-1) or a high (4.8 mmol l-1) concentration of 2-propenyl ITC (CAS Number 1476-23-9, 95% purity, Aldrich, Germany). The low concentration treatment was selected to mimic an approximate 100% conversion of the total expected GSL yield of the cultivars into 2-propenyl ITC. The high concentration treatment was chosen to demonstrate the effect of an artificially high ITC concentration on the nematode community.

Soil sampling and nematode extraction

Bulk soil samples were taken on day 3, 59 (just before biofumigation), 60 (one day after biofumigation), 81 and 116 (Fig. 1). Each sample consisted of 30 cores (Ø 2 cm) taken from the top 20 cm following a grid pattern of 3 x 10 m per plot. Samples were then mixed thoroughly, sieved over a 5 mm grid to remove stones and large organic debris and stored at 4°C.

Nematodes were extracted from 250 ml aliquots of the bulk soil in an Oostenbrink elutriator (Oostenbrink, 1960). Nematode density was determined by counting two subsamples of 5 ml out of 100 ml at low magnification under an inverted microscope. After counting, the subsamples were poured back. One half of each suspension (50 ml) was used for microscopic analysis of plant-parasitic nematodes. For this purpose, nematodes were fixed by replacing the water with DESS solution (a solution containing dimethyl sulfoxide (DMSO), disodium EDTA and NaCl, see Yoder et al., 2006) to a total volume of 10 ml. The remaining 50 ml of the suspension was further processed for quantitative PCR (qPCR) analysis to quantify free-living nematode taxa.

Nematode community analysis

Nematode suspensions were split, and one part was used for the morphological identification of plant parasites to genus using an inverted microscope (magnification 40x). The remaining part was lysed, purified, and analysed by quantitative PCR as described in Vervoort et al. (2012). Nematode taxon selection was based on two microscopic analyses of the overall nematode community composition: just before the start of the experiment (May 2010) and on day 81 of the experiment (September 2010).

Based on microscopic nematode biodiversity assessment, 15 taxa were selected for qPCR analysis (this selection covered 83% of the identified free-living genera, see supplementary Table S1). For the suborder Dorylaimina and the family Mononchidae, cluster-specific primers

D1, D3 and M3 were used according to Holterman et al. (2008). For the family Plectidae, separate primers were used targeting either *Anaplectus* or *Plectus* (the only other representative of this family in our field). Many nematode families were represented by a single genus (see supplementary Table S1). Within this experiment, two families were represented by multiple genera: Cephalobidae (five genera) and Dorylaimidae D1 (two genera).

Data analysis

To compare the plant GSL, C and N content on the day of biofumigation (day 59) and the concentrations of the GSLs incorporated into the soil between the four *B. juncea* cultivars ($n = 4$), data was subjected to an ANOVA analysis followed by a least significant difference test (LSD). Differences in nematode densities (total or taxon) between the treatments per each sampling time were tested using a Kruskal-Wallis test. The same test was applied to compare relative changes in nematode densities due to incorporation of plant material between the four *B. juncea* cultivars and the wheat control plots (day 59 – 60; total or taxon). With regard to the comparison of densities of free-living nematode taxa between day 59 and 60, 12 samples were analysed (instead of 16) due to the loss of four samples during processing for qPCR analysis. The Kruskal-Wallis test was also used to check for differences in relative change in nematode densities (total or taxon) between the treatments in the wheat plots from day 59 to 60, day 60 to 81, and day 60 to 118. Significant changes in nematode density (total or taxon) between different sampling times were determined by using either a paired *t*-test (data were log (x+1) transformed) or by a Wilcoxon Signed Rank test. The relationship between the quantity of incorporated GSLs, plant C and N, and changes in nematode densities (total or per taxon) in the *B. juncea* plots were analysed using linear regression. For the wheat plots, linear regression was used to determine if there was a relationship between the relative change in nematode densities (between day 59 and 60) and the two directly applied ITC concentrations. For all tests, differences were considered significant when $P < 0.05$. Data were analysed using SPSS 19 (IBM Corporation).

Results

Points of departure: Glucosinolate contents of *Brassica juncea* plant material and characterization of nematode community

Just prior to biofumigation (day 59), the glucosinolate (GSL) contents of the shoots and roots of the individual Indian mustard cultivars were determined (Table 1). Sinigrin (2-propenyl GSL) was the most abundant glucosinolate in the *B. juncea* plants. Between the four cultivars, only minor differences in GSL contents were observed. As compared to two other cultivars, *B. juncea* cultivar Terraplus showed a slightly higher concentration of indole-GSLs in the shoots (Table 1). Among the Indian mustard cultivars no significant differences were observed in sinigrin concentration, the C and N contents of the plant parts, or the total plant biomasses.

With regard to the nematode communities, both the plant-parasitic and the free-living fraction were characterised. In total 33 genera were identified, of which eight were plant parasites (supplementary Table S1). Five of these plant-parasitic nematode genera, known to feed exclusively on higher plants, were included by microscopic analysis; *i.e.* two ectoparasites (*Trichodorus* and *Tylenchorhynchus*), two sedentary endoparasites (*Heterodera* and *Meloidogyne*)

Table 1. Plant biomass and tissue concentrations of relevant elements (mean ± SD, n = 4) from four *Brassica juncea* cultivars just prior to biofumigation. GSL: glucosinolate, dw: dry weight, Mg: megagram (= metric ton), ha: hectare. Sinigrin is the common name for 2-propenyl GSL.

		***B. juncea* cultivar**			
		Terrafit	**Terratop**	**Terraplus**	**ISCI-99**
Shoot	**GSL, total**(μmol g^{-1})	24.2 ± 7.3	16.9 ± 2.9	14.2 ± 2.7	24.8 ± 8.0
	Sinigrin (μmol g^{-1})	22.6 ± 7.2	15.2 ± 2.7	12.3 ± 2.7	22.9 ± 8.1
	Indole GSLs (μmol g^{-1})*	1.5 ± 0.1 ^{a}	1.7 ± 0.3 ^{ab}	1.8 ± 0.2 ^{b}	1.9 ± 0.3 ^{ab}
	N (% dw)	1.5 ± 0.1	1.7 ± 0.4	1.3 ± 0.5	1.8 ± 0.4
	C (% dw)	83.4 ± 2.0	84.3 ± 2.0	82.3 ± 1.3	82.2 ± 2.7
	Biomass (Mg dw ha^{-1})	5.9 ± 2.0	6.9 ± 2.3	6.4 ± 2.3	8.8 ± 1.9
Root	**GSL, total** (μmol g^{-1})	20.2 ± 9.8	16.5 ± 7.6	25.9 ± 15.8	33.2 ± 10.1
	Sinigrin (μmol g^{-1})	15.9 ± 9.5	10.9 ± 6.7	18.5 ± 14.0	27.2 ± 9.5
	Indole GSLs (μmol g^{-1})	4.3 ± 0.4	5.6 ± 1.3	7.5 ± 2.1	6.1 ± 0.8
	N (% dw)	1.9 ± 1.1	1.7 ± 0.6	2.1 ± 1.1	2.2 ± 0.8
	C (% dw)	76.6 ± 1.9	68.8 ± 8.1	75.3 ± 7.0	75.2 ± 5.8
	Biomass (Mg dw ha^{-1})	0.9 ± 0.3	1.5 ± 0.6	1.0 ± 0.4	1.0 ± 0.1
Whole plant	**Biomass** (Mg dw ha^{-1})	6.9 ± 1.8	8.4 ± 2.5	7.5 ± 2.5	9.9 ± 2.0

* $P < 0.05$ (different letters indicate significantly different groups)

and one migratory endoparasite (*Pratylenchus*). Microscopic analysis revealed that *Tylenchorhynchus* was the most dominantly plant-parasitic genus with densities of 590 ± 379 individuals per 100 g dry soil (average ± stdev, *n* =120, present in all samples). The second most dominant plant parasite was *Trichodorus*, present in 97% of all samples with an overall average density of 107 ± 79 individuals per 100 g dry soil. *Heterodera*, *Pratylenchus* and *Meloidogyne* were only incidentally present at low densities of respectively 11 ± 28 (26%), 6 ± 15 (29%) and 3 ± 10 (11%) individuals per 100 g dry soil (average ± stdev; % samples present).

Free-living nematodes were analysed on the basis of unique SSU rDNA motifs using quantitative PCR (Vervoort et al. 2012). Preliminary analyses revealed that some taxa – Alaimidae, Aphelenchoididae, *Cruznema*, Dorylaimidae D3 and Mylonchulidae – were present in less than 50% of all samples. To allow paired comparisons between subsequent sampling times, only taxa present in at least 50% of soil samples were included for further analysis. These target taxa, two plant-parasitic genera and ten free-living nematode taxa (Fig. 2), were taken into consideration for the overall analyses.

Growing season

Both at the start (day 3) and the end (day 59) of the growing season, total nematode densities (Fig. 1) and the densities of individual nematode taxa (qPCR data for free-living nematodes and microscopic counts for plant parasites) were comparable for all cultivars. Although not significantly different, total densities in the wheat plots appeared to be relatively low during the growing season (Fig. 1). As no significant differences were observed in GSL, C and N contents of the *B. juncea* cultivars, and in the absence of clear qualitative or quantitative differences between the nematode assemblages in the individual plots, we decided to combine all *B. juncea*

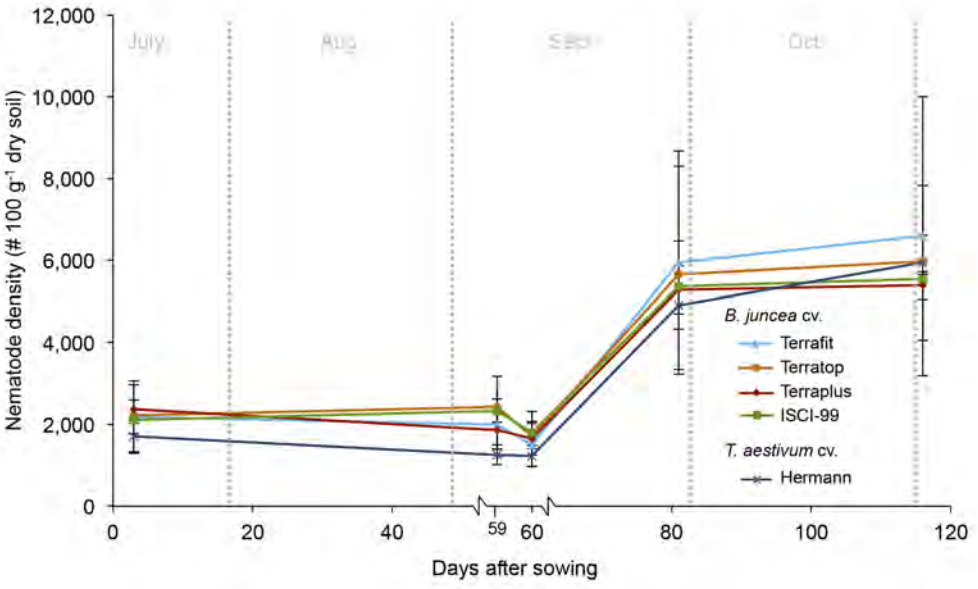

Figure 1. Total nematode density (analysed by microscope) per 100 g dry soil during the growing season (day 3 and 59) and after biofumigation (day 60, 81 and 116) with four *Brassica juncea* cultivars and wheat (Triticum aestivum). Data represents averages ± stdev (n=4).

samples of each sampling time point for the analyses at family and genus level. Although no overall change in nematode densities was observed in between day 3 and 59, significant shifts were detected for individual taxa (Fig. 2). A significant decrease in densities of Monhysteridae (Fig. 2B, bacterivores) and Mononchida M3 (Fig. 2A, carnivores) was paralleled by an increase in densities for Aphelenchidae (Fig. 2A, fungivores) and *Tylenchorhynchus* (Fig. 2A, plant parasites). In wheat plots, the only change between these two sampling times was seen for the Mononchida M3, of which density decreased significantly during the growing season (paired *t*-test, $P = 0.012$).

Biofumigation: direct effects on nematode community

The amount of plant GSLs, C and N incorporated into the soil was calculated for each plot of the *B. juncea* cultivars (Table 2). Overall, only the estimated concentration of sinigrin in the topsoil differed significantly between cultivars; topsoils with chopped ISCI-99 plants were exposed to a two times higher sinigrin concentration. This was due to the combined effect of slightly (but not significantly) higher GSL content in combination with a slightly (but not significantly) higher shoot biomass (Table 1).

Mulching, in fact mechanical de-compartimentalisation of plant tissues, followed by incorporation into the topsoil resulted in a significant decrease in the total nematode density in the *B. juncea* plots (n = 16, paired *t*-test, P=0.005). Apart from Monhysteridae and Mononchida M3

(Holterman et al., 2008), the density of the free-living taxa decreased significantly between day 59 and 60 (Fig. 2). Remarkably, no significant changes were observed for the two plant-parasitic genera (Fig. 2). In the untreated wheat plots, we did not observe a significant change in total nematode density between days 59 and 60 (n = 4, paired *t*-test 0.059). Fig. 1 could suggest for a difference in the degree of change in total nematode densities in the time interval day 59 - day 60 between the wheat plots on the one hand and the *B. juncea* cultivars on the other, but this difference was not significant.

Table 2. Calculated concentrations of relevant plant substances from *Brassica juncea* and *Triticum aestivum* cultivars in the topsoil (upper 20 cm) after biofumigation. For the determination of these concentrations, distinct values given for the plant parts (shoots and roots; Table 1) were transformed into either moles or weight and added together. For each of the cultivars, mean calculated concentrations in top soil ± SD of two types of glucosinolates (GSL), sinigrin (2-propenyl GSL) and indole GSLs, as well as plant C and N and total dry plant biomass are given (n = 4).

		B. juncea cv.				*T. aestivum* cv.
	g^{-1} dry soil	Terrafit	Terratop	Terraplus	ISCI-99	Hermann
Sinigrin**	nmol	55.8 ± 7.9[a]	52.5 ± 12.5[a]	54.5 ± 12.8[a]	91.4 ± 9.1[b]	nd
Indole GSLs	nmol	5.3 ± 1.2	9.3 ± 1.6	8.9 ± 3.5	9.1 ± 3.2	nd
C	µmol	189.6 ± 52.1	231.6 ± 73.6	208.2 ± 68.7	269.4 ± 56.7	nd
N	µmol	2.9 ± 0.8	4.2 ± 1.9	3.1 ± 1.6	5.4 ± 2.1	nd
Biomass	mg	2.8 ± 0.8	3.4 ± 1.1	3.1 ± 1.0	4.0 ± 0.9	3.0 ± 0.9

** $P < 0.01$ (different letters indicate significantly different groups)

Mulching-induced hydrolysis of GSLs should result in a rapid release of ITCs in the topsoil (Gimsing and Kirkegaard, 2006, Gimsing et al., 2009). Hence, we investigated whether the concentrations of GSLs in the topsoil (Table 2) could be related to the degree of change in nematode densities immediately before and directly after biofumigation. The same approach was used to test if there was a relationship between nematode density changes and the ITC concentrations in the soil of the wheat plots. For both *B. juncea* treatments and wheat treatments, no such relationships could be identified (data not shown).

