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Nematode response to nitrogen and phosphorus in grasslands, assessed by microscopy and molecular methods

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Nematode response to nitrogen and phosphorus in grasslands, assessed by microscopy and molecular methods

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Abstract

Although nematode community analysis is considered to be a useful environmental indicator, a major impediment to its wider adoption is the need for detailed morphological identification. Molecular techniques offer an alternative to this time-consuming traditional way. With both directed molecular T-RFLP approach and traditional microscopy for nematode analysis, this study characterized the responses of belowground nematode diversity and other associated soil processes to P fertilization in a long-term grassland trial and to inorganic N and cattle slurry application in short-term grassland plot.

P fertilization (> 40 years) increased the proportion of bacterial-feeding nematodes, while decreased that of fungal-feeding nematodes, indicating a shift from fungal to bacterial pathways in more intensively managed systems. In addition, the microbial PLFA profiles confirmed fungal biomass and fungal-bacterial ratio decreased, but soil microbial biomass C, N and P increased, with P fertilization.

Over the short-term period trial (< 2 years), nematode abundance increased significantly with slurry application, but not with inorganic N application. Inorganic N significantly increased bacterial-feeding nematodes, and decreased omnivorous ones, while slurry had no effects. Compared with slurry, higher inorganic N increased the herbage production and enhanced N uptake, indicating a faster N flux following inorganic N fertilization.

The traditional and molecular techniques yielded comparable results on profiling nematode community. The rapid, easy and replicable T-RFLP approach was suitable for the routine monitoring of the soil nematode community, which helped to advance the characterization of soil biodiversity and monitoring the soil quality. Further, real-time PCR assessment was used to determine the correlation between SSU rDNA copy number and body size of four free-living nematode species. No correlation was found for *Caenorhabditis elegans*, showing it is eutelic. The three other species (*Panagrolaimus detritophagus*, *Anatonchus tridentatus* and *Aporcelaimellus obtusicaudatu*, however, are non-eutelic, showing that rDNA copy number increased significantly with body size.

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Declaration

I declare that I am the author of this thesis; that the work of which this is a record has been done by myself, except where specifically stated in the text and acknowledgements, and all references cited have been consulted. This work has not been previously accepted for a higher degree.

Xiaoyun Chen

Chapter 1.   General introduction

1.1. The role of grasslands in terrestrial ecosystems

Grasslands, including steppes, savannas and prairies, are important terrestrial ecosystems covering about a quarter of the Earth's land surface (Bardgett and Cook, 1998). Also grasslands account for about 34% of the total global terrestrial organic carbon storage and store 7.3–11.4% of soil organic C (Jobbagy and Jackson, 2000). Grasslands generate soil systems that are different from those of forests and other vegetation types, even from the same parent material. A key feature of grasslands is a high turnover of shoot and root biomass leading to a large pool of labile organic matter at the soil surface (Bardgett and Cook, 1998). While the potential effects of global N enrichment on these ecosystems are currently best understood within an aboveground context, as an increase in production and a decrease in plant species diversity (Fynn and O'Connor, 2005; Suding *et al.*, 2005), it is recognized that in most terrestrial ecosystems the belowground biota supports a much greater diversity of organisms than does the aboveground biota (Wardle, 2006). Because of more intensive management, such as fertilization and overgrazing, grassland systems both above-ground and below-ground have experienced rapid shifts in composition, functioning, and services driven primarily by human disturbance (White *et al.*, 2000).

Grasslands are also a potential source of pollution, either through leaching (NO_3^-), gaseous emissions (N_2O) or surface runoff (P). For example, Hyde *et al.* (2006) assessed longer term studies to estimate the annual emission of N_2O from Irish fertilized and grazed grassland which represented between 3.17% and 7.22% of applied N. These emissions are the products of biological activity in the soil. Intensive grassland management aims to achieve a uniform sward through slurry and fertilizer applications to unify soil nutrient content, while grazing management aims to unify spatial and temporal exploitation of the field. The effects and consequences of these changes in above-ground heterogeneity on the soil biodiversity responsible for important ecosystem services are largely unknown.

1.2. Grassland management in Ireland

Grasslands are the main agricultural land use in Ireland, with permanent grassland the largest agricultural land use option, accounting for 80% of land area (Rath and Peel, 2005). But even within these systems there is a range of management options from unstocked, low input and organic systems through to intensively stocked pastures with applied slurry and

fertiliser. New economic drivers, such as abolition of milk quota and rapid increase in prices of milk and cereals, will encourage localised intensification of agricultural land use with potential effects on soil biodiversity and function.

Projections indicate that the world population may increase from about 6 to 8.2–9.3 billion inhabitants in 2030 (Nakićenović, 2000). Food production will have to increase to meet the increasing demand for the growing population, while with increasing prosperity and falling production costs dietary patterns may shift towards a higher share of meat and milk. The decrease in costs of animal products is related to the increasing share of production in efficient mixed and industrial production systems and decreasing share of traditional pastoral systems (Delgado, 1999). Grassland management affects the production and utilization of grass. Grassland over use and land conversion into grasslands are driven by the demand for forage production since significant portions of world milk (27%) and beef (23%) production occur on grasslands managed solely for those purposes (Seré and Steinfeld, 1996). Despite the rapid increase of ruminant production by 40% in the 1970–1995 period, the global area of grassland has increased by only 4% (FAO, 2001). Under this situation, the intensification of grassland, such as intensive fertilized, over-grazing and mowing, has been established for maximum production.

In many developed countries, much of commercial farming operates under the influence of increasingly multifunctional expectations by consumers. Such farming must thus be sustainable within a range of economic, social, and environmental criteria. For example, the European Union (EU) Common Agricultural Policy (CAP) reform agreement provides direct payments [commonly referred to as single farm payments (SFP)] to farmers who comply with a range of requirements relating to the environment, animal welfare, and food safety. This involves major changes and uncertainty in business conditions and operating practices for many farmers (Schnepf and Hanrahan, 2011).

In most agricultural systems, N, P and other nutrients are essential elements as mineral fertilizers for planting and animal growth. Use of nitrogen fertilizer is considered essential as a management tool for systems involving grazing livestock for intensive dairy production. A recent UK survey (Jarvis, 1999) reported that mean yearly N application rates on dairy farm swards amounted to 281 kg N ha^{-1} (varying from 100-689 kg N ha^{-1}). Such high N fertilization rates allow for large grassland and forage production, sustaining high animal densities (Jarvis *et al.*, 1989). In Ireland, mean stocking rate was 202 kg organic-N ha^{-1} deposited by grazing livestock, while overall fertilizer-N use on the farms decreased from 266 to 223 kg N ha yr^{-1} during the study, with the rate of fertilizer-N in the first

application each year decreasing from 49 to 33 kg N ha^{-1}, while the rate of fertilizer-N applied for first cut silage production also fell from 106 to 96 kg N ha^{-1} (Humphreys *et al.*, 2008). It is important that farmers manage their production systems to minimize N losses between application to the soil and uptake by the plants (Humphreys *et al.*, 2009). Farmers must also remain cognizant of P losses since small losses (of the order of 1 kg P ha^{-1} year^{-1}) are adequate to promote increased plant growth in rivers and lakes (Tunney *et al.*, 2000). A suitable P application strategy (e.g. Culleton *et al.*, 2000a; 2000b) is essential to minimize surpluses and the long-term accumulation of soil P on farms.

