

Impact of trophic ecologies on the whereabouts of nematodes in soil

Casper W. Quist



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on the whereabouts of nematodes in soil**

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Thesis committee

Promotor

Prof. Dr Jaap Bakker
Professor of Nematology
Wageningen University & Research

Co-promotor

Dr Johannes Helder
Associate professor, Laboratory of Nematology
Wageningen University & Research

Other members

Prof. Dr Wietse de Boer, Wageningen University & Research
Dr Sofie Derycke, Royal Belgian Institute of Natural Sciences, Brussels, Belgium
Prof. Dr Michael Bonkowski, University of Cologne, Germany
Dr Christien Ettema, CEO Shades of Green

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Casper W. Quist

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CHAPTER

General introduction

Casper W. Quist

Soil biodiversity and the soil food web

If the upper layer of the Earth's crust contains sufficient minerals and organic material to support the presence of diversified forms of life, it is called *soil*. The composition of the living soil communities depends on the soil characteristics, and varies for example with the soil acidity, nutrient availability and soil texture. Soils are among the most biodiverse habitats on Earth. Among soil inhabitants, bacteria are considered to be the most diversified group (Fierer *et al.*, 2007). A single gram of soil contains on average 1 to 10 billion individual cells (Raynaud and Nunan, 2014), comprising up to 50,000 species (Roesch *et al.*, 2007). Next to the great abundances of bacteria, underground ecosystems are inhabited by many other creatures, such as archaea, fungi, viruses, protists, nematodes, tardigrades, mites, springtails and earthworms (Fierer *et al.*, 2007, Bardgett and Van Der Putten, 2014). Hence, soil life is highly diverse, and ecologically intricate due to myriad of biotic interactions that take place. To better understand the complex phenomena that take place in soil, often a food web approach is used (Moore and de Ruiter, 2012). Food webs are diagrams of biological communities highlighting the trophic interactions between resources and consumers. Common soil food webs discriminate four trophic levels. Each trophic level includes groups of species that feed on species from an adjacent trophic level. For example, plant roots (trophic level (TL) 0; primary producers) are eaten by plant-parasitic nematodes (TL1), which in turn might be eaten by omnivorous nematodes (TL2) and predatory nematodes (TL3) (See Fig. 1.1). Within each trophic level functional groups are defined, in which species are placed together based on their similar feeding preferences, reproduction rates and predators (Holtkamp *et al.*, 2008). In food web diagrams, the nutrient flow between functional groups can be expressed in amounts of carbon or nitrogen. Food web interactions are key in understanding community structure, dynamics, and stability, because trophic interactions are crucial for the survival of both resource and consumer (Hastings *et al.*, 2016). Hitherto, understanding about how soil organisms interact and the factors that determine their distribution are, to a great extent, based on observations on important pests and diseases in agriculture (Moore and de Ruiter, 2012). Yield losses due to intensive agriculture has led to an increased societal awareness of the major threats of soil degradation and the need for increased understanding of soil functioning. At the same time, with the advent of molecular techniques, such as quantitative PCR and Environmental DNA sequencing, new avenues are open towards an improved understanding of interactions between components of soil food webs (Orgiazzi *et al.*, 2015).

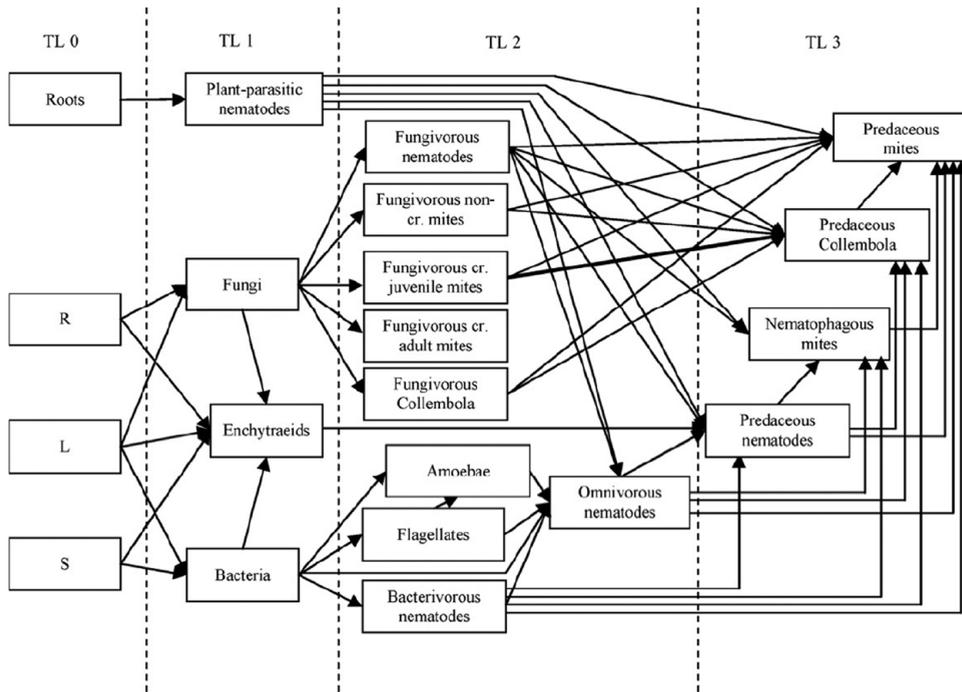


Figure 1.1. A soil food web diagram (Holtkamp *et al.* 2008) with arrows representing feeding links that are pointing from the prey to the predator. TL = trophic level, R: recalcitrant organic matter, L: Labile organic matter, S: Soluble sugar and cr: cryptostigmatic.

Soils under threat

Healthy soils create essential circumstances for plants to grow, via the delivery of so-called ecosystem services (Brussaard, 2012). The quality of some ecosystem services depends on degrees of carbon sequestration and nutrient cycling, processes that are governed by soil biota (De Vries *et al.*, 2013). To achieve a healthy functioning of soil ecosystems, a basal (often poorly defined) level of soil biodiversity has to be maintained and enhanced. Due to human activities, soil biodiversity is under threat. Among all land-use types, intensive agriculture is responsible for the largest environmental impact on biodiversity (Newbold *et al.*, 2015, Tsiafouli *et al.*, 2015, Levers *et al.*, 2016). This can be illustrated by Tsiafouli and co-authors (2015) who examined biodiversity in soil food webs from grasslands, extensive, and intensive rotations in four agricultural regions across Europe. Their results show that intensification of agricultural practices results in less complex soil food webs. Mainly due to a steady growth of the human population, intensification of agriculture is occurring across the world. The bright side might be that improved insights in the ecological functioning of soils will enable the design of land-use systems that serve human needs, while minimizing environmental impacts (Bender *et al.*, 2016).

The rationale behind nematode communities as bio-indicators

To facilitate monitoring of the biological condition of soil, various proxies have been identified, such as earthworms (Pansu *et al.*, 2015), mycorrhizas (Jansa *et al.*, 2014), collembolans (Nelson *et al.*, 2011), and nematodes (Bongers and Ferris, 1999, Neher, 2001). Recently, a group of 39 soil ecologists evaluated 27 potential cost-effective and policy-relevant bio-indicators for monitoring soil biodiversity and ecosystem functioning (Griffiths *et al.*, 2016). They found that none of the individual indicator groups included in their study was sensitive to all of the differences in land-use intensity. Therefore, they concluded that for a proper assessment of the biological condition in soil multiple indicators should be taken into account. Among all potential indicators, the molecular assessment of nematodes received the highest weighted score from a logical-sieve method (see (Ritz *et al.*, 2009)). The authors noted that molecular methods for nematodes are relatively advanced compared to other soil faunal indicators (see (Floyd *et al.*, 2002, Holterman *et al.*, 2008, Vervoort *et al.*, 2012, Rybarczyk-Mydłowska *et al.*, 2012)). The high bio-indicative potential of nematode communities might be explained by their representation at all three trophic levels of the soil food web (Holtkamp *et al.*, 2008) (Yeates, 2003). Practical advantages are their abundance and diversity in virtually all types of soil as well as their relatively easy and efficient extractability from soil (Oostenbrink, 1960, Verschoor and de Goede, 2000). Individual taxa show distinct sensitivities towards various kinds of environmental stressors (Bongers and Ferris, 1999). Communities of this trophically diverse group of soil fauna should preferably be studied at family or genus level, and not at the level of functional groups, as previous reports showed that this taxonomic resolution is required for understanding the impact of plant communities or land use (Porazinska *et al.*, 1999, Neher *et al.*, 2005, Viketoft and Sohlenius, 2011).

Importance of revealing belowground spatial patterns of nematodes towards increased understanding of soil life

Terrestrial nematodes are classified as microfauna. Of all terrestrial nematodes, 99% have body widths between 10 and 55 μm and body lengths between 150 and 1,500 μm (Mulder and Vonk, 2011). Microfaunal organisms are mainly passive dispersers. By means of wind, water and animal phoresis, they can cross long distances. It should be noted that passive dispersal is highly random and non-specific (Ettema *et al.*, 2000). Typical active migration velocities of nematodes through soil, range from 0 - 3 cm per day, depending on the species and the presence of external stimuli (Wallace, 1958, Wallace, 1960, Moore *et al.*, 2010, Bal *et al.*, 2014).

Factors that drive distribution of soil biota operate at different spatial scales. Therefore, spatial distribution of soil biota is usually described at three or four scales (Ettema and Wardle, 2002, Berg, 2012). At each spatial scale, patterning is defined by abiotic and biotic soil characteristics and the scale dimensions depend on the body size of the organismal group of interest (Ettema and Wardle, 2002, Martiny *et al.*, 2006). In this thesis three nested scales of horizontal nematode distribution are defined, microscale, mesoscale and metascale.

Microscale is the spatial level at which individual nematodes during their life cycle search for food, mate and multiply, are exposed to predators, and cope with abiotic stressors such as locally unfavorable temperature and moisture conditions (microplot - scale dimensions: 5 to 50 cm).

At the mesoscale, nematodes are exposed to comparable abiotic conditions. At this scale, general soil parameters (*e.g.* pH, organic matter, bulk density, texture, major mineral concentrations) as well as land-use history (*e.g.* farming system or vegetation type) are similar (plot or field - scale dimensions: 1 – 1,000 m). Horizontal patterning of nematodes at this scale was found in multiple studies. Often these spatial patterns at mesoscale are the result of large-scale landscape gradients, such soil carbon and cultivation practices (Ettema and Wardle, 2002). However, in other studies the spatial variation was only partly explained by soil resource gradients. For example, when the patchiness of bacterivore *Chronogaster* was examined at species level in the same wetland, the results pointed at an important role of largely unpredictable, local variations in humidity on the mesoscale distribution of individual bacterivorous nematode species (Ettema *et al.*, 2000).

At the metascale, multiple habitat types can be distinguished, each of them showing dissimilar soil properties and land-use histories (landscape - scale dimensions: some km).

Whereas our insights in the responses of nematode communities to land-use and plant species are improving rapidly, our knowledge of the spatial variability of soil biota is lagging behind. Most papers focus on nematode feeding types (Robertson and Freckman, 1995, Vikeftoft, 2013), on restricted number taxa within a feeding type (Ettema *et al.*, 1998, Ettema *et al.*, 2000), or on individual plant-parasitic nematode species (Been and Schomaker, 2006).

Geostatistical modelling is a potential powerful approach to reveal spatial patterns in soil (Ettema and Wardle, 2002). To optimally benefit from this modelling approach an optimized sampling design was used that includes a large number of samples. A reliable spatial model requires about 100 data points per object. Analysis of underlying driving factors of spatial aggregations requires multiple spatial models. State-of-the-art molecular techniques provided the opportunity to analyse high numbers of soil

samples, and thereby opening new options for studying the spatial patterning of soil biota. Belowground distribution patterns of a wide range of nematode taxa from all trophic groups will shed light on the underlying processes of pattern formation and might add to understanding of the overwhelming biodiversity in soil.

Outline of this thesis

The overarching aim of the work described in this thesis was to explore the potential of nematode communities as an indicator group for the biological condition of soils. Therefore, the whereabouts of nematode taxa were studied, within and between trophic groups and in soils conditioned by various plant species and/or farming systems.

The impact of several invasive plants on native vegetation is relatively well investigated (Hejda *et al.*, 2009). So far, the belowground effect of invasive plant species has received far less attention. In *Chapter 2* the belowground impact of *Solidago gigantea*, an invasive plant across Europe that originates from North-America, is investigated. Nematode communities and fungal biomass were examined in adjacent invaded and uninvaded patches, in two invaded ecosystems: semi-natural grasslands and riparian floodplains. Based on the significant impact on the vegetation, we hypothesized that this exotic plant might affect key components of the soil food web as well.

Soil life is essential for nutrient cycling, carbon storage and disease suppressiveness, and organic agriculture holds the promise to manage soil organisms in a more durable manner than conventional farming. However, it is largely unknown how soil organisms are affected by organic farming practices. Soil communities are known to strongly respond to crops types and other short term factors (Berkelmans *et al.*, 2003). Components of the soil food web that indicate long-term effects of land-use might be used to assess the condition of various land management types. In *Chapter 3* it is shown that differences in soil management systems are mirrored in compositional changes in nematode communities. The long-term impact of three farming systems (conventional, integrated and organic) on nematode communities was investigated at *De Vredepeel*, an experimental farm in the southeastern part of The Netherlands.

Knowledge on belowground distribution patterns will shed light on the underlying processes of pattern formation and might add to understanding of the overwhelming biodiversity in soil. Further, detailed information on belowground spatial variability is a prerequisite for the design of soil sampling strategies with predictable accuracies.

In *Chapter 4*, the microscale patchiness of 45 nematode taxa (at family, genus or species-level) in arable fields and semi-natural grasslands, on marine clay, river clay or sandy soils is investigated. From each microplot five replicate composite samples were collected. It was expected that an increase of the number of cores per composite sample would result in more accurate detection, as previously shown for some obligate plant-parasitic nematode species. This appeared not to hold for the free-living and facultative plant parasitic taxa under investigation here. Also nematode feeding preferences, land management and soil type were expected to affect variability of replicate soil samples.

For *Chapter 5* over 1,200 composite soil samples were collected and 35,000 qPCR reactions were run for detailed mapping of spatial distribution patterns of 45 nematode taxa (at family, genus and species level) across the Netherlands at mesoscale. State-of-the-art geostatistical analysis methods were used to reveal distribution patterns. Soil type and land-use were hypothesized to be important drivers for differences in nematode community composition and spatial distribution between fields. In this final experimental chapter, the intra-field variation is used to assess the contribution of stochasticity for belowground patterning of nematodes.

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2 CHAPTER

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

Casper W. Quist *

Mariëtte T.W. Vervoort *

Hanny H.B. van Megen

Gerrit Gort

Jaap Bakker

Wim H. van der Putten

Johannes Helder

* Authors contributed equally to this work
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Abstract

Apart from relatively well-studied aboveground effects, invasive plant species will also impact the soil food web. So far, most research has been focusing on primary decomposers, while studies on effects at higher trophic levels are relatively scarce. Giant goldenrod (*Solidago gigantea*), native to North America, is a widespread and common invasive species in most European countries. We investigated its impact on plant communities and on multiple trophic levels of the soil food web in two contrasting habitats: riparian zones and semi-natural grasslands. In 30 pairs of invaded and uninvaded plots, floristic composition, pH, fungal biomass, and the densities of 11 nematode taxa were determined by using a quantitative PCR-based method. In the two habitats, the invader outcompeted both rare and dominant plant species. Belowground, *S. gigantea* invasion reduced pH, increased overall fungal biomass as well as the density of a single lineage of fungivorous nematodes, the family Aphelenchoididae. The densities of two other, phylogenetically distinct lineages of fungivorous nematodes, Aphelenchidae and Diphtherophoridae, were unaffected by the local increase in fungal biomass. Apparently this plant species induces a local asymmetric boost of the fungal community, and only Aphelenchoididae were able to benefit from this invader-induced change. The alternative explanation – the results are explained by a subtle, *S. gigantea*-induced 0.1 - 0.2 units decrease of pH – seems unlikely, as pH optima for nematode taxa are relatively broad. Thus, apart from readily observable aboveground effects, the invasive plant species *S. gigantea* affects fungal biomass as well as a specific part of the fungivorous nematode community in a soil type-independent manner.

Key-words: fungal biomass, invasion ecology, molecular analysis, nematode community, quantitative PCR

Introduction

The successful establishment of exotic species in a given habitat 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 become exceptionally abundant in their new environments (for terminology see Pyšek *et al.* 2004). 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 characteristics such as stand height and cover are major determinants for invasiveness. The number of studies focusing 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. For example, 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 and Phillips 2001, Belnap *et al.* 2005, Chen *et al.* 2007).

Due to the enormous biodiversity and the high number of trophic relationships, there are myriad interactions between plants and soil microbial communities (Porazinska *et al.* 2003). Nematodes constitute an informative bio-indicator group for soil food web functioning, owing to their omnipresence in pores between soil aggregates, their trophic diversity, and their high degree of interconnectedness within the soil food web (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). Assemblages of this trophically diverse micro-faunal group should preferably be studied at family or genus level, and not at the level of feeding guilds, as previous reports showed that this taxonomic resolution is required for understanding the impact of plant communities or land use (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. Here, we applied a recently developed set of quantitative PCR

(qPCR)-based molecular assays (Vervoort *et al.* 2012), that 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. *Solidago gigantea* forms 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 *S. gigantea*-dominated plant communities. However, it is hard to predict whether these are specific or more widespread consequences of invasion by *S. gigantea*, and little is known about possible follow-up changes at higher trophic levels in the soil food web.

In this study, we investigated the belowground impact of *S. gigantea* on the fungal biomass and the nematode community composition in riparian zones and semi-natural grasslands (characterised by river clay and sandy soils respectively). By including two contrasting environments, we were looking for generic effects of invasion by *S. gigantea* on multiple trophic levels of the soil food web. In a mesocosm experiment, Scharfy *et al.* (2010) showed both bacterial and fungal biomass to be affected by *S. gigantea*. If this were true in other soil types and under natural conditions, these shifts should be reflected in changes in the bacterivorous and fungivorous nematode community.

Material and methods

Sites of study

Within an area of approx. 200 km² covering parts of the Dutch provinces Utrecht and Gelderland, ten sites were selected from two habitat types commonly invaded by *S. gigantea*: riverbanks of the Rhine and the Walloon and semi-natural grasslands on Pleistocene sandy soils (Table S2.1). In this area, the presence of *S. gigantea* have been reported since 1912 (Te Linde and Van den Berg 2003). In riparian habitats, *S. gigantea* is mainly spread by surface waters, which carry (fragments of) plants that can sprout under favourable conditions elsewhere (Weber and Jakobs 2005). Beekeepers and gardeners introduced *S. gigantea* to the semi-natural grasslands under investigation.

All selected sites met the following criteria: 1) *Solidago gigantea* occurred in well-defined patches in the plant community, 2) soil and plant communities showed no signs of disturbances caused by foraging wildlife or mowing, 3) sites that belong to the same habitat type were comparable in plant community, pH and humidity.

Soil 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 × 2 m) plots; one plot dominated by *S. gigantea* invaded plot and one uninvaded plot. 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 (Ø 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 earlier, the nematode diversity of all sites of this study was assessed microscopically (for details see Table S2.2).

Plant community analysis

In each plot ($n=60$) a relevé was made; all species of higher plants were recorded and the proportion of each species in the vegetation was estimated according to a modified Braun-Blanquet scale (Barkman *et al.* 1964, Table S2.3). Community characteristics were determined by calculating the species richness (S) and the Shannon diversity (H') as described by 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 methodology (Vervoort *et al.* 2012, Vervoort *et al.* 2014).

Microscopic analysis (of samples collected in August 2011) was used to assess the nematode community composition for invaded and uninvaded plots in each of the habitat types. Communities were characterised by the morphological identification (till genus level) of 100 individuals per sample (soil from under invaded plant communities and native plant communities was analysed separately for each site ($n=20$; for details see s S2.1 and S2.2). 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. Within the orders Dorylaimida and Mononchida, cluster-specific primers D3 and M3 were used according to Holterman *et al.* (2008). For the family Plectidae, separate primers were used targeting either *Anaplectus* or Plectidae except *Anaplectus*.

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, lysate purification and subsequent qPCR reactions – using 11 nematode taxon-specific primer combinations – were performed as described by 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, which does not occur in plant or animal cells (Gessner and Schmitt 1996, 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, plant communities, and nematode densities were analyzed 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 (total) were square root-transformed; and all other variables (ergosterol and nematode taxon densities) were log-transformed. The log-transformation was applied after addition of a constant (0.05 for ergosterol, and 0.5 for nematode densities with the exception of Dorylaimida D3) to push data away from the lower bound zero. Mixed linear models were used, because multiple

observations from the same site and/or plot pair within sites are not necessarily uncorrelated. The fixed part of the mixed model contained main effects of habitat and invasion and their interaction. Besides the residual error, random effects are introduced for sites and for plot pairs (within sites), so that total error variance is split into variance components for sites and for plot pairs within sites, and residual variance. 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 uninvaded plots per habitat, together with a statement about the significance of the difference between invaded and uninvaded plots.

Results

Changes in native plant communities 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 plant communities, plant-species richness (S) and diversity (H') were significantly lower compared to native plant communities ($P < 0.001$; Table 2.1 and Fig. 2.1). Common native species largely determining the plant community (e.g. *Jacobaea vulgaris*, *Holcus lanatus*, *Achillea millefolium*, *Dactylis glomerata*, and *Plantago lanceolata*; Table S2.3) were nearly absent in invaded plant communities. Relatively rare species such as *Achillea ptarmica*, *Epipactis* sp., *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*) thrived rather equal in invaded patches (Table S2.2).

Impact of *S. gigantea* invasion on soil acidity and moisture content

Overall, a comparison of pH of soils from uninvaded *versus* invaded plots revealed slight but significantly lower pH in invaded soils ($P < 0.001$; Table 2.1). Soil moisture content tended to be lower in invaded plots, but this effect was not significant ($P = 0.077$; Table 2.1). In general, the soil pH under semi-natural grasslands was ≈ 1.5 units lower (Table 2.2) and more variable as compared to the riparian plots ($P < 0.001$; Tables 2.1, 2.2). 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 2.1).

Table 2.1. Summary of ANOVA F_{df} 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 (neighbouring 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	
	$F_{1,8}$	<i>P</i>	$F_{1,28}$	<i>P</i>	$F_{1,28}$	<i>P</i>
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
Dorylaimida D3	6.25	0.037	1.89	0.181	2.47	0.127
Mononchida 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 <i>Anaplectus</i>)	0.93	0.362	0.12	0.731	1.94	0.174
<i>Anaplectus</i>	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 Dorylaimida D3 and Mononchida M3 (see Holterman *et al.*, 2008).

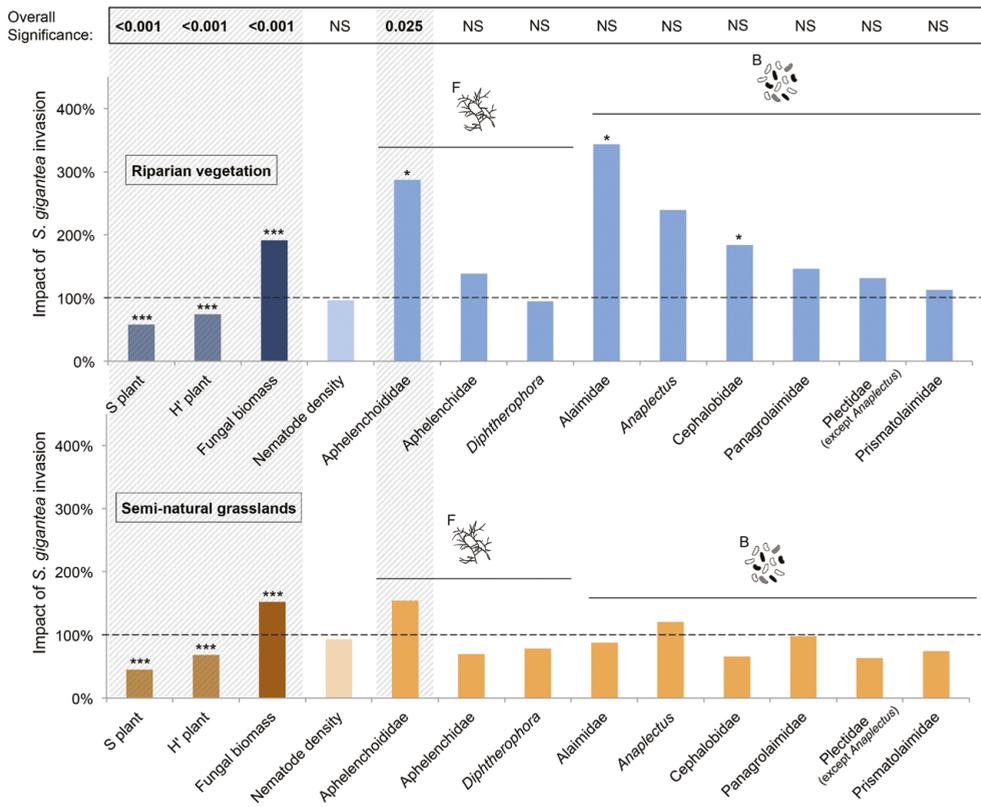


Figure 2.1. Impact of *Solidago gigantea* invasion in two habitat types, riparian vegetation and semi-natural grasslands, on plant-species richness (S_{plant}), plant-species diversity (H'_{plant}), fungal biomass, total nematode density, and the densities of three fungivorous ('F') and six bacterivorous ('B') nematode taxa. Impacts are expressed as the percentage of the (back transformed) mean values in invaded plots as compared to uninvaded plots (no change = 100%). For each of the two habitats, significant differences between invaded and uninvaded 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 habitat types taken together) are given in top part of this figure (expressed as P values). A shaded background is used to highlight significant variables.

Impact on soil fungal biomass

Overall, soil from invaded plots contained significantly higher amounts of fungal biomass as compared to uninvaded plots ($P < 0.001$; Table 2.1 and Fig. 2.1). Fungal biomass was approximately twice as high in soil collected from *S. gigantea* invaded plots, in comparison to plots with native plant communities (Table 2.2).

Changes in nematode assemblages upon *S. gigantea* invasion

Overall, total nematode densities (determined microscopically) were similar in neighbouring invaded and uninvaded soils. However, when we measured the impact of *S. gigantea* 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 (Data not shown) using an additional, *A. fragariae*-specific molecular assay (Rybarczyk-Mydlowska *et al.* 2012). Two other fungivorous taxa, Aphelenchidae and Diphtherophoridae (the latter represented in these two habitats by a single genus, *Diphtherophora*), did not show a difference in density between uninvaded and invaded soil (Fig. 1). For the predatory nematode family Mononchida M3 (see Holterman *et al.* 2008) and bacterivorous Cephalobidae, a significant interaction was observed between habitat type and invasion of *S. gigantea* (Table 2.1), showing that the nature of their response to invasion is habitat-type dependent.