As it is hard to assess the transformation efficiency of GSLs to ITCs in the topsoil *in situ*, the direct effects of 2-propenyl ITC on nematode assemblages were studied in small areas (4 m2) within the wheat plots. 2-propenyl ITC was directly applied to the topsoil in two concentrations. 'Low' treatment plots received a dosis comparable to that produced by cultivars Terrafit, Terratop and Terraplus treatments, whereas the high concentration is equivalent to twice the amount produced by ISCI-99. Both the applications of low and high concentrations of 2-propenyl ITC did not result in any difference in the relative increases or decreases in the total nematode density, or in the densities of individual taxa between day 59 and 60 (Table 3).

Biofumigation: prolonged effects on nematode community

Three weeks after biofumigation (day 81), the total nematode densities had increased significantly as compared to day 60, one day after incorporation of the Indian mustard or wheat plants

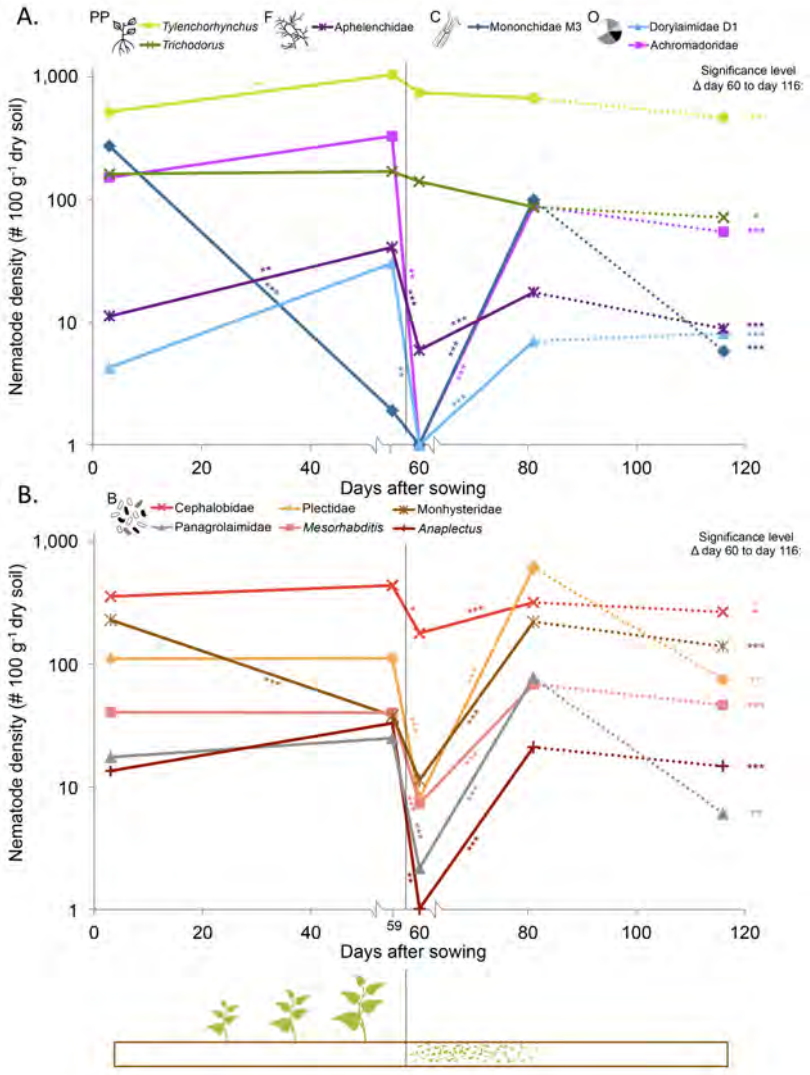

Figure 2. Biofumigation effects on the densities of 12 nematode taxa. PP: plant parasites, F: fungivores, C: carnivores, O: omnivores (panel A), B: bacterivores (panel B) Biofumigation took place on day 59. Significant changes between two connected time points are indicated with asterisks (*P < 0.05, **P < 0.01 and ***P < 0.001). Dashed lines are used to connect data from day 81 and 116. Taxon densities at day 116 were compared to densities one day after biofumigation (60), of which significances are shown by asterisks on the right part of the panels A and B.

(Fig. 1, paired *t*-test, $P < 0.001$). Analyses at the level of individual taxa revealed an increase for all groups of free-living nematodes under investigation (Fig. 2). However, the densities of the plant-parasitic taxa *Trichodorus* and *Tylenchorhynchus* showed no significant change (Fig. 2A). Only for Cephalobidae, we found a significant correlation between the degree of increase and the total quantity of biomass incorporated (R2 = 0.56, $F = 12.49$, $P = 0.005$).

Two months after biofumigation (day 60 compared to day 116), the population increase for free-living taxa as observed three weeks after biofumigation (day 81) was still visible, although less pronounced. In contrast to these taxa, the population densities of the plant-parasitic taxa *Tylenchorhynchus* and *Trichodorus* decreased. The relative increase of Monhysteridae was positively correlated with the total amount of plant biomass that had been incorporated (R2 = 0.46, $F = 8.36$, $P = 0.016$). Apart from short-term direct effects of 2-propenyl ITC (Table 3), we also looked for possible long-term consequences. However, no significant differences in total nematode density or densities of individual nematode taxa between treated and non-treated wheat plots between day 60 and 81 (all $P \geq 0.123$) or day 60 and 116 (all $P \geq 0.077$) were detected.

Table 3. Impact of low and high 2-propenyl isothiocyanate (2-p ITC) concentrations on total nematode abundance and the levels of individual nematode taxa in non-GSL (wheat) control plots expressed as the difference in density between before and after biofumigation (Δ density [day 60 – day 59]; Δ individuals per 100 g dry soil). 'Control' reflects the effect of incorporation fragmented wheat material into the top-soil, whereas 'Low' and 'High' present the combined effects of 2-p ITC and wheat incorporation. Low: 48.8 ± 1.6 nmol 2-p ITC g-1 dry soil; high: 195.1 ± 6.5 nmol 2-p ITC g-1 dry soil.

		Treatment			*P* value[c]
	Trophic group[a]	Control	Low	High	
Nematode density[m]		-27.2 ± 100.1	-185.3 ± 213.6	-107.9 ± 494.9	0.694
Achromadoridae[q]	O	-25.1 ± 87.8	-38.1 ± 75.5	-38.1 ± 75.5	0.938
Anaplectus[q]	B	-2.6 ± 3.8	-2.7 ± 4.3	-3.2 ± 3.7	0.825
Aphelenchidae[q]	F	-11.4 ± 8.2	-9.4 ± 7.5	-12.3 ± 6.8	0.967
Cephalobidae[q]	B	-33.2 ± 89.0	-1.0 ± 122	-55.5 ± 148.2	0.735
Dorylaimidae D1[q]	O	-27.5 ± 34.4	-27.4 ± 34.6	-27.3 ± 34.6	0.980
Monhysteridae[q]	B	-7.2 ± 32.4	-17 ± 19.4	-16.7 ± 19.4	0.944
Mononchidae M3[q]	O	-9.5 ± 17.2	-0.9 ± 9.8	-5 ± 21.3	0.546
Panagrolaimidae[q]	B	-4.2 ± 3.0	-4.7 ± 3.9	-4.5 ± 5.1	0.938
Plectidae[q,b]	B	-82.0 ± 142.5	-80.7 ± 142.9	-82.4 ± 142.7	0.943
Mesorhabditis[q]	B	-26.0 ± 23.2	-24.5 ± 20.5	-29.2 ± 22.6	0.837
Tylenchorhynchus[m]	PP	-129.3 ± 156.8	-31.8 ± 287.8	-301.6 ± 300.9	0.437
Trichodorus[m]	PP	-26.7 ± 92.9	-55 ± 70.2	-39.3 ± 48.7	0.794

[a] B: bacterivorous, F: fungivorous, C: carnivorous, O: omnivorous, PP: plant-parasitic

[b] Plectidae except for the genus Anaplectus.

[c] Kruskall-Wallis test

[m] Analysed microscopically

[q] Analysed by quantitative PCR

Discussion

Soil fumigants, among the most rigorous and non-discriminative measures that can be taken to control soil-borne pathogens including plant-parasitic nematodes, have even been banned in many countries or will be banned in the near future. Hence, there is a strong need for alternative, more sustainable control methods. One of the relatively new approaches exploits the presence of glucosinolates (GSLs) in many Brassicaceae. GSLs are produced by these plants to prevent herbivory by phytophagous insects. Here, we investigate the effects of various Indian mustard cultivars on targeted and non-targeted fractions of the nematode community.

Relationship between GSLs (ITCs) and biofumigation effects on different nematode taxa

Based on the GSL contents of the plants just prior to incorporation, GSL concentrations in the topsoil were calculated. The reportedly high producing cultivar ISCI-99 indeed produced the highest yield of GSLs. Overall, the total incorporated biomass and GSL levels of the four cultivars were comparable with the levels previously reported for Indian mustard cultivars (*e.g.* Hartz et al., 2005, Morra and Kirkegaard, 2002).

One day after biofumigation, no significant differences in densities of individual nematode taxa were detected between the three commercial Indian mustard cultivars on the one hand, and the high producer ISCI-99 on the other. We are aware that the calculated final GSL concentrations in the topsoil are a precursor of the bioactive ITCs, and that several factors co-determine the efficiency of the desired conversion. Upon incorporation of GSL-containing plant material into the topsoil, the degree of cell disruption of the material (Morra and Kirkegaard, 2002) and the activity of myrosinase as influenced by temperature and pH (Van Eylen et al., 2007) can have a considerable effect on the release efficiency of ITCs. However, as mulching and incorporation took place by a standardised procedure and on the same day, a substantial difference in conversion efficiencies between cultivars seems unlikely.

To address the issue of the possible low ITC release, we included treatments in which two concentrations of 2-propenyl ITC were directly applied to wheat plots. However, also in this "positive control" experiment no differential change in nematode taxa between the low and the high 2-propenyl ITC concentration and untreated wheat subplots could be detected (Table 3). If 2-propenyl ITC is released or directly applied to the soil, its volatility is influenced by soil texture and temperature (Price et al., 2005), its degradation rate depends on the activity of the *in situ* soil microbial community (Gimsing et al., 2009, Warton et al., 2003), and its degree of sorption is affected by the organic matter fraction in the soil (Gimsing et al., 2009).

Translation of toxicity levels for pathogens derived from *in vitro* settings to soil systems is not straightforward. In one *in vitro* study with autoclaved silica sand, Zasada and Ferris (2003) showed that the LC50 values of 2-propenyl ITC and of the soil fumigant metam sodium were similar for the plant-parasitic nematodes *Tylenchulus semipenetrans* and *Meloidogyne javanica*. In a follow-up greenhouse study, Zasada and Ferris (2004) amended 0.7 to 2.9% (w/w biomass levels) *B. junce*a into a soil (inoculated with either *T. semipenetrans* or *M. javanica*) theoretically corresponding to the LC50 and LC90 levels determined *in vitro* while correcting for the estimated release and conversion efficiency of GSLs. They showed that the amount of GSLs added to the

soil through the plant material had to be up to 70% higher as compared to the *in vitro* experiment in order to achieve the same results. In our study, the concentration of the highest 2-propenyl ITC treatment added to the soil of the wheat plots was about 2.5 and 12 times higher than the expected LC50 values for the two plant-parasitic nematodes mentioned before. However, we did not observe any effect on nematode densities in our field experiment. An explanation could be that *T. semipenetrans* and *M. javanica* are more susceptible to 2-propenyl ITC than the taxa occurring in our field experiment, but – more likely – other unknown parameters might hamper the translation of results from *in vitro* or greenhouse experiments (*e.g.* Lord et al., 2011, Zasada and Ferris, 2004) into field conditions with biofumigation protocols that will robustly reduce (plant-parasitic) nematode populations.

Non-ITC-related effects of biofumigation on different nematode taxa

If the observed reduction in total nematode density as well as the decreased densities of most free-living nematode taxa (eight out of ten studied taxa) one day after biofumigation are unrelated to GSL or ITC concentrations, other explanatory factors should identified. These decrements may be related to a combination of mechanical stress (tillage) and or the release of compounds other than ITC from the plant material. Little is known about sensitivities of individual nematode genera or families to the mechanical stress of tillage, as most studies focus on the overall effect of tillage (including enrichment) at trophic group levels (*e.g.* Fu et al., 2000, Timper et al., 2012, Treonis et al., 2010). However, Fiscus and Neher (2002) demonstrated distinct sensitivities of nematode genera for the direct and indirect effects of tillage by performing a canonical analysis of data from two field experiments. The categories of sensitivity appointed to different nematode genera in their study only partially explain our results. For instance, the genus *Achromadora* (Achromadoridae) showed a significant decrease on day 60 (Fig. 2A), while *Monhystera* (Monhysteridae), which is placed into the same tillage sensitivity category as *Achromadora*, did not (Fig. 2B). In many studies on the effects of tillage on nematode communities, data are analysed at trophic group level, while the original data, at genus level, are not included (*e.g.* Lenz and Eisenbeis, 2000, Rahman et al., 2007, Timper et al., 2012). Reviewing primary data from tillage studies performed could give insight in the differential effects of tillage on nematode genera.

Next to ITCs, non-glucosinolate derived, sulphur-containing compounds are released during the incorporation of *Brassica* plant material in topsoil. In *B. juncea,* these sulfides are generally less toxic than ITCs but are present in higher concentrations (Bending and Lincoln, 1999). Wang et al. (2009) demonstrated that high concentrations of dimethyl disulfide and methylsulfide could be related to suppression of pathogens. However, as we did not observe any difference in the degree of nematode decrease between *B. juncea* and wheat plots, we cannot confirm this assumption for our data. We conclude that the observed responses of the free-living nematode taxa one day after biofumigation are mainly due to mechanical disturbance.