Cattle slurry is an important asset on livestock farms as it reduces the need to use chemical fertiliser reducing variable costs. Slurry is typically composed of 50% ammoniacal N which is plant available and 50% organic N which is released more slowly to the plant providing a long-term supply of nitrogen (Hoekstra *et al.*, 2011). Animals housed over the winter period in Ireland can produce up to 300 l livestock unit (LU) of slurry per week (Nitrates Directive, 2006), over the 2.5- 5 month housing period. This equates to roughly 3,100 – 6,200 l of slurry being produced during housing. The nutrient content of slurry is highly variable (Martinez-Suller *et al.*, 2010) and should be recycled safely to land after the winter housing period. Large animal farms produce substantial amounts of slurry, which can potentially causes environmental problems. The amount of liquid manure depends on animal species, its age, kind of nutrition and on the method of husbandry (i.e. animals housed with or without litter). Cattle give about 10-20 litters of liquid manure a day containing, when stored properly, 1-3% of dry matter, 0.3-0.6% N, 0.5-0.8% K and 0.04-0.08% P (Wesolowski, 1999).

In pasture systems specifically, the combination of plant defoliation due to grazing, deposition of manure and urine, and compaction caused by mechanical clipping and trampling alters the soil chemical and physical environment (Mills and Adl, 2011).

1.3. Grassland biodiversity

Grassland management is about more than just production, and above-ground management has brought a series of threats, such as loss of soil organic matter, erosion, compaction and contamination, which affect grassland sustained utilization and grassland biodiversity. For example, high fertilizer inputs effects not only limit plant diversity but also leads to tremendous changes in the chemical, biological, and indirectly, physical status of soil (Plantureux *et al.*, 2005). It is now well established that large ruminants are able to enhance

plant diversity at low stocking rates, but decrease it at higher stocking rates (Olff and Ritchie, 1998). The proposed European Soil Framework Directive is formulated towards a sustainable use of soil. It emphasizes that the protection of soil biodiversity is necessary for sustainable land use and the provision of ecosystem services that contribute to the livelihood for land users (Creamer *et al.*, 2010).

Over the past several decades, many ecologists have focused on trying to understand why different communities or ecosystems differ in the diversity of organisms that they contain (e.g. MacArthur and Wilson, 1967; Grime, 1973; Tilman, 1982). Historically, most of the effort devoted to addressing this question in terrestrial ecosystems has focused on aboveground plant and animal species. However, it is well recognized that in most terrestrial ecosystems the belowground biota supports a much greater diversity of organisms than does the aboveground biota (Torsvik and Ovreas, 2002).

While the vast majority of species of soil biota have yet to be described, several key groups (bacteria, fungi, nematodes and insects) almost certainly contain several hundreds of thousands to millions of species globally (De Deyn and Van der Putten, 2005). This diversity is also apparent at local scales; for example, a few grams of soil may contain a few 1000 species of bacteria and several hundreds species of invertebrates (Wardle *et al.*, 2002). In spite of this critical contribution to global biodiversity, soil organisms have only weakly engaged the attention of taxonomists compared with other groups like higher plants and vertebrates (Decaëns *et al.*, 2006). The weakness of current taxonomic knowledge is of particular concern since the profile of below-ground taxa remains very low in systematic and taxonomy journals (Decaëns *et al.*, 2008). The scarcity of taxonomic expertise and of standardized sampling methods and designs has often been proposed to explain this trend (Andre *et al.*, 2002; Decaëns *et al.*, 2008). Another explanation is the lack of wider public interest in below-ground biota. This is highlighted by the relationship between the amount of scientific literature dedicated to a given taxon and its representation on the internet (which reflects public interest) (Wilson *et al.*, 2007). When compared with above-ground biota, soil animals are poorly represented both on the internet and in the scientific literature and, for a given number of websites, also tend to have a lower scientific presence (Decaens, 2010). Consequently, the less fashionable soil organisms are receiving relatively less scientific attention than the high-profile above-ground animals. As highlighted by Wilson *et al.* (2007), the existence of such taxonomic gaps defeats ecologists' claimed objective to construct generalizations. A wider research scope is thus needed to both wildlife management and teaching should consciously include less studied groups such as soil biota.

Raising awareness of soil biota is a key strategy that must be encouraged in the international arena through diverse initiatives to raise the profile of soil biological diversity. In historic dimensions, traditionally managed grasslands have been extensively utilized by mowing and grazing and have received relatively little artificial fertilizer. These semi-natural grasslands provided a wide range of habitats supporting a high biological diversity (Eriksson *et al.*, 1995; Partel and Zobel, 1999). But intensive grassland management including high nutrient input and increased defoliation frequency on the one hand and the abandonment of marginal grassland on the other has led to a dramatic decline in observed grassland biodiversity over the last decades (Janssens *et al.*, 1998; Hansson and Fogelfors, 2000). There is evidence that grassland management by grazing livestock at moderate levels contributes to the maintenance of plant diversity by reducing the abundance of competitive dominant species (Collins and Schwartz, 1998; Olff and Ritchie, 1998). Several studies have furthermore shown that mowing at moderate cutting intensity maintained plant species richness in grasslands (Hansson and Fogelfors, 2000; Fischer and Wipf, 2002). For both management regimes, grazing as well as cutting, it has been well established that high nitrogen fertiliser applications generally decrease species diversity (Jacquemyn *et al.*, 2003; Maurer *et al.*, 2006).

Nowadays, species-rich grasslands can only be maintained if farmers receive compensation payments against intensification by means of agri-environment subsidies or if the intensification in management is not cost-effective (Hodgson *et al.*, 2005). Up to now, a variety of agri-environment schemes have been applied in European countries to preserve and enhance biological diversity in agricultural systems (Kleijn and Sutherland, 2003), but their efficiency has been questioned (Kleijn *et al.*, 2001). Recent studies have indicated that management agreements that include financial compensation for farmers are failing to prevent the process of diversity loss (Balmford *et al.*, 2002; Ferraro and Kiss, 2002).

In most cases, intensive grass production from grasslands appears to be incompatible with maintaining a high level of biodiversity. The key question is how to restore biodiversity in intensive grasslands while limiting the technical and economical consequences? Two aspects arise here, 1) the relative lack of knowledge on grassland biodiversity, and 2) the objectives of biodiversity enhancement and its evaluation. In order to enhance the efficiency of management measures prescribed in agri-environment schemes, there is an urgent need to determine the underlying factors that control grassland plant species and soil biodiversity in managed grasslands, to understand the interaction between above-ground and below-ground diversity. For example, Benizri and Amiaud (2005) found decreases of

plant biodiversity with increasing nitrogen fertilization treatments in a grassland trial, and also bacteria extracted from rhizosphere of highly N fertilized plots showed a lower catabolic ability. The authors emphasized that it could be linked to a lower diversity in root rhizodeposit exudates in the fertilized treatment. Microbial community and soil process responses in grassland are less well known, except in a few cases, e.g., abundance of mycorrhizal fungi and the C: N ratio of microbial biomass tend to decline with N fertilization (Johnson *et al.*, 2003; Treseder *et al.*, 2004). With a greater comprehension of soil biodiversity, its function and the connection with above-ground diversity in grasslands, grassland management strategies can be developed directed at manipulating the soil biota to encourage a greater reliance on ecosystem self-regulation than on artificial inputs such as fertilizers and pesticides (Yeates and King, 1997).