When considering the two habitat types separately, differences between uninvaded and adjacent invaded soil were more pronounced in the riparian habitats than in semi-natural grasslands (Fig. 2.1, Table 2.2). While in riparian soils the densities of four out of eleven families differed significantly between invaded and uninvaded plots, this was observed for only one taxon in semi-natural grasslands (Table 2.2). 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. 2.1, Table 2.2). Other bacteria feeders did not show a consistent response. Mononchida M3, a family of predatory nematodes, was significantly more abundant in invaded riparian plots as well. In semi-natural grasslands, we found significantly higher densities of omnivorous Dorylaimida D3 in invaded plots; other taxa did not show a significant response (Table 2.2).

Table 2.2. 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 Dorylaimida D3 and Mononchida 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
Aphelenchoidea	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
Dorylaimida 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
Mononchida 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 <i>Anaplectus</i>)	87.8	156.0	277.5	115.1	204.7	364.3	168.5	299.7	533.6	107.2	190.6	339.2
<i>Anaplectus</i>	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

Analysis of samples taken in August 2011 and analysed microscopically, showed that in general nematode diversity was similar for both habitats. 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 (Table S2.2).

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 uninvaded 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. 2.1).

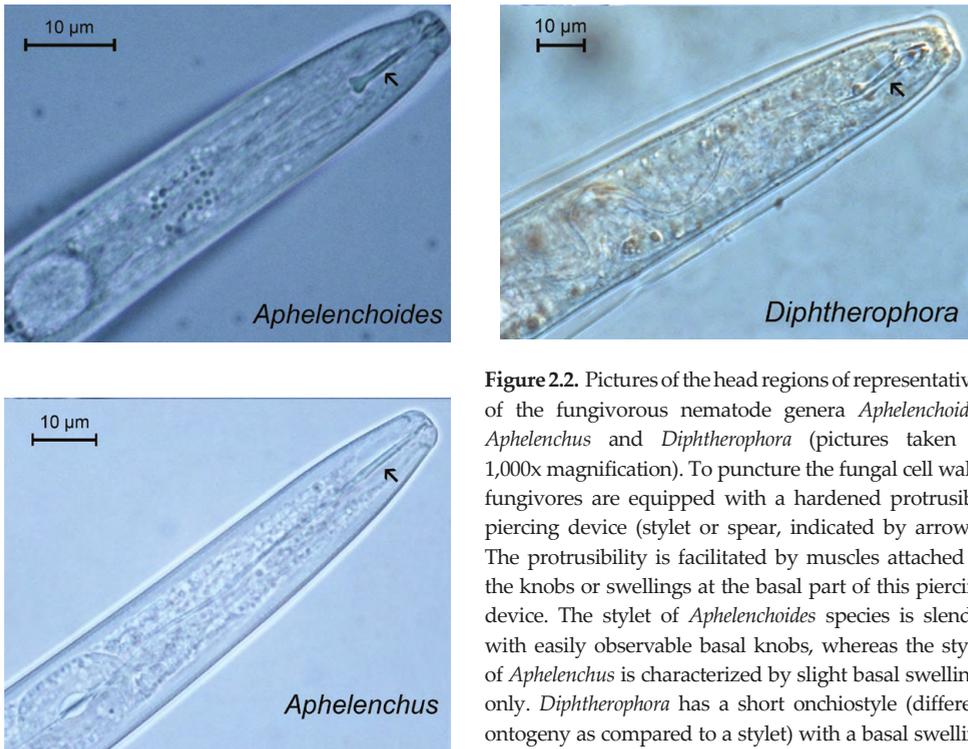


Figure 2.2. Pictures of the head regions of representatives of the fungivorous nematode genera *Aphelenchooides*, *Aphelenchus* and *Diphtherophora* (pictures taken at 1,000x magnification). To puncture the fungal cell walls, fungivores are equipped with a hardened protrusible piercing device (stylet 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 protrusibility of *Aphelenchooides* species is slender with easily observable basal knobs, whereas the stylet of *Aphelenchus* is characterized by slight basal swellings only. *Diphtherophora* has a short onchiostyle (different ontogeny as compared to a stylet) with a basal swelling of the onchiostyle extension.

No systematic effect was observed on the bacterivorous nematodes. 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.

Alternatively, it might be suggested that the local presence of dense *S. gigantea* stands is the result of a locally distinct fungal community. However, this would contradict the results of an extensive mesocosm experiment performed by Scharfy *et al.* (2010) in which *S. gigantea* was added to a number of experimentally assembled plant communities using soils that had not been covered by *S. gigantea*.

Effect of *Solidago gigantea* on soil acidity

The slightly lower pH in invaded plots (0.1 – 0.2 units) 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 (Chapuis-Lardy *et al.* 2006, Herr *et al.* 2007). In our study, only small differences in pH were measured, *i.e.* on average 0.1 units, which seem unlikely to explain the observed changes in soil biota, and more specifically, the increase of one of the three lineages of fungivorous nematodes (Aphelenchoididae). It is noted that a higher pH in riparian zones (appr. 1.5 unit), resulted in a higher Aphelenchoididae density (Table 2.2). Furthermore, 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 in the riparian and semi-natural grassland habitats, respectively. This impact is relatively high; in a study of Hejda *et al.* (2009), an overall reduction of plant-species richness 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, and in addition, *S. gigantea* was found to be impacted by limited extent 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 impact 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 (Vanderhoeven *et al.* 2006, Scharfy *et al.* 2009) From June onwards, the stems and leaves of *S. gigantea* can become increasingly dense and compete successfully for light (Weber and Jakobs 2005, Banta *et al.* 2008). Moreover, *S. gigantea* releases large amounts of furanoid compounds and acidic compounds in the rhizosphere (Weber and Jakobs 2005). In the case of *Solidago canadensis*, (Yuan *et al.* 2013) uncovered a relationship between the allelochemical content of the plants and their ability to compete with native plant species. Allelochemical compounds produced by *S. gigantea* may also play an important role in its competitiveness and could affect not only resident plant species but also belowground communities.

Despite the success of *S. gigantea*, not all plants were negatively affected. We observed a rare and exotic parasitic plant *Cuscuta 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.

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

Despite the fact that both habitats differ in soil type, floristic composition, and land use history, we found significant overall 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.

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. In Fig. 2.2, 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. 2.2) 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 *Tyloaimophorus*, 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 (Rössner and Nagel 1984, Ruess and Dighton 1996). 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.

Beside overall effects, we observed habitat-type dependent changes for some of the nematode taxa. Increased densities of two bacterivorous nematode families, Alaimidae and Cephalobidae, were exclusively observed in riparian vegetation. The over four times higher density of Alaimidae in the riparian zones as compared to the semi-natural grasslands suggests that these river clay soils are a preferred habitat for Alaimidae. Possibly, Alaimidae under near optimal conditions are more responsive to environmental changes such as a drastic change in the plant community. Cephalobidae, a widespread and abundant family of bacterivores, significantly increased in invaded riparian vegetation whereas their numbers decreased in invaded patches in grasslands (significant interaction effect). The family Cephalobidae was represented by six identical genera in both habitats (see Table S2.2). The significant impact of *S. gigantea*'s presence can be explained by a change in the abundance of a single or several different genera. Interpretation of the responses for this family requires the development and use of genus-specific assays in future studies.

Conclusion

In Europe, the colonisation of *S. gigantea* represents a hazardous factor at the plant community and at 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 nematode communities in *S. gigantea*-invaded soils are significantly different from neighbouring soils under the native flora. Remarkably, the observed two-fold increase of fungal biomass in soil under *S. gigantea* patches, did not result in a general, more or less even density increase in fungivorous nematodes, but rather in the specific boost of a single lineage, the Aphelenchoididae. Recent experimental data point at distinct food preferences for individual lineages of fungivorous nematodes (Vervoort *et al.* 2012), and a specific stimulation of a part of the fungal community would be a plausible explanation for the results presented in this paper. In order to better understand the belowground effects of *S. gigantea* in Europe, we currently work on the establishment of causal links between plant invader-induced changes in the composition of bacterial and fungal communities and shifts in the composition of bacterivorous and fungivorous nematode assemblages.

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Supporting information

Additional supporting information can be found online:
<https://sites.google.com/site/phdthesiscasperquist>

3 CHAPTER

Organic farming practices result in compositional shifts in nematode communities that exceed crop-related changes

Casper W. Quist *

Maarten Schrama *

Janjo J. de Haan

Geert Smant

Jaap Bakker

Wim H. van der Putten

Johannes Helder

* Authors contributed equally to this work
published in Applied Soil Ecology 2016, 98, 254-260

Abstract

Intensification of conventional agriculture has resulted in a decline of soil ecosystem functioning. Organic agriculture intends to manage soil biota in a manner that is more geared towards adequate cycling of nutrients with minimal losses. Ecological interpretation of agricultural practices-induced shifts in primary decomposers, bacteria and fungi, is non-trivial due to their enormous biodiversity. Bacterivorous and fungivorous nematodes feed selectively on these microorganisms, and we intended to test whether farming system effects are mirrored in compositional changes in nematode communities. Therefore, we analysed the impact of three farming systems, conventional (ConMin), integrated (ConSlu) and organic (Organic), on nematode communities in the southeastern part of The Netherlands on a sandy soil with 3-5% organic matter. Effects of each farming system were assessed for four different crops (barley, maize, pea or potato) by a series of taxon-specific quantitative PCRs (qPCR). Changes in community structure analysed by nonmetric multidimensional scaling (NMDS) showed that organic farming resulted in specific shifts in nematode community composition exceeding crop-related assemblage shifts. Three out of thirteen quantified nematode taxa showed significant farming system effects. Strongest effects were observed for the (putative) bacterivore *Prismatolaimus*, which was relatively common in Organic fields and nearly absent in ConMin and ConSlu fields. A reverse effect was observed for *Pristionchus*; this necromenic bacterivore and facultative predator made up about 21% and 7% of the total nematode community in respectively ConMin and ConSlu fields, whereas it was nearly absent from Organic fields. The observed farming system effects suggest that specific nematode taxa might be indicative for the impact of farming practices on soil biota.

Key-words: Organic farming, effective organic matter, *Prismatolaimus*, *Pristionchus*, microscopic analysis, quantitative PCR, bio-indicators, soil health

Introduction

Soil organisms are essential for the decomposition of organic matter from plant or animal origin (Janzen, 2006). In (agro-)ecosystems plants benefit from the biological degradation of various types of organic matter as soil biota mediate the bio-availability of *e.g.* carbon, nitrogen and phosphorous. Other ecosystem services delivered by soil biota are the build-up of soil organic matter, the improvement of soil structure (Six and Paustian, 2014), and the promotion of disease suppressiveness (Van Bruggen and Semenov, 2000; Wagg *et al.*, 2014). Intensification of agriculture has led to a decline of soil biodiversity (Tsiafouli *et al.*, 2014) and a general decline in soil ecosystem functioning (de Vries *et al.*, 2012). Organic agriculture aims at more sustainable food production through application of multiple types of organic fertilizers and strong reduction of pesticide use (Mäder *et al.*, 2002). Long-term effects of organic farming generally result in higher organic matter levels (Gattinger *et al.*, 2012), increased soil biodiversity and aboveground pest suppression (Birkhofer *et al.*, 2008; Mäder *et al.*, 2002).

To evaluate effects of farming on soil quality, various biotic indicators of soil quality have been identified (Doran and Zeiss, 2000). However, due to the overwhelming biodiversity, and the poor ecological characterisation of numerous constituents, it is hard to relate composition, diversity and abundance to ecological functioning of soils (Giller *et al.*, 1997; Thiele-Bruhn *et al.*, 2012). The use of nematodes as bio-indicators to monitor the impact of farming strategy and crop types has received some attention (Berkelmans *et al.*, 2003; Neher, 1999; Van Diepeningen *et al.*, 2006). Nematodes are present in high densities in virtually any soil, and their communities are species-rich with representatives in all trophic layers of the soil food web. Moreover, nematodes show distinct sensitivities towards various kinds and levels of environmental stressors (Bongers, 1990; Bongers and Ferris, 1999; Yeates, 2003). Despite these advantageous biological characteristics and the fact that nematodes can be easily separated from the soil matrix, the use of nematodes as indicators of soil quality is not widespread. This is mainly due to difficulties with identification as a result of the scarcity of informative morphological characters. Routine microscopic analyses are therefore time-consuming and require ample training. The resolution offered by the ribosomal DNA (rDNA) locus is relatively high, thus enabling DNA-based identification of nematode communities. Several quantitative (q) PCR-based methods have been developed for the characterisation of nematode assemblages (Floyd *et al.*, 2002; Holterman *et al.*, 2008; Vervoort *et al.*, 2012), but hitherto, such methods have not been frequently used for impact assessments.

Long-term (>10 years continued treatment) effects of organic and conventional farming practices on nematode assemblages have been investigated in various

experimental settings. Studies showed a negative effect of tillage on food web complexity (Ugarte *et al.*, 2013), an increase in overall abundance of nematodes in response to organic matter inputs (Li *et al.*, 2014), and strong correlations between soil nutrient status and the number of bacterivores (Berkelmans *et al.*, 2003; Pan *et al.*, 2010). Crop type was shown to affect composition of nematode assemblages to a larger extent than farming system (Berkelmans *et al.*, 2003; Neher, 1999). In four locations in North Carolina (USA), Neher (1999) investigated in detail the effects of conventional and organic farming practices during more than eight years of farming strategy transition. Three free-living nematode families were shown to be more abundant in organically managed soils; Plectidae, Prismatolaimidae and Tylencholaimidae. Due to our superficial knowledge about the feeding preferences within these groups, plausible mechanistic explanations for the promotion of these trophically distinct families by organic farming are lacking. At the same time, these studies illustrate the potential for using nematode communities to test the impact farming systems on the soil biological condition.

Here, we investigated effects of farming system and crop species on nematode assemblages in a long-term field experiment. Based on the results of a biodiversity inventory with 51 taxon-specific qPCRs, 15 abundant and trophically diverse nematode taxa were selected. qPCR analyses revealed significant impact of crop species and farming system on the nematode community composition. As a verification, subsamples were analysed microscopically in parallel, and this independent methodological approach gave similar, though less pronounced results. On top of the crop-related effects, organic farming practices resulted in significantly higher *Prismatolaimus* and *Diphtherophora* levels, whereas a strong opposite trend was observed for *Pristionchus*. Finally, possible explanations for the observed farming system-related shifts in nematode communities are presented.

Materials and methods

Study site

The Vredepeel farm is located in the southeastern part of the Netherlands (Oceanic climate (Cfb); 600 – 700 mm precipitation year⁻¹, mean temperatures of 11 °C) on a sandy soil (93.3% sand, 4.5% silt, 2.2% clay) with moderately high organic matter (OM) levels (3-5%) and high to very high phosphorus contents (~2.2 mg kg⁻¹). In 2001, three different farming strategies were installed. Organic farming fields received the highest organic matter inputs (cattle manure and crop residues) and no pesticides were applied (Organic: 3,050 kg effective organic matter (EOM) ha⁻¹ yr⁻¹; EOM as defined by Sukkel *et al.* (2008)). The two types of conventional farming

differed in the type and quantity of EOM application. In the ConSlu system, mineral fertilizers were applied in combination with pig and cattle slurry (1,950 kg EOM ha⁻¹ yr⁻¹), and the ConMin system is based on the application of mineral fertilizers only (1,250 kg EOM ha⁻¹ yr⁻¹, mainly crop residues). Nutritional regimes in each of the systems was designed to keep the total P and K input constant (≈ 50 and 220 kg ha⁻¹ yr⁻¹, respectively), while the active N input in the Organic treatment was 45% of the N inputs in the ConMin and ConSlu treatments. ConMin and ConSlu both received about 180 kg active N ha⁻¹ yr⁻¹.

Set-up field experiment

The field trials are based on a six-year crop rotation with (1) potato, (2) pea, (3) leek, (4) barley, (5) sugar beet (in ConMin and ConSlu) or carrot (in Organic), and (6) maize. For this study, samples were collected from fields with potato, pea, barley and maize. In total 12 rectangular experimental fields (each 180 m by 15m or 18 m) were sampled; four fields for each of the three farming systems. The overall layout of this field experiment is shown in Fig. S1. With regard to the design of this field experiment it should be noted that the European organic farming directive (SKAL) did not allow us to use a completely randomized block design. Therefore, the organic fields had to be placed in one block, whereas the two conventional treatments were mixed on the remaining two blocks. Additional studies have shown that differences between the organic and conventional farming systems could not be related to position effect (see Fig. S2 and S3).

Soil sampling and nematode extraction

Sampling took place on the 1st of May 2013, just prior to the growing season and presumably an ideal period to measure farming system effects, as preceding crop-effects have eroded during the winter period. In each field ($n = 12$) samples were collected along six virtual lines parallel to the short end of the rectangle. The spacing between the parallel lines was 30 m. Along each virtual line, one composite soil sample was collected consisting of 12 equidistantly-taken cores (\varnothing 1.5 cm, depth: 20 cm). Immediately after sampling, the resulting 72 (= 6 from each of the 12 fields) composite soil samples were stored at 4°C. Soil samples were homogenised thoroughly and nematodes were extracted from a 100 g subsample using an Oostenbrink elutriator (Oostenbrink, 1960). This amount was chosen because samples smaller than 100 g are less likely to reflect the true community (Wiesel *et al.* 2015), and since Verschoor and coworkers (2000) found that nematodes were more efficiently extracted from small (50 g) than from large samples (250 g). Nematode suspensions were split into two equal portions. One portion was analysed by a series of quantitative PCR assays, a subsample of the second half was analysed microscopically.

Microscopic analysis of nematode communities

Nematode suspensions were fixated in 8 ml 5% formaldehyde (Seinhorst, 1962) in 38 out of 72 samples. For this study at first 100 individuals were identified to genus level. For taxa represented by fewer than five individuals in any of the samples, another batch of 100 nematodes was examined. To estimate the nematode density 1/10 of each sample was counted under a dissecting microscope.

Quantitative PCR-based analysis of nematode communities

Nematode suspensions were concentrated and lysed (Holterman *et al.*, 2006). DNA extracts were purified using a glass fibre column-based procedure (Ivanova *et al.*, 2006; Vervoort *et al.*, 2012). For a nematode biodiversity check, 1 μ l subsamples were taken from each of the purified DNA extracts, and mixed. All purified DNA extracts were stored at -20 °C until further use.

Overall purified DNA extracts were used as template in qPCR using 51 nematode taxon-specific primer sets. Overall, 33 taxa were shown to be present, and 15 abundant taxa were selected for further analysis (Table S1). In total 17 primer sets were used: 15 taxon-specific, one to assess total nematode density and one external control (to compensate for losses during sampling handling). qPCR reactions were executed and Ct values (the number of PCR Cycles that were needed to reach a threshold) were converted to nematode densities by making use of the known linear relationships between Ct values and $^{10}\log$ (number of target nematodes) (Vervoort *et al.*, 2012). The absolute value of the first mathematic derivative of the melting curve was checked to confirm the correct nature of the amplicon. N/A (non applicable) was used to indicate that no (correct) amplicon was formed.

Data analysis

Generalized linear models (GLZ) with a Poisson-distributed dependent variable were used to separate effects of farming system and crop as well as their interaction. The outcome of both identification methods was compared. To meet the assumption of normality of residuals, standardized residuals were tested on normality using a Kolmogorov–Smirnov test. To inspect whether the different farming system and crop type affect nematode community compositions, nonmetric multidimensional scaling (NMDS) analyses were conducted on qPCR as well as microscopy data. Degrees of stress in NMDS plots indicate the reliability of the outcome, where a lower s-stress corresponds to a higher reliability (Oksanen, 2015). The dissimilarity matrices in the three different analyses were based on field-mean abundances of all taxa within the community. A Bray-Curtis dissimilarity metric was used to determine

distances between the sampling points, for which the metaMDS function was used (R vegan package; Oksanen, 2015; R Development Core Team, 2011). Spearman's ρ was used as a nonparametric measure to determine correlations between densities of taxa obtained by microscopy and qPCR. All statistics were done using Statistica 9.0 and R version 2.15.1.

Table 3.1. Farming systems: conventional farming with mineral fertilizer (ConMin), conventional farming with mineral fertilizer supplemented with pig slurry (ConSlu), and an organic farming with high organic matter inputs (cattle manure) and no mineral fertilizer (Organic). Different letters indicate significant differences ($P < 0.05$). EOM stands for effective organic matter, i.e. the amount of organic matter still present one year after incorporation into the soil. This is assessed by using standard parameters for every type of organic matter (Sukkel *et al.*, 2008).

Farming system	Annual input (kg EOM ha ⁻¹ yr ⁻¹)	Organic matter (% \pm SE)	Significance	Moisture (% \pm SE)	Significance
ConMin	1,250	4.5 \pm 0.1	A	7.1 \pm 0.3	A
ConSlu	1,950	5.1 \pm 0.1	B	7.5 \pm 0.3	A
Organic	3,050	5.7 \pm 0.2	C	10.3 \pm 0.4	B

Results

Effects of prolonged exposure to distinct farming systems

Twelve years of three different organic matter input regimes (ConMin, ConSlu and Organic) had significant influences on soil abiotic conditions (Table 3.1). Regarding effective organic matter (EOMs) inputs, it should be noted that the annual 1,250 kg EOMs input per hectare in the regime with “just mineral fertilizer” (ConMin) consists of an estimation of the average input of crop residues. Also for the other regimes, estimated inputs in the form of crop residues were taken into account. Prolonged input of additional organic matter, pig slurry in case of ConSlu, and cattle manure in case of Organic, resulted in significant differences in organic matter contents, being lowest in ConMin and highest in Organic (Table 3.1). Only for the Organic fields, a significant increase in the moisture content was detected (Table 3.1).

Nematode assemblages affected by farming system and crop

To make a selection of the most abundant nematode taxa, lysates from nematode assemblages were analysed using 51 taxon-specific primer combinations (Table S3.1). Thirty-three taxa were shown to be present. Based on density, trophic diversity, and molecular detectability (some classical taxonomic groups such as the family Rhabditidae appeared to be poly and/or paraphyletic, and cannot easily be detected using molecular methods), 15 taxa abundant taxa were selected for further

analysis. Nematode community compositions as determined by taxon-specific qPCRs show that nematode assemblages in the organic farming were distinct from the ones found under conventional farming irrespective of the current crop (Fig. 3.1A). No clear distinction was observed between the two types of conventional farming: fields receiving solely mineral fertilizer (ConMin), or fields receiving pig slurry in addition to mineral fertilizer (ConSlu). Analysis of in parallel-generated microscopic community data did not confirm this farming system-based separation of the nematode assemblages (Fig. 3.1B).

qPCR data revealed clear crop related effects on community composition. Distinct nematode assemblages were observed in potato and maize fields (Fig. 3.1C) and rather similar communities in barley and pea fields, which were positioned between communities of potato and maize. Microscopic data showed similar, though less pronounced separations of crop-specific nematode assemblages (Fig. 3.1D). Abiotic factors (organic matter and moisture contents) were positively correlated with the nematode communities in the organic system, as illustrated by the arrows in the NMDS plots (Fig. 3.1A, C). A similar, but less pronounced, trend was observed for the microscopic data (Fig. 3.1B, D).

Responses of individual nematode taxa to farming system and crop

Total nematode densities were not different between farming systems, but significant effects were found for crop type and farming system \times crop type interactions (Table 3.2). qPCR data showed significant effects of farming system on six out of thirteen nematode taxa (Table 3.2). Most notably Pristionchidae (a family harboring a single genus, *Pristionchus*) was relatively common in Organic fields whereas nearly absent from both conventional systems. Among the fungivores *Diphtherophora* was slightly but significantly more abundant in the organic fields, whereas the two other fungivorous taxa, Aphelenchidae and Aphelenchoididae, were not affected by the farming system. Remarkably, *Pristionchus* a necromenic bacterivore and facultative predator was significantly more abundant in ConMin (Table 3.2). No significant effect of farming system was found for the dominant plant-parasitic taxa, *Pratylenchus* spp. and *Tylenchorhynchus*.

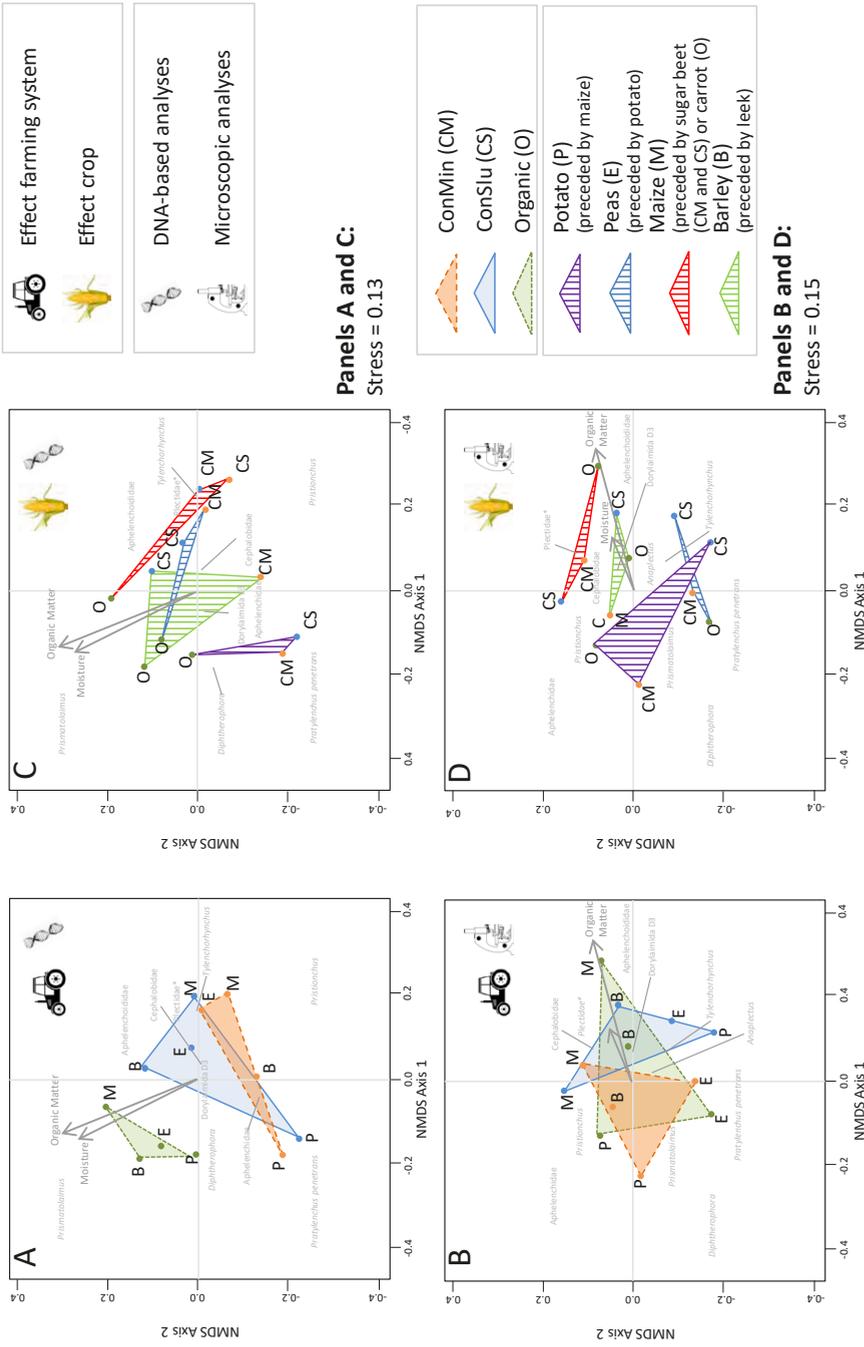


Figure 3.1. Nematode community compositions per farming system (indicated by tractor icon; A and B) and crop type (indicated by a maize cob; C and D) based on qPCR data (indicated by a DNA strand; A and C) and microscopic data (indicated by a microscope icon; B and D). Dissimilarities between community compositions were determined by Nonmetric multiple scaling (NMDS). Goodness of fit is expressed by a stress value. Polygons in different colors indicate farming system and crop type.