Subsequent effects of biofumigation on nematode taxa density

Effects on nematode densities three weeks and two months after incorporation of mulched Indian mustard into the topsoil are supposedly unrelated to the release of ITCs. All densities of free-living nematode taxa had increased, while the numbers of plant-parasitic taxa declined.

These results correspond to earlier findings by Valdes and coworkers (2012), who studied the nematode density before biofumigation and 6 weeks after. As also mentioned in their study, the increase in the density of free-living nematode taxa is most likely related to the incorporation of plant biomass, *i.e.* green manuring. Our results suggest that all free-living nematode taxa were able to benefit from this green manure within a timespan of weeks to months. Gruver et al. (2010) revealed that the quality of the plant material was major determining factor for the subsequent (> 6 weeks) effects of biofumigation on the nematode community. In their study, they used various *Brassica* species with distinct C/N ratios. Our experiment concentrated on the effects of various cultivars of a single *Brassica* species with consequently low variation in plant biomass and C/N ratios, and, hence, no differential, green manure-related effects were observed.

We ascribe the decrease of plant-parasitic nematodes after biofumigation (three weeks and two months) to the mere absence of host plants. This option is also considered in the study by Valdes et al. (2012), in which a decrease in potato cyst nematodes (PCNs) was observed 6 weeks after biofumigation with yellow mustard. However, encysted PCNs can survive for months if not years in absence of a host, and – more likely – this decrease was attributed to green manure related biotic suppression. Stirling et al. (2001) showed that the density of *Tylenchorhynchus* was very low in bare fallow soil and when planted with sugarcane could return to very high numbers. This indirectly suggests that a fraction of the population can survive without a host for a long period, and due to its short reproduction cycle it can re-establish high densities whenever a host is available again.

Conclusions

Our results demonstrate that 'biofumigation', the release of isothiocyanates due to incorporation of mulched plant material from four distinct *B. juncea* cultivars, did not directly affect plant-parasitic or free-living nematode populations. The observed short-term (day 1 after biofumigation) reduction in nematode densities was non-distinct from the effects of wheat incorporation, and therefore unrelated to *B. juncea*-derived GSLs. Also, direct application of relatively high concentrations of 2-propenyl ITC (up to around 480 mol ha-1 resulting in 195 nmol g-1 dry soil) did not result in shifts distinct from those observed in untreated wheat plots for the plant-parasitic and free-living nematode taxa under investigation. We therefore conclude that the observed direct and subsequent effects of biofumigation on the nematode community were mainly attributable to a combination of tillage and green manuring, and not to the effects of *B. juncea*-produced ITCs *per se*.

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Chapter 5

In comparison to its parental line and four other conventional cultivars, GM waxy starch potato has no distinct detectable effect on the soil food web as revealed by nematode community analysis

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Abstract

Since the first introduction of genetically modified (GM) crops in 1996, there has been intensive debate about the impact of this new and powerful technique on the environment. Soils constitute highly complex and biodiverse habitats, and – compared to aboveground parts of the ecosystem – little attention has been paid to possible adverse effect of GM crops on its ecological functioning. Here we investigated belowground impact of a marker-free GM potato (Solanum tuberosum, cultivar Modena) blocked in its amylose biosynthesis pathway. In a follow-up on studies concentrating on two organismal groups residing in the first trophic of the soil food web, bacteria and fungi (İnceoğlu et al. 2010, Hannula et al. 2012), we investigated possible side-effects of the waxy starch GM potato 'Modena' on higher trophic levels. For this, we concentrated on the free-living nematode community, as this organismal group is represented in multiple trophic levels. A field experiment was performed at two sites in which nematode communities were analysed from plots grown with the GM variety Modena, its parental conventional isoline, and four other conventional potato cultivars, each at two time points. Although quantitative PCR-based analysis of eight nematode taxa revealed clear location and sampling-time effects, no GM waxy starch potato-related changes were observed in the parts of the nematode community examined here. In combination with results from previous studies on other components of the soil food web, we tentatively conclude that the GM potato cultivar Modena has no observable GM-related effects on the soil food web during its growing season.

Introduction

Since the initial commercialization of genetically modified (GM) crops in 1996, the area planted with these crop varieties has increased from 1.7 million to 160 million hectares in 2011 (Privalle et al., 2012, James, 2011). At a worldwide scale, this acreage is asymmetrically distributed; about 2/3 of this area is located in USA, Brazil and Argentina (James, 2011). As a result of considerable public concern about the safety with of this new technology, the overall GM crop area in Europe is very small.

Before commercialisation, newly developed GM crops are subjected to risk assessment. Although the amplitude and stringency of these testing procedures differ per region and country, most schemes focus on the safety of such GM crops (*i.e.* harvested plant parts, its derivatives, and the remains) for use as food and feed for humans or livestock. In addition, GM crops are often tested for their possible adverse effects on the environment (Privalle et al., 2012). Such an environmental risk assessment may include the effects of GM crops on soil biota (Icoz and Stotzky, 2008).

Depending on the nature of the genetic modification(s), GM plant parts could affect biological soil functioning through changes in the plant's root physiology, root exudates, and litter quality (Powell, 2007). Several studies have focused on the direct effects of GM crops on bacterial and fungal communities, as main organismal groups that use GM crop exudates or remains as primary food source, during the growth season of GM crops (*e.g.* (Gschwendtner et al., 2011, Girlanda et al., 2008, Hannula et al., 2010, Hannula et al., 2012, Weinert et al., 2009). Although it is conceivable that subtle disturbances at the first trophic level of the soil food web might trigger more robust secondary effects at higher trophic levels, only few studies paid attention to effects of GM crops on higher trophic levels of the soil food web (Powell, 2007).

Due to their trophic diversity and differential sensitivities to environmental disturbances, the free-living nematode community constitutes an informative component of soil food webs in arable fields (Neher, 2001, Bongers, 1990). Qualitative and/or quantitative changes in the composition of nematode assemblages can be indicative for the impact of agricultural practices (Mulder et al., 2003), and for the differential effect of plant genotypes on soil biota. Changes in nematode communities are not only induced by crop rotation and/or changes in the composition of the plant community (Wardle, 2002) but can even be visible amongst varieties of a single crop species (Palomares-Rius et al., 2012).

Nematodes have occasionally been used as indicators for the impact of GM crops on belowground systems (*e.g.* Höss et al., 2011, Griffiths et al., 2006, O'Callaghan et al., 2008). The microscopic analysis of nematode assemblages from environmental samples by microscope requires a high level of taxonomic expertise and is invariably time-consuming. The recent development of molecular assays for the quantitative analysis of nematode communities based on taxon-specific sequence motifs in the small subunit of the ribosomal DNA (Vervoort et al., 2012) greatly facilitates the processing of large number of environmental samples in a relatively short time span.

In this study, we concentrated on the possible belowground side effects of a marker-free GM potato (*Solanum tuberosum*) cultivar modified in its starch production. "Marker-free" refers to the absence of a plant-transformation marker. Normally starch in potato consists of amylose and amylopectin in a ratio of about 1:5 (Broothaerts et al., 2007), but tubers of the

cultivar Modena (BASF Science co. GmbH, city, Germany), produce amylose-free starch. This was accomplished by the inhibition of the transcription of the granule-bound starch synthase (GBSS) gene, which is essential for amylose production. The resulting amylose-free starch, so-called waxy starch, is clearer, stickier and retrogradates slowly as compared to normal potato starch. These characteristics make waxy potato starch an attractive ingredient for a range of industrial applications. Because of the nature of this particular trait, the directed inhibition of a pathway in the carbohydrate household and the absence of a plant-transformation marker (De Vetten et al., 2003), its potential effects should be sought in changes in the plant's carbohydrate metabolism. This could have consequences for the composition and/or quantity of exudates released in the rhizosphere.

Our objective was to compare the nematode communities in the close vicinity of roots, stolons and developing tubers from various potato varieties, including the GM Modena and Karnico, the latter being parental line of Modena. In order to frame potential GM trait-related effects in the background of variation due to conventional genotype variation (Griffiths et al., 2007), we included four conventional potato cultivars, two with high and two with low starch content. Belowground effects were studied in two experimental fields at two time points during the growth season. Densities of individual nematode taxa were monitored using a recently developed quantitative PCR-based method (Vervoort et al., 2012).

Materials and Methods

Study sites and experimental design

The experiment was performed at two experimental fields ('VMD' and 'BUI'), located at approximately 10 km distance of each other in the province of Drenthe, the Netherlands. Soil from VMD was characterized as a sandy peat soil (organic matter: 26%, pH (H2O): 5.0) and the soil from field BUI as loamy sand (organic matter: 6%, pH (H2O): 5.0). The water retention ranged from 40-46% at VMD to 25-29% at the BUI location (Hannula et al., 2012). At each field, six different potato (*Solanum tuberosum*) cultivars were grown; two cultivars have relatively high growth rates and a low starch content: 'Premiere' (P) and 'Désirée' (D), four cultivars with a relatively low growth rate and high in starch content: 'Aveka' (A), 'Aventra' (Av), 'Karnico' (K), and an amylose-free potato cultivar named Modena (M). Each field consisted of four replicate plots per cultivar (24 plots in total), which were distributed using a randomized complete block design. Each plot contained 28 plants divided over four ridges.

Soil sampling and nematode extraction

At both experimental fields, soil samples were taken at two time points in the growth season, on July 12th (flowering stage) and August 15th in 2011(senescence stage). Per plot, a composite soil sample was taken, consisting of 16 cores (Ø 2 cm, depth 20 cm) taken from the four potato ridges in each plot, and was stored at 4 °C until further use. Nematodes were extracted from 100 ml bulk soil using an elutriator (Oostenbrink, 1960). Nematode densities were determined in the resulting nematode suspensions (100 ml, in tap water) by counting two subsamples (5 ml each) under a low magnification inverted microscope. Subsamples were poured back after counting and the total nematode suspensions were further processed for quantitative PCR (qPCR) analysis.

Nematode analysis

Nematode suspensions (n = 96) were used for DNA extraction and lysate purification. Purified DNA extracts were used as a template in qPCR using nematode taxon-specific primer combinations (for details see Vervoort et al., 2012). A microscopic analysis of the nematode biodiversity at the two locations (sampling time: July 2010, see Supplementary Table S1) was used to select the most informative taxon-specific PCR primer combinations. In total, 12 primer combinations specific for free-living taxa were selected for qPCR analysis of the samples. Of these 12 analysed taxa, four taxa, Anatonchidae, Dorylaimidae D3, Metateratocephalidae and Mylonchulidae, were present in less than 50 % of all samples and were not included in the full data analysis (Table 1).

Data analysis

Nematode densities were analysed using mixed linear models (using PROC MIXED of the SAS software system version 9.2, see (Littell et al., 2006)). With the exception of the total nematode densities, all data were transformed to obtain approximate normal distributions of residuals as required for valid statistical inference. Cephalobidae densities were square root-transformed; and all other nematode taxon densities were log-transformed. The log-transformation was applied after addition of a constant (0.5 in general, and 5 for Monhysteridae). The fixed part of the mixed model contained main effects of cultivar, location and time and their two-way interactions. The random part of the model consisted of random effects for blocks, individual plots, and residual error. In addition, because of the special interest in the comparison of the starch-modified potato (Modena) with the parental isoline (Karnico), the differences in nematode densities between the two groups were estimated and tested (by approximate t-tests), averaged over time and locations, and location-specific averaging over time alone, using tailor-made ESTIMATE statements of PROC MIXED within the larger mixed model.

Results

In a field experiment designed to detect possible environmental effects of the knock out of a granule-bound starch synthase (GBSS) gene, a gene encoding an essential enzyme in amylose biosynthesis, in potato, no significant differences in nematode densities (both overall and at individual taxon level) between the waxy starch GM Modena and the non-GM parental cultivar Karnico could be detected (t-test statistic for difference Karnico-Modena in total nematode density t30 = -0.28, P = 0.782; for individual taxa t-test statistic t30 ranges from -1.20 to -0.10, all P ≥ 0.238). In addition, no significant cultivar effects were observed for both total and taxon-specific nematode densities (Table 1).

The variables ‘sampling time’ and ‘location’ strongly affected both the overall nematode densities and the levels of several nematode taxa (Table 1). On the first sampling date, total nematode density was similar for both locations (Fig 1. and Table 2). Nematode density at the VMD location remained comparable over time, while at the BUI location total nematode density was lower on the second sampling date compared to the first (Table 2 and Fig. 1). The qPCR-monitored taxa included in this analysis encompassed five bacterivorous (B), one fungivorous (F), one omnivorous (O) and one carnivorous taxon (C). Although present in over

Table 1. Summary of ANOVA (Fdf and associated P values) for differences in total nematode density (100 g-1 dry soil, analysed by microscope) and the density of 8 individual nematode taxa (100 g-1 dry soil, analysed by quantitative PCR) between six different potato (*Solanum tuberosum*) cultivars. The belowground impact of the genetically modified, waxy starch cultivar Modena was compared with its parental line Karnico, and four other cultivars: Aveka, Aventra, Désirée, and Premiere at two locations and at two time points. Both main and interaction effects were determined based on mixed models fitted to the variables (see materials and methods, significant P values (< 0.05) in bold).

		Main effects						Interaction effects					
		Cultivar		Location		Time		Cultivar*Location		Cultivar*Time		Location*Time	
	Trophic[a]	$F_{5,30}$	*P*	$F_{1,30}$	*P*	$F_{1,41}$	*P*	$F_{5,30}$	*P*	$F_{5,41}$	*P*	$F_{1,41}$	*P*
Nematode density [M]		1.25	0.313	15.33	**0.008**	11.73	**0.001**	2.58	**0.047**	0.56	0.726	9.41	**0.004**
Achromadoridae	O	0.16	0.976	8.96	**0.024**	39.20	**<0.001**	0.89	0.499	0.09	0.993	1.17	0.286
Aphelenchidae	F	1.79	0.145	51.73	**<0.001**	7.48	**0.009**	1.01	0.427	1.80	0.134	15.51	**<0.001**
Cephalobidae	B	1.73	0.158	10.28	**0.019**	1.73	0.158	0.69	0.632	0.79	0.566	46.15	**<0.001**
Monhysteridae	B	1.51	0.218	0.46	0.524	1.58	0.216	0.34	0.884	1.09	0.382	1.34	0.253
Mononchidae M3	C	1.20	0.333	172.66	**<0.001**	1.05	0.310	0.95	0.463	0.47	0.798	0.67	0.418
Panagrolaimidae	B	0.31	0.905	0.16	0.704	1.42	0.240	2.39	0.061	1.89	0.116	0.09	0.764
Plectidae*	B	1.28	0.297	3.23	0.122	22.49	**<0.001**	0.15	0.978	1.26	0.302	27.72	**<0.001**
Prismatolaimidae	B	0.95	0.465	20.59	**0.004**	2.31	0.136	0.38	0.862	2.75	**0.031**	0.07	0.799

[a] Main trophic groups assigned primarily as in Yeates et al., 1993; B: bacterivorous, F: fungivorous, C: carnivorous, O: omnivorous.