1.4. Nematodes as an indicator in grasslands

Nematodes are one of the most abundant groups of soil invertebrates. More than four out of five metazoan individuals on earth are nematodes, often reaching several millions per square metre (Bongers and Bongers, 1998). Soil nematodes are believed to have profound effects on soil processes, such as residue decomposition, nutrient release through high turnover rates and their influence on the composition and activity of soil microflora (Ingham *et al.*, 1985; Moore *et al.*, 1988). Based on model calculations, approximately 30% of the annual N mineralization in Lovinkhoeve agricultural soil was due to the contribution of bacterivorous nematodes (de Ruiter *et al.*, 1998).

The diversity of fauna in grassland soils is very rich. In general, the nematodes, microarthropods and annelids are the most abundant in terms of both number and biomass. Nematodes are very abundant in grassland soils, where population densities can be as high as 10 million m^{-2} in highly productive lowland grasslands (Yeates and King, 1997) or as low as 1 million m^{-2} in acidic, infertile upland grassland soils (Bardgett *et al.*, 1997).

Soil nematodes have been used as indicators of overall ecological condition because of the wide range of feeding types and the fact that they seem to reflect the successional stages of the systems in which they occur (Ferris *et al.*, 2001). Furthermore, nematodes are sensitive to environmental perturbation and changes in their distribution and activity are diagnostic of changes in soil health (Ferris and Bongers, 2006). They are also the most abundant of the soil metazoa. Nematode species occurring in soils encompass a wide variety of feeding strategies, including many free-living species that feed on soil microbes (bacteria or fungi).

Microbial-feeding nematodes are among the most important consumers of bacteria and fungi in many systems, and their interactions with microbial decomposers affect ecosystem processes such as decomposition and nutrient cycling. Plant-feeding nematodes, omnivorous nematodes and predators also play an essential role in ecosystem functioning, and they are recognized that they may be capable indicators of environmental health, soil quality, and ecosystem resilience.

In the following chapters, I will review the molecular techniques used on nematode identification, and compare with morphological identification (Chapter 2) and then morphology and molecular comparison on fertilization grasslands (Chapter 3) and molecular technique on nematode community (Chapter 4) about the grassland management, mainly different fertilization schemes, and its effects on nematode community structure in Irish grassland.

1.5. Aims

Considering the central role of nematode in the soil ecosystem, the aims of this research were to:

- Use soil nematodes as an indicator for the assessment of grassland biodiversity under different grassland fertilization management.
- Evaluate a molecular tool, based on T-RFLP to investigate grassland soil nematode assemblages, by comparison with traditional light microscopy methods of nematode identification.
- Develop a better understanding of grassland soil food webs.

Chapter 2. Literature Review

－A comparison of molecular methods for monitoring soil nematodes and their use as biological indicators

2.1. Introduction

2.1.1. Nematodes as biological indicators for soils

In recent years, interest has been shown by soil scientists and ecologists in measuring soil quality, particularly since the drafting of the Soil Framework Directive and the increased national requirements for soil monitoring (Creamer *et al.*, 2010). Soil quality is a combination of the physical, chemical and biological properties that contribute to soil function. Indicators of soil quality should be responsive to manage practices, integrate ecosystem processes, and be components of existing, accessible data bases (Knoepp *et al.*, 1998). Such indicators must be quantified to document the improvement, maintenance or degradation of soil quality (Larson and Pierce, 1992), represent different aspects of soil quality in different ecosystems (Elliott, 1997), and strive to monitor or measure three basic functions or parameters: 1) soil structure development; 2) nutrient storage and 3) biological activity (Elliott, 1997).

Soil invertebrates are recognised as useful indicators as most are highly sensitive to perturbations and disturbances, for example, earthworms have been used to indicate soil properties (Blackshaw *et al.*, 2006) and soil pollution (van Gestel *et al.*, 2009); nematodes for environmental monitoring (Bongers, 1990; Bongers and Ferris, 1999); macroinvertebrates for soil heavy metal pollution (Nahmani and Rossi, 2003; Nahmani *et al.*, 2006); and collembola for the restoration of environmental conditions (Zeppelini *et al.*, 2009). Nematodes have been used as indicators of overall ecological condition because of the wide range of feeding types and the fact that they seem to reflect the successional stages of the systems in which they occur (Bongers, 1990; Ettema and Bongers, 1993; Yeates and Bongers, 1999; Ferris *et al.*, 2001). Furthermore, nematodes are sensitive to environmental insults, and changes in their distribution and activity are diagnostic of changes in soil health (Gupta and Yeates, 1997; Ritz and Trudgill, 1998; Bongers and Ferris, 1999; Ekschmitt *et al.*, 2001; Ferris *et al.*, 2001; Fiscus and Neher, 2002; Yeates, 2003), and also they are the most abundant of the soil metazoa (Ekschmitt *et al.*, 2001). Nematode species occurring in soils encompass a wide variety of feeding strategies (Yeates *et al.*, 1993), including many

free-living species that feed on soil microbes (bacteria or fungi). Microbial-feeding nematodes are among the most important consumers of bacteria and fungi in many systems (Griffiths *et al.*, 1999), and their interactions with microbial decomposers affect ecosystem processes such as decomposition and nutrient cycling (Freckman, 1988).

2.1.2. Limitations to routine monitoring

The identification of soil fauna often requires a high degree of taxonomic expertise (Andre *et al.*, 2001; Coleman *et al.*, 2004). Furthermore the time spent on identification (with the corresponding costs) makes it difficult to produce results over a relatively short period of time with affordability. This is particularly true for the nematodes. Identification of all individuals to the species level is time-consuming (Lawton *et al.*, 1998), so the characterisation of nematode communities continues to be resolved more coarsely than at the species level (i.e. genus, family, trophic group) (Porazinska *et al.*, 1999; Porazinska *et al.*, 2007), leaving ecological analysis potentially ambiguous or superficial (Yeates and Bongers, 1999). There is also a constraint where identification of species is only possible from adult specimens which usually represent only a small percentage of the overall nematode assemblage (Griffiths *et al.*, 2002).

So in spite of many advantages of using nematodes as biological indicators, identification even to functional group relies on highly trained experts (Ritz *et al.*, 2009). A possible solution is to find an appropriate surrogate molecular method allowing empirical assessment of soil fauna biodiversity. This is especially the case as currently training in classical taxonomic techniques is in decline while that in molecular methods is increasing.

2.1.3. Molecular methods for fauna identification.

Andre *et al.* (2002) highlighted the need for the development and consistency of methods in soil faunal monitoring, commenting that molecular techniques for community analysis are now widely used in soil microbiology and have greatly expanded knowledge on soil microbes. Molecular methods provide an alternative to traditional morphological identification for routine assessment of described species. Their application has enabled profiling of environmental samples of soil microbial populations, overcoming the need to culture and identify bacteria and fungi from complex mixtures (Amann *et al.*, 1995) and similarly potentially reducing the taxonomic expertise currently required to characterise microfaunal communities. New, high-throughput sequencing technologies provide an opportunity to generate very large amounts of sequence data in a very short time and at low

cost. One of the most important applications of those molecular methods is the ability to identify large numbers of species from complex communities (Opik *et al.*, 2008).