For a few other taxa, farming system-related shifts could not be confirmed by microscopic analysis (criterion: $P \leq 0.05$). The omnivorous Dorylaimida D3 (mainly *Thonus*, *Enchodelus*, *Eu-*, *Epi-* and *Prodorylaimus* (see Holterman *et al.* 2008, hard to distinguish from other Dorylaimids in routine micropic analyses) was more abundant in organic fields. Among the bacterivores, representatives of the opportunistic genus *Mesorhabditis* were significantly more abundant in the ConSlu fields, whereas Cephalobidae were present at higher densities in the ConMin fields (Table 3.3).

Eleven out of 13 taxa (including total nematode densities) showed a significant response to crop type, irrespective of the nematode community analysis method used (Table 3.2). Total nematode densities were significantly higher in barley and maize than in pea and potato (Table 3.3). This difference can be attributed mainly to the significantly higher densities of relatively abundant taxa *Pristionchus* and Cephalobidae in these crops. In comparison to the farming systems, the impact of crop on fungivorous taxa was reversed: both Aphelenchidae and Aphelenchoididae numbers were increased in a crop-specific manner, whereas *Diphtherophora* remained unaffected (Tables 3.2 and 3.3). Regarding plant parasitic taxa, *Tylenchorhynchus* densities increased in maize and pea fields. *Pratylenchus* spp. were more abundant in pea and potato fields (Tables 3.2 and 3.3).

Potato fields typically had relatively high levels of the omnivores group Dorylaimida D3. Barley and pea fields were characterized by relatively high densities of *Filenchus* group 3 (predominantly root-hair feeders and/or fungivores) and fungivorous *Aphelenchoides* species. Other remarkable observations were the low incidence of *Pristionchus* species in pea fields, and – to a lesser extent – low *Mesorhabditis* densities in potato (Table 3.3). Significant interaction effects were common, illustrating that, for the majority of nematode taxa, crop response is farming system dependent. The total nematode densities are significantly distinct between the four crops (Tables 3.2 and 3.3). As samples were collected early in the growing season, observed changes could relate to the previous crop, the kind of cover crop used during wintertime, and the actual crop (and not solely to the actual crop).

Table 3.2. Effects of farming system, crop and the interaction between both variables on the nematode community. Nematodes communities were analysed using taxon-specific molecular assays (n=72), and approximately half of the samples were analysed microscopically in parallel (n=38). Single letters are used to indicate food preferences of nematode taxa: (f): fungivores, (b): bacterivores, (pp): plant parasites, (omni): omnivores. *Pristionchus* (*): qPCR specifically detected representatives of the genus *Pristionchus*, in case of microscopic analysis all members of the family Neodiplogasteridae were included. *Pratylenchus* (**) was analysed at species level, microscopic analysis allowed for quantification at genus level only. For all taxa, generalized linear models (GLZ) with a Poisson-distributed dependent variable were used to analyse densities. Only P values ≤ 0.10 are given, P values ≤ 0.05 are given in **bold**.

Nematode taxon	MOLECULAR ANALYSIS			MICROSCOPIC ANALYSIS		
	Farming system	Crop	Farming system * Crop	Farming system	Crop	Farming system * Crop
Aphelenchoididae (f)	NS	P = 0.02	P < 0.001	NS	P = 0.013	P < 0.001
Aphelenchidae (f)	NS	P = 0.01	P = 0.004	NS	P < 0.001	P = 0.02
Cephalobidae (b)	P = 0.01	P < 0.001	P < 0.001	P = 0.056	P < 0.001	P = 0.08
Plectidae minus <i>Anaplectus</i> (b)	P = 0.07	P = 0.06	P < 0.001	NS	P = 0.07	P = 0.01
<i>Anaplectus</i> (b)	NS	P < 0.001	P = 0.05	NS	P = 0.02	NS
<i>Filenchus</i> (pp & f)	P = 0.07	P = 0.003	NS	P = 0.02	P < 0.001	P = 0.07
<i>Pristionchus</i> (*) (b)	P < 0.001	P < 0.001	NS	P = 0.02	P = 0.001	NS
<i>Diphtherophora</i> (f)	P = 0.01	NS	P < 0.001	P < 0.001	P = 0.03	P = 0.006
<i>Mesorhabditus</i> (b)	P = 0.002	P < 0.001	P < 0.001	P = 0.08	P < 0.001	P < 0.001
Prismatolaimidae (b)	P < 0.001	P < 0.001	NS	P < 0.001	P < 0.001	P < 0.001
<i>Tylenchorhynchus</i> (pp)	P = 0.08	P < 0.001	P = 0.08	NS	P < 0.001	NS
<i>Pratylenchus</i> (**) (pp)	P = 0.07	P < 0.001	P < 0.001	NS	P = 0.002	P = 0.02
Dorylaimida D3 (omni)	P < 0.001	P < 0.001	P = 0.02	NS	P = 0.10	NS
Total count	NS	P < 0.001	P < 0.001	NS	P < 0.001	P = 0.002
<i>**Pratylenchus penetrans</i>	P = 0.07	P < 0.001	P < 0.001	Routine light microscopic analysis (= no morphometrics) does not offer sufficient resolution to distinguish individual <i>Pratylenchus</i> species		
<i>P. crenatus</i>	P < 0.001	P < 0.001	P = 0.002			
<i>P. neglectus</i>	Only present in block 2 (See Fig. S1)					

Table 3.3. Densities of individual nematode taxa as determined by quantitative PCR. Densities are expressed as number of individuals per 100 g soil (fresh weight). With regard to crop it should be that nematode communities were sampled early in the growing season. In case of barley, radish was grown as cover crop in wintertime, in maize plots barley was used as cover crop, in pea plots ryegrass was grown as cover crop, and in case of potato the cover crop was barley.

	Farming system						Crop							
	ConMin (n=24)		ConStu (n=24)		Organic (n=24)		Barley (n=18)		Maize (n=18)		Pea (n=18)		Potato (n=18)	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Organic matter (w/w %)	4.5	0.5	5.1	0.5	5.7	0.7	5.1	0.8	5.2	0.7	5.1	0.9	5.0	0.6
Moisture content (w/w %)	7.1	1.4	7.5	1.5	10.4	1.8	8.3	2.2	8.6	1.4	8.9	2.7	7.7	1.9
Nematode taxon														
Aphelenchoideidae (f)	37.4	93.9	19.7	21.7	42.8	89.3	60.0	104.7	12.4	10.6	7.5	8.6	53.4	101.4
Aphelenchidae (f)	0.6	0.7	1.2	1.8	0.8	1.9	0.5	0.5	1.2	2.1	0.5	0.5	1.3	2.1
Cephalobidae (b)	236.8	122.7	188.7	123.7	204.1	92.4	292.2	104.3	273.1	58.4	120.2	56.1	154.1	116.8
Plectidae (except <i>Anaplectus</i>) (b)	2.9	2.6	3.6	3.4	4.3	3.1	3.7	2.8	4.1	3.4	3.3	1.9	3.4	4.0
<i>Anaplectus</i> (b)	34.1	24.4	31.8	39.1	28.4	30.7	34.0	24.4	55.7	46.5	17.6	13.0	18.6	15.2
<i>Filenchus</i> group 3 (pp & f)	7.2	7.9	1.2	2.5	13.1	17.3	14.0	19.0	3.3	5.1	10.5	9.6	1.0	2.0
<i>Prisionchus</i> (b)	616.4	1802.5	145.9	221.7	2.5	6.4	594.7	2001.4	353.0	695.7	9.9	17.5	62.0	167.0
<i>Diphtherophora</i> (f)	0.9	1.0	0.7	1.0	1.6	1.8	1.1	1.1	1.3	2.2	0.8	0.8	1.1	1.0
<i>Mesorhabditis</i> (b)	6.1	6.0	12.1	7.9	7.4	4.4	13.0	8.6	7.5	4.8	9.5	6.0	4.0	3.0
Prismatolaimidae (b)	0.1	0.5	0.1	0.1	19.3	30.6	8.8	27.3	9.8	22.1	4.0	16.3	3.3	8.9
<i>Tylenchorhynchus</i> (pp)	3.7	2.9	2.6	2.4	1.7	1.5	1.7	1.5	4.1	3.0	3.7	2.3	1.1	1.1
<i>Pratylenchus</i> (pp)**	30.3	62.9	16.5	12.5	15.2	13.9	9.6	7.9	8.3	10.7	44.6	69.2	20.3	12.8
<i>Dorylaimida</i> D3 (omni)	66.7	69.0	43.2	40.7	116.6	116.7	37.2	40.7	90.9	77.8	50.9	49.9	123.2	126.5
Total nematode densities	2959	3131	2134	1478	2352	982	3211	3186	3614	1585	1777	998	1325	720
** <i>Pratylenchus penetrans</i>	9.1	14.3	8.9	10.1	5.9	6.8	7.4	7.0	2.3	5.4	4.8	7.2	17.4	14.7
<i>P. crenatus</i>	19.9	62.43	2.4	2.7	9.2	12.2	2.2	2.5	5.9	10.4	31.2	70.4	2.8	5.0
<i>P. neglectus</i>	1.3	3.1	5.2	11.6	0.1	0.2	0.1	0.2	0.1	0.3	8.6	12.5	0.0	0.0

(In)consistencies between qPCR and microscopy-based quantitative community analyses

To see whether qPCR-based analyses could be confirmed by the most common approach for nematode community characterization, microscopic analysis, nematode suspensions were split and analyzed with both methods. Due to practical limitations, viz. the time required to microscopically analyse nematode community samples, not all 72 samples were analyzed. Half of the samples ($n = 38$) were investigated using both methods. From the 42 test results, 83% led to similar or comparable P-values and hence concurrent conclusions about effects of farming system, crop types or interactions of these (Table 3.2, see Table S3.2). Poor correlation coefficients were found for (occasionally) rare taxa such as *Aphelenchoides*, Plectidae, *Anaplectus*, *Aphelenchus*, *Pristionchus*, *Diphtherophora* and *Tyololaimophorus* (Table S3.2 and Fig S3.4). Doubling of efforts by microscopy resulted in structurally higher correlations (t-test for dependent samples: $P = 0.03$) (Table S2).

Discussion

Long-term organic farming resulted in overall shifts in the nematode community; three out of 13 nematode taxa under investigation showed a significant farming system effect, while differences between the two conventional practices were less pronounced. Strongest effects were observed for *Prismatolaimus*, which was relatively common in organic fields and nearly absent in conventional fields, and *Pristionchus*, which was abundant in conventional fields and nearly absent in organic fields. Crop type affected more nematode taxa than farming system; 11 out of 13 nematode taxa showed a significant response to crop. Significant interaction effects for numerous taxa suggested that the effect of crop type is farming system dependent.

The absence of farming system-related effects on total nematode densities

Total nematode densities in ConMin, ConSlu and Organic fields were not significantly different. This corresponds with data presented by Van Diepeningen *et al.* (2006) and Pan *et al.* (2010), but contrasts with findings in numerous other studies where higher nematode densities were reported for organic systems (for review see Hole *et al.*, 2005). Apparently, a long-term increase of OM inputs by organic farming does not necessarily result in increased nematode densities. Our results suggest that crop type is a major short-term determinant of total nematode abundances (reflecting the steep density increase of a few taxa only). As noted before, differences in total nematode densities - significantly higher under barley and maize than under peas and potato - should be considered as a combined effect of the preceding crop, the

cover crop during wintertime and the current crop. Such (combined) crop effects were found for almost all taxa under investigation. Likewise, large crop effects were reported by Berkelmans *et al.* (2003) and Neher (1999) implying that crop type is a major driver of nematode abundances.

Possible biological explanations for the farming system-specific impact on individual nematode taxa

For three taxa pronounced farming system effects were observed: relatively steep increase in *Prismatolaimus* densities, as well as a slight increase in the *Diphtherophora* levels were found in soils under organic management, whereas an opposite trend was observed for *Pristionchus*. Higher levels of *Prismatolaimus* in fields under organic management for a prolonged period were previously reported by Neher (1999) in a range of locations varying in organic matter content (1.9 - 5.6%) and soil type (both sand and clay sites were represented). Yeates *et al.* (1997) also found higher densities of this particular genus under organic management on silt and loam, however an opposite tendency was observed on sandy soils. Usually, members of the genus *Prismatolaimus* are considered to be bacterivores, but the presence of teeth in the stoma could point at more non-selective feeding habits (Ferris *et al.*, 1996), suggesting that they may be facultative predators as well. Various reasons could underlie the increased presence of *Prismatolaimus* under organic management; they could be sensitive to one of more of the pesticides applied to the conventional fields, or they could be stimulated indirectly by the higher organic matter content in the fields under the organic management. The fact that a similar phenomenon has been described now by various laboratories under a range of soil conditions could justify additional efforts to ecologically understand this relationship. As compared to *Prismatolaimus*, only slightly higher *Diphtherophora* densities were observed in fields under organic farming. Similar observations have been reported before: in a meta-analysis on the effects of a range of disturbances in terrestrial nematode communities, Zhao and Neher (2013) reported significantly lower levels of *Diphtherophora* in fields under conventional cultivation (as compared to e.g. low tillage).

Multiple explanations can be proposed for the low *Pristionchus* densities in fields under organic management. *Pristionchus* is described as a bacterivore and facultative predator (Yeates, 1993). Members of this genus live in close association with beetles (mainly Scarabaeidae), and their main food source consists of bacteria that invade the corpus of the beetle upon its death (Rae *et al.*, 2008). So far, *Pristionchus* has been sampled mostly by collecting beetles in the field. In a recent paper on the impact of soil carbon increase on nematode assemblages in a subtropical arable soils, *Pristionchus* was identified as one of the most responsive genera (Ito *et al.*, 2015).

In our experiment, the organic matter contents (OM%) under the three farming systems differed significantly: Organic fields showing the highest OM%, and ConMin fields the lowest. Serobyán and co-workers (2014) showed the ecological advantage of the ability of *Pristionchus pacificus* to develop different mouth forms. Shortage of bacterial food triggered the formation of individuals with a mouth form more suitable for a predatory lifestyle. The unusual trophic flexibility of this genus could be advantageous in more disturbed habitats such as, in this research, the ConMin fields. As an alternative explanation, the reduced levels of *Pristionchus* could be associated with the non-use of insecticides in the Organic fields. Although less plausible, we cannot rule out the possibility that a higher abundance of scarab beetles, with which *Pristionchus*' life cycle is closely associated, resulted in emigration of *Pristionchus* from the Organic fields.

Farming system versus crop-related effects on nematode communities

Significant interaction effects (farming system × crop type) were shown for 6 of 13 nematode taxa. This illustrates that the observed effects of crops on nematode communities are largely farming system dependent. Both qPCR and microscopic analysis revealed interaction effects for all fungivores under investigation, and by qPCR data for individual *Pratylenchus* species. No consistent interaction effects were detected for the remaining 7 taxa including *Pristionchus*, and the polyphagous plant parasite *Tylenchorhynchus*. From studies such as Neher (2000) and Berkelmans and coworkers (2003), it is known that crops have large effects on individual nematode taxa and the nematode community as a whole.

It should be underlined that the effects of farming system reported in this paper were observed in Spring, at the very beginning the growing season. Moreover, the experimental fields were located in an area with sandy soils, and fields were exposed to temperate, relatively moist Northwestern European climate conditions. An indication that our observations are not necessarily bound to this soil type and/or climate zone only, was recently presented by Ito *et al.* (2015). In their study the impact of increased soil carbon levels on nematode communities was studied on an Andosol under humid subtropical conditions. Monitoring nematode assemblages over eight years revealed a similar trend compared to the present study: the nematode genera *Prismatolaimus*, *Pristionchus*, and *Pratylenchus* were mostly affected by increased total soil carbon (Ito *et al.*, 2015). These results suggest that amongst the two main organic farming-typifying practices – higher and qualitatively distinct organic matter inputs and the non-use of pesticides – the first one might be the most relevant explanatory factor for the observed nematode community shifts.

Conclusion

Our results show that prolonged exposure of agricultural fields to three different farming systems, conventional, integrated and organic, resulted in nematode community shifts that exceeded the crop-related changes. Relatively high levels of *Prismatolaimus* and – to a lesser extent – *Diphtherophora* were observed in soil under organic management, whereas high *Pristionchus* densities were associated with conventional farming practices. It remains to be investigated whether these changes can be attributed to specific organic matter input, the non-use of chemical pesticides or other measure that are typical for organic farming.

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Supporting information

Additional supporting information can be found online:
<https://sites.google.com/site/phdthesiscasperquist>

4 CHAPTER

Feeding preference as a main determinant of microscale patchiness among terrestrial nematodes

Casper W. Quist
Gerrit Gort
Christian Mulder
Ruud H.P. Wilbers
Aad J. Termorshuizen
Jaap Bakker
Johannes Helder

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Abstract

Soil biota are responsible for essential ecosystem services such as carbon storage, nutrient cycling and water retention. However, assessment of the condition of soil biota is hampered by the overwhelming diversity. With representatives in all trophic levels of the food web, nematode communities can be used as bio-indicators. Accurate assessment of nematode assemblages requires insight in the distribution of specimens with distinct food preferences. With the availability of taxon-specific quantitative-PCR assays, distribution patterns of multiple nematode groups can be investigated simultaneously. Here, microscale patchiness of 45 nematode taxa was studied on 12 sampling sites (each with four adjacent microplots) located on arable fields or semi-natural grasslands ('system'), and on marine-, river clay or sandy soils ('soil type'). From each microplot five composite samples were collected. Contrary to our expectations, an increase of the number of cores per composite sample did not result in more accurate measurements, and apparently the levels of microscale patchiness of the taxa are low compared to what has been reported for oligophagous plant-parasites. System and soil type did not affect microscale distribution. To investigate the level of patchiness in more detail, detection probability (DP) and variability of abundances were calculated. Common and widespread bacterivorous and fungivorous taxa had $DP \geq 90\%$, confirming low level of microscale patchiness. With DPs of 40-70%, predators and most omnivores showed degrees of local clustering. An overview of mean variabilities of abundances is presented that offers insight in how feeding preferences impact the microscale distribution both between and within trophic groups.

Key-words: nematode community, quantitative PCR, bio-indicators, spatial distribution, microscale, trophic group

Introduction

Soil biota are responsible for ecosystems services such as nutrient cycling, carbon fixation, water retention, detoxification of a variety of wastes, and specific and general disease suppressiveness (Janzen 2006; Six & Paustian 2014; Wagg *et al.* 2014). The societal relevance of soil contrasts with our fragmentary understanding of the functioning of soil biota (Fitter 2005). Though technically demanding, the generation of a full inventory of soil life is feasible, but the resulting data are ecologically barely interpretable. Various proxies for soil quality have been identified, such as earthworms (Pansu *et al.* 2015), mycorrhizas (Jansa *et al.* 2014), collembolans (Nelson *et al.* 2011) and nematodes (Bongers & Ferris 1999; Neher 2001). Several motives plea in favour of nematode communities as bio-indicators, such as their representation in all major trophic levels of the soil food web (Holtkamp *et al.* 2008), their species diversity within each trophic level (Yeates 2003), their abundance and diversity in virtually any soil, and their extractability from soil samples. In a recent overview paper on cost-effective and policy-relevant indicators for the soil biological condition by 39 European soil ecologists, nematodes received the highest score among a selection of 30 types of potential indicators (Griffiths *et al.* 2016).

The main operational reason that hampers the use of nematodes as bio-indicators, seems to be the lack of informative morphological characters. Therefore, microscopic characterization of nematode communities is laborious, requires ample expertise, and typically relatively small subsamples are analyzed (Wiesel *et al.* 2015). These practical hurdles prompted the development of quantitative (q)PCR-based methods, which have increased the capacity to characterize nematode communities (Floyd *et al.* 2002; Holterman *et al.* 2008; Rybarczyk-Mydłowska *et al.* 2012; Vervoort *et al.* 2012; Quist *et al.* 2016). Griffiths *et al.* (2016) identified that the molecular analysis of nematode assemblages was the preferred method for the assessment of the soil biological condition.

Insights into the spatial distribution of nematodes will greatly contribute to our understanding of the ecological functioning of individual taxa. At the same time, this knowledge can be applied to design soil sampling strategies with predictable accuracies. Horizontal spatial distribution of soil biota has been described at two (Ferris *et al.* 1990), three (Ettema & Wardle 2002) or even four nested scales (Berg 2012). At each spatial scale, patterning is defined by abiotic and biotic soil characteristics and the dimensions of the chosen scales predominantly depend on the body size of the organismal group of interest (Ettema & Wardle 2002; Martiny *et al.* 2006). Nematodes belong to the soil microfauna (animals with body width of < 100 μm) as 99% of all soil nematodes have body widths between ~ 10 and $55 \mu\text{m}$ and body lengths between ~ 150 and $1500 \mu\text{m}$ (Mulder & Vonk 2011). Microfaunal

organisms are mainly passive dispersers. Typical active migration velocities range from 0 - 3 cm per day; exposure to external stimuli however, can result in a 10-fold higher migration speed (Bal *et al.* 2014; Moore *et al.* 2010; Wallace 1958, 1960).

Here we propose three nested scales of horizontal nematode distribution: microscale, mesoscale and metascale. *Microscale* is the spatial level at which individual nematodes search for food, mate and multiply, are exposed to predators, and cope with abiotic stressors such as locally unfavorable temperature and moisture conditions (microplot – scale dimensions: 0.05 to 1 m). Major drivers of heterogeneity of nematode microscale distribution are the size and nature of soil aggregates, and the local spread of food and predators. At *mesoscale*, nematode communities are exposed to comparable abiotic conditions and land use (plot or field – scale dimensions: 1 – 1,000 m). Horizontal patterning at mesoscale is probably mainly driven by plant inputs and soil texture modifiers such as tunneling soil fauna and tillage practices. At the *metascale*, multiple connected mesoscale habitats are involved (landscape – scale dimensions: > 1,000 m). At this scale, distribution patterns are mainly driven by dissimilarities in land use and soil properties.

So far, most research on spatial distribution of nematodes concentrated on mesoscale distribution; in arable fields focusing on individual plant-parasites (Been & Schomaker 2006; Duncan & Phillips 2009; McSorley & Parrado 1982; Seinhorst 1982), and in natural areas on trophic group level (Robertson & Freckman 1995; Simmons *et al.* 2008). Information about the microscale distribution of nematode taxa is scarce, and almost fully restricted to obligate plant parasites (Rossi *et al.* 1996, Been & Schomaker 2006) in agricultural systems. Microscale distribution of non-plant parasitic nematodes at family level and lower, received even less attention. Viketoft (2013) used a geostatistical approach to determine practical ranges (= limits of spatial dependence) of plant-parasites, bacterivores, fungivores and omnivores/predators in a 6.6 × 4.2 m plot, located in a semi-natural grassland (smallest distance between sampling points was 0.1 m). The estimated patch sizes were around 1 m, and these were independent of the trophic preference. Microscale patterns of four physiological stages entomopathogenic nematodes, *Steinernema feltiae* and *S. affine*, were studied in great detail (5 × 5 cm samples from 0.25m² plots) by Spiridonov and co-workers (2007). They revealed that levels of aggregation were negatively related with time after emergence from insect hosts.

Keeping in mind that trophic groups consist of evolutionary independent lineages with comparable food preferences as a common denominator (e.g., Quist *et al.* 2015), distinct degrees of patchiness can be expected when nematode communities are analyzed at lower taxonomic levels (Neher *et al.* 2005; Porazinska *et al.* 1999; Quist *et al.* 2014). The aim of the present study was to investigate the optimal methodology to assess and define micro-patchiness of plant parasitic, bacterivorous, fungivorous,

omnivorous and predatory nematodes, with representatives of all colonizer-persister (*c-p*) groups. According to their ecological characteristics, nematode families are assigned to one of the five *c-p* groups (Bongers 1990). A highly standardized qPCR-based detection approach allowed for quantitative detection of individual nematode taxa. Variations in microscale patchiness were subsequently used to design soil sampling strategies with a known level of accuracy. To this end, 12 sampling sites were selected, and in each sampling site four adjacent microplots (0.25 and 1.0 m²) were defined. Five composite samples (a thorough mixture of multiple soil cores) were collected from each microplot. The number of cores (diameter: 1.5 cm, depth: 0 – 20 cm) per composite sample was different in each of the four microplots: 3, 6, 12 or 24. Samples were collected from arable fields with crop rotation, and in semi-natural grasslands with high plant diversity, as well as from three soil types – marine clay, river clay and sand. We expect higher degrees of patchiness in natural fields with high plant diversity as compared to the arable fields, reflecting differences in plant diversity and management practices. Further, based on studies on the distributions of oligophagous parasites of higher plants (Been & Schomaker 2006; McSorley & Parrado 1982), we hypothesized that more cores per composite sample would result into a reduction of the degree of variation between replicate composite samples. We examined how the variation in microscale patchiness was related to the trophic ecology of the taxa under investigation. At least at higher taxonomic levels nematodes show limited biogeography (e.g. Finlay 2002), implying that most families and genera investigated here can be found on all continents. Therefore, the insights presented here may translate to other geographical regions, providing broader relevance for our understanding of the spatial distribution, sampling and ecological relevance of the Nematoda in soils.