[M] Total nematode density was determined by microscope prior to DNA extraction for qPCR analyses.

* Plectidae except the genus *Anaplectus*.

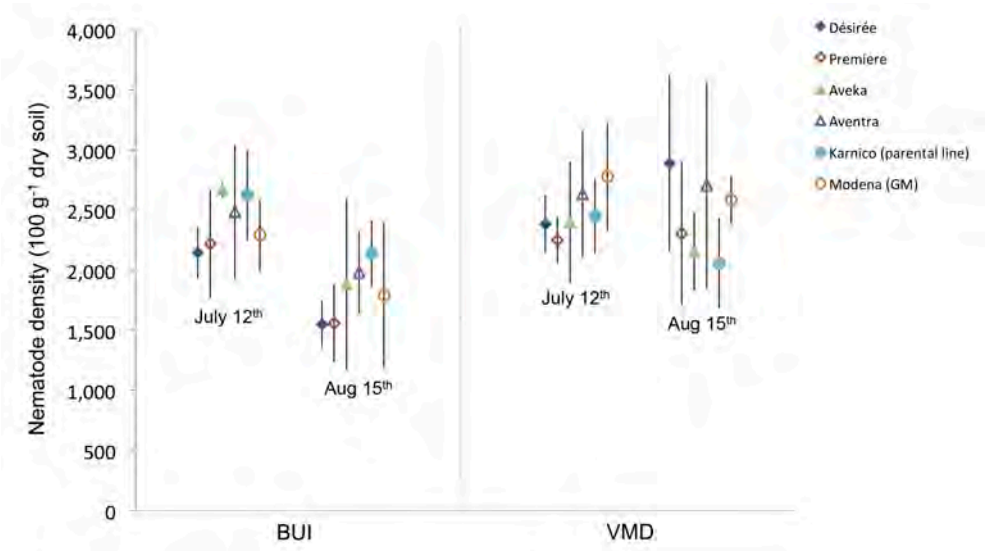

Figure 1. Nematode density (100 g-1 dry soil) in soil planted with six different potato (*Solanum tuberosum*) cultivars, including the genetically modified waxy starch variety Modena of one of the other five included conventional cultivars (i.e. Karnico), measured at two time points during the growing season (the 12th of July and the 15th of August) at two experimental fields (BUI and VMD).

50% of all samples (n = 96, both locations combined), the densities of the taxa Aphelenchidae (fungivore), Panagrolaimidae (bacterivore), Mononchidae M3 (predator) and Dorylaimidae D3 (omnivore) were relatively low as compared to the other taxa (on average below 10 individuals per 100 g dry soil)

both locations, the bacterivorous families Plectidae and Cephalobidae were the most abundantly and consistently present. 'Location' had a significant effect on densities of five out of the eight taxa covering all occurring feeding habits (Table 1): the densities of Achromadoridae (O), Aphelenchidae (F), Cephalobidae (B) were higher at the BUI location (Table 1 and 2, *P*-values: 0.024, <0.001 and 0.019, respectively), while the overall difference in (both time points combined) the density of the taxa Mononchidae M3 (C) and Prismatolaimidae (B) was higher at the VMD location (Table 1 and 2, *P*-values: <0.001 and 0.004, respectively).

Time of sampling had a significant effect on the densities of the taxa Achromadoridae, Aphelenchidae and Plectidae (Table 1). For the families Aphelenchidae, Cephalobidae and Plectidae, there was an interaction of time and location (Table 1). At both locations, Achromadoridae increased both in abundance (Table 2) and occurrence (BUI: from 76% to 97%. VMD: from 50% to 88%). As mentioned before, the predominantly fungivorous family Aphelenchidae was overall present in relatively low numbers and showed a decrease in abundance (Table 2) as well as occurrence (100% to 58%) at the BUI location and an increase in abundance (Table 2) and slightly in occurrence (38% to 50) at the VMD location. For the bacterivorous family Cephalobidae the average density decreased over time for Location BUI, but increased for

VMD (table 2), resulting in a significant interaction (P=<0.0001, Table 1). Another relatively abundant bacterivorous family, the Plectidae, showed a strong effect of time, and its density was higher at the second sampling time at both locations. This increase was much larger in location VMD than in BUI, resulting in a significant location*time interaction.

Table 2. Descriptive statistics (overall average ± standard deviation) of untransformed total nematode density (100 g-1 dry soil, analysed by microscope), and density of eight nematode taxa (100 g-1 dry soil, analysed by quantitative PCR) in two experimental fields (BUI and VMD) at which the belowground impact of the GM waxy starch cultivar Modena was compared to five other potato cultivars at two time points during the growth season. In addition, the percentage of samples in which the taxon was detected is given per location. Nematode taxa are defined as by (De Ley et al., 2006), except for Mononchidae M3 (see (Holterman et al., 2008)).

	BUI			VMD		
	July 12th	Aug 15th	%	July 12th	Aug 15th	%
Nematode density	2406 ± 380	1818 ± 453	100	2483 ± 391	2451 ± 586	100
Achromadoridae	41.3 ± 101.1	348.1 ± 683.2	87.5	4.2 ± 9.8	40.6 ± 56.2	68.8
Aphelenchidae	5.9 ± 4.3	2.8 ± 4.4	79.2	0.5 ± 0.7	0.9 ± 1.2	43.8
Cephalobidae	441 ± 150.7	150.4 ± 146.9	100.0	143.4 ± 123.2	232.5 ± 140.5	100.0
Monhysteridae	35.5 ± 50.4	33.9 ± 38.9	70.8	21.9 ± 17.8	68.6 ± 76.1	75.0
Mononchidae M3	0.5 ± 1	0.5 ± 0.9	29.2	6.1 ± 5.2	7.3 ± 4.6	97.9
Panagrolaimidae	0.8 ± 0.6	1.7 ± 2.8	75.0	0.8 ± 0.6	1.4 ± 2	62.5
Plectidae*	44.5 ± 40.8	111.7 ± 192.6	95.8	76 ± 150.4	1594.4 ± 2091.1	100.0
Prismatolaimidae	1.4 ± 2.9	3.1 ± 6.5	68.8	14.5 ± 22.5	58.9 ± 104.1	93.8

* Plectidae except for the genus *Anaplectus*.

Discussion

Introduction of genetically modified (GM) crop varieties is invariably preceded by an extensive risk assessment. Apart from testing the safety of resulting products on the end-consumers, humans or livestock, this risk assessment also includes the testing for possible adverse effects on the environment. In this study, we measured the belowground effect of a GM waxy starch potato cultivar, as compared to its untransformed parental line and four other conventional potato cultivars with different starch contents during their growing season. Nematodes are trophically diverse, and changes in the bacterial or fungal community will be observable as corresponding shifts in a subset of the bacterivorous and fungivorous nematode taxa. In this field experiment, densities of different nematode taxa were determined at two locations and two time points. Overall, neither a cultivar effect (taking all six cultivars into consideration), nor a genetic modification related shift (direct comparison between Karnico and the derived waxy starch cultivar Modena) was detected in the total nematode density or in the densities of the individual taxa. However, clear effects of sampling time and of location on densities of nematode taxa were observed.

We did not observe any effect of the plant genotype and genetic modification on the nematode taxa included in this study. This result corresponds to the outcomes of studies that focussed on

the effects on the microbial community in the same experimental fields (Hannula et al., 2010, Hannula et al., 2012b, İnceoğlu et al., 2010, İnceoğlu et al., 2011). In these studies, differences in the fungal or bacterial community between Karnico and Modena were either not significant or transient and fell within the range of variation observed for the other potato cultivars. For both, the major determining factors were growth stage of the plant and soil type, and not the genotypic constitution of the potato cultivars included in this research (Hannula et al., 2012, İnceoğlu et al., 2010). Hence, the currently presented nematode community composition data point at the same direction as previously presented data on the bacterial and fungal community suggesting that factors such as plant development and soil type affect the composition of the soil food web, whereas – within this particular set of six potato cultivars – the impact of plant genotype is not significant.

Here, two soil types representing the two types of starch potato production areas in The Netherlands were included. Overall, we observed a strong effect of location on the total nematode density and several of the monitored nematode taxa. The overall differences in nematode community composition between the two sites ('location effect') can almost fully be attributed to the difference in soil structure as the two other major determinants, temperature and precipitation / evaporation, hardly differed between the two experimental sites (distance between sites just 10 km, no natural barriers between sites). Although located relatively close to each other, the soils of the two experimental fields differed considerably in their organic matter (OM) content (VMD: 26% OM, BUI: 6% OM). In a survey on the impact of various crops (92 fields in North Carolina, USA) on the nematode community composition, no direct correlation was found between organic matter contents – ranging in these fields from 0.6 to 45.7% – and the relative abundances of trophic groups (Neher and Lee Campbell, 1994). Alternatively, the observed location effects could be attributed to differences in the soil particle sizes and water retention of the two soils, as nematodes are dependent on the water film between soil particles for their movement and reaching their food sources. (Neher et al., 1999) studied the effects of soil moisture content on nematode communities. Their results showed the genus *Prismatolaimus* to be consistently present in high moisture containing soils, which corresponds to our results. In the case of the family Cephalobidae, for which we see a similar pattern in our study, their study also showed a dependency on soil moisture content, however this effect interacted with the month of sampling (Neher et al., 1999).

The results of our field experiment confirm the relevance of the inclusion of multiple soil structures, and showed that neither of the two distinct nematodes assemblages was affected to a detectable degree by the nature of the potato cultivar and or genetic modification. Soil texture is one of the major determinants of the nematode community (*e.g.* Ronn et al., 1995), and possible community-disturbing effects of GM crops should therefore be investigated in the range of soil types relevant for the crop of interest. As we also detected effects of sampling time, it seems favourable to include different sampling times during the growing season when monitoring GM crop effects. In arable fields, seasonal effects such as plant development, rainfall or temperature can have an impact on the soil faunal composition (Wardle, 2002, Sohlenius and Boström, 2001).

The GM potato variety Modena had no detectable effect on the nematode community in our field study. It must be underlined that the results of this study concern the effect of a specific GM-trait, actually the knock down of a specific carbohydrate metabolism pathway,

and are not translatable to other modifications or crops. Within our experimental set up with two experimental fields and two sampling moments, only soil type and sampling time had a significant effect on the native nematode community.

Investigations on possible unwanted or unexpected side effect on soil biota including betaproteobacterial, fungal and nematode communities all indicate that this particular GM waxy starch potato variety has no measurable consistent GM-related effect on one of these organismal groups (İnceoğlu et al., 2010, Hannula et al., 2010, Hannula et al., 2012). Based on the relevance of these groups in terms of biodiversity, biomass and trophic connectedness, we tentatively conclude that the waxy starch GM potato cultivar Modena has no significant effect on the soil food web that could be related to its GM nature.

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Chapter 6

General discussion

Mariëtte T.W. Vervoort

General discussion

The use of indicators for assessing the biological functioning of ecosystems is common practice. Nematodes meet several requirements that make them suitable as a bio-indicator group of the soil ecosystem. In comparison to other major soil inhabitants such as bacteria and fungi, they are easily extractable from soil, occupy central positions in the soil food web and have differential sensitivities to both natural and anthropogenic disturbances. However, the microscopic identification of nematodes demands taxonomic expertise and a considerable investment of time. This thesis presents the results of the first field applications of a molecular tool for the quantitative and qualitative monitoring of nematode assemblages. It shows the impact on the nematode community of different types of disturbances, either directly or indirectly related to anthropogenic practices. In this chapter, several aspects concerning the molecular method, the use of nematode taxa as indicators and the challenges and future opportunities for this group of organisms in soil ecological research will be discussed.

A molecular approach to nematode community analysis

As described in Chapter 1, multiple molecular methods have been developed over time for the analysis of nematode communities (Chen et al. 2010). Even without the sequence technology available to us today, Van Der Knaap et al. (1993) demonstrated the value of molecular methods in nematode identification by showing the ability to distinguish species of handpicked individuals based on their produced band patterns using random amplification of polymorphic DNA (RAPD-PCR). Twenty years later, we have arrived at the point of being able to perform directed metagenomic studies using high-throughput sequencing (Porazinska et al. 2009). Nevertheless, data processing and analysis of these large (around 10,000 reads per sample) high-resolution datasets, takes a considerable amount of time. Although high-throughput sequencing delivers a vast amount of information, this type of data may not be required for monitoring studies and its laborious nature may at this stage form a practical limitation for the experimental set up. For monitoring studies, methods such as terminal restriction fragment length polymorphism (T-RFLP) (Donn et al. 2012) or a quantitative PCR (qPCR) based approach (Vervoort et al. 2012) may be more practical and will allow for more intense sampling schemes. In other words, the method of choice for nematode community analysis will very much depend on the underlying research question(s). Directed (T-RFLP and qPCR) and undirected (high throughput rDNA sequencing) have their inherent pros and cons and suitability will depend on the precision, resolution and scale required to comply with the aims of the study in which it will be applied.

Approaches based on quantitative PCR (qPCR) and Terminal-Restriction Fragment Length Polymophism (T-RFLP)

In this thesis, we monitored the nematode community using a qPCR-based approach. So far, most nematological studies using qPCR have designed probes for a specific species or a particular group and or required pre-selection and prescreening of environmental nematode samples (Jones et al. 2006, Toyota et al. 2008, Derycke et al. 2012, Green et al. 2012). Thereby these studies still relied, to a certain degree, on microscopic analysis. A distinctive property of the assays used in the studies presented in this thesis, is their development based on a relatively

versatile, phylum-wide SSU ribosomal DNA framework (Holterman et al. 2006, Van Megen et al. 2009), currently covering over 2,800 sequences. Both the availability of this sequence framework as well as the accompanying rDNA clone library (thousands of *Escherichia coli* clones harbouring nematode rDNA fragments) permits for the development and testing of taxon-specific qPCR assays. The rDNA framework is mined to define *in silico* taxon-specific DNA motifs and to identify close non-targets. It is noted that potential false positives are not necessarily taxonomically related to the target (see Chapter 1). Subsequently, relevant recombinant rDNA fragments are used to test the specificity of assays for which – in a first selection round – cloned rDNA fragments are selected (Holterman et al., 2008). In a second selection round, the most promising primer combination were tested on real nematodes. Primers developed and tested in this way are applicable for use in complex DNA backgrounds without the necessity of pre-selection or pre-screening. Although it is not a necessity, for the studies in this thesis we decided to determine the nematode diversity by microscope, as well. In this way we were able to select a set of assays covering the majority of the free-living nematode diversity at each location. In the meantime, we have developed approx. 70 taxon-specific (family, genus or species level) assays. In future studies, we can now use this set to perform a molecular pre-screening of nematode diversity and composition.