In addition to more rapid high throughput discrimination requiring less specialised skills, molecular techniques may also readily allow identification of cryptic species and juveniles (Blouin, 2001; Powers, 2004), although care needs to be taken with identifications made from a single gene target especially where taxa are currently represented by few confirmed sequences. Amplification and sequencing of diagnostic regions (i.e. rapidly evolving regions of SSU rDNA and LSU rDNA coding for the small and large subunit of rRNA, respectively) of single nematode specimens has resulted in the development of extensive public DNA sequence databases that are available for blast-match searching (Ye *et al.*, 2007) and phylogenetic comparison (Blaxter *et al.*, 1998; Griffiths *et al.*, 2006). Although DNA-based databases are strongly biased towards plant-parasitic nematode taxa (De Ley *et al.*, 2005), the utility of these searches for identification of free-living taxa that comprise the majority of soil nematodes is continuously improving. Recent publications on the phylogeny of terrestrial nematodes now make the identification of nematodes, and their ecological function, far more robust (Holterman *et al.*, 2008).

2.2. Molecular methods for nematode community analysis

Vanderknapp *et al.* (1993) used an arbitrarily primed PCR technique to differentiate closely related bacterial-feeding nematode species (from agar culture) that could not be morphologically distinguished, and suggested that the technique could be used in an ecological context. It would, however, require PCR-amplification of individual nematodes with at least three different primer sets and could not identify the nematodes without considerable calibration. Since that early example, more practical solutions have been developed.

2.2.1. DNA Barcoding or sequencing

DNA barcoding, based on the sequencing of a small segment of the genome, in the form of a specific sequence which carries both the species-specific and phylogenetic information of an organism, and which provides taxonomic identification for a specimen, is a technique that should be applicable to all organisms (Floyd *et al.*, 2002; Blaxter *et al.*, 2004; Hamilton *et al.*, 2009; Powers *et al.*, 2009). DNA barcodes can be used in the identification of unknown specimens, to assist the phylogenetic placement of unknown taxa through

comparison with known reference sequences, and to enable the definition of molecular operational taxonomic units (MOTUs). Theoretically, this should allow rapid and high-throughput identification, either of individual organisms or of sequences isolated from an environmental DNA sample. DNA barcoding has been used to perform surveys of nematodes, tardigrades and other meiofauna in terrestrial and marine habitats (Floyd *et al.*, 2002; Waite *et al.*, 2003; Blaxter *et al.*, 2004; Hamilton *et al.*, 2009; Powers *et al.*, 2009). For example, Floyd *et al.* (2002) used PCR amplification products from 166 individual cultured specimens to analyse MOTUs of 74 randomly sampled individuals from their study site of a hill farm grassland ecosystem. They also developed a simplified system that would permit diversity and abundance estimation of nematodes in soils, and then suggested modifications to make the method applicable to community analysis.

Hamilton *et al.* (2009) extracted faunal DNA directly from soil samples, and then used PCR with metazoan specific primers and sequencing to characterise micro- and meso-faunal community composition. The technique captured the more abundant faunal groups (nematodes, Collembola, Acari, tardigrades, enchytraeids) and provided sufficient taxonomic resolution to describe the overall structure of the soil faunal communities, although the nematodes were only separated into two major taxonomic classifications (Chromadorea and Enoplea). Powers *et al.* (2009) estimated nematode diversity and nematode distribution among soil, litter, and understorey habitats based on MOTU analysis in a tropical rainforest.

The effectiveness of barcoding is dependant on the identity of the standardised gene region that is selected. To date, barcoding has been tested most extensively in the animal kingdom using a 648-bp region of the cytochrome *c* oxidase 1 (*CO1*) gene (Seifert *et al.*, 2007). Much of the research undertaken on soil and marine nematodes has used the 18S rRNA gene although in the case of Bhadury *et al.* (2006) a range of gene targets were used, including *CO1*, but reliable PCR amplification was only obtained from the 18S rRNA gene (Floyd *et al.*, 2002; Bhadury *et al.*, 2006). More recently the *CO1* gene was used successfully to identify filarial nematodes (Ferri *et al.*, 2009). An advantage of the 18S rRNA gene is the large amount of sequence data available, but there may be future developments to align nematode barcodes with those used for other animal phyla.

The next step in using a sequencing approach is the application of pyrosequencing or other of the so-called 'next generation' sequencing technologies. This approach is almost untried for the analysis of nematode communities, although Porazinska *et al.* (2007) presented results strongly supporting the suitability of 454-technology for identification of all

nematode individuals from environmental samples. Porazinska *et al*. (2009) assessed the suitability of massively parallel sequencing technologies (GS FLX Roche ╱ 454 Life Sciences) for analysis of artificially-assembled nematode diversity from metagenomic samples, involving 41 diverse reference nematodes of known identities and densities. He revealed that although neither rDNA section recovered all nematode species, the use of both SSU rDNA and LSU rDNA loci improved the detection level of nematode species from 90 to 97%. They also used seven datasets, which represent three, independent, in-house PCR amplifications and independent sequencing replicates, that come from replicated experiments using the same multi-nematode species DNA template, and identical amplification and sequencing conditions to test the reproducibility of these metagenetic experiments. Their results suggested that both qualitative and quantitative data were consistent and highly reproducible. Variation associated with in-house PCR amplification or emPCR and sequencing were present but the representation of each nematode was very consistent from experiment to experiment and supported the use of read counts to estimate relative abundance of taxa in a metagenetic sample (Porazinska *et al*., 2010). At this point, however, the use of the distribution reads for inferring the relative abundances of species within a nematode community is premature. However, the use of such technology for monitoring is probably inappropriate as real strength lies in the ability to extract enormous amounts of information from relatively few samples (Poinar *et al.*, 2006), whereas the requirements for biological monitoring are for relatively little information from enormous numbers of samples.

In the paper, van Megen *et al*. (2009) made a selection of *ca* 1200 (nearly) full-length SSU rDNA sequences from representatives throughout the phylum Nematoda. It is the most species rich and diverse nematode tree based on molecular data published so far and, it is biased towards terrestrial nematodes living in moderate climate zones. It is, still incomplete even at the ordinal level, and it is based on only a single gene. Nevertheless, it provides numerous insights into the evolutionary relationships within the Nematoda in all its trophic and ecological diversity. This framework can be used to (quantitatively) detect single targets in highly complex DNA backgrounds, such as specific plant parasites in a soil community.

2.2.2. PCR-DGGE

Several research teams have already attempted to analyse soil or marine nematode communities using denaturing gradient gel electrophoresis (DGGE) (Waite *et al.*, 2003;

Foucher *et al.*, 2004; Bhadury *et al.*, 2006; Okada and Oba, 2008). PCR-DGGE has been used to estimate nematode diversity in soil, by detecting nematode taxa as different bands of equal size PCR products that are separated according to their DNA sequence on a denaturing gradient gel.

Waite *et al.* (2003) also used DGGE to analyse nematode communities from DNA directly extracted from a single gram of soil. This latter approach gave a community 'finger-print' which differed between sites, but from which it was not possible to infer much about the genetic diversity or relative abundance of any particular taxa (Waite *et al.*, 2003). Foucher *et al.* (2004) extracted nematodes from soils prior to DNA extraction, a method that allows larger soil samples to be analysed (100-200g soil, more representative of environmental conditions than 1g soil) and minimises the risks of amplifying non-target organisms, but similarly without making any analysis of the taxa present or their abundance from the molecular data. Sato *et al.* (2009) examined the relationship between the damage to radish by *Pratylenchus penetrans* and the soil nematode community structure analyzed by PCR-DGGE, suggesting that the damage to radish might be predicted from the soil nematode community structure. An evaluation of the soil nematode community structure by PCR-DGGE could be used to distinguish soils that could suffer low damage from those that could suffer high damage.