Materials and Methods

Sampling sites and sample collection

Composite samples were collected during March 2012 and March - April 2013, just before the growing season. Eight fields were sampled across The Netherlands in five arable systems and three grasslands with high plant diversity on three soil types; marine clay, river clay and sand (see Table 4.1, S1 and Fig. S1). Abiotic soil characteristics were determined by Blgg AgroXpertus (Wageningen), a NEN-EN-ISO 17025 certified service laboratory, using standardized procedures. Visually homogeneous sampling sites were localized approximately in the middle of the selected fields. At each of the sampling sites, four adjacent microplots were defined (Fig. 4.1). In the first microplot, composite samples consisted of 3 cores (Ø 1.5 cm,

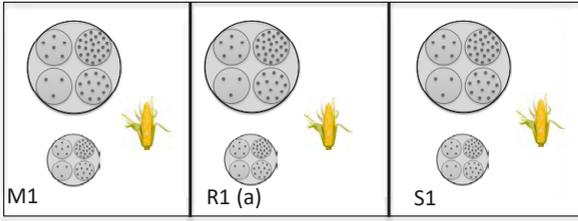
depth: 0 – 20 cm), in the second of 6 cores, in the third of 12 cores, and in the fourth of 24 cores. Five composite samples were collected from each microplot. In 2012 composite samples were taken from three arable fields with two sampling sites in each field approximately 10 m apart: microplots at the first sampling site were 1 m² and in the second 0.25 m². In 2013 composite samples were collected from three arable fields and three semi-natural grasslands with high plant diversity, where one sampling site was chosen per field with 0.25 m² microplots only. This selection of sampling sites includes a variety of abiotic conditions, management and plant diversity, thereby allowing us to investigate the effects of sampling in relation to the micro-patchiness of an ecologically wide range of nematode taxa. In total 240 composite samples were stored at 4°C immediately after sampling.

DNA extraction and qPCR-based analysis

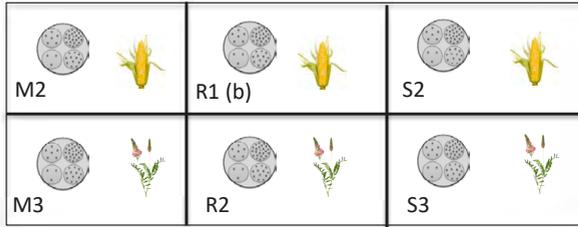
Within one week after sampling, composite samples were homogenised thoroughly and nematodes were extracted from a 100 g subsample using an elutriator - cotton wool filter method (Oostenbrink 1960). All nematode suspensions were concentrated and the DNA was extracted by a lysis buffer including mammalian DNA as an external standard (to monitor losses due to sampling handling and DNA purification) as described by Vervoort *et al.* (2012). Thereafter DNA extracts were purified using a glass fiber column-based procedure (Ivanova *et al.* 2006). All purified DNA extracts were stored at -20°C.

To assess nematode biodiversity per sampling site, 1 µl aliquots of all purified DNA extracts from a given site were combined, and mixtures were analysed. Initial analysis with 59 nematode taxon-specific primer combinations generated insight in the nematode biodiversity per sampling site (Table S2). Depending on the known biodiversity, between 24 and 33 primer sets were selected for quantification of specific taxa in each sample of a given sampling site. A separate qPCR assay was used to assess total nematode density. To generate factors that compensate for DNA losses during sampling handling, DNA levels of the external control were quantified after purification. Quantitative PCR reactions were executed and C_t values were converted to qPCR counts: nematode densities were approximated by making use of the known linear relationships between C_t values and ¹⁰log of the number of target nematodes. The maxima of the negative first mathematic derivative of the melting curves were checked to confirm the correct nature of the amplicons (Vervoort *et al.* 2012; Quist *et al.* 2016).

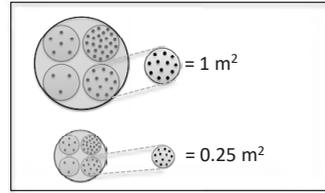
March 2012



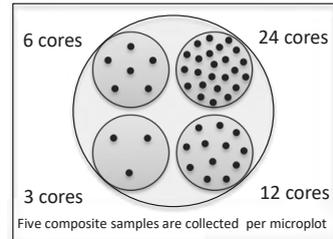
March - April 2013



Two sampling surfaces



Sampling site with four adjacent microplots



 Arable  Natural

Figure 4.1. Experimental design to investigate the effect of sampling surface and number of cores, in different soil types and systems, on detection probability and quantification accuracy. Fields on marine clay (M1, 2, 3), river clay (R1 (a and b), 2) and sandy soil (S1, 2, 3) in arable and natural systems (with corresponding icons), were sampled in March 2012 and March - April 2013. Per sampling site ($n = 12$, two per field in M1, R1(a) and S1), one in each of the other fields), four adjacent microplots were sampled, indicated in the scheme as grey circles with circular microplots inside. In March 2012 microplots of two surfaces (0.25 and 1 m^2) were sampled at two sampling sites per field. In March-April 2013 four adjacent microplots (0.25 m^2) were sampled at one site per field. Per microplot, five composite samples containing 3, 6, 12 or 24 cores were collected.

Table 4.1. Localization of fields under investigation. Soil samples were collected from arable fields (just before the onset of the growing season), and from semi-natural grasslands with high plant diversity (*). Soil types are indicated by 'm' (marine clay), 'r' (river clay), or 's' (sand) (See also Table S1 and Fig. S1). For each of the fields, five major abiotic soil characteristics were determined: pH, total nitrogen, total phosphorus, organic matter % and clay content (% soil particles < 2 µm).

Field	GPS coordinates	Soil pH (pH-CaCl ₂)	Total nitrogen (mg N / kg dry soil)	Total phosphorus (mg P ₂ O ₅ / kg dry soil)	Organic matter (%)	Clay (%)	Crops rotation scheme / dominant plant species
Schoondijke (Zeeland; m)	51°19' N 3°31' E	7.5	980	1,540	2.6	17	Potato, onion, sugar beet, wheat
Lelystad (Flevoland; m)	52°32' N 5°33' E	7.2	1,410	1,410	2.8	17	Potato, onion, sugar beet, wheat
*Lauwersmeer (Friesland; m)	53°20' N 6°09' E	7.2	2,360	1,440	5.0	17	<i>Holcus lanatus</i> , <i>Agrostis stolonifera</i> , <i>Ranunculus repens</i> , <i>Trifolium pratense</i>
Houten (Utrecht; r)	52°02' N 5°09' E	6.7	1,760	1,910	3.5	25	Corn
*Millingerwaard (Gelderland; r)	51°52' N 6°00' E	7.0	2,050	1,930	3.8	19	<i>Brassica nigra</i> , <i>Solidago gigantea</i> , <i>Calamagrostis epigjos</i> , <i>Eriogon annuus</i>
Wageningen (Gelderland; s)	51°59' N 5°39' E	5.7	720	1,310	2.8	2	Potato, barley, sugar beet, wheat, corn, grass (2 consecutive years)
Sint Kruis (Zeeland; s)	51°16' N 3°30' E	5.3	1,490	1,440	3.8	2	Potato, <i>Lolium perenne</i> (2 consecutive years), barley, wheat, beans
*Mossel (Gelderland; s)	52°03' N 5°45' E	5.6	950	1,200	3.2	2	<i>Agrostis capillaris</i> , <i>Jacobaea vulgaris</i> , <i>Achillea millefolium</i> , <i>Holcus lanatus</i> , <i>Plantago lanceolata</i>

Table 4.2. Overview of main characteristics of nematode taxa detected by qPCR assays. Trophic group is given by single capitals: B, bacterivores; E, entomopathogens; H, herbivores; O, omnivores; P, predators and U, unicellular eukaryote feeders. For feeding preferences we adhered to Yeates *et al.* (1993). Distribution of individual nematode taxa over sampling sites: R, rare: present in < 25% of the sampling sites; C, common: present in > 25% - 75% of the sampling sites, or W, widespread: present in > 75% of the sampling sites. Attributed cp value, a 1-5 colonizer-persister scales defined at family level by Bongers (1990), as well as phylogenetic position (Clade 1-12; Holterman *et al.* 2006) are provided.

Trophic group	Rare, Common, Widespread	cp value	Clade	Family	Taxon targeted by qPCR
B	W	1	10	Panagrolaimidae	<i>Panagrolaimus</i>
B	R	1	9	Rhabditidae	<i>Cruzinema</i>
B	W	1	9	Rhabditidae	<i>Mesorhabditis</i>
B	W	2	11	Cephalobidae	Cephalobidae
B	W	2	6	Plectidae	<i>Anaplectus</i>
B	W	2	6	Plectidae	Plectidae***
B	W	2	5	Monhysteridae	Monhysteridae
B	R	3	7	Teratocephalidae	<i>Teratocephalus</i>
B	R	3	6	Aphanolaimidae	<i>Aphanolaimus</i>
B	R	3	6	Metateratocephalidae	Metateratocephalidae
B	R	3	5	Diplopeltidae	<i>Cylindrolaimus</i>
B	R	3	1	Prismatolaimidae	<i>Prismatolaimus</i>
B	C	4	1	Alaimidae	<i>Alaimidae</i>
B / P	W	1	9	Neodiplogastridae	<i>Pristionchus</i>
E	R	1	10	Steinernematidae	<i>Steinernema</i>
E	R	1	9	Heterorhabditidae	<i>Heterorhabditis</i>
F	W	2	12	Aphelenchidae	Aphelenchidae
F	W	2	10	Aphelenchoididae	Aphelenchoididae
F	C	3	1	Diphtherophoridae	<i>Diphtherophora</i>
F	R	3	1	Diphtherophoridae	<i>Tyololaimophorus</i>
F / H	C	2	12	Tylenchidae	<i>Basiria</i>
F / H	R	2	12	Tylenchidae	<i>Ditylenchus</i>
F / H	W	2	12	Tylenchidae	<i>Filenchus</i> group 3**
H	R	2	12	Tylenchidae	<i>Aglenchus</i>
H	R	2	12	Tylenchidae	<i>Coslenchus</i>
H	R	2	12	Tylenchidae	<i>Filenchus</i> group 1**
H	R	2	12	Tylenchidae	<i>Filenchus</i> group 2**
H	R	2	12	Tylenchidae	<i>Tylenchus</i>
H	C	3	12	Belonolaimidae	<i>Tylenchorhynchus</i>
H	R	3	12	Pratylenchidae	<i>Pratylenchus crenatus</i>
H	R	3	12	Pratylenchidae	<i>Pratylenchus neglectus</i>

Trophic group	Rare, Common, Widespread	cp value	Clade	Family	Taxon targeted by qPCR
H	R	3	12	Pratylenchidae	<i>Pratylenchus penetrans</i>
H	R	3	12	Pratylenchidae	<i>Pratylenchus thornei</i>
O	R	4	2	Qudsianematidae	Dorylaimida PP2*
O	C	4	2	Qudsianematidae / Nordiidae	<i>Dorylaimida PP1*</i>
O	W	4	2	Qudsianematidae / Nordiidae / Dorylaimidae	Dorylaimida D3*
P	R	3	1	Tobrilidae	<i>Tobrilus</i>
P	R	3	1	Tripylidae	<i>Tripyla</i>
P	C	4	2	Anatonchidae	<i>Anatonchus</i>
P	R	4	2	Mononchidae	Mononchida M2*
P	W	4	2	Mononchidae	Mononchida M3*
P	C	4	2	Mylonchulidae	<i>Mylonchulus</i>
P	C	5	2	Nygolaimidae	<i>Dorylaimida D9A*</i>
P	R	5	2	Nygolaimidae	<i>Dorylaimida D9B*</i>
U	C	3	3	Achromadoridae	<i>Achromadora</i>

* See Holterman *et al.* 2008

** See Helder *et al.* manuscript in preparation

*** Except for the genus *Anaplectus*

Data analysis

Detection probability (DP) is defined here as the chance that a given taxon is present in a given composite sample, provided this taxon is known to be present at that site. Hence, DP is a qualitative measure, a fraction that can be calculated per microplot by the number of times a taxon is present among the five replicates. To determine the effects of system, soil type, surface and number of cores per composite sample on DP, relatively common or widespread taxa were analyzed. Taxa present in < 25% of all sampling sites ('rare taxa') were excluded because of the low number of data points. The fraction of composite samples showing presence of a taxon (out of the five composite samples per microplot) was analyzed using generalized linear mixed models (Littell 2006) with a binomial distribution and a logit link function. As the DP is expected to be related to the number of cores, we hypothesized that an increase in the number of cores per composite sample would result in higher DP. DP could also be related to factor surface (0.25-1 m²; larger surface was expected to be related to higher detection probability) and field traits (system: arable field - semi-natural grassland; soil type: marine clay, river clay, sand). Random effects of fields were introduced into the model to allow for correlations among multiple observations on the same field. For some taxa the DP could not be related to field traits as they were undetectable in some soil types or systems (Table S3).

The second quantity of interest was the variability of nematode abundance among the five composite samples per microplot. This variability was quantified as the IQR (Inter Quartile Range) of transformed qPCR counts (using the natural logarithm (\ln) of $(y+0.1)$, y being the primary qPCR count). We choose the IQR instead of the variance, because the IQR is less sensitive to outliers. To relate this measure of variability to the four experimental factors (number of cores: 3,6,12 and 24; system: arable and natural; soil type: marine clay, river clay and sand; surface: 0.25 and 1 m²), we fitted mixed models (Littell 2006), using transformed IQR ($\ln(\text{IQR}+0.1)$) as response, which we named tIQR. The transformation of IQR was needed to obtain more normally distributed responses, as required for mixed models. The mixed models contained random effects for fields and sampling sites per field, as multiple observations from the same field and sampling sites were modelled. Fixed effects were introduced for the four above-mentioned experimental factors. Mixed models were fitted per nematode taxon. For each nematode taxon, microplots with only qPCR counts of zero were excluded from the analysis. To facilitate the interpretation of the results, the mean tIQR per taxon – obtained from mixed models by averaging over all factors – was back-transformed to a relative scale factor (RSF). RSF is the multiplication factor required to go from the first (Q1) to the third quartile (Q3) of qPCR counts within a set of five composite samples per plot (by definition RSF is > 1).

Mean DP and RSF with 95% confidence intervals (CI) per nematode taxon were estimated and plotted for comparison among taxa (Fig. 4.2 and 4.3). The mean DP was estimated using a generalized linear model with binomial distribution and logit link, ignoring all experimental factors (motivated by results from analysis described above). The mean RSF with 95% CI was estimated by back-transformation of results of mixed model analyses for tIQR, averaging over levels of factors surface and cores only.

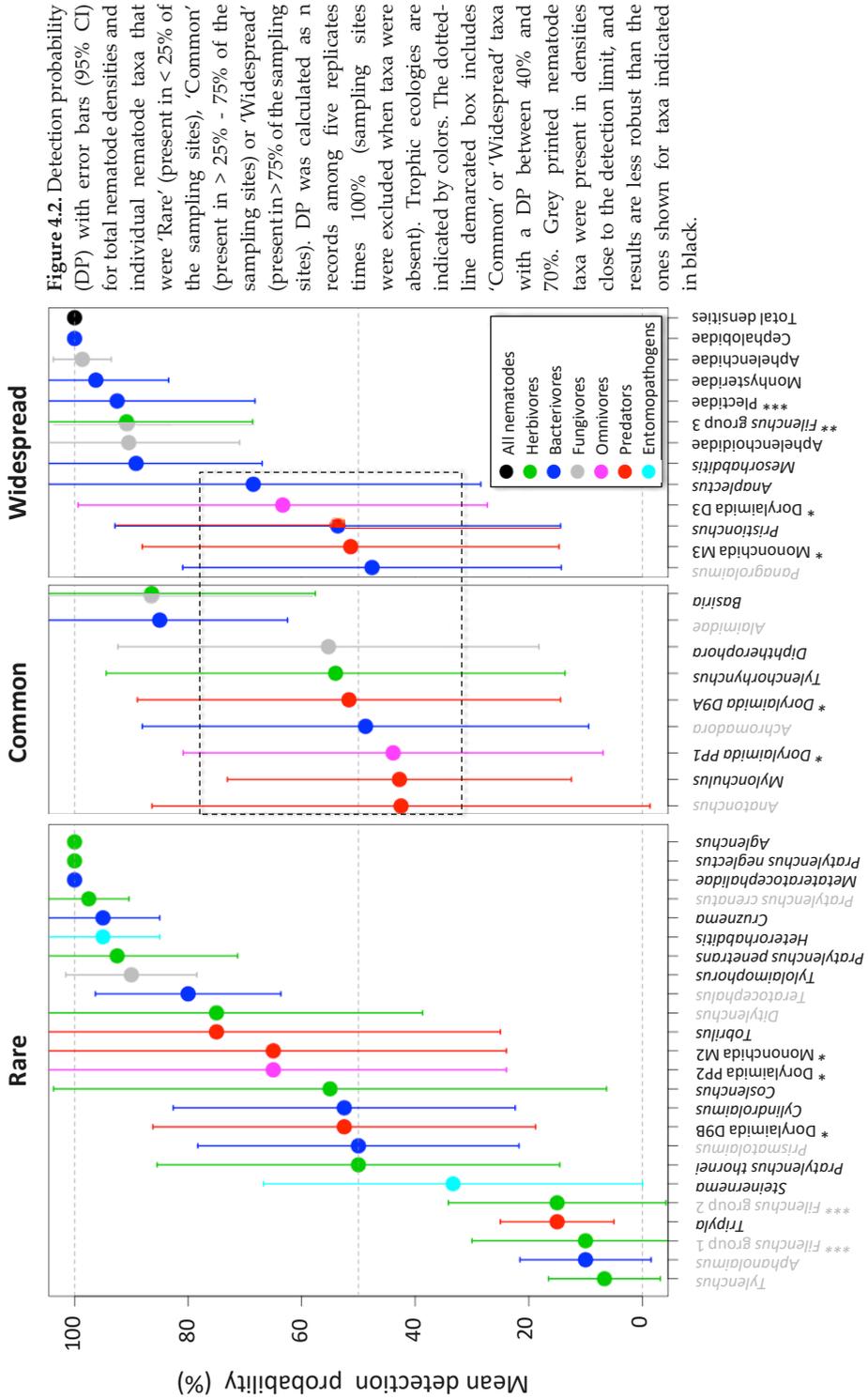
Finally, the relationship between number of replicate composite samples and accuracy of nematode quantification per nematode taxon was studied. To this end, we pooled variability estimates from all separate microplots, ignoring differences in number of cores (motivated by results, showing no effect of number of cores, see our Results section), using mixed models for log-transformed qPCR counts ($\ln(y+0.1)$) for reasons of simplicity. Assuming a log-normal distribution of the qPCR count, the coefficient of variation (CV) for the back-transformed mean qPCR count equals with the variance of the mean log transformed qPCR count. Parameter σ^2 is the within-microplot-variance component, estimated from the mixed models. In this way, the number of replicate composite samples can be calculated to obtain a given CV for back-transformed mean log qPCR count. Alternatively, the CV can be calculated given the number of replicate composite samples.

Results

To investigate the micro-patchiness among terrestrial nematodes, microplots were sampled in nine fields that varied in land management and soil type (Fig. 4.1, Table 4.1). We investigated 45 nematode taxa, with representatives from all major trophic groups (bacterivores, fungivores, omnivores, predators, plant parasites and entomopathogens; Table 4.2). It is noted that most taxa are determined either at family or at genus level. Genus level is used if the corresponding family is not monophyletic, or in cases a given family harbours only a single genus. The first option is exemplified by the fungivorous Diphtherophoridae; this family can only be detected by measuring the two constituting genera, *Diphtherophora* and *Tyololaimophorus*, individually. Entomopathogenic nematodes are an example of the second option; Heterorhabditidae and Steinernematidae each harbour a single genus, namely *Heterorhabditis* and *Steinernema*. Some plant parasites such as *Pratylenchus* are measured at species level as the genus is non-monophyletic (e.g. Rybarczyk-Mydłowska *et al.* 2014). The selected taxa belong to 27 nematode families residing in 10 out of the 12 major nematode clades (Holterman *et al.* 2006). On the basis of their ecological characteristics, nematode families have been grouped on a colonizer-persister scale (cp 1-5, Bongers 1990). In the current selection of nematode taxa, all cp categories are represented (Table 4.2).

Effect of the number of cores per composite sample on qualitative and quantitative detection

No significant effects of the number of cores per composite sample on detection probability (DP) were found for the 'common' or 'widespread' taxa under investigation (Tables 4.3 and S3). Also for the other variables, plot size, soil type, and system, rarely an impact on the DP was observed. Plot size was shown to have significant effects in two instances only: the necromenic bacterivore / predator *Pristionchus* had higher DP in 1 m² plots, whereas the omnivorous group Dorylaimida D3 had higher DP in 0.25 m² plots ($P < 0.001$ and $P < 0.01$, respectively; see Table S3). Also, no effects of soil type and system on DP of any of the taxa under investigation were found. It is noted that not all variables could be tested for all nematode groups. A few taxa received the label 'rare' as they were exclusively detected in either sand (e.g., *Pratylenchus crenatus*, *Cruznema*) or clay soils (e.g. *Pratylenchus thornei*, Dorylaimida D9B). Probably these taxa would not have received this label in case a single soil type was considered.



qPCR counts were used to obtain inter quartile ranges (IQR) for quantitative analysis. Again, variability of densities of individual nematode taxa was not significantly influenced by system, by sample surface or by the number of cores per composite sample (Table S4). Soil type affected variabilities of densities of herbivores (including all small Tylenchidae) at trophic group level, which were less variable in sand than in clay ($P < 0.05$). Abundances of *Basiria* (Tylenchidae) were lower and less variable in sand than in clay. Abundances of the herbivore *Tylenchorhynchus* were higher and less variable in sand than in clay (respectively Table S2 and S4).

Table 4.3. Effect of the number of soil cores per composite sample (3, 6, 12 or 24) on the detection probabilities of 'common' (C) or 'widespread' (W) nematode taxa (see Table 4.2). df (n): degrees of freedom, numerator, and df (d), denominator, F-values and P-values are given. $P \leq 0.05$ is considered to indicate significant differences and are given in **bold**. For taxa present in (nearly) all samples no test result could be generated by generalized linear mixed models. For details about statistical results see Table S3 and about statistical analysis of data see Material and Methods.

Taxon	Effect cores per composite sample			
	df (n)	df (d)	F-value	P-value
<i>Panagrolaimus</i>	3	36	1.50	0.23
<i>Mesorhabditis</i>	3	36	0.38	0.76
Cephalobidae	3	36	Present in all samples	
<i>Anaplectus</i>	3	33	0.91	0.44
Plectidae (except <i>Anaplectus</i>)	3	36	Present in nearly all samples	
Monhysteridae	3	36	Present in nearly all samples	
Alaimidae	3	36	Present in nearly all samples	
<i>Pristionchus</i>	3	33	1.28	0.30
Aphelenchidae	3	36	Present in nearly all samples	
Aphelenchoididae	3	33	0.77	0.52
<i>Diphtherophora</i>	3	26	0.14	0.93
<i>Filenchus</i> group 3	3	36	0.1	0.96
<i>Basiria</i>	3	20	0.54	0.66
<i>Tylenchorhynchus</i>	3	26	1.73	0.19
Dorylaimida PP1*	3	26	3.06	0.05
Dorylaimida D3*	3	36	2.12	0.12
<i>Anatonchus</i>	3	17	1.09	0.38
Mononchida M3*	3	33	0.16	0.92
Dorylaimida D9A*	3	15	0.41	0.75
<i>Mylonchulus</i>	3	26	0.95	0.43
<i>Achromadora</i>	3	9	1.69	0.24

* See Holterman *et al.* 2008

As no significant effects of the numbers of cores per composite sample were detected for the taxa under investigation (Table 4.3, Table S3 and Table S4), the mean DP and relative scale factor (RSF) with 95% confidence interval (CI) were calculated over all microplots (hence regardless of the number of cores) for comparison among individual taxa and to study how the variation in microscale patchiness was related to trophic group, c-p value and phylogenetic position.

Nematode detection probabilities

Within nematode communities individual taxa were shown to differ in both distribution across the fields of study (e.g. rare, common or widespread) as well as in mean DP with 95% CI (given they were present) (Fig. 4.2). To illustrate this, three examples are briefly discussed. The fungivorous nematode family Aphelenchidae was shown to be ‘widespread’ (present in > 75% of the sites, right panel), and the mean DP – the expected chance of detecting Aphelenchidae in a composite sample – was close to 100%. The predatory Dorylaimida D9A (including the genera *Aquatides*, *Clavicaudoides*, and *Nygolaimus* (Holterman *et al.* 2008)) was ‘common’ as it occurred in 25 – 75% of the sites (middle panel), its mean DP was close to 50% with a wide 95% CI. The left panel of Fig. 4.2 shows the ‘rare’ taxa (here defined as present in < 25% of the sampling sites). The entomopathogenic genus *Steinernema* was present at three sampling sites only, and at these sites its mean DP was around 35%.

Among the ‘widespread’ taxa, bacterivores were overrepresented. Aphelenchidae and Aphelenchoididae, two out of the four main fungivorous lineages resided in the ‘widespread’ category as well. Also *Filenchus* group 3 (Tylenchidae) and omnivore Dorylaimida D3 were shown to be widespread. Among the ‘common’ taxa another set of bacterivores was found (*Anaplectus*, Alaimidae and *Achromadora*), together with the fungivore *Diphtherophora*, the supposedly root-hair feeder *Basiria*, and an omnivore group referred to as Dorylaimida PP1 (see Holterman *et al.* 2008). Further, predatory nematodes (Mononchida M3, Dorylaimida D9A and *Mylonchulus*) and an obligate plant-parasite (*Tylenchorhynchus*) were common in our sites. Almost half of all ‘rare’ taxa were herbivores, whereas bacterivores were underrepresented in this category. Mononchida M2, Dorylaimida D9B (dominant genus: *Paravulvius*) and *Tripyla* were among the more rarely detected predators. The acidophilic fungivorous genus *Tyololaimophorus* was detected only once, in an arable field on sand. Representatives of the entomopathogenic genera *Steinernema* and *Heterorhabditis* were ‘rare’ and they were almost exclusively found in semi-natural grasslands.