Next to qPCR-based assays, T-RFLP, a molecular fingerprinting method, has been successfully applied for the monitoring of nematode communities in field studies (Donn et al. 2012, Griffiths et al. 2012). For this method, DNA is extracted from soil samples and SSU rDNA is amplified using nematode-specific primers. This complex amplicon is then digested by taxon-specific restriction enzymes, resulting in fragments of different lengths. The relative abundance of each fragment size detected, portrays the abundance of a specific group of nematodes present in the initial sample. The identification of the peaks measured can be accomplished by the beforehand production of a location-specific sequence database; a strategy termed 'directed T-RFLP' (Donn et al. 2012). The necessity of producing location-specific sequence databases combined with the relatively complex sampling handling – amplified of nematode DNA is separated on a capillary sequencer upon restriction by a range of enzymes – could be considered as limitations of this method. Nevertheless, (Donn et al. 2012) showed that this method can reveal changes in the community related to agricultural practice.

The link between microscopic and molecular-based analyses

Our knowledge of nematode ecology is largely based on microscopically acquired data. It is therefore important to find a way to link results produced by a molecular method to those produced by microscope (Chen et al. 2010). However, every molecular method has its own efficiency and precision, dependent on factors such as the extraction efficiency, the occurrence of PCR bias or the precision of the calibration curves used. For microscopic analysis the composition of a sample is generally determined by the analysis of a subsample (often the first 150 adults in a mass slide) of the total community and consequential extrapolation. For a number of species, the scarcity of informative morphological characters for non-adult life stages, often these individuals are discarded from further analysis, introduces an error margin of its own. The direct comparison of data produced by different methods is therefore not as straightforward as it may seem. Nevertheless, it may be possible to qualitatively compare the results

obtained by different methods, for instance, in terms of their ability or sensitivity to detect certain changes or differences in a community. While determining which method is overall the best to use is difficult, at least in this way it will become clear what type of information each method can provide.

Nematode taxa as indicators for human-related disturbances

Especially since the start of soil cultivation, anthropogenic activities have had an impact on the aboveground and the belowground biodiversity and functioning of ecosystems (Bardgett and Hefin Jones 2012). Soil ecological research has in different ways contributed to our understanding of the consequences of these disturbances. For the soil ecosystem, the nematode community has been used as an environmental indicator of the impact of different types of disturbances directly or indirectly related to human practices such as tillage, pollution, climate change, invasive plant species, GM crops (Powell 2007). To a certain degree, all chapters in this thesis concern a different type of disturbance directly or indirectly related to human practices; recovery after prolonged use for arable farming (Chapter 2), the impact of agricultural management practices (Chapter 4), the effect of an introduced invasive plant species (Chapter 3), and the possible unintended effects of a GM crop (Chapter 5). These field studies were performed in both semi-natural (Chapter 2 and 3) and agricultural (Chapter 3 and 4) systems. Inherently, an agricultural system is exposed to disturbances of a different character and at a different frequency compared to a natural system. However, it is not only the nature and frequency of a disturbance that determines its effect, but also the pre-disturbance state or history of the soil in which it occurs (Grandy et al., 2012). In this section, I will first discuss the relative degree of impact by different types of disturbances on the nematode community and the soil food web. Finally, I will discuss the potential of individual nematode taxa as indicators for specific types of disturbances.

The impact of land-use change on the soil food web

In Chapter 2 of this thesis, we monitored the nematode community of an ex-arable field and its adjacent beech forest and showed that these differed considerably both spatially and throughout time. Apart from the differences in vegetation between the two areas, the soil pH as well as soil structure were distinct. The ex-arable field studied in Chapter 2 was previously included in a study focused on the succession and development of above and belowground communities after abandonment (*e.g.* Kardol et al. 2005, Van Der Wal et al. 2006, Holtkamp et al. 2008). For this purpose, the soil food web was monitored for a chronosequence of abandoned agricultural fields sharing the same soil type and located within a region in the Netherlands. The results of these studies showed that when a field is abandoned after a period of cultivation, there is an initial short period of change in the nematode as well as soil community. This short period of change is most likely related to the release of physical stress from agricultural practices, such as tillage (Kardol et al. 2005), which for instance in Chapter 4 but also in other literature (*e.g.* Fu et al. 2000) has been shown to have a strong effect on the nematode community. However, the chronosequence studies showed that in the decades following this response there is a difference in the speed of aboveground and belowground succession, with the development of the nematode community as well as the overall structure of the soil food web lagging behind

when compared to that of the plant community. Other studies concerning belowground recovery after cultivation have shown similar results (Buckley and Schmidt 2001). Overall, this demonstrates that effects of land use, the combination of *e.g.* liming, fumigating, fertilizing, tilling the soil, can have a considerably effect on an ecosystem that lingers over a period of decades. Hence, for the assessment of ecosystem recovery, the monitoring of aboveground succession only doesn't provide us with the full picture with regard to ecosystem recovery; inclusion of the belowground part – the soil ecosystem – is essential (Young et al., 2005).

Plant identity effects belowground

Plant species can differ in their effects on the soil ecosystem and its inhabitants. These differences cannot only be ascribed to variation between species in their nutrient uptake or their quantity of input into soil, but also to differences in the quality of the resource input, *e.g.* carbon to nitrogen ratio, lignin or cellulose content or due to differences in the production of different secondary metabolites (Wardle 2002). Next to the quantity and character of resource input and output, the presence of a plant species can also partially determine microclimate conditions, affecting *e.g.* soil moisture retention or soil temperature through their height and density (Ehrenfeld et al., 2001). For the nematode community, De Deyn et al. (2004) showed that plant identity is a determining factor; in this relationship resource quality has shown to be more influential than resource quantity.

Ehrenfeld et al. (2001) showed that invasive plants species can change soil properties and that their effects on the soil ecosystem differ from those caused by native plant species. Moreover, effects may persist after the invasive plants have been removed and these areas can thereafter remain more sensitive to reinvasion (Eviner et al., 2010). This ability of invasive plant species may contribute to their invasiveness and demonstrates the closeness of the relationship between above- and belowground communities. In Chapter 3, we studied the local effect of invasion by the plant species *Solidago gigantea* (Giant goldenrod, native in North America) above and belowground and observed habitat-independent effects on different groups of soil organisms. The observation that *Solidago gigantea* is able to locally alter nutrient cycling (Chapuis-Lardy et al. 2006, Vanderhoeven et al. 2006, Herr et al. 2007) could be related to the shift in the soil community that we observed. Apparently *S. gigantea* is to a certain degree able to change the soil food web in such a way that it benefits its own growth, potentially through the quality of its litter or root exudates and or through the exudation of secondary metabolites. The latter can be via *S. gigantea's* release of furanoid and acidic compounds into its rhizosphere (Weber and Jakobs 2005), which are potentially toxic for both native plants as well as present soil organisms. There are strong indications that allelochemicals can play a large role in the invasiveness of plant species. Studies in which activated coal was added to the soil to bind toxic compounds from an invasive plant species resulted in cover reduction of the invader (Kulmatiski and Beard 2006, Eviner et al., 2010). This exemplifies how recent insights in the process by which different invasive species can affect the soil ecosystem may lead to the development of strategies for limiting invasion (Baer et al., 2012). However, at this moment our general knowledge of plant-specific effects belowground is too limited for this purpose. Studies concerning invasive plant species may be a way to serve both the need for nature conservation strategies concerning invasive plant species as well as our need to learn more about plant species-specific effects on the soil ecosystem.

While in Chapter 3 we focused on the effect of an exotic plant species and compared it to native vegetation, in Chapter 5 the effects of different varieties of another exotic plant species in Europe were assessed, namely potato. Moreover, we included what may be considered as an exotic version of this exotic plant species, namely a genetically modified (GM) variety of a conventional potato cultivar, altered in its starch production. The variation of nematode communities under these varieties was monitored to see if the effects of the GM variety on the soil ecosystem differed from those of conventional varieties. In our study, no differences in nematode assemblages under all the included varieties of potato were observed, whether it being a GM, a low producing-, or a high starch-producing conventional cultivar. Naturally, these results cannot be generalized to other GM crops as the character of GM crop traits can be very distinct and should therefore be assessed for each modification individually. Moreover, our study was limited to a single growing season and the results can therefore not account for possible long-term effects.

Individual nematode taxa as indicators for specific disturbances

The nematode community has often been used to study the effects of different measures or disturbances in agricultural systems, such as fertilisation (Ferris and Bongers 2006), the effects of tillage (Fiscus and Neher 2002) or nematicides (Timper et al. 2012) etc. Nematode community indices are widely applied in most of these studies. The most commonly used nematode community indices are based on the so called *cp*-scaling (colonizer – persister), which distinguishes nematode families based on their life strategies, and – related to this – their sensitivity to stress (see Chapter 1). In this thesis, community indices were not applied and we focused mainly on taxon-specific responses to disturbances. This choice has two underlying reasons: firstly a number of families distinguished in classical nematological community analyses such as Rhabditidae, Nordiidae or the Qudsianematidae appeared to by para- and/or polyphyletic (*e.g.* Van Megen et al. 2009). The second reason is that the use of nematode indices typically involves the lumping of data, and generalizations concerning nematode life strategies. Already in 1996, Yeates and Van Der Meulen suggested that the elucidation of so-called "key populations", individual taxa for which the population size can be linked to specific soil conditions or processes, could be more valuable than the use of general nematode indexes. Several recent studies have started to work towards this elucidation by combining datasets and the application of statistical tools (Fiscus and Neher 2002, Zhao and Neher 2013, Zhao et al. 2013).

Zhao and Neher (2013) demonstrated the potential value of meta-analysis of existing data to elucidate consistently responding nematode genera to different types of disturbances. For example, their results (based on data from 7 studies) showed that the genera *Diphtherophora* (Clade 1, fungivore), *Prismatolaimus* (Clade 1, bacterivore) and *Tylenchorhynchus* (Clade 12, a plant parasite) were present in lower densities in standard cultivation fields compared to fields managed by using conservation tillage, suggesting these taxa to be more sensitive to physical disturbance. A high sensitivity of *Prismatolaimus* to physical disturbance in combination with a legacy of tillage may partially explain the contrast in the number of *Prismatolaimus* between ex-arable field and beech forest that we observed in Chapter 2. However, for *Tylenchorhynchus* we monitored short-term sensitivity to tillage and we did not see a significant decrease in abundance between right before and after the soil was tilled while most of the other taxa did

respond (Chapter 4). Zhao and Neher (2013) mention several discrepancies between their overall findings and the results of certain studies. These differences may originate from the variation in soil characteristics, crop types and weather conditions of the individual experiments that were grouped in order to have enough data to perform the analyses.

While Zhao and Neher (2013) show the potential of using meta-analysis studies to elucidate individual taxon indicators, their study also reveals its current limitations. Data of suitable studies is often analysed and presented in papers on the level of trophic groups, although the microscopic analyses often resulted in community characterisation at genus level. In addition, requesting raw data from authors may not always be efficient. The latter is illustrated by the study of Zhao and Neher (2013) in which they received the primary community composition data underlying 21 out of the 48 requested papers. Another factor that complicates data comparison is the fact that research groups often use different nematode extraction methods. Extraction methods can differ in their efficiency of extracting nematodes of different sizes and mobility and can also vary in their suitability for different soil types (Nagy 1996, Yeates and Bongers 1999). The variation introduced by different extraction methodologies may therefore complicate comparison of results from different studies. In the same way, future use of different molecular methods may also complicate the performance of meta-analysis studies and the identification of single taxon indicators for specific disturbances. To achieve this identification, standardised sampling, extraction and analysis methodologies within the nematological research community may increase the use and acceptance of nematodes as bio-indicators.

Challenges in nematode ecology: Scaling up and zooming in

By lifting the practical limitations of microscopic analysis, it is now possible to analyse the nematode communities of large numbers of soil samples at an unprecedented rate and, eventually, at a resolution of our choice. Producing elaborate datasets can reveal new information about nematode ecology as well as soil ecology in general. Nevertheless, our limited knowledge of for instance nematode feeding habits and spatial and temporal variation at high resolutions may hamper progress. It is therefore not only important to gain the possibility to scale up sample sizes (*e.g.* by using a molecular method) but also to zoom in on nematode ecology. Therefore, in this section, I will first discuss the necessity of high-resolution information on nematode feeding habits and the possibilities of molecular tools to contribute to this. Secondly, I will discuss the relevance of gaining more insight into the spatial and temporal patterns of different nematode taxa.

The assignment of nematode feeding types and differential intra-guild feeding preferences

Throughout this thesis, the occurrence or density changes of different nematode taxa are discussed on the basis of their assigned feeding habit or the results of observational studies. However, most feeding types assigned to nematode taxa are inferred from their morphology supplemented with fragmented experimental data and/or anecdotal observations introducing a degree of uncertainty with regard to their real food preferences (Yeates 2003). To illustrate this point the family Tylenchidae can be taken as an example. In soil samples members of this family harbouring five subfamilies and over 50 genera often constitute more than 30% of the

total nematode community (Ferris and Bongers 2006). A genus level their feeding habits are far from clear, and they are generally either assigned to be herbivores (algae, mosses, lichen or higher plants) or fungivores or both (Yeates 2003).

Next to the uncertainty that resides in the assignment of feeding groups, differential feeding preferences within a trophic group, *e.g.* the preference of nematode taxa for specific groups of bacteria or fungi as a food source, are often not defined. In this thesis, both the results from Chapter 2 and 4 show variation in the response of nematodes assigned to the same feeding guild, which is most likely indicative for differences in their food preference. So far, most studies concerning food preference of nematodes have been performed on medium plates in the lab (*e.g.* Bilgrami 1993, Ruess and Dighton 1996, Okada and Kadota 2003, Hasna et al. 2007). However, it has been shown that the results from feeding preference studies performed on agar plates can be different to those performed in soil (Okada and Kadota 2003).