From a soil monitoring point of view, the difficulty in relating bands to particular functional groups or taxa would be a disadvantage. Although PCR-DGGE is used for bacterial biodiversity in the Dutch Soil Monitoring Network (Rutgers *et al.*, 2009), just the number and intensity of bands are used. This method lacks the taxonomic or functional assessment that makes other nematode community analyses so useful.

2.2.3. PCR-TRFLP

Terminal restriction fragment length polymorphism (TRFLP) analysis is a highly reproducible method in which fluorescently labelled products are sized and quantified in an automated DNA sequencer (Liu *et al.*, 1997). TRFLP analysis can either be designed using a pre-existing sequence library to select restriction enzymes to yield diagnostic peaks of interest (directed TRFLP) so that different taxa result in distinct restriction fragment (RF) peaks. Alternatively, it can utilise an essentially random digestion to give a 'fingerprint' with no taxonomic information (random TRFLP). Initially developed for bacterial communities, similar approaches are now available for nematode identification (Donn *et al.*, 2008).

Donn *et al.* (2008) demonstrated TRFLP based on a single, randomly chosen enzyme digest to be sufficient to discriminate between nematode communities extracted from five habitats (arable agriculture, sand dune, coniferous forest, permanent pasture and moorland). Hermann et al. (2008) also used random TRFLP analysis to assess the community structures of protozoa and nematodes, using group-specific PCR primers, showing the potential of TRFLP procedures to differentiate the community structures based on the response of the soil biota to organic inputs (Hermann *et al.*, 2008). Gibb *et al.* (2008) analyzed soil mite assemblages using TRFLP, and found that the mite community was represented by a series of DNA fragment lengths that reflected mite sequence diversity. Their approach was to use a random TRFLP coupled with a reference sequence database of identified mites, but the current paucity of soil mite sequences in the public databases was a limiting factor (Gibb *et al.*, 2008). Another approach is directed TRFLP, where sequence information is first obtained, and the sequences are analysed to find an enzyme combination that would separate the taxa present to provide relevant ecological information (Donn *et al.*, 2008). As a result every peak in the community profile can be allocated to a nematode taxon at a level allowing inference of function from the putative trophic group (Donn *et al.*, 2008). The main advantage of TRFLP is the ability to easily compare data from different sequencer runs unlike DGGE where comparison between gels is difficult (Moeseneder *et al.*, 1999; Nunan *et al.*, 2005). The inclusion of a size standard with every sample allows accurate sizing of fragments and thus comparison across electrophoretic runs (Marsh, 1999). Fragments can be sized accurately up to 700 bp; beyond this a small degree of error may be associated with sizing up to 1000 bp (Osborn *et al.*, 2000). Automated sample loading with a capillary sequencer reduces variability between runs and the digital output allows rapid, objective data analysis (Osborn *et al.*, 2000). Community fingerprints from TRFLP can also be translated into taxonomic information especially with a directed approach (Donn *et al.*, 2008; Gibb *et al.*, 2008). The ability to perform TRFLP in 96 or 384 well plate format, coupled with the electronic data output and automated fragment calling software means it is a truly high throughput technique with rapid data analysis. This permits large scale temporal and spatial studies with the replication required to lend statistical power to the analysis and make the technique ideal for monitoring purposes.

2.2.4. Real-time PCR

Real-time quantitative PCR-based detection is more rapid, specific and sensitive than conventional PCR (Bustin, 2002; Schaad and Frederick, 2002; Gachon *et al.*, 2004), and

widely used for the quantification of species of particular economic importance. Real-time PCR was, for example, able to detect DNA at a concentration eight times lower than conventional PCR (Gachon *et al.*, 2004). Potato cyst nematodes, for example, can be distinguished by morphological differences and their ability to reproduce on various hosts (Nowaczyk *et al.*, 2008; Quader *et al.*, 2008), but real-time PCR methods for the simultaneous detection and quantification of species of potato cyst nematodes have been developed (Bulman and Marshall, 1997; Bates *et al.*, 2002). The real-time PCR method was more rapid, reliable and easier to interpret, especially when few cysts were available, than morphological and reproduction tests and so represent a convenient alternative for studies with a limited amount of starting material (Bustin, 2000).

Jones *et al.* (2006a) used a combination of low power microscopy and taxon-specific real-time probes, which was the first time showed that 18S probing successfully assigned all nematodes to their specific taxonomic group, and then real-time PCR, using multiplexed probes and primers specific to that subset of taxa, could further diagnose nematode taxonomy down to the species level. The development of real-time PCR to assess nematode taxonomic identity within the microbial-feeding nematodes on the Konza Tallgrass Prairie enabled the identification of 19 microbial-feeding nematode taxa across four families, and positively assigned the majority of nematodes to genus/species groups 96% and 83%, respectively (Jones *et al.*, 2006b).

Sato *et al.* developed a direct quantification method, consisting of soil compaction and real-time polymerase chain reaction (PCR), for the soybean cyst nematodes *Heterodera glycines* (Goto *et al.*, 2009) and *Pratylenchus penetrans* (Sato *et al.*, 2010) in soil. In the proposed method, nematodes at any stage, including cyst, egg and vermiform, were destroyed by compaction to release their DNA and their numbers are quantified by real-time PCR with primers specific to the nematodes. In addition, Sato *et al.* (2011) reported this method for *Pratylenchus penetrans* in the soils of radish fields, to reveal the relationship between density of *P. penetrans* and damage to radish caused by *P. penetrans* in order to evaluate the economic threshold in radish cultivation. Their results suggest that the combination method has an advantage in the estimation of damage to radish by the nematode.

A current development is towards a DNA-based version of the Maturity Index (Bongers, 1990), made possible as many nematode families appear as monophyletic groups in a phylogenetic tree (Powers, 2004). Primers designed to quantitatively monitor each of these

families within a nematode community will provide a community analysis based on molecular rather than morphological traits (Neilson *et al.*, 2009).

2.3. Comparison of molecular vs. morphological technique

Demonstrating a clear correlation of the analytical results between those obtained by conventional morphological methods and those by developing molecular methods is of key importance in the transition from the current mainly morphological approaches to future molecular scenarios. Molecular techniques need to reflect the relative abundances of the particular species or functional guilds (Yeates and Bongers, 1999).

Griffiths *et al.* (2006) combined morphological identification and molecular sequencing to establish the potential for analysing nematode communities by molecular biological characterization, but found that nematodes from the Rhabditida and Tylenchida were under-represented in the molecular characterisation compared to the morphological method. Better relationships between the quantification of nematode types and the amount of nematode DNA, at least the total biomass of each type, rather than abundance of each type, made it difficult to use some molecular data to calculate nematode assemblage as ecological indices. In their study of mite communities, Gibb *et al.* (2008) argued that there will never be complete convergence between the two methods (molecular and morphological) because there are just too many steps where 100% recovery or efficiency is not achieved with either methodology. The reasons for this are varied and often unknown, but can occur anywhere from the point of faunal or DNA extraction through to a bias in PCR amplification. DNA from all organisms present in a sample may not be amplified with the same efficiency or sub-sampling inherent in morphological methods may prevent all individuals from being identified (Wang *et al.*, 2004).