The dotted-line demarcated box in Fig. 4.2 was drawn to emphasize ‘Common’ or ‘Widespread’ taxa with a DP between 40% and 70%. Omnivores, predatory nematodes are overrepresented in this box. Also the two nematode taxa that predominantly live

in phoretic association with insects, *Pristionchus* and Panagrolaimidae, are in the demarcated box. Six out of the twelve taxa in the demarcated box belong to the stress-sensitive nematode orders Dorylaimida and Mononchida (Holterman *et al.* 2008), taxa belonging to cp groups 1, 2 or 3 are present but under-represented in this box.

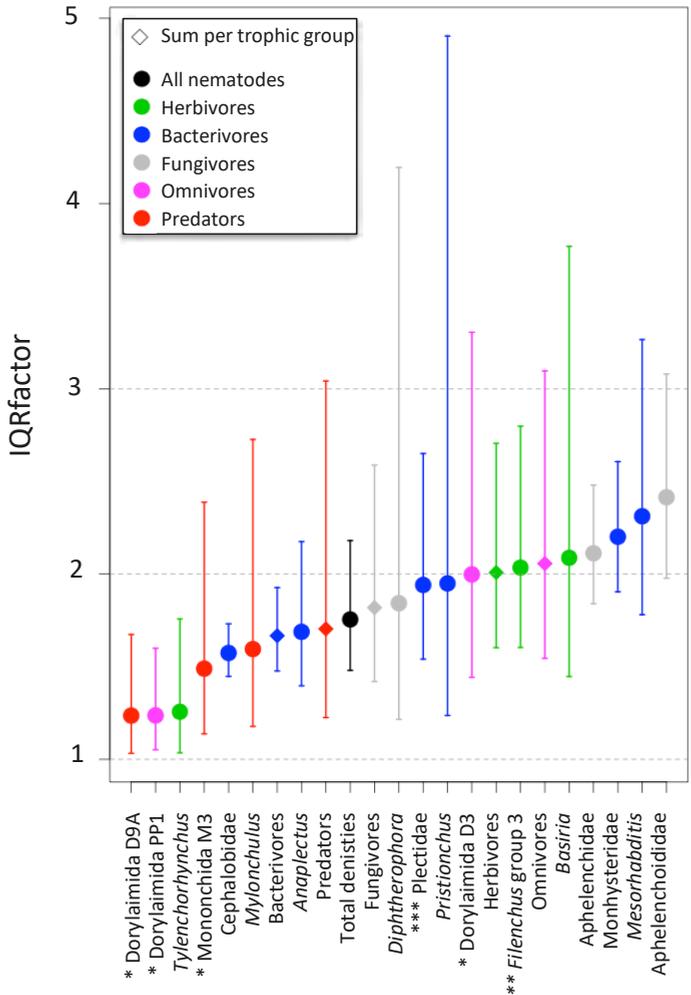


Figure 4.3. Variability of nematode densities for common and widespread taxa and trophic groups over three soil types (marine clay, river clay and sand) in six arable and three natural sites, with plots of 0.25 and 1 m² and by taking five replicate composite samples per plot with 3, 6, 12 or 24 cores per composite sample. This variability of densities is expressed as a relative scale factor (RSF). The interquartile range (IQR) for nematode densities was calculated per microplot and transformed (tIQR) (see Material and Methods for details about statistical analysis). To facilitate the interpretation of the results, the mean tIQR per taxon – obtained from mixed models by averaging over all factors – was back-transformed to obtain the RSF. This is the multiplication factor to go from the first (Q1) to the third quartile (Q3) of qPCR counts within the set of five composite samples per plot. Error bars (95% CI) show the range of plausible values of the relative scale factor. The colour of the dots indicates trophic group. Diamonds show the sum per trophic group.

Variability of nematode densities

Widespread and common taxa displayed a wide range of variabilities of abundances, here quantified by the mean RSF with 95% CI (Fig. 4.3). Differences in mean RSF can be attributed to a combination of spatial variability (within and between microplots and sampling sites), and measurement errors (sum of variation caused by the protocol, from nematode extraction till qPCR reactions). Within the bacterivores, different degrees of variability of abundances were seen: Cephalobidae and *Mesorhabditis* respectively had the lowest and the highest mean RSFs. Based on the width of the 95% CI, bacterivorous taxa can be roughly divided into two categories: the first category includes taxa that have a narrow 95% CI, representatives of this category are e.g. Cephalobidae, *Anaplectus* and Monhysteridae. The second type includes taxa that have wide 95% CI, such as *Pristionchus*, *Mesorhabditis* and Plectidae (excluding *Anaplectus*). Predators usually had a relatively low mean RSF with wide 95% CI. Comparison of variability of taxa within all trophic groups resulted in significantly different levels of RSF, except for the fungivores, (Table S4). For the majority of widespread and common nematode taxa the RSF was around two (so Q3 is about twice the value of Q1). Cephalobidae showed the narrowest 95% CI, so we are most certain about the mean RSF of this family; other taxa such as *Pristionchus* and *Diphtherophora* had relatively wide 95% CI, indicating a high level of uncertainty about the mean RSF.

Optimal sampling to reach increased detection probabilities and more accurate estimates of nematode densities

Contrary to our expectations increased numbers of cores per composite sample above three did not result in increased DP or more accurate assessments of nematode densities of any taxon under investigation. In addition, neither system nor soil type influenced the DP and IQR. Analysis of increased numbers of *replicate* composite samples however, results in higher DP as well as in more accurate quantification. A prediction of the expected DP when different numbers of replicate composite samples (n) are analysed is obtained by using DP_{mean} from Fig. 4.2: $DP_{\text{expected}} = (1 - (DP_{\text{mean}})^n) * 100\%$. Table 4.4 shows the number of composite samples that should be collected from a microplot in order to get a desired CV for common and widespread taxa with well-detectable densities (substantially above the qPCR detection limit). For this reason, four of the 'common' and 'widespread' taxa in grey font in Fig. 4.2, *Anatochus*, *Achromadora*, *Alaimidae*, and *Panagrolaimus* are not represented in Table 4.4. In this table, nematode taxa are arranged by predicted quantification accuracies, and this arrangement mirrors the results presented in Fig. 4.3.

Discussion

Contrary to our expectations, increasing the number of cores per composite sample did not affect the detection probability nor the variability of nematode densities, pointing at relatively low degrees of microscale patchiness. Comparison of the distribution of nematode taxa across our study sites show that being rare, common or widespread, was strongly related to feeding type. Most plant-feeding and entomopathogenic nematodes reside in the rare category, reflecting their dependence on the availability of specific hosts and/or preferences for specific site characteristics. Most bacteria feeders and two out four fungivores reside in the widespread category, and seem to be either less picky or consume a very commonly available food source. Predators and omnivores were mainly found in the common category. Detection probabilities were related to feeding behaviour as well: common and widespread bacterivorous and fungivorous taxa had $DP \geq 90\%$, confirming the low levels of microscale patchiness. Predators and most omnivores however, showed degrees of local clustering based on their lower DPs (between 40% and 70%). Variation in densities however were highly taxon dependent and not related to trophic group, cp-group or phylogenetic position. For common and widespread nematode taxa of this study, we made predictions about the number of composite samples that should be collected from microplots to get desired quantification accuracies.

Increase of the number of cores per composite sample doesn't affect the detection probability nor the variability of nematode densities

Except for the Dorylaimida PP1, an increase of the number of cores per composite sample above three does not result in a higher DP nor in less variability of abundances among replicated samples (IQR). Studies on microscale patchiness of nematodes are rare, and vary in scope. In some studies, the microscale distribution of nematodes in general is investigated (Klironomos *et al.* 1999), others concentrate on major trophic groups (Viketoft 2013), whereas a third category focuses on individual genera or species (e.g. Been & Schomaker 2006; Rossi *et al.* 1996). In a semi-natural grassland in south-central Sweden, Viketoft (2013) observed small and similar ranges for four major trophic groups, *viz.*, plant, fungal and bacterial feeding nematodes and omnivores / predators) ($\approx 1m$). In this study, density data from 7 (fungivores) up to 27 genera (bacterivores) were lumped and subsequently analyzed. Reports on microscale distributions of individual genera or species almost exclusively focus on oligophagous plant-parasites such as *Globodera pallida*, *Heterodera* spp., and four tropical plant parasites in agro-ecosystems (Been & Schomaker 2006; Rossi *et al.* 1996; Fenwick 1961). For these plant parasites with narrow host ranges, the observed microscale patchiness was mainly attributed to host plant distribution,

root architecture and developmental stage of host plant (Rossi *et al.* 1996). Seinhorst (1988) detected low degrees of patchiness for two polyphagous plant parasitic species; the beet cyst nematode *Heterodera schachtii* (host range includes over 200 plant species including 80% of the Chenopodiaceae and Brassicaceae, Steele 1965) and the stem nematode *Ditylenchus dipsaci* (appr. 500 host plant species; Janssen 1994). Although the number of well-documented examples is limited, polyphagy among plant-parasitic nematodes seems to be associated with low levels of microscale patchiness. To the best of our knowledge, no well-documented examples have been published on the microscale-distribution of free-living nematode at genus or species level in bulk soil. Detailed information about the distribution of the bacterial and fungal communities at this spatial scale might be indicative for the distribution of bacterivorous and fungivorous nematodes. Members of these trophic groups show food preferences (e.g. Shtonda & Avery 2006, Moens *et al.* 1999, Quist *et al.* 2014), but too little is known about the nature of these preferences under field conditions to make any statement about the anticipated distribution of individual nematode taxa. Hence, the observed low degree of patchiness of polyphagous plant parasites matches with previous findings. With regard to the microscale distribution of free-living nematodes, the scarcity of information hampers a more detailed reflection on our finding.

Technical and biological factors affecting the accuracy of nematode density assessments

Ideally, we would have analyzed total DNA extracts of the 100 g soil subsamples directly. However, there is currently no routine protocol available that allows the handling of such large quantities of soil. The Oostenbrink elutriator – cotton wool filter method for the extraction of nematodes from the soil matrix is relatively efficient (Nijs & Van den Berg 2013), but variations have been reported with regard to the yield for individual nematode genera. This might for example have resulted in an under-estimation of the Plectidae and the Alaimidae concentrations (Verschoor & de Goede 2000).

A biological factor that introduced some bias in our results are soil type-dependent density differences. This can be illustrated by the widespread and common bacterivorous family Cephalobidae. In marine clay soils, densities of 40-100 individuals per 100 g soil were found, whereas typically 300-750 individuals were observed in sandy sites. The reverse was observed for the root-hair feeder *Basiria*. This genus was systematically present in higher densities in marine and river clay as compared to sandy soils (see Table S2). In both cases no significant effect of soil type on the microscale distribution was observed (and for further analysis results were lumped), but we might have found effects of numbers of cores on taxon detectability and quantification if more replicate samples were studied per soil type.

Table 4.4. Taxon-specific estimation of the number of times a composite soil sample should be collected from a microplot in order to get a given CV%, as well as the estimated CVs for a given number of composite samples collected from a microplot. Mean coefficients of variation (CV) were calculated per taxon, aiming to estimate sampling accuracies when different numbers of replicates are analyzed (see Material and Methods for details about statistical analysis).

Taxon	Estimated number of replicates per microplot to reach a given CV (%)				Estimated CV with <i>n</i> replicates				
	100%	50%	25%	10%	1	2	3	5	10
Dorylaimida PP1	1	1	1	3	16%	11%	9%	7%	5%
Dorylaimida D9A	1	1	1	3	17%	12%	10%	7%	5%
<i>Tylenchorhynchus</i>	1	1	1	6	24%	17%	13%	10%	7%
<i>Mylonchulus</i>	1	1	2	7	26%	18%	15%	11%	8%
Cephalobidae	1	1	2	7	26%	18%	15%	11%	8%
Total nematode densities	1	1	2	8	27%	19%	15%	12%	8%
<i>Basiria</i>	1	1	2	11	33%	23%	19%	14%	10%
Plectidae (except <i>Anaplectus</i>)	1	1	2	11	33%	23%	19%	15%	10%
<i>Anaplectus</i>	1	1	2	12	36%	25%	20%	16%	11%
<i>Diphtherophora</i>	1	1	2	12	36%	25%	20%	16%	11%
Mononchida M3	1	1	3	13	36%	25%	20%	16%	11%
Monhysteridae	1	1	3	13	37%	26%	21%	16%	11%
Aphelenchidae	1	1	3	14	38%	27%	22%	17%	12%
<i>Mesorhabditis</i>	1	1	3	16	41%	28%	23%	18%	12%
<i>Filenchus</i> group 3	1	1	3	18	44%	30%	25%	19%	13%
Aphelenchoididae	1	1	4	22	49%	34%	27%	21%	15%
Dorylaimida D3	1	2	6	32	61%	41%	33%	26%	18%
<i>Pristionchus</i>	1	3	8	48	78%	52%	42%	32%	22%

* See Holterman *et al.* 2008

** See Helder *et al.* manuscript in preparation

*** Except for the genus *Anaplectus*

A third factor that could have affected our results is the increase of the noise to signal ratio for quantitative PCR data from nematode taxa present at very low densities. Low numbers of target individuals (typically 1-10) per 100 g soil result in high C_t values with a relatively high technical noise (Karlen *et al.* 2007). It is noted that taxa present in low densities can be present in almost all samples ('widespread'). Nematode taxa that generally occur in low densities are presented in grey font in Fig. 4.2, and these results should be appreciated accordingly.

Explanations for the contrasting DP between trophic groups of nematodes

To explain the differences in DP of predatory, omnivorous and insect associated nematode taxa on the one hand, and the higher mean DP of bacterivorous, fungivorous and putative root-hair feeders on the other hand, we will concentrate on taxa in the categories 'common' and 'widespread' (taxa in black font in Fig. 4.2). For these taxa, we assume that the mean DP are mainly related to spatial variability at microscale: higher DP would correspond to more evenly distributed taxa, while lower DP would point at a degree of patchiness for a taxon. Four out of the twelve 'common' and 'widespread' taxa with a relatively low DP were predators (below 60%). This set of predators resides at the top of the food chain of the soil food web, and, as a consequence, they are present at low densities. Hence, these predators could – by matter of chance – be absent in some of the composite samples. If this were correct, it would have resulted in lower DP.

Six out of the twelve taxa in the demarcated box in Fig. 4.2 belong to the stress-sensitive nematode orders Dorylaimida and Mononchida (Holterman *et al.* 2008). Representatives of these groups have a relatively thin and permeable cuticle, and as such they are more affected by local chemical and/or physical stressors than most other categories of terrestrial nematodes. This is further illustrated by the Maturity Index, an ecological index that categorizes terrestrial nematode families into five so-called *c-p* groups (*c*, colonizer; *p*, persister) (Bongers 1990). All Dorylaimida and Mononchida taxa considered here belong to the *c-p* groups 4 and 5; the most stress sensitive categories. Hence, chemical and/or physical micro-heterogeneity might explain the observed patchiness as well.

The only two nematode taxa that predominantly live in phoretic association with insects, *Pristionchus* and Panagrolaimidae, are situated in the demarcated box in Fig. 4.2. *Pristionchus* varied enormously between sampling sites: at some sites *Pristionchus* was present in high densities in each of the five replicates, whereas on other sites this genus was present in low densities and was detected in a fraction of the replicates only. Recently, *Pristionchus pacificus* populations were shown to harbour bacterivorous as well as predatory individuals (Serobyán *et al.* 2014). The at least occasional predatory life style of *Pristionchus* (indicated in Fig. 4.2 by a partially red and blue sphere) fits in the overrepresentation of predatory nematodes in this specific category. Another noteworthy explanation for the observed microscale patchiness relates to the remarkable behaviour of *Pristionchus* that results in the formation 'Dauer towers', a strategy to efficiently reach beetle hosts. These towers consist of up to a thousand individuals (Penkov *et al.* 2014), and would result in extreme micro-patchiness. *Pristionchus* has a necromenic association with scarab beetles. Numerous members of the family Panagrolaimidae live in association with

bark beetles (Massey 1974). Results on Panagrolaimidae, however, should be viewed with caution as densities of representatives of this family were close to the detection limit. It could be envisaged that close association with insects results in some degree of patchiness at microplot scale for both taxa.

Distribution of plant-parasites

Mainly because of their dependence of a specific host plant, most herbivorous taxa were detected in only a few fields. However, the polyphagous root and root hair feeders *Tylenchorhynchus*, *Basiria* and *Filenchus* group 3 were common or widespread. *Tylenchorhynchus* constitutes a polyphagous genus feeding on cytoplasm of root-hairs and epidermal cells (Brinkman *et al.* 2008). Tylenchidae, such as *Basiria* and *Filenchus*, are usually labelled as epidermal cell and root-hair feeders that might use lower plants or algae as an alternative food source. The fact that *Filenchus* group 3 and *Basiria* are commonly present in combination with high DP (~ 90%) was shown to be different from other Tylenchidae (such as *Aglenchus*, *Coslenchus* and *Filenchus* groups 1 and 2), and similar to common and abundant bacterivores and fungivores. This observation suggests that *Filenchus* group 3 and *Basiria* feed on omnipresent resources such as fungi and/or algae.

Distribution of bacterivores and fungivores

Individual bacterivorous and fungivorous taxa showed considerable variation in terms of commonness and DP. Taxa showing relatively high DP, such as the bacterivorous Cephalobidae, Monhysteridae, Plectidae (except *Anaplectus*), *Mesorhabditis*, Metateratocephalidae and *Cruznema*, and fungivorous Aphelenchidae, Aphelenchoididae and Tyloilaimophorus probably feed on ubiquitous food resources, whereas other microbivores *Anaplectus*, *Pristionchus*, *Cylindrolaimus* and *Diphtherophora* might be more selective with regard to their food preferences.

Differences in quantification accuracies of 'common' and 'widespread' nematode taxa and their use as bio-indicators

Nematodes at taxon as well as trophic group level showed relative scale factors (RSFs) ranging from 1.2 (Dorylaimida D9A, a predator) to 2.4 (the fungivorous family Aphelenchoididae). Considering common and widespread taxa only, we observe a positive correlation between RSF and the variation in nematode densities. Further, a narrow 95% CI points at relatively small differences in variability of densities between sampling sites, and/or larger sample size, and *vice versa* for wide 95% CI.

Mesorhabditis, Aphelenchoididae and *Basiria* showed relative high RSF, suggesting that their densities – as compared to other taxa – show a degree of patchiness, which might be related to aggregation near certain food sources that are not ubiquitous at microscale. The lowest RSF was found for Dorylaimida groups D9A and PP1 (see Holterman *et al.* 2008), and *Tylenchorhynchus*. This suggests very low spatial variability for these taxa. However, densities of these taxa tended to be low in our microplots, in which these taxa were usually detected in only one or two of five replicates, thereby pushing the RSF towards 1. For the remaining common and widespread taxa, the RSF and 95% CI show their degree of microscale patchiness and (dis)similarity of this patchiness between fields, respectively. *Pristionchus* and *Diphtherophora* had the widest 95% CI, indicating relative large differences in variability between sampling sites, and apparently *Pristionchus* and *Diphtherophora* are sensitive to site-specific characteristics. Recently, Ito and coworkers (2015) demonstrated that *Pristionchus* was one of the most responsive taxon to soil management practices under humid subtropical conditions in Japan. Effects of soil management on *Diphtherophora* have been studied in a multivariate meta-analysis of datasets collected across the world, *Diphtherophora* appeared to be one of the genera that was consistently reduced by cultivation (Zhao & Neher 2013). However, we did not observe a significant effect of ‘system’ (arable fields *versus* semi-natural grasslands) for these two genera (Table S3).

Cephalobidae, probably the most abundant family in terrestrial habitats in temperate climate zones, showed a remarkably narrow 95% CI. This family of bacterivores harbors highly common genera, such as *Eucephalobus*, *Acrobeloides*, *Cervidellus* and *Cephalobus*, which, when considered individually, might show more variability.

Conclusions and outlook

Studies on microscale distribution of non-plant parasitic nematode taxa are scarce and it is largely unknown how nematode distribution patterns are related to nematode traits such as feeding type, cp-value and phylogenetic position. For all nematode taxa included in this study, detection accuracies were not affected by an increase of the number of cores per composite sample above three. Notwithstanding this observation, a certain level of spatial variability within and between trophic groups was demonstrated upon analysis of detection probabilities and variability of abundances. The detection probability of several predatory and omnivorous taxa deviated from 100%, and points at a degree of local clustering. The variation of detection probabilities and variability of abundances expressed by individual taxa can be explained by field effects, whereas no significant effect of soil type and land-use system was found. It is noted that this conclusion will probably not hold for oligotrophic plant parasites, a category of nematodes that was not included in this study.

Hence, to get insight in the presence or absence of a given nematode group in a given area, a relatively simple soil sampling strategy can be used for taxa within nematode communities at microplot level provided it is homogeneous in terms of land use and/or vegetation type. Accurate quantitative analysis may require the analysis of multiple composite samples, and the number was shown to be taxon-dependent, and – evidently – dependent on the level of accuracy required. Recently, we demonstrated distinct food preferences for individual lineages within a trophic group (Quist *et al.* 2014), and more detailed insights in trophic relationship between nematodes and other organismal groups in the soil food web will contribute to a better ecological understanding of shifts in nematode communities in bulk soil and the rhizosphere.

In this research we used a quantitative, real-time PCR-based approach as we aimed to generate both qualitative and quantitative information about the spatial distribution of terrestrial nematode taxa. DNA metabarcoding approaches have been developed to assess the biodiversity of soil Metazoa (*e.g.* Capra *et al.* 2016), but it should be noted that high throughput sequencing approaches are not yet suitable for quantitative analyses of nematode assemblages.

Recently, nematodes received the highest score among a selection of 30 potential indicators (Griffiths *et al.* 2016). The detailed insights in the spatial distribution of nematodes at microplot scale presented here, teach us about the impact of trophic preferences on the spatial distribution of individual nematode taxa, and also will allow for the design of statistically sound soil sampling strategies. This will bring the robust mapping of effects of anthropogenic and non-anthropogenic physical, chemical or biological disturbances on soil life within reach.

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Supporting information

Additional supporting information can be found online:

<https://sites.google.com/site/phdthesiscasperquist>

5 CHAPTER

**Whereas soil type and management drive
belowground composition of nematode communities
among fields, within-field distributions are
predominantly determined by stochasticity**

Casper W. Quist

Gerrit Gort

Paul Mooijman

Dick J. Brus

Sven van den Elsen

Olga Kostenko

Mariette T.W. Vervoort

Wim H. van der Putten

Jaap Bakker

Johannes Helder

Manuscript in preparation

Abstract

Insight into spatial distribution patterns of soil organisms at a nested series of scale levels is required for the proper understanding of soil food web functioning, and will contribute to our comprehension of factors that drive soil biodiversity. In this study we examined belowground distribution patterns of 48 nematode taxa at mesoscale level in 12 visually homogeneous fields (each 100 x 100 m) on three soil types (marine clay, river clay and sand) and two land-use types (arable and natural grasslands) across The Netherlands. A sampling scheme was optimized for geostatistical analysis. Over 35,000 nematode-taxon specific qPCR assays allowed us to quantitatively analyse nematode taxa at family, genus or species level in over 1,200 soil samples. Multivariate analysis showed soil type and land use-related differences in the nematode community composition. Data from all nematode taxa revealed a wide range of degrees of spatial variabilities (parameters: range and spatial variance $\sigma^2_{\text{spatial}}$ estimated using Bayesian geostatistical analysis with INLA – integrated Nested Laplace Approximation). No general effects were found of soil characteristics or nematode traits such as cp-value, trophic group, or fresh weight on these spatial distribution parameters. The relatively high percentages of unexplained spatial variability, 92.5% of the variation of the range-parameter, and 74% for $\sigma^2_{\text{spatial}}$ point at a major role of stochasticity for variability of nematode densities within fields. This study provides empirical evidence for stochastic processes being the dominant factor in pattern formation among terrestrial nematodes at mesoscale level in visually homogeneous agricultural and semi natural fields. It is noted that these insights can be applied for the design soil sampling strategies for nematode communities at field level with predictable accuracies.

Key-words: Terrestrial nematodes, bio-indicator, quantitative PCR, spatial distribution patterns, niche theory, neutral theory, geo-statistics, INLA

Introduction

Soil life is characterized by myriad interactions between overwhelming numbers of organisms that together drive the flow of carbon and nutrients in terrestrial ecosystems (Bardgett & Van Der Putten 2014; Wall *et al.* 2015). Environmental DNA sequencing is providing us with new perspectives on the biodiversity of inconspicuous soil inhabitants (Orgiazzi *et al.* 2015). For a proper understanding of the ecological functioning of soils, it should be noted that up to 80% of the microbial cells, representing around 50% of the biodiversity, could be dormant awaiting ecologically better conditions (Lennon & Jones 2011). Despite recent progress in our understanding about the functioning of complex soil communities, it is still highly fragmented (Bardgett & Van Der Putten 2014).

Mainly due to its high and poorly understood biological complexity, bio-indicators are frequently used to assess the condition of soil ecosystems. Nematode communities have potential to function as a bio-indicator group as they are represented in all major trophic levels of the soil food-web (Holtkamp *et al.* 2008). Moreover, nematodes are highly abundant in virtually any soil, can easily be separated from the soil matrix and show limited biogeography (most genera are found worldwide (Finlay 2002). Extraction from the soil matrix includes a step that requires active movement, and this excludes the co-extraction of dormant individuals. For some time, the use of nematodes as bio-indicator has been hampered by the limited number of informative morphological characters. More recently this practical obstacle has been relieved by quantitative PCR-based analysis methods that make use of taxon-specific DNA motifs (Floyd *et al.* 2002; Holterman *et al.* 2008; Vervoort *et al.* 2012b; Quist *et al.* 2016)

The bio-indicative value of nematode communities has been demonstrated in a range of studies. The composition of nematode communities was shown to be affected by the types of fertilizers, and by the application of herbicides and insecticides (Neher & Olson 1999). Specific shifts were shown to occur as a result of prolonged exposure to organic and conventional agricultural practices (Neher 1999a; Berkelmans *et al.* 2003; Ito *et al.* 2015; Quist *et al.* 2016). Evidently, plant species also have a distinct impact on nematode communities in the rhizosphere (Bezemer *et al.* 2010; Viketoft & Sohlenius 2011; Quist *et al.* 2014).

Most studies focus on the differences in distribution and abundances of nematodes between experimental plots with contrasting conditions. Typical surfaces for such plots are 10 -100 m² (Ito *et al.* 2015; Viketoft & Sohlenius 2011; Bezemer *et al.* 2010), and the collection of up to six soil cores per plot allows to reasonably assess the condition of the nematode community. For robust nematode community assessment at mesoscale level (scale dimensions: 1 – 1,000 m, see Chapter 4), more detailed information on belowground patterning *within* fields (typically 1 ha) is

required. Knowledge of spatial distribution patterns of nematodes with distinct trophic preferences will contribute to our understanding of factors that maintain and regulate soil biodiversity (Ettema & Wardle 2002). Insights into the spatial distribution of nematode taxa within homogeneous fields are also needed to design soil sampling strategies with predictable accuracies.