Ideally, as was already stated by Yeates et al. in their overview of feeding habits in 1993, feeding habits and preferences of nematode taxa should be assessed per ecological setting. Even though today this task is still daunting, the application of molecular methods may be able to contribute to this goal. This can be done by a direct approach which is demonstrated by (Read et al. 2006), who studied the feeding behaviour of soil micro-arthropods by sequencing and performing PCRs on the gut content of individuals taken directly from field samples. For this approach, the use of PCR and next generation sequencing allow for identification of feeding associations with an unparalleled precision level and can thereby reveal trophic links which would have been impossible to uncover without molecular methods (Pompanon et al. 2012). Another indirect approach would be the associational divergence of information on feeding relationships from large datasets encompassing high-resolution measurements of groups of soil organism from multiple trophic levels.

Spatial and temporal variation of nematode taxa

Although spatial and temporal variation of aboveground populations has been elaborately studied, this is not the case for belowground communities (Ettema and Wardle 2002). For belowground organisms, progression in this field has been hampered by the inability to process large numbers of samples and distinguish communities of soil biota at high resolutions. Therefore, we are often unable to interpret this type of variation in data and are limited in our ability to extract the information it contains (Ettema and Wardle 2002). Nevertheless, spatial and temporal variation patterns could provide us with information on species coexistence, their relation to abiotic factors and differential sensitivities to disturbances (Levin and Paine 1974). In addition, the fundamental knowledge of the spatial and temporal heterogeneity of nematode taxa in different soils and systems will also improve sampling schemes of future studies. It may also reveal that, depending on the research question, certain nematode taxa are more suitable for use as indicators compared to others. Extensive sampling combined with molecular analyses of the nematode community has the potential to provide us with more insight into spatial as well as temporal variation. At this moment, our group is producing a dataset consisting of the output of molecular analyses of nematode taxa for over 1000 soil samples. With this dataset the patchiness (horizontal spatial variation) of free-living nematode taxa in arable fields will be determined for different soil types.

Conclusions

The results presented in this thesis demonstrate that the use of taxon-specific quantitative PCR-based assays is a suitable method for the monitoring of nematode assemblages in field experiments. This method has a number of advantages: no subsampling is required, analysis is life stage independent, and it does not require microscopic pre-screening or pre-characterisation of samples or sampling sites. The results of these field experiments show that even though changes in the plant community, such as the invasion by an exotic plant species, can have distinct effects on the soil food web, these changes, however, are small compared to the impact of land use practices. Furthermore, the results indicate that nematode genera belonging to the same trophic guild can respond differentially to natural and anthropogenic disturbances, and, moreover, this refined community analysis provides us with new ecological insights. The implementation of molecular methods for the monitoring of nematode communities allows the formation of large, high-resolution datasets, which will contribute to an improved understanding about nematode ecology and, thereby, about soil ecology in general.

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Supplementary data

Chapter 2 - Supplementary Table S1. Nematode densities (average ± standard error) in numbers of individuals per 100 ml elutriated soil at different times in a former arable field and its adjacent pristine beech forest. Feeding guilds are given in capitals: B: bacterivore, F: fungivore, FP: facultative plant parasite, O: omnivore, P: predator. The weeks are defined as number of weeks after March 17.

i	Teratocephalidae		Prismatolaimidae		Plectidae		Cephalobidae		*Anaplectus*	
	B		B		B		B		B	
Wk	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'
0	190±121	144±79	13±7	2245±1159	100±27	33±5	328±79	166±62	40±16	0±0
2	15±12	12±12	12±7	733±74	27±11	2±2	248±100	4±0	8±5	0±0
4	52±29	9±8	5±4	1031±302	55±31	3±1	232±35	39±35	37±11	0±0
7	5±5	45±33	3±2	1833±1833	44±41	13±12	222±172	51±30	12±10	0±0
9	35±30	13±6	11±6	2949±2598	55±54	0±0	139±49	93±82	49±22	0±0
13	0±0	0±0	17±16	881±311	0±0	63±63	5±1	83±67	21±19	0±0
15	0±0	0±0	128±98	494±375	0±0	0±0	889±610	6±5	261±141	0±0
17	0±0	0±0	2±1	710±169	0±0	0±0	157±35	5±1	40±25	0±0
19	36±13	1±0	4±2	1107±1107	41±22	0±0	62±33	10±8	22±22	0±0
21	148±83	270±267	96±28	2735±601	645±412	86±85	306±148	2±2	935±730	0±0
23	84±79	0±0	10±8	669±669	91±59	0±0	337±220	0±0	308±188	0±0
25	145±50	652±340	13±5	3039±1907	294±98	18±14	338±162	5±3	69±23	0±0
27	12±12	0±0	28±16	33±32	180±87	0±0	271±99	2±2	18±11	0±0
29	76±76	404±209	20±7	4±4	354±157	10±6	189±85	14±2	154±120	0±0
33	62±41	550±223	87±53	542±73	662±370	163±66	370±126	125±63	218±185	5±5
35	978±459	568±204	130±76	2077±861	942±270	298±118	524±157	94±30	38±24	0±0
37	552±459	115	12±8	845	506±263	96	279±122	34	42±16	0
39	252±180	496±195	126±53	1217±1123	1714±870	68±31	1298±342	53±36	203±126	0±0

ii	Alaimidae		Metateratocephalidae		Monhysteridae		Aphelenchoididae		Aphelenchidae	
	B		B		B		F/FP		F/FP	
Wk	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'
0	0±0	0±0	0±0	550±410	393±94	468±185	0±0	0±0	4±4	0±0
2	24±23	0±0	0±0	0±0	156±36	52±28	0±0	0±0	4±3	0±0
4	22±19	0±0	67±67	94±11	414±160	108±108	0±0	0±0	11±4	0±0
7	0±0	0±0	0±0	591±591	133±125	112±37	0±0	0±0	2±2	0±0
9	0±0	0±0	0±0	1121	436±125	2900±2677	0±0	0±0	0±0	0±0
13	0±0	0±0	32±32	115±83	51±49	0±0	0±0	0±0	0±0	0±0
15	0±0	0±0	0±0	8±8	2139±1736	60±40	0±0	0±0	18±18	0±0
17	0±0	0±0	226±226	423±26	32±14	218±218	13±13	0±0	0±0	0±0
19	0±0	0±0	0±0	587±384	62±41	289±99	0±0	0±0	4±4	0±0
21	53±17	38±38	7±7	322±321	724±400	1196±172	0±0	0±0	70±25	0±0
23	35±24	0±0	0±0	3±3	279±88	409±409	0±0	0±0	30±4	0±0
25	27±9	0±0	47±47	82±69	221±64	459±169	2±2	0±0	36±10	0±0
27	106±52	0±0	30±30	1±1	18±11	0±0	2±2	0±0	45±16	0±0
29	27±13	0±0	68±68	182±85	69±12	0±0	2±1	0±0	39±23	0±0
33	83±60	50±29	295±295	145±78	174±162	164±59	35±35	14±9	45±13	0±0
35	92±45	23±23	0±0	134±56	114±38	580±521	90±41	1±1	71±26	0±0
37	219±103	0	0±0	22	362±128	384	3±2	0	29±8	0
39	316±166	9±7	267±166	189±35	1114±445	128±128	35±19	8±6	55±15	1±1

iii	*Tylolaimophorus*		*Diphtherophora*		Dorylaimidae		Mononchidae		Mylonchulidae	
	F		F		O		P		P	
Wk	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'	'Field'	'Forest'
0	8±8	101±87	1±1	0±0	1±1	6±5	0±0	0±0	0±0	0±0
2	81±78	82±82	1±1	0±0	6±6	0±0	0±0	0±0	0±0	0±0
4	8±7	19±18	1±0	0±0	3±2	8±8	0±0	0±0	0±0	0±0
7	0±0	5±2	9±6	0±0	0±0	6±6	16±12	0±0	0±0	0±0
9	40±40	12±6	26±15	0±0	0±0	4±3	9±7	2±2	2±1	4±4
13	0±0	721±669	0±0	0±0	0±0	0±0	4±2	0±0	1±1	1±1
15	189±189	250±7	8±6	0±0	0±0	0±0	3±3	0±0	1±1	0±0
17	0±0	590±554	6±6	0±0	10±10	0±0	17±12	0±0	1±0	9±3
19	3±3	119±87	2±2	0±0	108±85	0±0	2±1	0±0	0±0	1±1
21	4±4	157±149	36±21	0±0	349±141	182±182	22±14	0±0	0±0	0±0
23	22±21	21±2	19±5	0±0	43±43	5±5	43±37	3±3	0±0	0±0
25	0±0	114±13	43±30	0±0	74±74	37±28	48±44	0±0	3±3	0±0
27	15±11	8±8	36±21	0±0	8±8	60±60	1±1	0±0	0±0	0±0
29	261±150	1007±598	23±13	0±0	71±71	48±2	5±3	4±4	0±0	0±0
33	16±9	74±16	11±5	0±0	28±28	66±39	8±8	2±2	0±0	0±0
35	22±15	11±3	30±13	0±0	379±41	396±107	5±1	5±3	0±0	0±0
37	5±3	70	29±20	0	93±57	83	8±8	3	0±0	0
39	2±2	472±61	10±5	0±0	189±114	125±16	6±2	1±0	0±0	0±0

Chapter 3 - Supplementary Table S1. Study site locations and descriptions.

	No.	Site	Soil type	Coordinates	Year of introduction
Semi-natural grassland sites	1	Planken Wambuis	Pleistocene sandy soil	52^0 01' 45.64" N 5^0 47' 53.50" E	1982
	2	Reinaerde Den Dolder	Pleistocene sandy soil	52^0 08' 59.53" N 5^0 14' 35.14" E	2000
	3	Plantage Willem III	Pleistocene sandy soil	51^0 58' 48.62" N 5^0 31' 08.47" E	1995
	4	Hollandseweg Wageningen	Pleistocene sandy soil	51^0 58' 49.89" N 5^0 40' 59.84" E	before 2005
	5	Reijerscamp	Pleistocene sandy soil	52^0 00' 47.49" N 5^{0}46 '08.64" E	2006
Riparian vegetation sites	6	Duffeltdijk (Walloon)	Riverclay	51^0 51' 58.11" N 6^0 00' 35.47" E	~ 1950
	7	Center of Millingerwaard (Walloon)	Riverclay	51^0 52' 26.39" N 6^0 00' 31.46" E	~ 1950
	8	Ewijkse plaat (Walloon)	Riverclay	51^0 52' 47.36" N 5^0 44' 52.17" E	~ 1950
	9	Blauwe kamer West (Rhine)	Riverclay	51^0 56' 40.22" N 5^0 36' 19.90" E	after 1950
	10	Blauwe kamer East (Rhine)	Riverclay and sand	51^0 56' 32.56" N 5^0 37' 09.54" E	after 1950

 - **Supplementary Table S2.** Floristic composition of semi-natural grasslands at five sites, all with three plot-pairs composed of an invaded *Solidago gigantea* patch (I) and a corresponding adjacent vegetation (U) (see Table S1). All vascular plant species were recorded and valued on an ordinal scale, based on abundance or coverage (numerical transformation of Braun Blanquets' scale).

Site number	1						2						3						4						5					
Plot-pair number	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15	
State	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I
Plantspecies																														
Achillea millefolium	3		3		2		4		7		6	2	2				3													
Aegopodium podagraria							2	5		3		3																		
Agrostis capillaris	7		8		9		4		3		3		9	3	8	4	5		9	8	8		4	3	2		2			
Arrhenatherum elatius																					2									
Artemisia vulgaris							2	2		2		2	2		2		3	2												
Bromus hordeaceus															2															
Bryophyta spp.	7	9	3	2		4	9		9		9	9		8	7	7		7	4					5	8	9	9	9		
Calystegia sepium																					3	3	2	2						
Calluna vulgaris					3																									
Cardamine sp.				1	1	3							4	3		3		3									3			
Centaurea jacea							2		1			1																		
Cerastium fontanum												3			2										4					
Chenopodium album															1															
Cirsium arvense																									2	3	3	2	5	2
Cirsium vulgare																											1			
Conyza canadensis			2																								3			
Crataegus monogyna			1														1													
Crepis capillaris	3		3		2								2		5		2										2			
Cytisus scoparius													2		3		3													
Dactylis glomerata							7	4	1	2									3	2	6		2	1	2		1		2	
Daucus carota	2	2	2					2			1																			
Deschampsia flexuosa	3		3		4																									
Elytrigia repens	4				2					3	2								5	8	9	5	9	5		1	3		4	4
Epilobium sp.																											2			
Equisetum arvense									3	2	2	4																		
Fallopia convolvulus													2				2													
Festuca rubra					3	2			3			3							7						6		6		4	3
Filago vulgaris																											1			
Fragaria sp.																			3											
Galium aparine																						5								
Galium mollugo							4	2	2	3	1	3																		
Geranium molle	3		3		3						2		4		3		3	3												
Geranium pusillum													1				1											1		
Glechoma hederacea								3		3							3		5	8	3	2	2	2						
Gnaphalium sylvaticum																									2	2				
Hieracium aurantiacum													2																	
Hieracium sabaudum	1				2																									
Holcus lanatus	6		3		4	3							5	5	3	3	9	3	2		3						3		4	

Holcus mollis									2		3																					
Hypericum perforatum	2		2	2	3		1					2	3		2																
Hypochaeris radicata									1		2		5		2		2		2								5		3		2	
Jacobeae vulgaris	3				4				2		1		2				3										5	3	7	3	3	3
Leontodon autumnale													1				1															
Leucanthemum vulgare							2		2																						5	2
Lotus corniculatus					2																											
Lolium perenne																											5					
Luzula campestris													2																			
Malva alcea																											1					
Medicago lupulina									2																							
Myosotis arvensis	3				2																											
Oenothera sp.									3	1	3				5																	
Plantago lanceolata	7		2				4		4	2	5	2					1		1	1							2					
Phleum pratense subsp. *pratense*																													1		7	
Poa sp.								3							3	6	3					4		3								
Poa annua	3	3	3		4	2					2								4		3				4	2					4
Pteropsida spp.																		1												2		
Ranunculus repens							2																									
Rubus caesius			2	1			1		1			1																				
Rumex sp.																			3													
Rumex acetosa			2		2		3	2																								
Rumex acetosella	1						2		2		3		3		2		2															
Saponaria officinalis									1			2																				
Scrophularia nodosa	1		2	1																												
Sonchus arvensis																													2			
Solidago gigantea	2	9	2	9		9	2	9	2	9	1	9		9		9		9		9		9		9		9	2	9	2	9		9
Stellaria media									3	2			2				3												2			
Taraxacum officinale	2		2		3	2							1														3		3		2	2
Tragopogon pratensis															2																	
Trifolium arvense							1		2		6				2																	
Trifolium pretense									3																							
Trifolium repens	3				2						3	1															5		1			
Urtica dioica						1																2	1	3								
Verbascum nigrum												2			2	2																
Veronica arvensis	2		3														3												2		3	
Veronica officinalis	2		3										2																			
Vicia hirsuta	2		2		1	1									2	1					1											
Vicia sativa															2																	
Viola arvensis			1																										2			

Chapter 3 - Supplementary Table S3. Floristic composition of riparian vegetation at five sites, all with three plot-pairs composed of an invaded Solidago gigantea patch (I) and a corresponding adjacent vegetation (U) (see Table S1). All vascular plant species were recorded and valued on an ordinal scale, based on abundance or coverage (numerical transformation of Braun Blanquets' scale).