There are more and more research works which have compared molecular and morphological techniques. Okada and Oba (2008) compared the results on nematode communities between DGGE and morphological identification. The correlation coefficients between the two methods were not extremely large (0.400-0.603), but they were always significant. Interestingly the authors argued that the analytical result obtained by morphology may not always reflect the 'true' community because only 150 individuals per sample were examined without replication, due to the laboriousness of the morphological identification (Okada and Oba, 2008). Wang *et al.* (2008) assessed the effect of copper contamination on soil nematode diversity by PCR-DGGE and morphological analysis, and found that

PCR-DGGE could give more information on nematode genera, and the intensity of the bands could reflect the abundance of nematode genera in the assemblage.

Hamilton *et al.* (2009) compared the results obtained using a DNA sequence-based molecular approach to results obtained using a traditional, microscopy-based approach and found that the results were broadly similar. However, since biases were inherent in both methods it is unclear which method provided a more accurate assessment of community composition. Although the sequencing approach was based on a relatively small number of sequences (65–88) the taxonomic identification sequencing can provide will be more accurate and consistent across research groups, facilitating effective comparisons of meso-faunal surveys (Hamilton *et al.*, 2009). Bhadury *et al.* (2008) also compared morphological data with PCR-DNA sequencing. They concluded that current taxonomy based on morphological characters detected using light-microscopy may be unable to discriminate possible species complexes and that the biodiversity of marine nematodes may often be underestimated due to the presence of morphologically cryptic species complexes.

2.4. The role of nematodes in monitoring schemes for soil quality and biodiversity

It is probable that any monitoring scheme will use a suite of indicators and not rely solely on any single biological indicator. For example the 'environmental assessment of soil for monitoring' (ENVASSO) project selected three separate tools, one representative from the macrofauna, mesofauna and microflora (Gardi *et al.*, 2009). The Dutch soil monitoring network (BISQ), lists 25 soil parameters of which the density and diversity of nematodes are included (Rutgers *et al.*, 2009); while in a framework for selecting candidate soil indicators, Ritz *et al.* (2009) identified nematodes as a valuable indicator in a suite, further differentiating between the morphological identification of nematodes (which is currently available) and a molecular technique (PCR-TRFLP) which would be ready for deployment in the short-term.

Although existing monitoring schemes successfully use morphological methods to assess nematode communities, such as the Biological indicator-system for Soil Quality (BiSQ) which was designed in 1997 for the Dutch Ministry of Housing, Spatial Planning and the Environment (Schouten *et al.*, 2002). The major disadvantage for morphology is the specialised taxonomic expertise needed and laborious nature of the task. Molecular approaches are developing rapidly and should become more common place once the

comparison between morphological and molecular methods is defined. The major advantage of a faunal approach is that a direct comparison between morphological and molecular methods can be undertaken, something not possible for microorganisms.

Chapter 3. Long-term effects of P fertiliser on nematode community structure and soil microbial community structure

3.1. Introduction

The chemical element phosphorus (P) is essential for plant and animal life. It is one of the least available of all essential nutrients in the soil and its concentration is generally below that of many other micronutrients (Zvomuya *et al.*, 2006). Low availability of P is a major constraint for crop production in many low-input systems of agriculture worldwide (Raghothama, 1999). Many soils in the world, when unfertilized, are relatively low in phosphorus, and this can have adverse effects on plant and animal health. Meanwhile, its low natural concentration and low solubility in the soil, makes it commonly a key growth-limiting nutrient in soils and waters (Sharpley *et al.*, 2001). Phosphorus therefore usually controls primary productivity in most natural ecosystems, both terrestrial and aquatic.

In Ireland, historically up to the Second World War soil P levels were extremely low. During the period of 1941-1946, P inputs to agriculture were lower than P removals. Lack of P limited plant and animal production up to the 1950s before the rapid increase in use of chemical P fertilizer in the 1960/70s, which was helped by a government subsidy on P fertilizer to correct P deficiency (Tunney, 1990). Use of P fertilizer then stabilized at just over 60 000 t ∕ yr from the early 1980s to the mid 1990s. Since 1996 there has been a downward trend in P fertilizer use, mainly because research indicated that P inputs were in excess of outputs and concerns that these P inputs were a contributory factor in eutrophication of surface waters (Foy *et al.*, 1997; Tunney, 2002). Usage has dropped in recent years to approximately 50,000t in 2000 (Culleton *et al.*, 2002).

In 2006, the Nitrate Directive (through Statutory Instruments) S.I. 378 (Anon, 2006), which demands that nutrient application rates do not exceed grass demand, nor result in nutrient losses that may have a negative impact on water quality, was implemented in Ireland, aimed at reducing nutrient losses from agriculture to water bodies, i.e. surface waters, groundwater and estuarine waters. This legislation introduced strict regulation of nutrient management on Irish farms. Although the fate of phosphorous when applied to soil remains something of an enigma (Fey, 1988), it is widely accepted that a combination of biological, chemical, physical, and environmental properties and processes, together with the history

and intensity of grassland use and management determine the form, dynamics and mobility of P in soil-plant-animal-water system (Condron *et al.*, 1996).

A long term trial in grassland, the Cowlands Trial experiment at Johnstown Castle Research Centre, Co. Wexford, was established in 1968 with replicate plots receiving different amount of P fertilizer, and has continued over since. The aim of the experiment was to determine the optimum rate of phosphorous (P) fertilizer application required for grassland grazed by beef cattle. In these plots, research has been done to assess the effects of P application on soil fertilization, nutrient loss, soil physical and chemical properties. For examples, Tunney *et al.* (1990, 2000, 2002, 2010) published a series of papers about the impacts of P fertilizer on the changes in Morgan's soil test P, P nutrient loss from soil to water, and live weight gain of mature beef animals over the ten years (1999-2008). They found that soil test P, as measured by Morgan's extractant, stabilized over the past 10 years on the three original treatments, while it has decreased significantly where P application was stopped on the highest P treatment and has increased significantly where P was applied on the zero P treatment. Kurz *et al.* (2005) assessed the effects of grassland management practices on the loss of nutrients from agricultural land to water bodies, and found there was a clear increase in P loss in overland flow from the low to the medium and high soil P sites. McGrath and Tunney (2010) determined that after 31 years P fertilizer application increased heavy-metal content in Cowlands grassland. Fu (2009) examined the spatial variation of soil test phosphorus (STP) at the Cowlands, and found a log-normal distribution, with a median of 5.3 mg L^{-1} and an arithmetic mean of 6.2 mg L^{-1}. Also he found statistically positive correlations between soil P and pH, soil P and Mg and soil P and K. King-Salter (2008) studied the response of arbuscular mycorrhizal fungi in the Cowlands to long-term phosphate fertilisation, and found that phosphate application had a significant effect on the composition of the plant community, and altered the arbuscular-mycorrhizal fungal community.

From the above work, most studies dealing with the application of P fertilizer have been interested in how it influences soil nutrient, plant performance, leaching losses and soil chemistry, and animal production. It is clear that little information is available on the consequences of historic P-fertilizer use on soil microbial community and soil nematodes, which are good indicators in soil nutrient decomposition and soil food web (Ferris *et al.*, 2001).

Better knowledge of factors affecting turnover of the microbial biomass, and the information on soil nematode community structure would improve current understanding of

nutrient fluxes in P-treated soils. The soil micro-fauna, i.e. protozoa and nematodes, are important consumers of the microbial biomass (Forge *et al.*, 2003), and are therefore indicative of microbial turnover and flux of nutrients through the soil food web. Soil animals may affect microbial communities directly, by feeding on various microorganisms, or indirectly, by mixing and channelling detritus, dispersing microbial propagules and excreting nutrient-rich wastes (Griffiths and Bardgett, 2000). Generally, the overall effects of these microbial faunal interactions are increased rates of nutrient turn-over and plant uptake of available nutrients (Ingham *et al.*, 1985; Bonkowski *et al.*, 2000).