Distribution of soil inhabitants with same or similar trophic preferences could be shaped by stochastic processes, deterministic processes, or a combination of both. According to the niche theory, communities are shaped by deterministic processes in which abiotic and biotic factors determine the relative abundances of species. Neutral processes include probabilistic dispersal and random changes in the relative abundances of species (ecological drift) (Hubbell 2001; Chase 2014). In other words: when neutral processes play a dominant role, variation in communities cannot be predicted by variations in environmental factors. Evidence is accumulating that niche and neutral processes operate at different spatial scales, and jointly regulate ecological communities (Chase 2014).

Only a few studies have been published on the relative importance of stochastic and deterministic processes in the distribution of soil biota at mesoscale level. (Bahram *et al.* 2016) investigated the patchiness of small soil eukaryotes in temperate forest on 64 x 64 m plots with a homogeneous vegetation. It was shown that the shared effect of environmental selection and spatial processes explained less than 10% of variance, suggesting a major effect of stochastic processes for pattern formation (Bahram *et al.* 2016). (Moroenyane *et al.* 2016) investigated the distribution patterns of nematodes in a heathland ('Fynbos') at mesoscale. Based on the analysis of five composite samples per hectare square, the authors observed co-occurrence of certain phylogenetically related nematode taxa, and they concluded that this pointed at a dominant role of deterministic processes in shaping the nematode community structure.

At nematode feeding type level, patchiness was observed in a 48 ha corn field despite the homogenizing effects of yearly tillage and mono-cropping for decades. Edaphic factors collectively explained < 30% of the variability of bacterivorous, fungivorous and omnivorous/predatory nematode groups (Robertson & Freckman 1995). In a more detailed study, divergent spatiotemporal distributions of eight dominant bacterivores were found in a 0.7 ha recently restored riparian wetland. Six genera showed a degree of patchiness, whereas for two other bacterivores, no spatial dependence was observed. For the spatially structured bacterivores, no correlation with soil resource patterns could be determined (Ettema *et al.* 1998). However, when the patchiness of bacterivore *Chronogaster* was examined at species level in the same wetland, the results pointed at an important role of largely unpredictable, local variations in humidity on the mesoscale distribution of individual bacterivorous nematode species (Ettema *et al.* 2000).

In this study we examined belowground distribution patterns of 48 nematode taxa in 12 visually homogeneous fields (each 100 x 100 m) on three different soil types (marine clay, river clay and sand) and two land-use types (arable and natural grasslands). Within each field, soil communities were exposed to comparable abiotic conditions and uniform land use for at least 10 years. Over 12,00 composite soil samples were collected with a sampling design optimized for geostatistical modelling. Over 35,000 nematode taxon-specific qPCR assays were run to analyse the presence and abundances of the dominant nematode taxa in each field. Within-field variation of nematode taxa was studied in detail by geostatistical modelling. A Bayesian geostatistical analysis using SPDE (Stochastic partial differential equations) and INLA (Integrated nested Laplace approximations) was applied, a recently developed approach that is flexible regarding the handling of variable numbers of zeros in the primary data set, and required limited data processing time.

With the combined use of a sampling scheme optimized for geostatistical analysis, and a quantitative high throughput system for the characterization of nematode communities, we were able to provide a detailed overview of the densities and distribution patterns of a wide range of nematode taxa from different trophic groups, in different soil types and systems. We hypothesize that nematode community compositions will differ between fields as a result of contrasting soil type and/or land use histories. Further we expect taxon- and functional group-dependent degrees of spatial variability. We will assess the relative importance of various factors that determine the belowground spatial patterns. The relative importance of soil type and land use as factors determining nematode community composition and spatial distribution will be indicative for the role of deterministic processes as regulators of the spatial distribution of nematodes at mesoscale. Within-field patterning will be used to assess the contribution of stochasticity in soil biotic pattern formation.

Materials and Methods

Experimental design

In total 1,476 composite soil samples were collected during the winter-period between December 2012 and April 2013. Across The Netherlands, 12 areas were sampled: nine arable farmlands and three natural grasslands on three soil types; marine clay, river clay and sand (Table 5.1 and Fig S5.1).

All selected areas had been under one single management regime for at least 15 years. Taking into consideration prior knowledge about land-use history, disturbances, productivity and soil texture, a visually homogeneous surface (1 ha, 100 x 100m)

was demarcated within each area. These selected 1 ha squares are referred to as “field” in this study. The sampling design was optimized for geo-statistical analysis. The 96 microplots (a microplot is a circular surface of 0.25m^2) were positioned in a pattern that guaranteed a continuous range of distances between the microplots (ranging from 0.5 to 100 m). To facilitate soil sampling, each field was divided into 16 sub-plots (each $25 \times 25\text{ m}$). In or near the centre of each sub-plot (see details in Fig 5.1.), six microplots were placed along a transect. The direction of these transects was regularly alternated (see Fig. 5.1). To position the six microplots per transect, a Golomb-ruler was used and adapted to our needs (distances between microplots ranging from 0.5 m and 13.5 m). In six out of 12 fields, an additional 20 microplots were sampled in the centre (for details see Fig. 5.1 and Chapter 4). This resulted in a total of 1,272 microplots for nematode community analysis. Per microplot, a single composite sample was collected, *i.e.* a homogeneous mixture consisting of 12 soil cores ($\varnothing 1.5\text{ cm}$, depth: 20 cm).

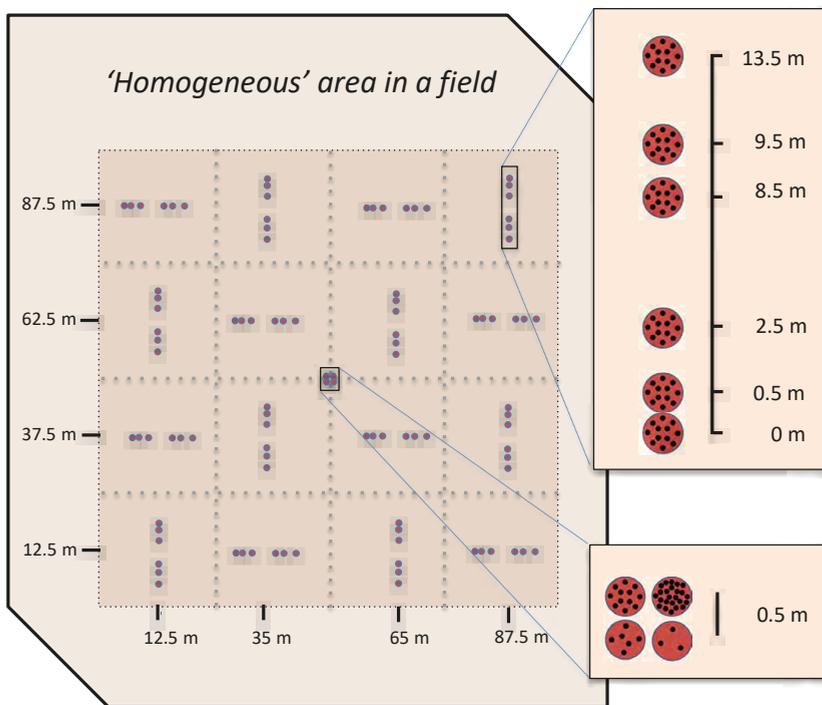


Figure 5.1. Sampling design optimized for geostatistical analysis. Twelve visually uniform hectares were selected within arable fields with crop-rotation ($n = 9$) and natural grasslands ($n = 3$) (see also Table 1 and Fig. S1). In or near the center of each subsurface ($25 \times 25\text{m}$; $n = 16$) a transect was positioned with six circular microplots of 0.25 m^2 . The position of these six microplots along the transect was determined by an adapted Golomb ruler to obtain a range of between-plot-distances from 0.5 to 13.5m without plot-pairs with the same distance apart. Composite samples were collected by mixing 12 soil cores ($\varnothing 1.5\text{ cm}$, depth: 20 cm). In six fields an additional 20 microplots were positioned in the center of the mesoplot.

Table 5.1. Major soil characteristics of the of fields under investigation. Soil samples were collected from arable fields (just before the onset of the growing season), and from semi-natural grasslands with high plant diversity (*). Soil types are indicated by 'm' (marine clay), 'r' (river clay), or 's' (sand) (See also Fig S1. For each of the locations, five major abiotic soil characteristics were determined: pH, total nitrogen, total phosphorus, organic matter % and clay content (% soil particles < 2 µm).

Field	GPS coordinates	pH (pH-CaCl ₂)	Nitrogen (mg N / kg dry soil)	Phosphorus (mg P ₂ O ₅ / kg dry soil)	Organic matter (%)	Clay (%)	Crops rotation scheme / dominant plant species
Schoondijke (Zeeland; m1)	51°19' N 3°31' E	7.5	980	1,540	2.6	17	Potato, onion, sugar beet, wheat
Draatbrug (Zeeland; m2)	51°18' N 3°27' E	7.5	1080	1,650	2.9	20	Potato, onion, sugar beet, wheat
Lelystad (Flevoland; m3)	52°32' N 5°33' E	7.2	1,410	1,410	2.8	17	Potato, onion, sugar beet, wheat
*Lauwersmeer (Friesland; m4)	53°20' N 6°09' E	7.2	2,360	1,440	5.0	17	<i>Holcus lanatus</i> , <i>Agrostis stolonifera</i> , <i>Ranunculus repens</i> , <i>Trifolium pratense</i>
Wageningen (Gelderland; r1)	51°57' N 5°38' E	6.5	1,780	2,260	5.6	32	Potato, barley, sugar beet, grass (2 consecutive years)
Cortenoever (Gelderland; r2)	52°06' N 6°12' E	7.0	1,230	1,460	2.6	7	Corn
Houten (Utrecht; r3)	52°02' N 5°09' E	6.7	1,760	1,910	3.5	25	Corn
*Millingerveerd (Gelderland; r4)	51°52' N 6°00' E	7.0	2,050	1,930	3.8	19	<i>Brassica nigra</i> , <i>Solidago gigantea</i> , <i>Calamagrostis epigejos</i> , <i>Eriogon annuus</i>
Wageningen (Gelderland; s1)	51°59' N 5°39' E	5.7	720	1,310	2.8	2	Potato, barley, sugar beet, wheat, corn, grass (2 consecutive years)
America (Limburg; s2)	51°25' N 5°58' E	5.4	1,090	1,200	3.6	2	Salsify, Potato, Iceberg lettuce, Chinese cabbage
Sint Kruis (Zeeland; s3)	51°16' N 3°30' E	5.3	1,490	1,440	3.8	2	Potato, <i>Lotium perenne</i> (2 consecutive years), barley, wheat, beans
*Mossel (Gelderland; s4)	52°03' N 5°45' E	5.6	950	1,200	3.2	2	<i>Agrostis capillaris</i> , <i>Jacobaea vulgaris</i> , <i>Achillea millefolium</i> , <i>Holcus lanatus</i> , <i>Plantago lanceolata</i>

For organic matter (OM) content measurement, one composite sample of 12 cores (\varnothing 1.5 cm, depth: 20 cm) was collected from each transect, resulting in 16 composite samples per field and 192 composite samples for the whole experiment. Abiotic soil characteristics (pH, total nitrogen, total phosphorous, and clay content) were determined by Blgg AgroXpertus (Wageningen), a NEN-EN-ISO 17025 certified service laboratory, using standardized procedures. For this, a single composite soil sample of 60 cores (\varnothing 1.5 cm, depth: 20 cm, using a standardized “W”-shaped sampling design) was collected from each of the fields. All composite soil samples were stored at 4°C immediately after sampling until further processed.

Nematode and DNA extraction, and qPCR-based analysis

Within two weeks after sampling, composite samples were mixed thoroughly and nematodes were extracted from a 100 g sub-sample using an elutriator (Oostenbrink 1960). Nematode suspensions were concentrated and DNA was extracted by a lysis buffer with an internal standard as described by (Vervoort *et al.* 2012a). Thereafter DNA extracts were purified using a glass fibre column-based procedure (Ivanova *et al.* 2006). To assess nematode diversity per field, a mixture was made of 3 μ l DNA extract of each sample per field, and analysed first. All purified DNA extracts were stored at -20°C awaiting further qPCR analyses.

The field-specific DNA mixture was used as template in qPCR using 59 nematode taxon-specific primer sets. Between 25 and 34 taxa were detected per field (Table S5.1), and depending of this nematode diversity assessment between 24 and 33 nematode taxon-specific primer sets were selected to study in each microplot of a given field. Further, primer sets were used to assess total nematode densities and measure the internal control. The internal control allows compensating for losses during sampling handling. Quantitative PCR reactions were executed and C_t values were converted to nematode densities by making use of the known linear relationships between C_t values and $^{10}\log$ (number of target nematodes). The maxima of the negative, first mathematic derivative of the melting curves were checked to confirm the correct nature of the amplicons (Vervoort *et al.* 2012a).

Data analysis

To examine whether system (arable or natural) and soil type (river clay, marine clay or sandy soil) had an effect on nematode community composition, we used multivariate principal component (PCA) and redundancy (RDA) analyses (in CANOCO version 5.03 (Smilauer & Leps 2014); Fig 5.2). The choice of linear methods was justified by the short length of gradients (less than 3.0). Significances in multivariate analyses were tested using a Monte Carlo permutation test with 999 restricted permutations.

The samples collected within one field were permuted at the field level to take into account that these samples are pseudo-replicates. The samples within fields were not permuted. For our analyses the additional 20 samples collected in the centre of six fields were not included. Three missing samples were replaced by the average values of the field to which they belonged. The nematode data were $^{10}\log$ -transformed prior to the multivariate analyses.

To quantify the spatial patterns of the abundance of each nematode taxon per field we applied a Bayesian geostatistical analysis method using SPDE (Stochastic partial differential equations) and INLA (Integrated nested Laplace approximations), as available in the R package R-INLA (Lindgren & Rue 2015). Because many nematode taxa showed relatively large number of absences at microplot level, we followed the method given by Krainski (2015), which describes a joint analysis of presence/absence scores as well as abundances. In the geostatistical analysis, the Matérn covariance function was used to quantify the spatial distribution (Minasny & McBratney 2005). In this way, we obtained for each nematode taxon within each field a set of estimated geostatistical parameters, which together describe the spatial variation. We focused on the level parameters mean density (given the presence of nematodes) and detection probability (DP), and the Matérn related variation parameters (1) range, (2) $\sigma^2_{\text{spatial}}$ and (3) σ^2_{nugget} . (1) The range parameter of this model expresses how quickly spatial correlations decay with distance. This parameter positively relates to patch size (Ettema and Wardle 2002). (2) The strength of the distance-decay relationship is reflected by $\sigma^2_{\text{spatial}}$. In other words $\sigma^2_{\text{spatial}}$ positively relates with the steepness of the patches. (3) The variance due to inherent measurement errors is given by the nugget (σ^2_{nugget}). Based on the geostatistical models, kriging maps were plotted to assess nematode densities in areas between the sampling points (Fig. 5.3). Distribution maps are shown from three dominant fungivorous taxa (Fig. 5.3A) and six bacterivorous taxa (Fig. 5.3B and C). To facilitate the comparison of distribution patterns between fields and nematode taxa from the same feeding type, estimated densities of a given nematode taxon/field combination were square root-transformed. A full color scale (from purple for low densities to red for high densities) was used in each of the fields separately.

We transformed the geostatistical parameters for further analysis, using the $^{10}\log$ transformation for all but the DP, which was arcsine square root transformed. The relationships between transformed geostatistical parameters and field characteristics were studied in two ways:

1) Per nematode species all possible subset regressions of transformed geostatistical parameters on field characteristics were performed using R package MuMIn (Barton 2016). Available field characteristics were land use, soil type, pH, N, P, organic matter and clay content. Notice that a maximum of only twelve observations (fields)

were available per parameter; we considered only those nematode species which occurred in at least nine fields. The best fitting models according to the corrected Akaike's Information Criterion (AICc) criterion were reported.

2) Overall analyses of transformed geostatistical parameters were performed, including observations from all nematode species, in which the relationships of each geostatistical parameter with both field characteristics and nematode characteristics were studied. In these analyses we applied mixed linear models for trait-environment relationships using a tiered forward model selection as described in Jamil *et al.* (2012). With this method repeated measurements per field (multiple nematode taxa) and per nematode taxon (multiple fields) were accommodated. The null model contains crossed random effects for fields (environments) and taxa. Next, it is determined whether the null model may be improved by inclusion of random field/trait trends by taxon, for fixed field trait trends and fixed nematode trait trends, and their interaction. The same set of the field traits as listed above was used. Nematode traits were trophic level, cp value, and three nematode fresh-weight related variables (weight, $^{10}\log$ -transformed weight, and an indicator for above-average weight). Based on the final mixed models pseudo- R^2 values for transformed geostatistical parameters were calculated, using and modifying methods introduced by Nakagawa & Schielzeth (2013) and Johnson (2014). In this way, we quantified 1) which percentage of the total variance could be explained by fixed and random effects (both nematode and field related), and 2) which percentage of the total variance could be explained by nematode-related fixed and random effects alone.

Results

Soil type and land use delineate nematode communities

In a comparison of nematode communities from 12 visually homogeneous fields (100 × 100m), the first two principal component axes explained about 37% of the variation in nematode community composition (Fig. 5.2). Nematode communities differed between natural and arable fields, but these differences were soil-type dependent (RDA: system type × soil type interaction: $F=135$, $P=0.02$; 36.9% adjusted explained variation). On sandy soils, nematode community composition of natural fields was clearly separated from those of arable fields in an unconstrained analysis (Fig. 2A). However, on soils with high clay content, independent of clay origin (marine or river), there was no difference in nematode community composition between natural and arable fields (Fig. 5.2A). The abundance of about 80% of all nematode taxa were higher in the fields on sandy soils as compared to the fields on marine or river clay (Fig. 5.2B). Among sandy soils, *Pratylenchus penetrans*, *Mesorhabditis* and

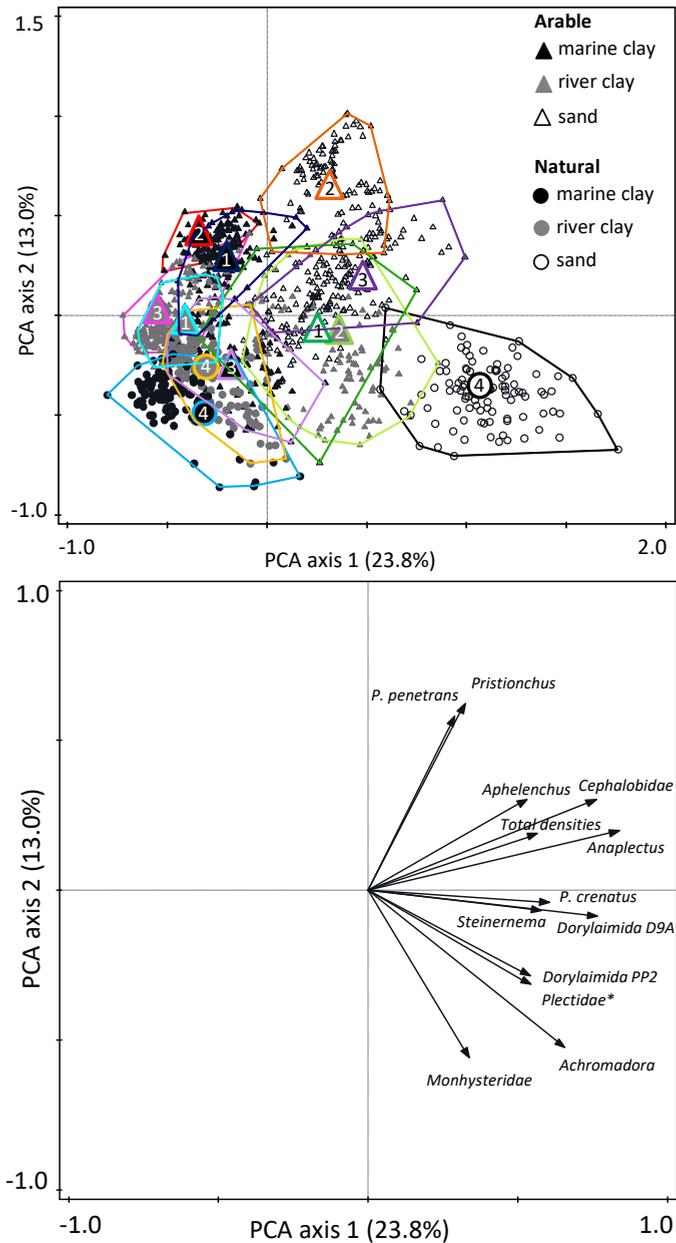


Figure 5.2. Ordination plots showing the first and second axis of a principal component analysis (PCA) of the nematode community composition. The percentage of variation explained by each of the axes is shown between brackets. A) Triangles depict arable fields and circles - natural fields. Black filled symbols depict marine clay soils, grey filled symbols - river clay soils and open symbols - sandy soils. Envelope is drawn around the samples collected from the same field. The color of the envelopes designates different fields (numbers indicate the field number which can be used to trace back specific information about the field in Table 1.). B) All nematode taxa with more than 30% fit are shown as arrows.

Pristionchus were more abundant in arable fields, whereas Monhysteridae, Plectidae, *Achromadora* and *Dorylaimida* PP2 were more abundant in natural fields (Fig. 5.2B). *Ditylenchus* and *Basiria* were present in higher densities on clay soil (data not shown).

Within field variation in community composition

Although fields were selected without visually observable environmental gradients, and elaborate composite samples were collected (12 soil cores per sample, see Chapter 4), PCA graphs demonstrate that the community composition within these fields shows substantial variation. This can be seen by comparing the surfaces of the envelopes that were drawn around the most peripheral data points from each field. The largest envelopes were found for the sandy fields, indicating highest intra-field variation for communities in sandy soils.

Within feeding groups, taxon-specific differences in densities and detection probabilities were observed (Table S5.2.1 and S5.2.2). Among fungivores, *Aphelenchus* and *Aphelenchoides* were more common and occurred in higher densities than members of the genera *Diphtherophora* and *Tyololaimophorus*. Among bacterial feeders, Cephalobidae, Monhysteridae and *Mesorhabditis* and Plectidae (except *Anaplectus*) were detected in all or nearly all samples, whereas other taxa such as *Anaplectus*, *Pristionchus*, *Prismatolaimus* and *Panagrolaimus* had lower detection probabilities. A similar trend was observed for most bacterivores and fungivores. Some herbivorous and predatory nematodes on the other hand were detected in lower numbers in sand than in clay (Table S5.2.1).

Belowground distribution patterns of dominant fungivorous and bacterivorous nematodes in different soil types and systems

To quantitatively describe nematode distributions within a field, three parameters from the Matérn model were determined, (1) the range, (2) $\sigma^2_{\text{spatial}}$ and (3) σ^2_{nugget} . Kriging maps were plotted to assess nematode densities in areas between the sampling points (Fig. 5.3). Distribution maps are shown from three dominant fungivorous taxa (Fig. 5.3A) and six bacterivorous taxa (Fig. 5.3B and C)

The degree of patchiness from nematode taxa varied between the fields and nematode taxa. The impact of soil type was apparently more prominent than the effect of land use. For most dominant fungivores and bacterivores, more similar patch sizes were observed within soil types than between soil types. For *Pristionchus* and Cephalobidae however, it was observed that within land use types the patch sizes are more similar than between land use (Fig 5.3.).

Gradually changing densities were observed for the fungivorous *Aphelenchus* in all arable fields, and also in the natural field on river clay (reflected by a long range and low $\sigma^2_{\text{spatial}}$). More spatial variability (reflected by a short range and a higher $\sigma^2_{\text{spatial}}$) was found for *Aphelenchus* in the grassland on marine clay and in the natural grassland on sandy soil (See also Table S5.2.3 and S5.2.4). As compared to *Aphelenchus*, the densities of *Aphelenchoides* were more variable (Table S5.2.1). Kriging maps of *Aphelenchoides* point at a lower levels of patchiness in marine clay, compared to river clay and sandy soils. *Diphtherophora* was found in lower densities as compared *Aphelenchus* and *Aphelenchoides*, and showed on average more spatial variability than *Aphelenchoides* (reflected by a higher $\sigma^2_{\text{spatial}}$).

An increasing level of patchiness among fungivores (*Aphelenchus* < *Aphelenchoides* < *Diphtherophora* (Fig. 5.3A and Table S5.2.3 and 5.2.4) was also observed at microscale level (0.25 – 1 m²; Chapter 4). For bacterivorous nematode families Cephalobidae, Monhysteridae and Plectidae (Fig. 5.3B) the kriging maps point at higher degrees of patchiness (reflected by shorter ranges) as compared to the genera *Mesorhabditis*, *Pristionchus* and *Anaplectus* (Fig. 5.3C). Similar to the fungivores, within bacterivorous taxa, substantial variation of patterning is found between fields, but no consistent significant differences were observed between soil types or systems.

Evaluation of factors underlying nematode patterning within the fields

The degree of patchiness of nematodes within visually homogeneous fields might be determined by nematode traits and site characteristics. At microscale level, dominant bacterivores and fungivores were shown to be more evenly distributed than dominant omnivores and predatory nematodes (Chapter 4).

All possible subset-regressions were carried out to evaluate effect of site characteristics (soil type, system, pH, total nitrogen, total phosphate, organic matter content and clay content; see Table 5.1) on the mean densities, detection probability and Matérn related parameters (range, $\sigma^2_{\text{spatial}}$ and σ^2_{nugget}) of dominant nematode taxa in the fields of this study (See Table 5.2). We expected that an increase in clay content would result in a reduced horizontal migration capacity. As a consequence a higher level of spatial aggregation was expected in clay compared to sand, the ranges would be shorter, and the $\sigma^2_{\text{spatial}}$ higher.