Site number	6						7						8						9						10					
Plot-pair number	16		17		18		19		20		21		22		23		24		25		26		27		28		29		30	
State	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I
Plantspecies																														
Achillea millefolium							2						2		2		2													
Achillea ptarmica					2																									
Agrostis stolonifera	3																													
Arctium lappa															1											1		7		
Arrhenatherum elatius					2																									
Artemisia vulgaris							2						2	2	4	2					2	2								
Brassica nigra			1	1												2	2		2	2	5		2	1	9		8		2	
Calamagrostis sp.							6	3	9	3	9	3													2					
Calystegia sepium			2	2	2																		3		2		9	3	2	2
Carex sp.	5	2	2		3	2																				1				
Cerastium fontanum			1														2													
Cirsium arvense	2	2	3		2						1				2	2		2	6		5	2	6	2	2	2	6	2	7	5
Cirsium vulgare																			2	2		1	1							
Conyza canadensis							1																							
Crataegus monogyna									2																					
Crepis capillaris							2																							
Cuscata gronovii																				2		1				1				1
Dactylis glomerata	2								3				7	3	8		7		2		5		3		5					
Daucus carota							2										2													
Elytrigia repens	5	4	9		9		7								6	5	5	3	8	6	7	5	9	5	8	5			7	
Epipactis sp.											1																			
Equisetum arvense			2			2																				2				
Eryngium campestre													8		5	2	2	2												
Euphorbia esula			1	2																										
Festuca rubra							7		2		2		9	2	8		8	2												
Galeopsis tetrahit																									3					
Galium aparine					2												1													1
Galium verum																3	4													
Glechoma hederacea	4	4	4	4	4		5	3	6		3	2	3	2	4	8	4	7	3	2			4	3	4	4	3	5	3	2
Heracleum sphondylium			2	1	1	1										2			2				2		1					
Holcus lanatus	3																													
Hypericum perforatum							1		1																					
Jacobeae vulgaris			2				3		2		2				4										1					
Lamium album																			3	2	6	2								
Lamium purpureum																					2	2								
Lycopus europaeus									1		2																			
Lysimachia nummularia	5	6																												
Lythrum salicaria	2				2	1																								

Matricaria recutita	1																															
Mentha aquatica	2		2		1		2		2																							
Odontites vernus subsp. *serotinus*	3		2																													
Pastinaca sativa											2																					
Persicaria amphibia	3		2		3																						1					
Plantago lanceolata	8		5				2		2						3		4															
Poa sp.																													3		4	
Poa annua											3								8	4	4											
Potentilla sp.							2																2	2								
Ranunculus acris																										2	2			2		
Ranunculus repens	2		2				3		2																							
Rubus sp.								1																								
Rubus caesius									2		6	3																				
Rumex sp.										3																	2		2			
Rumex crispus															1																	
Rumex obtusifolius																						2										
Senecio inaequidens																			2													
Solidago gigantea		9		9		9		9		9		9		9		9		9		9		9		9		9		9		9	1	9		9
Stachys palustris										1																	3		7	1		
Stellaria media													2																			
Tanacetum vulgare													2				2	2												1	2	
Taraxacum officinale	2	2																														
Trifolium pratense	3								1																							
Trifolium repens																			2													
Urtica dioica		2	4	2	3	3		3	2	2			2	3		2	4	4	3	5	3	2	3	3	7		6	2	5	3		
Vicia sativa									1								1															

Chapter 3 - Supplementary Table S4. Overall nematode diversity up to genus level of soil (depth: 25 cm) from five riparian sites ('Riparian') and five semi-natural grasslands ('Grassland') invaded by Solidago gigantea (microscopic analysis). The taxonomy of the nematode families is presented as according to (De Ley, Decraemer & Abebe 2006) and trophic groups are assigned as in (Yeates 1993). Plant parasitic nematodes are given in green. Genera marked by a q in the qPCR column were included in quantitative PCR analysis and for genera marked with both a q and r, a quantitative range was available (see Vervoort et al. under review for more details).

Genus	Family	Trophic	qPCR	Range	Riparian	Grassland
Achromadora	Achromadoridae	U			+	+
Acrobeles	Cephalobidae	B	q	r	+	+
Acrobeloides	Cephalobidae	B	q		+	+
Aglenchus	Tylenchidae	PP			+	+
Alaimus	Alaimidae	B	q	r	+	+
Amphidelus	Alaimidae	B	q		+	+
Amplimerlinius	Dolichodoridae	PP				+
Anaplectus	Plectidae	B	q	r	+	+
Anatonchus	Anatonchidae	P				+
Aphelenchoides	Aphelenchoididae	F	q	r	+	+
Aphelenchus	Aphelenchidae	F	q	r	+	+
Aporcelaimellus	Aporcelaimidae	P, O	q	r	+	+
Basiria	Tylenchidae	PP			+	+
Bastiania	Bastianiidae	B			+	+
Boleodorus	Tylenchidae	PP or F			+	+
Cephalobus	Cephalobidae	B	q		+	+
Cervidellus	Cephalobidae	B	q	r	+	+
Chiloplacus	Cephalobidae	B	q	r	+	+
Clarkus	Mononchidae	P	q	r	+	+
Coomansus	Mononchidae	P	q		+	
Coslenchus	Tylenchidae	PP			+	+
Criconematidae (Other genera)	Criconematidae	PP			+	+
Criconemoides	Criconematidae	PP				+
Cylindrolaimus	Diplopeltidae	B			+	+
Diphtherophora	Diphtherophoridae	F	q	r	+	+
Ditylenchus	Anguinidae	F or PP			+	+
Dorylaimoides	Mydonomidae	O				+
Dorylaimus	Dorylaimidae	O				+
Drilocephalobus	Osstellidae	B			+	+
Epidorylaimus	Qudsianematidae	O	q	r		+
Eucephalobus	Cephalobidae	B	q	r	+	+
Eudorylaimus	Qudsianematidae	P, O	q		+	+
Eumonhystera	Monhysteridae	B, S			+	+
Euteratocephalus	Metateratocephalidae	B			+	+
Filenchus	Tylenchidae	PP			+	+
Geomonhystera	Monhysteridae	B, S			+	
Globodera/Heterodera	Heteroderidae	PP				+
Helicotylenchus	Hoplolaimidae	PP			+	+
Hemicycliophora	Hemicycliophoridae	PP			+	+
Heterocephalobus	Cephalobidae	B	q	r	+	+
Laimydorus	Dorylaimidae	O				+
Lelenchus	Tylenchidae	PP			+	
Leptonchus	Leptonchidae	F				+
Longidorella	Nordiidae	PP				+
Longidorus	Longidoridae	PP			+	+
Malenchus	Tylenchidae	PP				+
Meloidogyne	Meloidogynidae	PP			+	+
Merlinius	Dolichodoridae	PP			+	+
Mesocriconema	Criconematidae	PP				+
Mesodorylaimus	Dorylaimidae	O			+	+
Mesorhabditis	Mesorhabditidae	B			+	+
Metateratocephalus	Metateratocephalidae	B			+	+
Microdorylaimus	Qudsianematidae	O			+	+
Mononchus	Mononchidae	P				+
Mylonchulus	Mylonchulidae	P			+	+
Nagelus	Belonolaimidae	PP				+
Nygolaimus	Nygolaimidae	P			+	+
Oxydirus	Belondiridae	PP, O				+
Panagrolaimus	Panagrolaimidae	B	q	r	+	+
Paramphidelus	Alaimidae	B	q	r	+	
Pararotylenchus	Hoplolaimidae	PP			+	+
Paratrichodorus	Trichodoridae	PP			+	
Paratylenchus	Paratylenchidae	PP			+	+
Paraxonchium	Aporcelaimidae	P, O			+	
Plectus	Plectidae	B	q	r	+	+
Pratylenchus	Pratylenchidae	PP			+	+
Prionchulus	Mononchidae	P	q	r	+	
Prismatolaimus	Prismatolaimidae	B	q	r	+	+

Genus	Family	Trophic	qPCR	Range	Riparian	Grassland
Pro/Mesodorylaimus	Dorylaimidae	O			+	+
Pseudhalenchus	Anguinidae	F				+
Psilenchus	Tylenchidae	PP			+	+
Pungentus	Nordiidae	PP, P, O			+	
Rhabditis	Rhabditidae	B			+	+
Rhabditophanes	Alloionematidae	B			+	+
Rhabdolaimus	Rhabdolaimidae	B			+	
Rotylenchulus	Rotylenchulidae	PP				+
Rotylenchus	Hoplolaimidae	PP			+	+
Scutylenchus	Telotylenchidae	PP			+	+
Sectonema	Aporcelaimidae	P/O				+
Seinura	Seinuridae	P			+	+
Steinernema	Steinernematidae	E			+	+
Teratocephalus	Teratocephalidae	B			+	
Theristus	Xyalidae	B, S or U			+	
Thonus	Qudsianematidae	P/O	q		+	+
Trichodorus	Trichodoridae	PP			+	+
Tylencholaimellus	Tylencholaimellidae	F			+	+
Tylencholaimus	Tylencholaimidae	F			+	+
Tylenchorhynchus	Belonolaimidae	PP			+	+
Tylenchus	Tylenchidae	PP/F				+
Tylocephalus	Plectidae	B	q	r	+	+
Tylolaimophorus	Diphtherophoridae	F			+	
Wilsonema	Plectidae	B	q	r	+	
Total # of genera					**79**	**73**

Chapter 4 - Supplementary Table S1. Estimation of the nematode diversity at genus level in the soil (upper 20 cm) of the experimental field based on two microscopic analyses of a mixed sample taken 44 days before sowing and on day 81 of the experiment. Genera further analyzed by microscope (m) or by quantitative PCR analysis (q) are marked. For genera marked with in the last column (r), also a quantitative range was available (for more details see Vervoort et al. 2012).

Family	Genus	Trophic group[a]	qPCR/Microscope	Range
Achromadoridae	*Achromadora*	O	q	r
Alaimidae	*Alaimus*	B	q	r
Aphelenchidae	*Aphelenchus*	F	q	r
Aphelenchoididae	*Aphelenchoides*	F	q	r
Aporcelaimidae	*Aporcelaimellus**	C		
Cephalobidae	*Acrobeles*	B	q	r
Cephalobidae	*Acrobeloides*	B	q	
Cephalobidae	*Cephalobus**	B	q	
Cephalobidae	*Eucephalobus*	B	q	r
Cephalobidae	*Heterocephalobus**	B	q	r
Diplogastridae	*Diplogaster**	B		
Discolaimidae	*Discolaimus*	C		
Dorylaimidae D1	*Mesodorylaimus*	O	q	r
Dorylaimidae D1	*Ecumenicus*	O	q	r
Dorylaimidae D3	*Thonus*	O	q	r
Mesorhabditidae	*Mesorhabditis*	B	q	r
Monhysteridae	*Eumonhystera*	B	q	r
Mononchidae M3	*Clarkus*	C	q	
Mylonchulidae	*Mylonchulus*	C	q	r
Neodiplogastridae	*Pristionchus*	B		
Panagrolaimidae	*Panagrolaimus*	B	q	r
Plectidae	*Anaplectus*	B	q	r
Plectidae	*Plectus*	B	q	r
Rhabditidae	*Cruznema*	B	q	r
Rhabditidae	Rhabditidae (s.l.)[‡]	B		
Belonolaimidae	*Tylenchorhynchus*	PP1	m	
Heteroderidae	*Heterodera*	PP4	m	
Meloidogynidae	*Meloidogyne**	PP4	m	
Pratylenchidae	*Pratylenchus*	PP3	m	
Trichodoridae	*Trichodorus*	PP1	m	
Tylenchidae	*Basiria*	PP2		
Tylenchidae	*Coslenchus*	PP2		
Tylenchidae	*Tylenchus*	PP5		

[a] B: bacterivorous, F: fungivorous, C: carnivorous, O: omnivorous, PP: plant-parasitic (1: ectoparasite, 2: epidermal cell and root hair feeder, 3: migratory endoparasite, 4: sedentary parasite, 5: algal, lichen or moss feeder).

*Genera only observed on day 81.

[‡] Family, not identified to genus.

Chapter 5 - Supplementary Table S1. Estimation of the overall nematode diversity at genus level in the soil (upper 20 cm depth) of the experimental fields BUI and VMD based on a microscopic analysis of two mixed samples (consisting of 20 randomly taken cores from each field) taken in July 2010 (one year before the experiment). Plant parasitic nematodes are shown in green. Genera further analysed by quantitative PCR analysis (q) are marked. For genera marked with in the last column (r), a quantitative range was available (for more details see Vervoort et al., 2012).

Family[a]	Genus	Trophic group[b]	qPCR	Range
Achromadoridae	Achromadora	O	q	r
Anatonchidae	Anatonchus	C	q	r
Anguinidae	Ditylenchus	PP		
Aphelenchidae	Aphelenchus	F	q	r
Aphelenchoididae	Aphelenchoides	F	q	r
Belonolaimidae	Tylenchorhynchus	PP		
Cephalobidae	Acrobeles	B	q	r
Cephalobidae	Acrobeloides	B	q	
Cephalobidae	Chiloplacus	B	q	r
Cephalobidae	Eucephalobus	B	q	r
Dorylaimidae D3	Thonus	O	q	
Hoplolaimidae	Helicotylenchus	PP		
Metateratocephalidae	Metateratocephalus	B	q	r
Monhysteridae	Eumonhystera	B	q	r
Monhysteridae	Monhystera	B	q	r
Mononchidae M3	Prionchulus	C	q	r
Mylonchulidae	Mylonchulus	C	q	r
Panagrolaimidae	Panagrolaimus	B	q	r
Paratylenchidae	Paratylenchus	PP		
Plectidae	Plectus	B	q	r
Plectidae	Wilsonema	B	q	r
Pratylenchidae	Pratylenchus	PP		
Prismatolaimidae	Prismatolaimus	B	q	r
Rhabditidae	Rhabditis	B		
Tylenchidae	Filenchus	PP		
Tylenchidae	Psilenchus	PP		
Tylenchidae	Tylenchus	PP		

[a] Taxonomy as according to De Ley et al., 2006 with exception of Mononchidae M3 and Dorylaimidae D3 (see Holterman et al., 2008).