In this chapter, soil nematode community as well as microbial communities and microbial biomass was observed in order to understand the cycling of P in Cowlands grassland. This study also evaluated the potential to use a Terminal restriction fragment length polymorphism (T-RFLP) technique to characterise nematode community structure. The specific objectives of the present study were to:

1) identify nematode abundance and diversity,

2) evaluate changes in nematode families, functional groups or communities through time using traditional morphology and a high-throughput molecular method, and

3) determine the driving factors of nematode community change in response to pasture management intensity.

The identification of specific nematode bioindicators would enable the evaluation of soil quality relatively efficiently and inexpensively. This information would be particularly useful in situations where permanent pasture is used, or where soil chemical analysis would provide limited agroecological resolution.

3.2. Material and methods

3.2.1. Site description and experimental design

The Cowlands Trial (Figure 3.1) was established in 1968 at Johnstown Castle Research Centre, Co. Wexford (52°18'N, 06°30'W). Monthly average precipitation and air temperature for the period of study are shown in Figure 3.2. Initially, three P treatments were set up, with fertilization rates of 0, 15 and 30 kg P ha^{-1} yr^{-1} (subsequently referred to P0, P15 and P30) in the form of calcium super phosphate. Nitrogen and potassium were applied evenly across all plots, at mean annual application rate of 240 kg N ha^{-1} as ammonium nitrate and 20 kg K ha^{-1} as potassium chloride. Each year, P and K were applied between late February and early March, and nitrogen was spread between March and September. With each P treatment, the 12 plots were divided between a low stocking rate of

2200 kg stock ha^{-1} and high stocking rate of 3300 kg stock ha^{-1}, each with six replicates. This regime continued unaltered from the establishment of the trial in 1968 through to 1998, but in 1999 the treatments were altered. From 1999 onwards all plots had the same stocking rate (3300 kg stock ha^{-1}) but, six of the 12 P0 plots now received 30 kg P ha^{-1} yr^{-1} (P0-30), six of the 12 P15 plots received 5 kg P ha^{-1} yr^{-1} (P15-5), and six of the 12 P30 plots received no P (P30-0). The N and K fertilisation regime on the plots continued unaltered, so that from 1999 onwards there were six P treatments, each with six replicate plots (See Table 3.1 for details, Culleton *et al.*, 2002).

3.2.2. Soil nutrient analysis

3.2.2.1. Soil samples collection and preparation

Soil samples were taken in May 2009, October 2010 and April 2011. Thirty cores in a stratified random design were taken using an auger to a depth of 10cm from each plot and mixed together to give a composite sample from each plot. Prior to nematode extraction and soil microbial analysis, all samples were stored at 4°C. Moisture content of fresh soil was determined by drying at 105°C for 12 h, while samples for nutrient analysis were dried at 40°C for 24 h.

At the first sampling time, only soil nematodes and microbial biomass were measured. Considering more useful information compared to nematodes community, total soil bacterial DNA and PLFA were measured from the second and third samples, as well as soil nematodes and microbial biomass. In order to understand the application on P on soil nutrient, the third soil samples were used to analyze soil total P, Morgan's P, Morgan's K and Morgan's Mg, pH and Lime requirement.

3.2.2.2. Soil analysis

Soil total P: The P analysis of a soil sample is carried out using British Standards ISO 11466:1995, extracted soluble in Aqua Regia (3:1 mixture of hydrochloric acid and nitric acid, which is used to dissolve the organic matrix of the soil). Following extraction, phosphorus is analysed colorimetrically using the chemical reaction between P and ammonium molybdate. Phosphorus is measured on a Camspec 230 UV spectrophotometer at 675 nm.
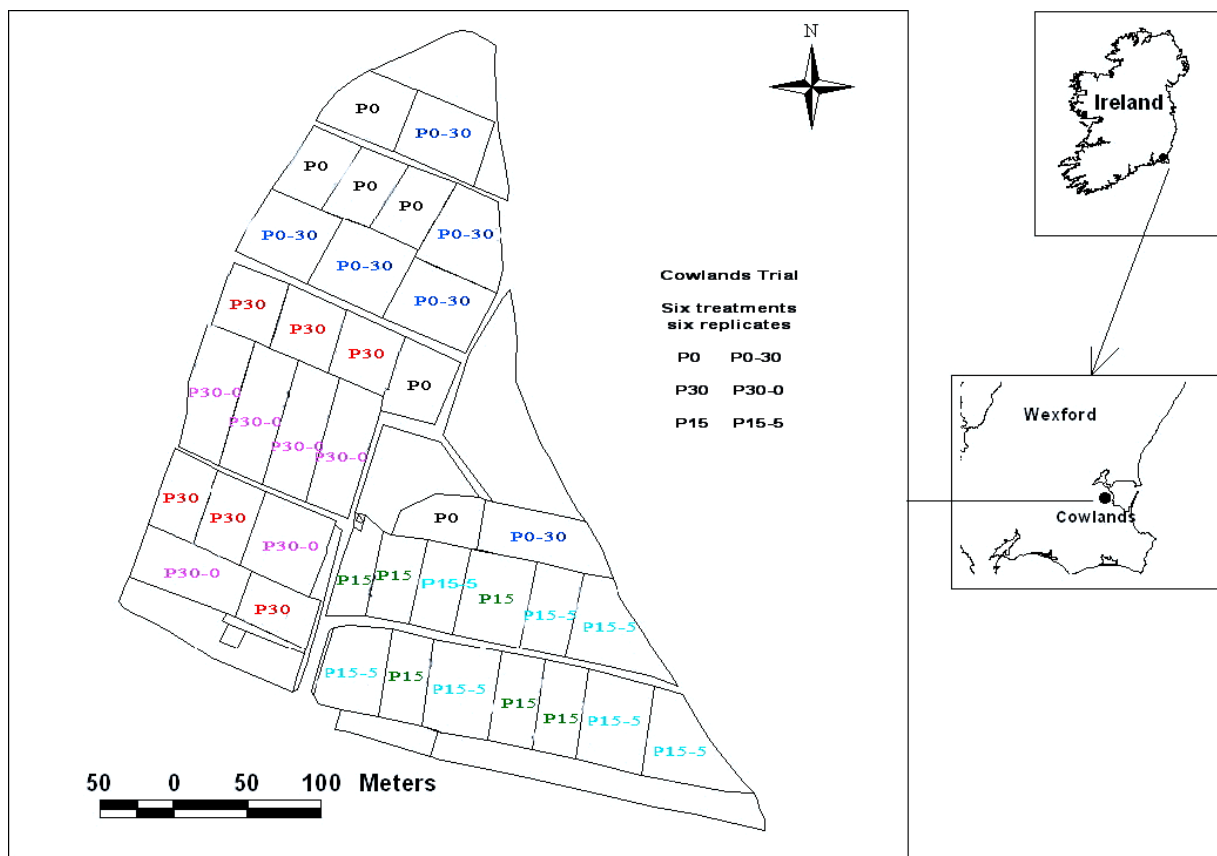
Figure 3.1. Map of the Cowlands trial experimental field (Wexford, Ireland), showing layout of six replicate plots for each of six P treatments. Treatments P0, P15 and P30 were originally high-stocking rate plots and have had constant P application and stocking rates since 1968. Treatments P0-30, P15-5 and P30-0 were originally low-stocking rate plots and P applications were changed in 1999, while stocking was increased to the high-stock level.