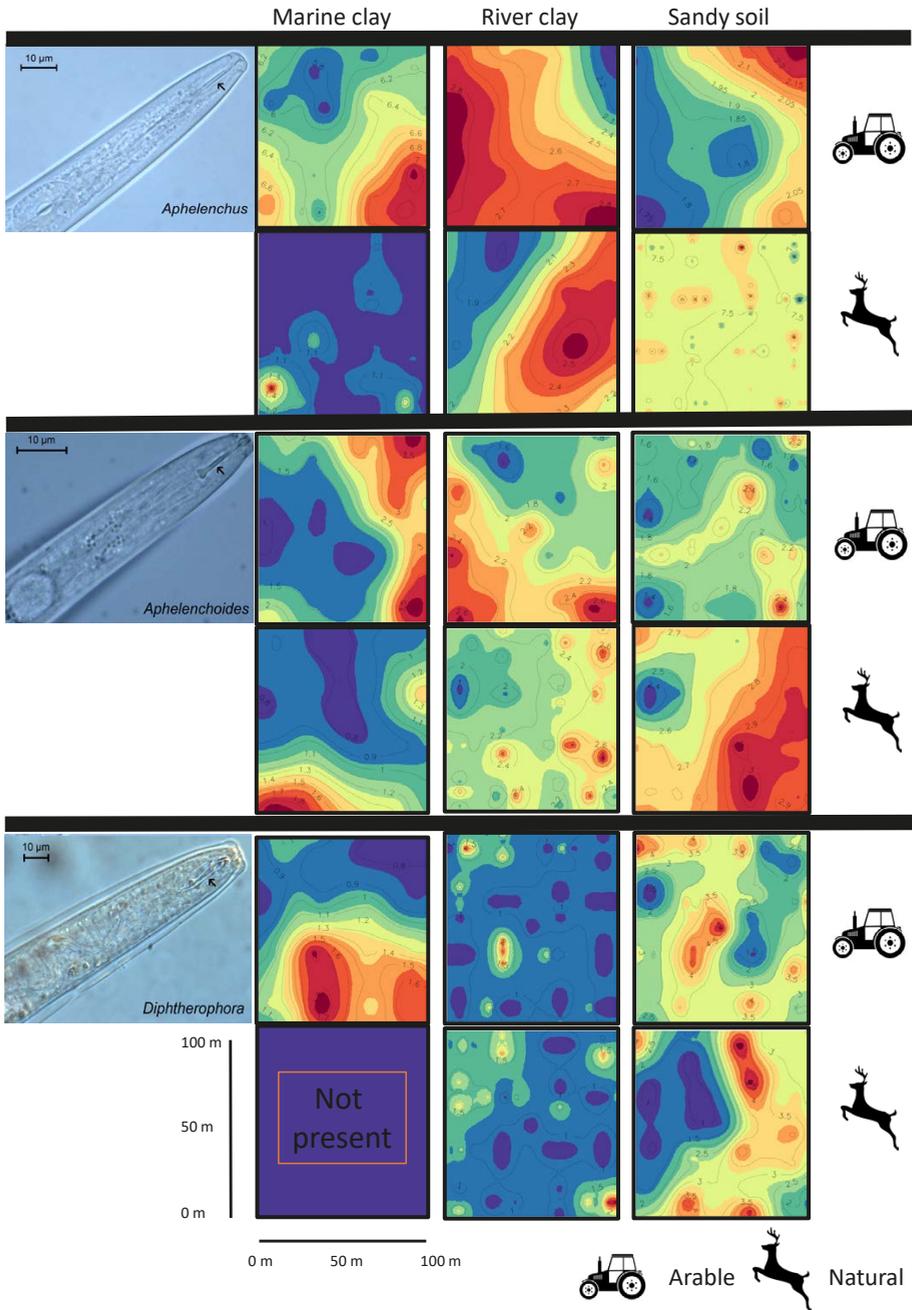


Figure 5.3A. Kriging maps of nematode densities of *Aphelenchus*, *Aphelenchoides* and *Diphtherophora*. To facilitate the comparison of distribution patterns between fields and nematode taxa from the same feeding type, estimated densities of a given nematode taxon/field combination were square root-transformed. A full color scale (from purple for low densities to red for high densities) was used in each of the fields separately.

In our analysis, no relation was found between ranges of spatial aggregation and clay content. A positive association was found between pH and total nematode densities as well as *Panagrolaimus* densities. This might be seen as an indirect effect of clay content, as pH positively correlates with clay content. Ranges of *Aphelenchus* and *Diphtherophora* were shorter at higher organic matter levels (Table 5.2). Analysis of the effect of abiotic soil conditions on $\sigma^2_{\text{spatial}}$ showed that total nematode densities, bacterivorous Cephalobidae and fungivorous *Aphelenchus* had higher $\sigma^2_{\text{spatial}}$ in more clayey soils. Plectidae and *Aphelenchus* had higher $\sigma^2_{\text{spatial}}$ at higher OM levels.

Effects of soil characteristics on σ^2_{nugget} were found as well. pH was negatively correlated to σ^2_{nugget} of *Aphelenchus* and Monhysteridae. A positive association was found between total P and the nugget of *Aphelenchus*, whereas this parameter was negatively associated with total nematode densities and Monhysteridae. The nugget of Cephalobidae and Monhysteridae was higher in clay than in sand. Mixed effects were observed between σ^2_{nugget} and the OM level.

In the mixed model analyses to detect species-environment relationships, relating geostatistical parameters to field and taxon characteristics, we found limited proof of systematic relationships beyond simple random effects for fields and taxa, and never systematic field - taxon interactions. The mean density showed taxon-specific lutum and nitrogen trends, which were negative overall (higher lutum or nitrogen resulted overall in lower density). No taxon traits showed associations w.r.t. mean density. DP also showed taxon-specific lutum effects, and overall negative trends for lutum, but also for cp (higher cp values showed lower detection probabilities). For the range and $\sigma^2_{\text{spatial}}$ variables no model improvements beyond the null model with simple random effects for fields and taxa were found. For σ^2_{nugget} a slight model improvement due to taxon-specific organic matter trends was found.

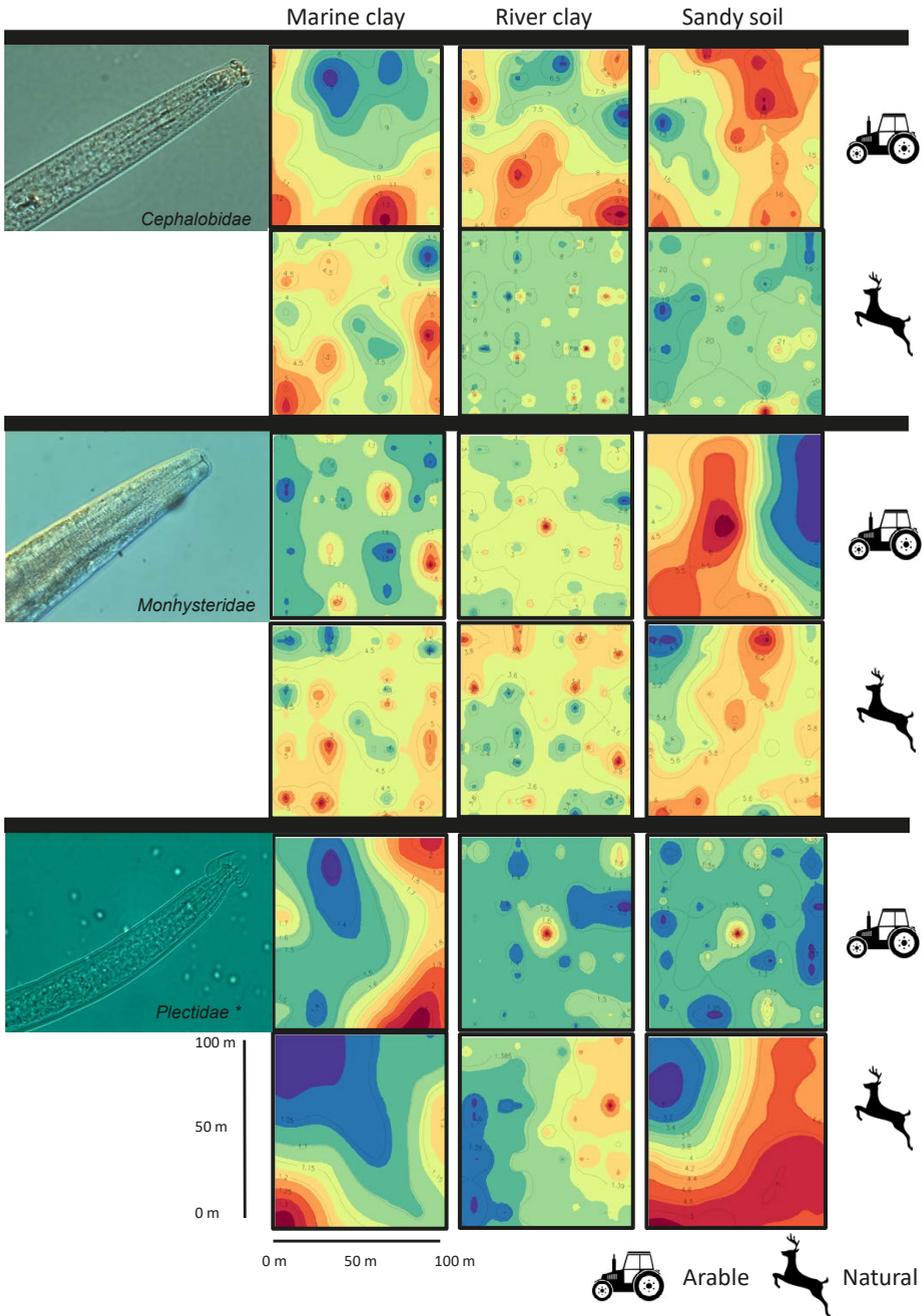


Figure 5.3B. Kriging maps of nematode densities of bacterivorous families *Cephalobidae*, *Monhysteridae* and *Plectidae** (except *Anaplectus*). To facilitate the comparison of distribution patterns between fields and nematode taxa from the same feeding type, estimated densities of a given nematode taxon/field combination were square root-transformed. A full color scale (from purple for low densities to red for high densities) was used in each of the fields separately.

Table 5.2. Outcome of all possible subset regressions of the effect of site characteristics on the range, the spatial structured variance and the nugget (microscale variation and/or measurement error) of the most dominant taxa of this study (present in at least nine fields). Reported are regression coefficients of standardized variables for the best models according to the AICc criterion. For factor soil type (3 levels) “+” is reported.

Nematode taxon	n	log(Range)								log(Sigma ²)								log(Nugget)								
		ic	system	soiltype	pH	Nt	Pt	OM	Clay	ic	system	soiltype	pH	Nt	Pt	OM	Clay	ic	system	soiltype	pH	Nt	Pt	OM	Clay	
Total nematode densities	12	1.6			0.4																					
<i>Anaplectus</i>	10	1.4																								
Cephalobidae	12	1.7																								
Dorylaimida PP1*	9	1.5																								
<i>Mesorhabditis</i>	12	1.5																								
Monhysteridae	12	1.5																								
<i>Panagrolaimus</i>	9	1.2																								
Plectidae (except <i>Anaplectus</i>)	12	1.3																								
<i>Pristionchus</i>	10	1.7																								
<i>Aphelenchoides</i>	12	1.5																								
<i>Aphelenchus</i>	12	1.6																								
<i>Diphtherophora</i>	9	1.4																								
<i>Filenchus</i> group 3	12	1.5																								
Dorylaimida D3*	12	1.4																								
Mononchida M3*	11	1.4																								
<i>Mylonchulus</i>	9	1.4																								

Quantification of deterministic or stochastic drivers of pattern formation

Pseudo-R²s were calculated to quantify the percentage of explained variance from the total variance of mean nematode densities, detection probabilities as well as of the spatial distribution parameters from the Matérn models (range, $\sigma^2_{\text{spatial}}$, and the σ^2_{nugget}). Pseudo-R²s were calculated for nematode related factors (weight, cp-value, trophic group) alone and or nematode and field related characteristics (soil type, system, and soil abiotic conditions) together (Table 5.3). Pseudo-R²-analysis pointed out that differences in mean nematode densities and detection probabilities can largely be explained by nematode traits and field characteristics (R² = 74.7% and 57.5%, respectively). The unexplained, random component was dominating in case of the three major parameters describing the nematode distribution patterns at the mesoscale level, the range (R² = 7.5%), $\sigma^2_{\text{spatial}}$ (R² = 24.2%), and the σ^2_{nugget} (R² = 35.5%). In other words, whereas variation in mean densities and DP can be largely attributed to nematode traits and field characteristics, distribution patterns were only to a limited degree explained by deterministic factors. For mean densities and DP, variation between fields was respectively much smaller and slightly smaller than between taxa. For the range, variability between fields was much higher than between taxa. For $\sigma^2_{\text{spatial}}$ and σ^2_{nugget} variabilities between fields and taxa were roughly equal.

Table 5.3. Pseudo R² values showing the % of variance of transformed geostatistical parameters explained by both field and nematode-related factors, and nematode related factors alone.

Variance explained by	Mean	DP	Range	$\sigma^2_{\text{spatial}}$	σ^2_{nugget}
Nematode and field fixed and random effects	71.8	57.5	7.5	24.1	35.5
Nematode related fixed and random effects	69.8	48.3	0.7	13.9	23.4

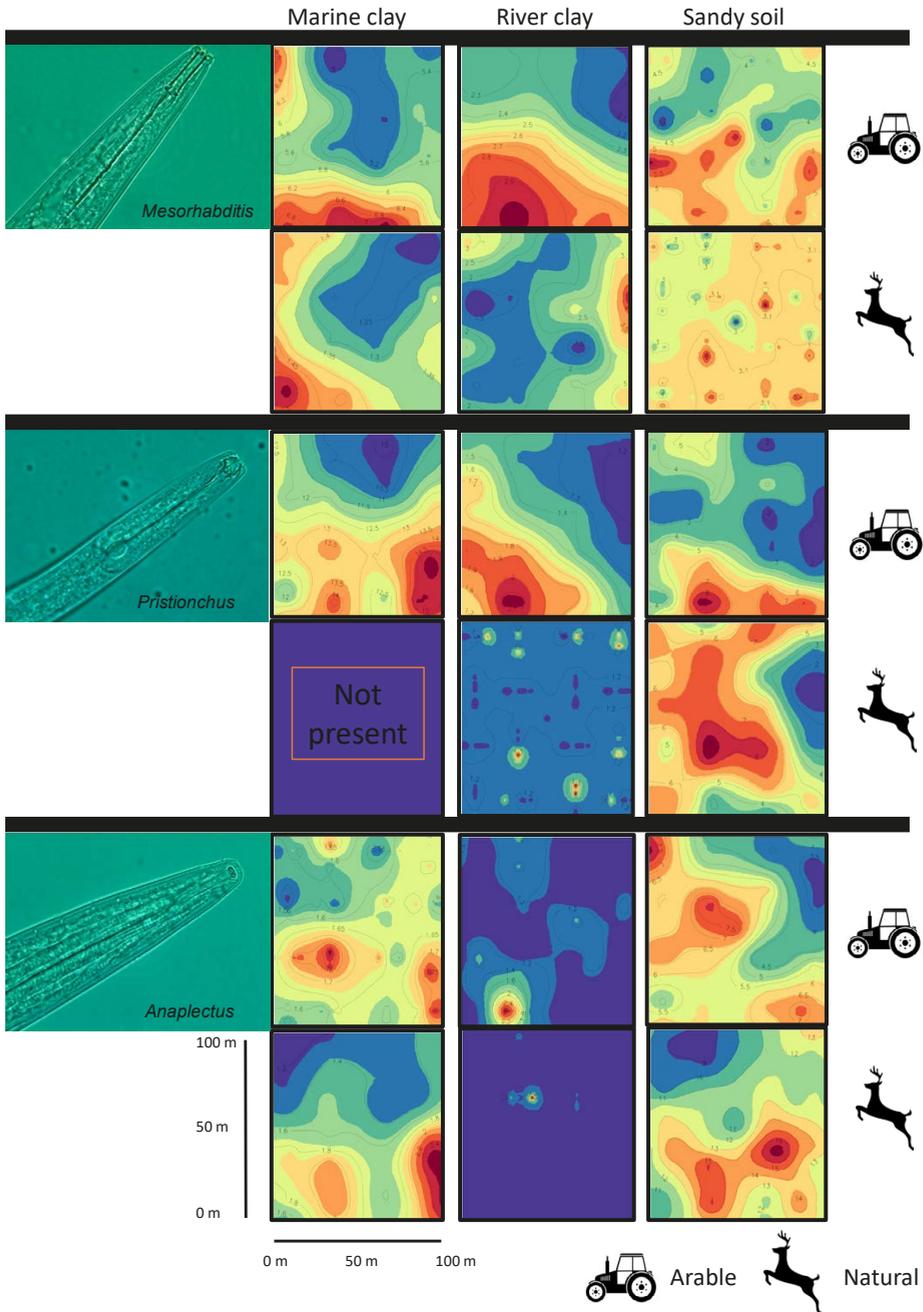


Figure 5.3C. Kriging maps of nematode densities of bacterivorous genera *Mesorhabditis*, *Pristionchus* and *Anaplectus*. To facilitate the comparison of distribution patterns between fields and nematode taxa from the same feeding type, estimated densities of a given nematode taxon/field combination were square root-transformed. A full color scale (from purple for low densities to red for high densities) was used in each of the fields separately.

Discussion

Our knowledge about the qualitative shifts in belowground communities caused by land-management and plant /crop species is rapidly increasing. As a logical, yet barely explored, next step we gained insight in the spatial variability of terrestrial nematodes at field / mesoscale level. Our results revealed the belowground distribution patterns of a wide range of plant-parasitic and free-living nematode taxa within visually homogeneous fields (1 ha each). This study showed that distribution patterns of terrestrial nematodes in areas without noticeable gradients are driven by neutral / stochastic processes, whereas soil type and management drive belowground composition of nematode communities among fields. A sampling scheme optimized for geostatistical analysis was designed, and this was combined with the power offered by quantitative molecular detection techniques. The combination allowed us to pinpoint the spatial patterns of 48 nematode taxa at mesoscale level by collection and qPCR-based analyses of over 1,200 composite soil samples from twelve locations including contrasts in soil type and land use.

Multivariate analysis confirmed important roles of soil type and land management with regard to the composition of nematode communities at mesoscale level. These results are in agreement with other studies with similar plot sizes (Neher 1999; Berkelmans *et al.* 2003; Quist *et al.* 2016). The soil type and management related differences in the nematode communities of this study and the densities of specific taxa underline the effects of environmental filtering and niche partitioning of nematodes (Ettema 1998). The impact of soil texture on the nematode community compositions have been documented before (Bongers 1994). In our fields, higher clay contents were associated with increased water holding capacities, and also pH, soil nutrients (N and P) and organic matter content were positively correlated with a fine soil texture (Quist *et al.* unpublished data). These abiotic characteristics linked to field with high clays contents resulted in significantly lower nematode abundances. Remarkably, the dominant nematode taxa analysed here were in general negatively related to soil nutrients and organic matter content.

Here we accumulated evidence that nematode taxa have different distribution patterns within the same field, irrespective of trophic preferences (*e.g.* herbivores, bacterivores, fungivores, omnivores, predators or entomopathogens), life history strategy (cp 1-5) or body weight. This reinforces the notion that nematode communities should preferably be studied at family or genus level, and not at the level of functional groups or life history strategy (Ettema 1998; Porazinska *et al.* 1999; Neher *et al.* 2005; Vikefto & Sohlenius 2011; Quist *et al.* 2014). The functional group approach might be justified in food web analysis (Moore & de Rooter 2012), but higher taxonomic resolution is required when the goal is to gain insight into nematode distributions and their responses to plants and land use.

Within-field variation of nematode communities and nematode densities is common in this and in other studies. In nematode ecology studies, coefficients of variation of replicate samples in are typically 100% or above (e.g. Ettema *et al.* 2000; Liang *et al.* 2005; Neher *et al.* 2005; Vikefto *et al.* 2009; Quist *et al.* 2014). This source of variation is usually ignored, or – if recognized – it remains unexplained. The geostatistical models show that this consistent source of variation is the result of the combined effects of spatial variability ($\sigma^2_{\text{spatial}}$) and technical variation (σ^2_{nugget}). The relative importance of $\sigma^2_{\text{spatial}}$ and σ^2_{nugget} is dependent the scale of sampling (expressed by the range of the model).

The belowground spatial patterns are the outcome of numerous processes which might be stochastic or deterministic in nature. High values for explained variance would point at deterministic drivers for pattern formation. Low values for explained variance would suggest that random dispersal and probabilistic birth and death events underlie the spatial variation, pointing at stochasticity as the dominant factor for patterning (Rosindell *et al.* 2012; Bahram *et al.* 2016). Psuedo- R^2 -s were calculated to evaluate the explained variance for mean nematode densities, detection probabilities as well as of the spatial distribution parameters from the Matérn models. For the range-parameter only 7.5% and for the $\sigma^2_{\text{spatial}}$ only 26% of the variation was attributed to the combined effects of nematode traits and site characteristics. The large unexplained component could be ascribed to (1) to technical errors, (2) unmeasured environmental variables or (3) to the role of stochastic processes for pattern formation.

- (1) Geostatistical modelling resulted in three parameters that describe distribution patterns: the range, the $\sigma^2_{\text{spatial}}$ and the σ^2_{nugget} . The latter describes the measurement error, which may be subdivided in microscale variation and technical variation (Diggle & Ribeiro 2007). Here composite samples were collected consisting of 12 soil cores each, thereby virtually excluding the effect of microscale variation (see details in Quist *et al.* under review). The σ^2_{nugget} in the current study is therefore interpreted as technical variation only. In sum, technical error was quantified by geostatistical modelling and we find on top of this various degrees of spatial variability (expressed by $\sigma^2_{\text{spatial}}$ and the range-parameter) for each taxon studied.
- (2) No major role of unmeasured variables is assumed, as the 1 ha plots were carefully selected on the basis of visual uniformity as supported by the expert opinion of the owners of the selected fields.
- (3) Hence, the relatively high percentages of unexplained spatial variability (92.5% of the variation for the range-parameter and 74% for $\sigma^2_{\text{spatial}}$), point at a major role of stochasticity.

When stochastic processes largely outweigh the effects of environmental factors, this points to a high level of ecological neutrality among taxa within a given trophic group. The neutrality concept contributes to our understanding of complex systems with extreme parsimonious models assuming that all species within a trophic group are equivalent and proposes that diversity arises from a balance between immigration, speciation and extinction (Chisholm & Pacala 2010; Rosindell *et al.* 2012). To explain spatial variability, neutral theory proposes that spatial variability arises from a balance between random dispersal, and probabilistic birth and death events (ecological drift). This model might result in a mosaic landscape (Chave *et al.* 2002), where each patch is in a different stage of succession. The stage of the patch can be close or far away from the competitive equilibrium (Ettema 1998), but within the boundaries set by the environment (see PCA in Fig. 1.). Hence, stochastic patch dynamics may be an important element to explain the variation in nematode community composition of microplots within the fields, and the great variety in degrees of spatial variability of nematode taxa.

Conclusion

A combination of optimized field sampling, DNA-based nematode community analysis and state-of-the-art geostatistical analysis methods enabled the generation of belowground “nemato-scapes”. Analysis of distribution patterns of nematodes within and between visually homogeneous fields provides new insights to soil food web functioning and biodiversity. Here we present empirical evidence that both deterministic and stochastic processes regulate nematode community composition, pattern formation and biodiversity. Soil type and soil management drive belowground composition of nematode communities among fields. However, in areas without noticeable environmental gradients and similar management practices, stochastic processes are the dominant factor underlying variations in soil communities. Detailed knowledge on belowground spatial distribution patterns will greatly contribute to the design of statistically robust sampling strategies for nematode communities at mesoscale level.

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Supporting information

Additional supporting information can be found online:
<https://sites.google.com/site/phdthesiscasperquist>

6

CHAPTER

General Discussion

Casper W. Quist

General discussion

Terrestrial nematodes have a high potential to serve as an effective and policy-relevant indicator group for ecosystem functioning and soil biodiversity (Griffiths *et al.* 2016; Stone *et al.* 2016). The work described in this thesis contributes to more reliable, robust and affordable assessment of nematode communities in terrestrial habitats. Here, nematode taxon-specific quantitative (q) PCR assays were used to pinpoint responses of nematode communities to invasive plants, and to various farming strategies. Furthermore, spatial distribution patterns were determined for over 40 nematode taxa representing three trophic levels of the soil food web, at both micro and mesoscale. In this chapter, I discuss the opportunities and challenges of the use of molecular tools in soil ecological research, the impact of trophic preferences on the whereabouts of nematodes, the use of nematode communities as indicator for soil condition and how this might be developed and applied to facilitate more sustainable ecosystem management.

Molecular techniques in soil ecology research

Microscopic identification and quantification of soil biota is time-consuming, and requires (increasingly rare) detailed taxonomic expertise. A quantitative PCR-based tool for the analyses of nematode assemblages (first described by Vervoort *et al.* 2012) was developed based on a phylum-wide framework consisting of over 2,500 small subunit (SSU) rDNA sequences (Holterman *et al.* 2006; Van Megen *et al.* 2009; Quist *et al.* 2015). This molecular technique allowed for detection and quantification of nematodes at species, genus or family level. Quantitative PCR assays are powerful to test the response of a selection nematode taxa, or determine community compositions. If all qPCR primer combinations available in our collection (around 70 taxon-specific assays) are applied to a sample, this will give a reasonable indication of the nematode biodiversity. It should be noted that a series of taxon-specific qPCR assays as used in this PhD thesis will provide quantitative information on the selected taxa only. For a full overview of the nematode biodiversity, other approaches such as high throughput sequencing of complex amplicons should be used. However, the translation of high throughput sequencing data into a quantitative characterization of communities is intrinsically troublesome. Asymmetric amplification of target DNAs is the main cause of this bias (Porazinska *et al.* 2009 and 2010). Hence, to assess the qualitative and quantitative effects of treatments on nematode communities, a combined approach of qPCR assays and rDNA sequencing techniques could be used. Currently, qPCR and environmental DNA sequencing approaches are sufficiently robust and affordable. Major challenges need to be faced with regard to

the analysis of large datasets that are associated with high-throughput sequencing. Molecular techniques open new avenues in soil ecological research. The contents of this PhD thesis as well as numerous recent papers in this field show that molecular identification and quantification techniques can be used to answer ecologically relevant questions, without the need for detailed 'classical' taxonomic expertise on the organismal groups under investigation. To fully exploit the information hidden in these large, environmental DNA datasets, biologists should invest more in bio-informatics skills and/or initiate relevant collaborations.

The impact of trophic ecologies on the whereabouts of nematodes

Within the trophic levels of soil food web diagrams, functional groups are defined in which species are placed together based on their similar feeding preferences, reproduction rate and predators (Holtkamp *et al.* 2008).

As compared to bulk soil, the nematode density in the immediate vicinity of plant roots is about five times higher. Consequently, even in portions as small as a single gram of rhizosphere soil, herbivores, bacterivores, fungivores, omnivores and predators can be detected simultaneously (CQ unpublished results). Representation of all three levels of the food web is a quite stable condition. Tsiafouli and co-workers (2015), found that abundances and diversity of the functional groups were hardly affected by intensification of agricultural practices, whereas the densities and diversity of other components of the soil food web such as earthworms, oribatid mites and collembolans were negatively affected. Although nematode communities were analysed at genus level in this study, it should be noted that higher aggregation levels were used for data analyses.