[b] Main trophic groups assigned primarily as in Yeates et al., 1993; B:bacterivorous, F: fungivorous, C: carnivorous, O: omnivorous, PP: plant parasitic.

Summary

Soil performs numerous functions, which allow us to produce food and feed and provide us with clean freshwater. These functions rely on the high diversity of organisms residing in soils. Within the high complexity of the soil food web, nematodes, worm-shaped animals belonging to the phylum Nematoda, are an informative group for assessing the status of a soil-dwelling community due to their ubiquity, abundance and trophic diversity. Although nematodes also possess several other assets favourable for a biological indicator of soil ecosystems (*e.g.* easy extractability, differential sensitivities to disturbances, ecological interpretability), their microscopic identification demands a considerable amount of expertise and time because of their relatively conserved morphology. Hence, the use of a molecular method for the identification and quantification of nematode assemblages has the potential to lift practical limitations and allows for more intensive sampling schemes. The aim of the research described in this thesis was to assess the suitability of molecular taxon-specific assays, developed on the basis of a phylum-wide molecular framework of 2,400 full-length small subunit ribosomal DNA sequences, for the monitoring of nematode assemblages in field experiments. The method was applied to monitor the impact of different types of disturbances on the soil food web, *i.e.* agricultural practices, invasive plant species and the effects of genetically modified crop (potato).

The second chapter of this thesis presents the background of the molecular method and the results of its first field application. It demonstrates the suitability of this method for use in extensive field experiments and the results of this study reveal distinct seasonal fluctuations between nematode genera classified to belong to the same feeding type group. A distinct response of nematode genera within trophic groups – taxonomically diverse groups of nematodes having the nature of their main food source as a common denominator – was also observed in the study described in Chapter 3. In this chapter, a study was conducted to investigate the impact of an invasive plant species called Giant goldenrod (*Solidago gigantea*, native to North America) on the plant community as well as on different trophic levels of the soil food web. In addition to monitoring the nematode community, pH and fungal biomass were measured in plots invaded or uninvaded by Giant goldenrod in two contrasting habitats. The results revealed that, in addition to outcompeting native plant species, this invader also reduced pH and increased fungal biomass in the soil of both habitats. Based on the results concerning the nematode community, the impact on the soil food web seemed to be selective since the local increase of fungal biomass appeared to benefit only one fungivorous nematode lineage of the three present in the field. This suggests that invasion by Giant goldenrod only stimulates one part of the fungal community.

Contrary to the preceding chapters, the effects of different disturbances on the nematode community were studied in an arable setting in chapters 4 and 5. Chapter 4 describes a field experiment in which the impact of biofumigation, a pest control measure, on the nematode community was assessed. Biofumigation is considered as an alternative for the use of synthetic fumigants and entails the incorporation of mulched brassicaceous plant material, which, upon de-compartmentalisation, releases general biocides called isothiocyanates. In our experiment, these compounds as well as their precursors could not be related to the effects observed for the nematode community. Therefore, changes in nematode assemblages are more likely to be related to the intense mechanical disturbance and green manure – the addition of a large

quantity of fresh plant material to the topsoil – rather than the release of isothiocyanates from the plant material

Chapter 5 presents a field experiment in which the possible belowground side effects of a waxy starch GM potato, a genetically modified plant blocked in its amylose biosynthesis, were investigated. The nematode community was monitored during the growing season of this GM variety, its parental line and four other conventional potato cultivars in two experimental fields. Although we observed clear effects of location and time, no GM-related effects were observed on the nematode community. Our results, in line with previous studies concerning the microbial community, indicate there are no observable, non-transient effects related to this particular GM trait on the soil food web during the growing season.

Overall, the results presented in this thesis demonstrate that, first of all, the developed molecular approach is suitable as a tool for the quantitative monitoring of nematode assemblages in field experiments, and, secondly, how a molecular monitoring method based on nematode taxon-specific DNA motifs can be exploited to get new insights into the ecology of terrestrial nematodes and – more in general – into the ecological functioning of this obscure, highly biodiverse and poorly understood habitat below our feet.

Samenvatting

De bodem vervult talrijke functies die ons onder andere de mogelijkheid geven om voedsel te produceren en die ons toegang bieden tot schoon drinkwater. Deze functies zijn afhankelijk van de hoge diversiteit van organismen die in de bodem leven. Binnen de complexiteit van het bodemvoedselweb zijn nematoden, wormvormige dieren uit het fylum Nematoda, een informatieve groep voor het bepalen van de status van het bodemleven dankzij hun alomtegenwoordigheid, talrijkheid en trofische diversiteit. Hoewel nematoden nog meer eigenschappen bezitten die wenselijk zijn voor een biologische indicator voor bodemsystemen (bijvoorbeeld: gemakkelijke extractie uit grond, variatie in gevoeligheid voor verstoringen en ecologische interpreteerbaarheid van veranderingen), vereist het identificeren van nematoden met behulp van een microscoop een aanzienlijke hoeveelheid expertise en tijd. Dit laatste heeft alles te maken met de geconserveerde morfologie binnen dit fylum (*i.e.* ze lijken op elkaar). Om deze reden kan het gebruik van een moleculaire methode voor de identificatie en kwantificering van nematodengemeenschappen praktische beperkingen opheffen en het mogelijk maken om experimenten uit te voeren met intensievere monstername. Het doel van het onderzoek dat beschreven staat in dit proefschrift was om de geschiktheid van moleculaire taxon-specifieke assays, ontworpen op basis van een fylum-breed raamwerk bestaande uit 2.400 full-length small subunit ribosomal DNA sequenties, voor het monitoren van nematodengemeenschappen in veldexperimenten. De methode was toegepast om de impact op het bodemvoedselweb te bepalen van verschillende type verstoringen, namelijk agrarische toepassingen, invasieve plantensoorten en effecten van genetische gemodificeerde gewassen (aardappel).

In hoofdstuk 2 van dit proefschrift presenteer ik de achtergrond van de moleculaire methode en de resultaten van de eerste toepassing hiervan in veldcondities. Dit hoofdstuk demonstreert de geschiktheid van deze methode voor gebruik in extensieve veldexperimenten, ook laten de resultaten verschillen zien tussen de fluctuaties door het seizoen van nematoden genera behorende bij dezelfde trofische groep (gekenmerkt door hetzelfde type voedselbron). Een verschil in respons tussen genera van dezelfde trofische groep werd ook geobserveerd in het experiment beschreven in hoofdstuk 3. Dit hoofdstuk betreft een studie naar de impact van een invasieve plantensoort genaamd Late guldenroede (*Solidago gigantea*, afkomstig uit Noord-Amerika) op zowel de plantengemeenschap als op verschillende trofische niveaus van het bodemvoedselweb. Naast het monitoren van de nematodengemeenschap, werden pH en schimmelbiomassa gemeten in plots met en zonder invasie door Late guldenroede in twee verschillende habitats. De resultaten lieten zien dat deze invasieve soort, naast het verdringen van inheemse plantensoorten, lokaal ook leidt tot een lagere pH en hogere schimmelbiomassa in de bodem van beide habitat types. Gebaseerd op de resultaten van de nematodengemeenschap, lijkt het effect op het bodemvoedselweb selectief, omdat de toename van schimmelbiomassa enkel benut leek te worden door slechts een van de drie aanwezige fungivore nematode genera in deze locaties. Dit suggereert dat invasie door Late guldenroede slechts een specifiek gedeelte van de schimmelgemeenschap stimuleert.

In tegenstelling tot de voorgaande hoofdstukken, betreffen hoofdstuk 4 en 5 de effecten van verschillende verstoringen op de nematodengemeenschap in een agrarische setting. Hoofdstuk 4 beschrijft een veldexperiment waarin het effect van biofumigatie, een grondontsmettings-

methode, op de nematodengemeenschap werd bestudeerd. Biofumigatie wordt beschouwd als een alternatief voor het gebruik van synthetische fumigantia en betreft het incorporeren van fijngehakseld plantenmateriaal afkomstig van koolachtige gewassen (Brassicaceae) in de bodem. Bij dit proces komen stoffen vrij, isothiocyanaten, die een breed toxisch effect hebben.

In dit experiment gepresenteerd in hoofdstuk 4 werd geen relatie gevonden tussen de hoeveelheid isothiocyanaten of de uitgangsstoffen hiervan en de geobserveerde effecten van biofumigatie op de nematodengemeenschap. Deze effecten zijn waarschijnlijk gerelateerd aan de intense mechanische verstoring en de toevoeging van grote hoeveelheden plantenbiomassa aan de bodem, in plaats van het vrijkomen van isothiocyanaten van het plantenmateriaal.

In hoofdstuk 5 worden de resultaten gepresenteerd van een veldexperiment waarin gekeken is naar de ondergrondse effecten van een aardappelras dat genetisch gemodificeerd is om enkel zetmeel te produceren in de vorm van amylopectine (middels de blokkering van de synthese van amylose). In een veldexperiment werd de nematodengemeenschap gemonitord gedurende het groeiseizoen van deze GM variant, de uitgangs-cultivar en van vier andere conventionele aardappelcultivars in twee verschillende proefvelden. Hoewel we sterke effecten zagen van locatie en tijd, werden er geen observeerbare, consistente effecten waargenomen in relatie met dit specifieke GM kenmerk gedurende het groeiseizoen.

De resultaten gepresenteerd in deze dissertatie tonen aan dat de ontwikkelde moleculaire methode geschikt is voor het kwantitatief monitoren van nematodengemeenschappen in veldexperimenten. Daarnaast toont dit onderzoek dat de toepassing van moleculair monitoren gebruikt kan worden voor het vergaren van nieuwe inzichten wat betreft de ecologie van terrestrische nematoden en – in het algemeen – in het ecologische functioneren van de obscure, moeilijk te doorgronden, hoog biodivers habitat dat zich onder onze voeten bevindt.

Curriculum vitae

Mariëtte Theodora Wilhelmina Vervoort was born on the 23rd of May 1986 in Nijmegen, The Netherlands. She holds a MSc. in Plant Biology from Utrecht University. During her studies at Utrecht, she specialized in plant pathology and microbiology. In August 2008 she started her PhD at the Laboratory of Nematology, Plant Science group at Wageningen University. In May 2013 she started teaching at this department.

Acknowledgements

A PhD programme is often described as a road. In my case, I would like to take that comparison a step further and compare my PhD to the A12 between Utrecht and Wageningen. My relationship with the A12 is quite intense; I work next to it, I drive it every day and I live only 500 m away from it. Both cars I have driven since I started working in Wageningen (magnificent Toyota Starlets) probably know every bump and corner of the A12 by heart now. From the start to the end of my PhD, there has been a lot of construction work on this road, leading to traffic jams and stricter speed limits. Nevertheless, this work has finally resulted in an extra lane for the whole distance and, in the end, a smoother ride. So, yes, although kind of a cliché, I do believe I can compare my PhD to a freeway. Fortunately, there is one big difference between the two, because my metaphorical PhD car was always filled with all these people, without whom, I would never have made it to the end.

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PE&RC PhD Training Certificate

With the educational activities listed below the PhD candidate has complied with the educational 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 (5.6 ECTS)
- Glucosinolate profiles in different Brassica species (2008)
- Nematode community shifts (2008)
- GM crop risk assessment studies for soil ecosystems (2010)

Post-graduate courses (3 ECTS)
- Soil ecology: linking theory to practice: PE&RC (2010)
- Sampling in space and time for survey and monitoring of natural resources: PE&RC (2012)
- Introduction to R for statistical analysis: PE&RC (2012)

Laboratory training and working visits (4.5 ECTS)
- Barcode-based monitoring of nematode communities; BLGG (2008, 2009)
- Field experiment; Julius Kühn Institut, Münster (2010)
- Statistical data analysis; RIVM (2009-2012)
- Sample analysis; NIOO (2011)
- Nematode behavioural models; AMOLF (2012)

Invited review of (unpublished) journal manuscript (1 ECTS)
- Plant and Soil: hyperaccumlators and their associated nematodes (2012)

Deficiency, refresh, brush-up courses (3 ECTS)
- Nematodes in environmental studies/identification course (2009)
- Food web ecology; lectures

Competence strengthening / skills courses (4.5 ECTS)
- PhD competence assessment: PE&RC (2009)
- Afstudeervak organiseren en begeleiden; DO, WUR (2009)
- Career perspectives ; WGS, WUR (2011)
- Coaching of ACT groups; DO, WUR (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
- PE&RC Weekend (2008)
- PE&RC Day: expect the unexpected (2008)
- PE&RC Day: intelligent nature (2009)
- PE&RC Day: selling science (2010)
- PE&RC Day: innovation for sustainability (2011)

Discussion groups / local seminars / other scientific meetings (5 ECTS)
- Current themes in ecology: Plants-Insects-Microbes: an ecological dance for three (2008)
- Discussion group meetings: Experimental Evolution; Ecogenomics; Plant-Soil interactions (2008-2012)
- EPOS Meeting, interactions between plants and biotic agents; with presentation (2009)
- National Ecogenomics Day; with presentation (2009, 2010)
- Presentation at the Julius Kühn Institut Münster (2009, 2012)
- NOW ERGO Meetings; with presentation (2009, 2010, 2012)
- Congres Biodiversiteit: kansen voor een nieuwe economie (2010)
- Presentation at the RIVM, LER meeting (2010)

International symposia, workshops and conferences (5.1 ECTS)
- Society of Nematologists annual meeting; Portland, Oregon (2011)
- Nematodes as bio indicators; presentation: impact of four Indian mustard cultivars with distinct glucosinolate levels on free living and plant parasitic nematodes (2012)

Lecturing / supervision of practical's/ tutorials (3 ECTS)
- Introduction environmental studies (2008- 2011)
- Biology and management of plant pathogens insects and weeds I (2009)
- Food web ecology (2009)
- Nematology (2010)
- Ecological aspects of biological interactions (2010, 2011)

Supervision of a MSc student (3 ECTS)
- Belowground impact of invasive plant species *Solidago gigantea*

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