A substantial body of literature is published in the domain of nematode ecology that has used the "functional group" or the "functional guild" approach (Robertson & Freckman 1995; Berkelmans *et al.* 2003; De Deyn *et al.* 2004; Viketoft 2013). A "functional group" (in the context equivalent to "trophic group") is a group of nematodes with the same feeding habits. A "functional guild" should be considered as a refinement of the functional group concept, and is defined as "nematode taxa with the same feeding habits, and inferred function, in the food web" (Ferris *et al.* 2001). Examples of functional guilds are bacterivores (Ba_x), fungivores (Fu_x), carnivores (Ca_x) and omnivores (Om_x), whereby x can be 1-5 in the *colonizer-persister* (cp) scale. One of the underlying assumptions is that members of such a guild show "the same feeding habits". In the broad sense this might be correct, bacterivores feed (mainly) on bacteria (*etc.*), but for a better ecological understanding it should be realized that (at least) individual genera within this functional guild feed on distinct parts of the

bacterial community. Nematode within a functional guild should not be regarded as ‘indiscriminate grazers’, even not within a single cp group. The notion that at least for some ecological questions, such as the impact of crops, plant communities or land management, an essentially more detailed analysis of nematode assemblages is required, is more and more appreciated (Porazinska *et al.* 1999, Neher *et al.* 2005, Viketoft and Sohlenius 2011).

In Chapter 2 of this thesis we found that three fungivorous nematode taxa, *Aphelenchus*, *Aphelenchoides* and *Diphtherophora* (belonging to cp groups 2, 2 and 3, respectively), showed a differential response to an asymmetric boost of the fungal community. Whereas the members of the family Aphelenchoididae (Fu₂) benefitted from the increase in fungal biomass, the representatives of the family Aphelenchidae (Fu₁) could not. This example illustrates that taxa within a feeding type have distinct food preferences. This notion was confirmed in all following experiments of this PhD thesis (Chapter 3, 4 and 5). The results presented here underline that the functional guild approach might not be the preferred approach if we aim to gain insight into nematode distributions and their responses to plant (crop) species and land management.

Feeding habits of nematodes are usually based on the nematode mouth morphologies (Yeates *et al.* 1993). Attempts have been made to investigate food preferences of bacterivorous nematodes in more detail. This approach included the extraction from bacterial DNA from the gut of individual nematodes. Such analyses are intrinsically difficult (1) as the half-life of bacterial DNA in the nematode gut is short in relation to the time required for nematode extraction, (2) as it is hard to rigorously discriminate between bacterial attached to the outside of the nematode (cuticle) and bacteria that are present in the intestines, and (3) as the inclusion of proper negative controls is not straightforward at all. Hence, for most free-living nematodes, little is known about the nature of their food preferences *under natural conditions*. One might wonder whether the ability to grow certain nematode species *in vitro* could be informative with regard to their natural trophic preferences. The limited relevance of such observations can be illustrated by the bacterivore *Caenorhabditis elegans* that is usually grown on *Escherichia coli* (OP50), a bacterium that is found in the lower intestine of many endotherms. Obviously, information about the kind of microorganisms that can be used to grow certain nematodes *in vitro* is not always informative with regard to their trophic preferences under natural conditions.

Insight into spatial variation of nematodes reveals stochasticity as one of the explanations for overwhelmingly high biodiversity in soils

More than a century ago, Nathan A. Cobb, noted that “if all the matter in the universe except nematodes were swept away, our world would still be recognizable, and if we could then investigate it, we should find its mountains, hills, vales, rivers, lakes, and oceans represented by a film of nematodes” (Cobb 1915). Cobb probably used this statement to illustrate the ubiquity, the abundance and the speciose nature of the phylum Nematoda. Marine nematode communities are highly distinct from terrestrial assemblages. Although fresh water communities are related to their terrestrial equivalents, a number of typical fresh-water taxa make that river and lake communities indeed can be distinguished. Soil type is a major determinant of the composition of nematode communities in terrestrial ecosystems, but this impact of this parameter is moderately well characterized. In this thesis, first steps were made to describe and pinpoint “below-ground biological landscapes”. Optimized sampling schemes, high-throughput DNA barcode-based community characterisation as well as state-of-the-art geostatistical analysis methods were used to reveal the kind of belowground landscapes Cobb was referring to.

Insight in belowground spatial distribution patterns contributes to our understanding about the factors that maintain and regulate soil biodiversity (Ettema & Wardle 2002).

This thesis provides insight in the relative importance of deterministic and stochastic processes as regulators of terrestrial nematode community composition and pattern formations. The results of Chapter 5 show that soil type and soil management drive belowground composition of nematode communities among fields. The results also show that spatial distribution parameters were to only a small extent explained (7% for the range of spatial distribution, and 26% for the spatial variance) by the combined effects of nematode traits and site characteristics. Therefore, we conclude that within areas under a single management regime and without visually observable environmental gradients, stochasticity is the main driving factor causing variations in nematode communities. The important role of stochasticity for occurrence and densities of soil organisms might be another factor explaining coexistence of species (Ettema & Wardle 2002).

Stochastic patch dynamics might result in a mosaic landscape (Chave *et al.* 2002), where each patch within a given environment is in a different stage of succession. The stage of the patch can be close or far away from the competitive equilibrium (Ettema 1998). In Chapter 5 we found that nematode community composition was less variable in fields on clay than in fields on sandy soil. This suggests that the amplitude from the competitive equilibrium might be dependent on the environmental conditions of the object.

More detailed understanding soil biodiversity and soil functioning, will be gained when interactions at species level are investigated (Wardle 2006). The taxonomic resolution of the qPCR assays used in this thesis was occasionally till species level (plant parasites), but more often till family or genus level (free-living nematodes). Molecular assays at species level usually focus on economically important plant pathogens, such as individual cyst nematode species belonging to the genera *Globodera* or *Heterodera* species, on various lesion nematodes (members of the genus *Pratylenchus*) and on individual root knot nematodes (*Meloidogyne spp.*). For the detection and quantitative analysis of nematode at species level, we made use of the observation that a parasitic life style accelerates the rate of change of the ribosomal DNA (Holterman *et al.* 2006). However, the development of species-specific qPCR assays with a single, common annealing temperature is still a non-trivial process.

Nematode communities as indicators for soil condition in agro-ecosystems: current progress, challenges and future opportunities

In (semi) natural ecosystems, plant communities are highly informative with regard to the condition of terrestrial habitats. Plant species are relatively easy to identify and the condition of ecosystems can be estimated based on the presence of specific indicator plant species. In agro-ecosystems as well as in extremely dynamic habitats such as coastal areas, floodplains along rivers and arable systems where plants are regularly removed, the assessment of biological soil condition relies on biological indicators belowground.

Whereas macro-faunal organisms are negatively affected by common agricultural practices, the overall diversity and abundances of micro-fauna or even smaller forms of life was reported to be relatively stable (at the resolution level used in that study) (Tsiafouli *et al.* 2015). The lower abundances of macro fauna in agricultural field render them rather unpractical bio-indicator as large soil samples (> 1 kg) would be required for proper assessment. Though compositional shifts take place, nematodes are present in high densities even under the most disturbed agricultural conditions such as conventional banana plantations (Djigal *et al.* 2012) and intensive crop rotation systems (Neher *et al.* 2005). Hence, similar soil sampling strategies can be used to assess nematode communities, even under the harshest agronomic conditions.

In Chapter 3 we found two indicative nematode taxa for conventional and organic agriculture. The results described in Chapter 3 showed that organic farming causes specific shifts in nematode community composition, exceeding the usually large crop-related assemblage shifts. Strongest effects were observed for the (putative)

bacterivore *Prismatolaimus*, which was relatively common in organic fields and nearly absent in conventional and integrated farming. A reverse effect was observed for *Pristionchus*; this necromenic bacterivore and facultative predator made up about 7 – 21% of the total nematode community in integrated and conventional farming, whereas it was nearly absent from organic fields. The observed farming system effects suggest that specific nematode taxa might be indicative for the impact of farming practices on soil biota. High-throughput quantitative molecular analysis of nematode communities and sampling schemes with predictable accuracies facilitate monitoring studies to assess nematode taxon-specific preferences for soil conditions and management types.

Gaining insight into soil processes to facilitate sustainable food production

Belowground ecological intensification has been proposed as an approach to enhance ecosystem service delivery by integrating more biological processes into farming systems (Bommarco *et al.* 2013). Effective ecological intensification goes beyond the general biodiversity-function relations and will rather benefit from a targeted soil-biological engineering approach (Bender *et al.* 2016). Higher yields for example were obtained when living mulch of White clover (*Trifolium repens*) was introduced in corn fields because arbuscular mycorrhizal fungi were promoted by *T. repens*, that enhanced the phosphorous uptake of the corn roots (Deguchi *et al.* 2007; Deguchi *et al.* 2012).

To increase understanding of the functioning of soil organisms and the effect of biodiversity in soil, nematodes can serve as a model system. Given the fast development of affordable high throughput DNA sequencing methods, it is expected that Next Generation Sequencing (NGS) approaches will provide a more detailed characterisation of nematode assemblages in the near future. More detailed community characterisations might help to better estimate nematode species-area curves and assess co-existence of nematode species within and between trophic groups. With these future prospects in mind, it might be relevant to note that all DNA extracts collected in the framework of this PhD thesis were stored at -80°C.

Clearly, to gain understanding in soil processes that facilitate sustainable food production also other relevant functional groups such as bacteria, fungi, and protists should be taken into consideration (Griffiths *et al.* 2016). These groups can be investigated by the same molecular techniques as developed for nematode communities, NGS and quantitative PCR. Insights into the soil conditions and management strategies on the whereabouts of nematode communities and other relevant soil organismal groups will allow us to develop diagnostic assays to provide soil type- and crop-specific advices for the optimal and durable utilization of soil life.

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Summary

Soil life is highly diverse, and ecologically intricate due to myriad of biotic interactions that take place. Terrestrial nematodes have a high potential to serve as an effective and policy-relevant indicator group for ecosystem functioning and soil biodiversity. The work described in this thesis contributed to the robust mapping of nematode communities at scales relevant in both agronomic and environmental contexts. The overarching aim of the work described in this thesis was to contribute to a sound exploration of the potential of nematode communities as an indicator group for the biological condition of soils. Therefore, the distributions of a wide range of nematode taxa were studied, within and between trophic groups and in soils conditioned by various plant species and/or farming systems.

In Chapter 2 nematode taxon-specific qPCR assays were used to pinpoint responses of nematode communities to invasive plant species *Solidago gigantea* in two invaded ecosystems: semi-natural grasslands and riparian floodplains. Nematode communities and fungal biomass were examined in adjacent invaded and uninvaded patches. The dominant presence of the invasive plant causes a decrease of plant species-richness and diversity, and an about twofold increase of fungal biomass. Only the density of a single group of fungivorous nematodes (Aphelenchoididea) increased, whereas the densities of two other, phylogenetically distinct lineages of fungivorous nematodes, Aphelenchidae and Diphtherophoridae, were unaffected by the local increase in fungal biomass. Apparently *S. gigantea* induces a local asymmetric boost of the fungal community, and only Aphelenchoididae were able to benefit from this change induced by the invasive plant.

In Chapter 3 the outcome is shown of a test whether farming system effects are mirrored in compositional changes in nematode communities. The long-term impact of three farming systems (conventional, integrated and organic) on nematode communities was investigated at *the Vredepeel*, an experimental farm in the southeastern part of The Netherlands. The results showed that organic farming causes specific shifts in nematode community composition, exceeding the usually large crop-related assemblage shifts. Strongest effects were observed for the (putative) bacterivore *Prismatolaimus*, which was relatively common in organic fields and nearly absent in conventional and integrated farming. A reverse effect was observed for *Pristionchus*; this necromenic bacterivore and facultative predator made up about 7 – 21% of the total nematode community in integrated and conventional farming, whereas it was nearly absent from organic fields. The observed farming system effects suggest that specific nematode taxa might be indicative for the impact of farming practices on soil biota.

Knowledge of spatial distribution patterns of soil organisms with distinct trophic preferences will contribute to our understanding of factors that maintain and regulate soil biodiversity, and is essential information to design soil sampling strategies with predictable accuracies.

Chapter 4 deals with microscale patchiness of 45 nematode taxa (at family, genus or species-level) in arable fields and semi-natural grasslands, on marine clay, river clay or sandy soils. Contrary to our expectations, an increase of the number of cores per composite sample above 3, did not result in more accurate detection for any of the taxa under investigation (range of number of cores per composite sample: 3, 6, 12 or 24). Neither system nor soil type did influence microscale distribution. The insights in the spatial distribution of nematodes at microscale presented here, sheds light on the impact of trophic preferences on the spatial distribution of individual nematode taxa, and will allow for the design of statistically sound soil sampling strategies.

Chapter 5 shows belowground distribution patterns of 48 nematode taxa in 12 visually homogeneous fields (each 100 x 100 m) on three soil types (marine clay, river clay and sand) and two land-use types (arable and natural grasslands) across the Netherlands. Over 35,000 nematode-taxon specific qPCR assays allowed us to quantitative analyse nematode taxa at family, genus or species level in over 1,200 soil samples. A sampling scheme was optimized for Bayesian geostatistical analysis (Integrated nested Laplace approximations; INLA). Multivariate analysis show soil type and land-use related differences in the nematode community composition, which underline the effects of environmental filtering and niche partitioning of nematodes. All individual nematode taxa together show a wide range of degrees of spatial variabilities were found (expressed by the range-parameter and the spatial variance parameter ($\sigma^2_{\text{spatial}}$). No general effects were detected of soil characteristics or nematode traits (cp-value, trophic group, weight) on the spatial distribution parameters. The relatively high percentages of unexplained spatial variability – 92.5% of the variation for the range-parameter and 74% for spatial variance ($\sigma^2_{\text{spatial}}$) – point at a major role of stochasticity for variability of nematode densities within fields. This study adds empirical evidence that distribution patterns of terrestrial nematodes, in areas without noticeable gradients, are driven by neutral / stochastic processes, within the boundaries set by the environment.

In the final Chapter 6, I discuss the opportunities and challenges of the use of molecular tools in soil ecological research, the impact of trophic preferences on the whereabouts of nematodes, the use of nematode communities as indicator for soil condition and how this might be developed and applied to facilitate more sustainable ecosystem management.

Acknowledgements

Often a PhD project is compared to a road trip. But I believe that for me the journey to become a doctor is far more challenging than reaching the far destination by car. It will probably not be a surprise that I am advocating the comparison of the PhD track with a survivalrun instead. There are many similarities between the PhD and the survivalrun. In both situations, there is a high chance that you will run into obstacles for which you're not prepared. This makes you unsure if you will ever be able to finish. However, if you mess up with any obstacle, you are given multiple opportunities and usually provided with plenty of suggestions by people that want you to succeed. Furthermore, during both the Ph.D. and the survival sport, my closest colleagues and best friends have the strange habit to take great pleasure when finding out about my failures. Along the way, there are ups and downs, but you know you need to continue. Mainly the final obstacle is something beautiful and terrifying at the same time. I am grateful to all those amazing people who were always there for me, who helped me, who welcomed me into their group and collaborated with me.

First and foremost, I want to thank my co-promotor Hans Helder for his daily contributions and trust. Hans, you have checked literally each sentence of this Ph.D. thesis, each abstract that I have sent, each talk that I was going to present. You have been incredibly supportive, always in a good mood and full of bright ideas at any time. I feel very privileged having had you as a supervisor! I also want to thank my promotor Jaap for being so warm, friendly, wise, and for taking a mentor-role especially in the end of my Ph.D. I am also grateful to you for running the research group in such a way that it feels like we are a family within a safe environment with plenty opportunities. From all "brothers", "sisters", "aunts" and "uncles", I first need to thank uncle Sven for tempting me to start increasingly large research-projects at Nema, from when I was a BSc and MSc student to when I started a Ph.D. in your group. I could say many nice things about you for example about organizing yearly Boekel-weekends and how good you are in throwing objects, but we all know it is better for everyone that you don't receive too many compliments. Jet, I had such a great time with you on during our adventurous field expeditions, lab work with dangerous chemicals, and writing our groundbreaking paper, while singing the Trololo song for hours. You are an extremely open, hardworking and caring person. Almost like a mother to me. Now you are rapidly progressing to the point that you are seen as mother to all students at nematology, if you like it or not ;). King of qPCR Paul, I am very grateful to you for all your help in the lab and for being so patient when solving queries about qPCR results, primer sets etc. It is mainly because of you that I started to understand what I was doing. Queen of nematode taxonomy Hanny, thank you for sharing your enthusiasm for observing nematodes by the microscope. I appreciate you having me over in your garden room in Woudenberg until well after your retirement, analyzing slides for hours in a row.

Besides these and other fellow labmates, there were some people that gave extra color to my life as a young researcher. I want to thank David for his fellowship, it was great that you were always open to share thoughts, interested in food related activities and sports. Collecting soil samples with Maarten Schrama is like competing with the Duracell Bunny. I can not wait to have another romantic lunch meeting with you at our small wooden bridge. Gerrit Gort, your statistical help has been fundamental to this thesis, I feel so lucky that you were willing to help me. Wim van der Putten, thanks for your guidance that provided more depth to this Ph.D. thesis. Erik Sloomweg, from all the bright people that surround me, I know nobody who knows more about so many different things than you do, thanks for your endless support and being such a good colleague and friend.

Research is beautiful work, but at least for a Ph.D. it is usually restricted to one particular topic and working on it on your own for a long period of time is lonely. Luckily, I have had the chance to spend quite some time supervising lovely BSc and MSc students, coaching and giving field excursions to interactive groups students. This was a great experience and besides thanking all the students who were part of this, I want to thank the research group for giving me this opportunity. I am very grateful to Setareh, Roel and Lars for having me in their cosy flora-excursion-team in the French Pyrenees.

While writing these acknowledgements, I am a visitor at another institute. Although the atmosphere is excellent here, I realize again that coffee breaks at Nema are sublime events. Of course all of us make a good contribution to these thrice-a-day gatherings, but I want to thank some people in particular. Joost, Lisette for being punctual, Rikus for laughing so loud and clear that others will notice they are running late, Sven for not bothering telling a story more than once, the Rainbow-Casper (*El Casper con colores*) for having the best jokes, and of course Paula, for being so open, making every topic acceptable, so that slightly more reserved people feel free to open up as well. Let me also thank Basten and Mark for being so helpful to me when fighting R. And Octavia, you have been like an angel to me, by surprising me with pieces of cake on my desk.

Usually, sitting for a while behind a desk make my batteries become (over)loaded, thanks to all *Bomenduwvers* (Kroes, van der Ploeg, Menger, de Weert, Joosten, Drukker, van de Logt *et al.*) I was always accompanied doing sports.

Before I moved to Wageningen, I spent most of my free time working at a farm with apple, pear and sour cherry orchards. I want to thank Urbain and Jos van Waes for getting me interested all aspects of farming, biology in general, and basically teaching me how to work and have some responsibility. Finally, I want to thank my parents for being non-stop supportive in all possible ways.

Sydney (Australia), 19th March 2017

Curriculum vitae

Casper Willem Quist was born on the 23rd of March 1986 in Dirksland, the Netherlands. After finishing pre-scientific education in 2005, he moved to Wageningen to study Biology at Wageningen University. In 2009 he completed his BSc with the specialization Plant Biology. Within his Msc he dedicated six months to an internship at Corporación Bananera Nacional de Costa Rica (Corbana), under supervision of Prof. Dr Gert Kema. Together with Dr Eddy Weeda he wrote one chapter in book *Natuur als nooit tevoren* about exotic and invasive plants in relation with nature conservation. Further, under



Photograph by Lars Chatrou

supervision of Dr Jet Vervoort and Dr Hans Helder, he studied the effect of invasive species *Fallopia japonica* and *Solidago gigantea* on soil nematode communities and the native vegetation (which has led to a publication in *Oikos*). In 2011 he completed his MSc, and in the same year he started a PhD project at the laboratory of Nematology supervised by Dr Hans Helder.

During his PhD, he contributed to the development of reliable, robust and affordable qPCR-based assessments of nematode communities. These methods were applied by pinpointing responses of nematode communities to invasive plants, and various farming strategies. Further the methods were used elucidating spatial distribution patterns in arable and natural settings. He presented his work at five international conferences.

Casper supervised MSc and BSc thesis students, and was involved in several courses giving field excursions about landscape history, ecology and botany, supervising and coordinating lab work and coaching students in their group work and research projects.

In 2017, he received funding from the Hawkesbury Institute for the Environment (Western Sydney University) to temporally join the research group of Dr Uffe Nielsen to investigate the effect of long-term rainfall manipulations on vegetation and belowground communities in arid and semi-arid grassland ecosystems of Australia. Currently, Casper has a post-doc position at the Laboratory of Nematology.

List of publications

Peer reviewed articles

Feeding preference as a main determinant of microscale patchiness among terrestrial nematodes
Quist, C.W.; Gort, G.; Mulder, C.; Wilbers, R. H.P.; Termorshuizen, A.J.; Bakker, J.,
Helder, J. (2017) *Molecular Ecology Resources* XX. – p. XX – XX.

Organic farming practices result in compositional shifts in nematode communities that exceed crop-related changes

Quist, C.W. ; Schrama, M. ; Haan, J.J. de; Smant, G. ; Bakker, J. ; Putten, W.H. van
der; Helder, J. (2016) *Applied Soil Ecology* 98 . - p. 254 - 260.

Evolution of Plant Parasitism in the Phylum Nematoda

Quist, C.W. ; Smant, G. ; Helder, J. (2015) *Annual Review of Phytopathology* 53 . - p.
289 - 310.

Phytopathogenic Nematodes

Helder, J. ; Vervoort, M.T.W. ; Megen, H.H.B. van; Rybarczyk-Mydłowska, K. ;
Quist, C.W. ; Smant, G. ; Bakker, J. (2015)

In: *Principles of Plant-Microbe Interactions* / Lugtenberg, B., Springer, chapter 11 - p. 443

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

Quist, C.W. ; Vervoort, M.T.W. ; Megen, H.H.B. van; Gort, G. ; Bakker, J. ; Putten,
W.H. van der; Helder, J. (2014) *Oikos* 123 (7). - p. 837 - 845.

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

Vervoort, M.T.W. ; Vonk, J.A. ; Brotsma, K.M. ; Schütze, W. ; **Quist, C.W.** ; Goede,
R.G.M. de; Hoffland, E. ; Bakker, J. ; Mulder, C. ; Hallmann, J. ; Helder, J. (2014)
Soil Biology and Biochemistry 68 . - p. 200 - 207.

Exoten en invasieve soorten: nieuwe uitdagingen voor het natuurbeheer

Quist, C.W. ; Weeda, E.J. (2009)

In: *Natuur als nooit tevoren. Beschouwingen over natuurbeheer in Nederland / Schaminée, J., Weeda, E.J., Zeist : KNNV uitgeverij, (Vegetatiekundige monografieën 2) - p. 167 - 187.*

PE&RC Training and Education Statement

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



Review of literature (6 ECTS)

- DNA barcode-based monitoring in soil and its use in ecological studies

Post-graduate courses (6.4 ECTS)

- Sampling in space and time; PE&RC (2012)
- Soil, biodiversity and life: the contribution of soil to sustainability of life; PE&RC (2012)
- Introduction to R for statistical analysis; PE&RC (2012)
- Sampling in space and time for survey and monitoring of natural resources; PE&RC (2013)
- Spatial ecology; PE&RC (2014)

Laboratory training and working visits (1.8 ECTS)

- Barcoding-based monitoring of nematode communities; BLGG (2012)
- Long term effects of herbivores on upland grassland carbon pools; Soil and Ecosystem Ecology Group, University of Manchester (2015)

Invited review of (unpublished) journal manuscript (3 ECTS)

- Plant and Soil: selenium distribution in hyperaccumulator roots and its potential effects on nematode colonization (2012)
- Soil: technological advancements and their importance for nematode identification (2015)
- Ecology and Evolution: nematodes as a null model for species richness distribution across environmental gradients (2016)

Competence strengthening / skills courses (1.8 ECTS)

- PhD Competence assessment; WGS (2012)
- Voice matters; WGS (2012)
- Techniques for writing and presenting a scientific paper; WGS (2013)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.4 ECTS)

- PE&RC Day: innovation for sustainability (2011)
- PE&RC Introduction weekend (2012)
- PE&RC Day: extreme life (2012)
- PE&RC Day: biomimicry: unlocking nature's secrets (2013)
- PE&RC Day: optimization of science – pressure & pleasure (2014)
- PE&RC Day: one's waste...another's treasure? (2015)

Discussion groups / local seminars / other scientific meetings (5.7 ECTS)

- Second BE-Basic symposium; poster and oral presentation (2012)
- Ecogenomics day (2012)
- BE-Basic flagship 8 meeting; oral presentation (2012, 2013, 2014)
- Third BE-Basic symposium (2013)
- Fourth BE-Basic symposium; poster presentation (2014)
- Working group Soilborne pathogens and soil microbiology; oral presentation (2014) and (2015)
- Netherlands Annual Ecology Meeting; poster presentation (2015)
- Fifth Be-Basic symposium; poster presentation (2015)
- Nematologendag; oral presentation; Lelystad (2015)
- Plant-soil interactions discussion-group meetings (2016)
- Visit Bonkowski-lab; oral presentation; Cologne (2016)
- Plant-soil-microbe interactions for crop and pest management (2016)

International symposia, workshops and conferences (11 ECTS)

- International Symposium on Nematodes as Environmental Bio-Indicators; oral presentation; Ghent (2012)
- 6th International Congress of Nematology; oral presentation; Cape Town (2014)
- 54th Annual Meeting of the Society of Nematologists; oral presentation; East-Lansing (2015)
- British Ecological Society meeting; oral presentation; Edinburgh (2015)
- 32nd Symposium of the European Society of Nematologists; oral presentation; Braga (2016)

Lecturing / supervision of practicals / tutorials (46.8 ECTS)

- Ecological aspects of bio-interactions (2010-2016)
- Introductie omgevingswetenschappen (2011-2016)
- Webs of terrestrial diversity (2014-2016)
- Nematology / molecular tools course; Coimbra, Portugal (2011)

- Ecology I excursions (2012-2017)
- Oriëntatie plantenwetenschappen-excursion (2013-2016)
- Introductie plantenwetenschappen-excursion and practicals (2013-2016)

Supervision of MSc students

- Monitoring nematode communities in natural and arable soils
- Effect of organic farming on terrestrial nematode communities at the Vredepeel
- Effect of organic farming on terrestrial nematode communities across the Netherlands
- Insect-plant-nematode interactions in a field experiment

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