Table 3.1. Historical and current P applications and stocking levels for six treatments of the Cowlands trial. Each P treatment has 6 replicate plots.

| P treatment | 1968-1998 | | 1999-2009 | |
| | P application | Cattle stocking rate | P application | Cattle stocking rate |
	Kg ha^{-1} yr^{-1}	Kg ha^{-1}	Kg ha^{-1} yr^{-1}	Kg ha^{-1}
P0	0	3300	0	3300
P0-30	0	2200	30	3300
P15	15	3300	15	3300
P15-5	15	2200	5	3300
P30	30	3300	30	3300
P30-0	30	2200	0	3300
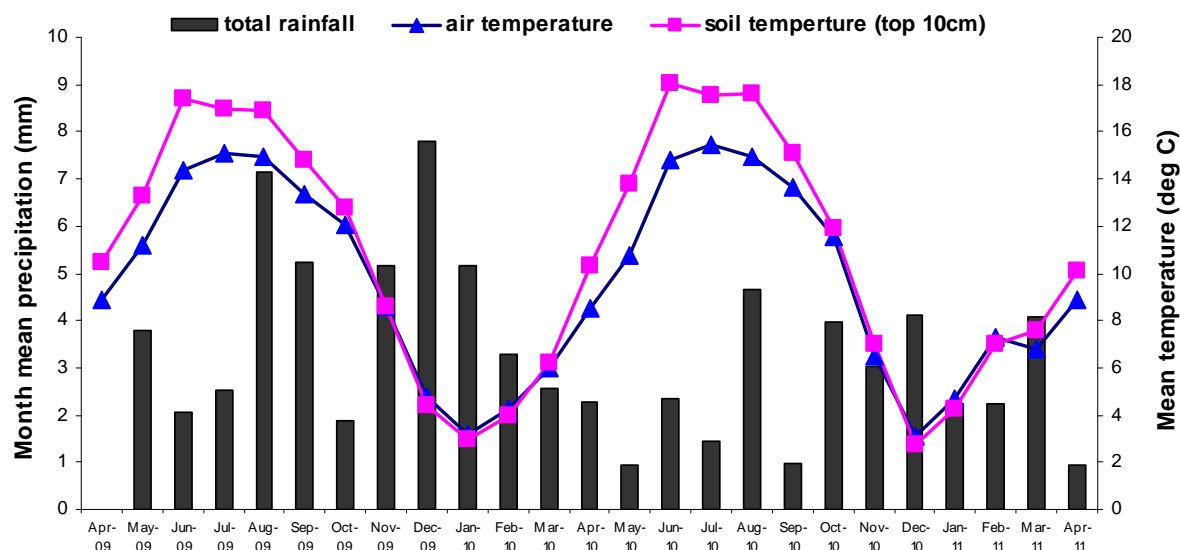
Figure 3.2. Mean precipitation, air and soil temperature per month from April 2009 to April 2011. Climatic data were provided by the monitoring station at Johnstown Castle, courtesy of Met Éireann (Glasnevin, Dublin, Ireland).

Morgan's P, Morgan's K and Morgan's Mg: Soil test P, K and Mg were determined using Morgan's extraction solution (10% sodium acetate in 13% acetic acid buffered at pH 4.8). A 8ml volume of soil was mixed with 40 ml of Morgan's extraction solution and shaken for 30 min. The suspension was filtered into an Istamec Kasette Track of disposable test tube. The Kasette Track was then placed on the automated Istamec Transporters for analysis.

Lime requirement (LR): Soil LR was measured by a pH buffer solution method (Shoemaker *et al*., 1961), in which 10g soil was mixed with 20 ml SMP buffer solution (1:2 soil: buffer), and stood for 15 min before recording pH with a Thermo Orion 420 pH meter.

pH: Soil pH was measured in deionised water (Thomas, 1996). 10 g oven-dried soils were mixed with 10 ml deionised water in a 100 ml beaker. After 10 min, pH was recorded with a Thermo Orion 420 pH meter.

3.2.3. Soil sampling and nematode extraction

Nematodes were extracted from 100 g composite samples using the Oostenbrink Elutriation method followed by Baermann funnel extraction over 48 h (Verschoor and de Goede, 2000).

The resulting nematode suspension was vigorously shaken and divided into two equal parts. Nematodes in one part were killed at 65°C for 4min, preserved by 0.4% formaldehyde, and

the first 150 individual identified microscopically according to (Bongers, 1988) for the 2009 samples, while for the nematode samples in 2010 and 2011, only nematodes in the P0, P15 and P30 treatments were identified. Nematodes in the second part were allowed to sediment, most of the overlying water pipetted off and the nematode pellet in approximately 300 µl water was stored frozen at -20°C until DNA extraction.

3.2.4. Nematode DNA extraction and amplification

DNA was extracted as described by Donn *et al*. (2008). Briefly, nematode samples stored in 2ml tubes were thawed and beaten for 2 min (Mini Beadbeater-8, Biospec Products, Bartlesville, USA) with 0.05 g 0.50-0.75 mm diameter sterile glass beads (Thistle Scientific, Glasgow, UK) in 1.2ml of extraction buffer from a PureLink PCR purification kit (Invitrogen, Paisley, UK). Following beating, samples were heated to 95°C for 3 min, and purified (PureLink PCR purification kit) as per manufactories' instructions. DNA eluted in 50 µl PCR elution buffer and then stored at -20°C. Small subunit ribosomal DNA (18S rDNA) was selectively amplified using the primers: Nem_SSU_F74 (AARCYGCGWAHRGCTCRKTA) and fluorescently labelled VIC-Nem_18S_R (GGGCGGTATCTRATCGCC) (Eurofins MWG Operon, Ebersberg, Germany) (Donn *et al.*, 2011). PCR was performed in a 50 µl final volume containing: 5 µl 10× PCR buffer (Bioline, London, UK); 4µl 50 mM $MgCl_2$; 4µl 10 pM of each PCR primer; 1 µl of 10 mM dNTP mix; 4 µL DNA template; 27.6µl distilled water and 0.4µL of 4 units Start *Taq* polymerase. All PCRs were performed on a G-STORM Thermal Cycler (Gene Technologies Ltd., Braintree, Essex, UK) with the following run parameters: one initial denaturation cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 51°C for 30 s, and 68°C for 1 min. A final elongation step was run at 68°C for further 10 min. Positive (DNA extracted from mixed nematodes, confirmed by preliminary study) and negative (distilled water) controls were included for each amplification series.

3.2.5. Directed T-RFLP

Terminal restriction fragment length polymorphism (T-RFLP) was applied to the amplified nematode community DNA. An enzyme mix was made consisting of 1 x NEBuffer4 (20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol (pH 7.9)), 100 µg ml^{-1} BSA (supplied with enzyme) and 2 units PleI per µl enzyme mix (all reagents from New England Biolabs, Hitchin, UK). 1 µl PleI enzyme mix was added to 10 µl PCR products which were digested at 37°C for 60 min, followed by

65°C for 20 min, to denature the enzyme. Products were then digested with 2 units BtsCI (diluted as above in 1 x NEBuffer4 with BSA) by incubating at 50°C for a further 1 h. Digest products were diluted 1 in 10 and 1 μl was mixed with 9 μl Hi-Di™ Formamide and 0.05 μl ROX labelled MapMarker 1000 (BioVentures, Murfreesboro, Tennessee, USA). Fragments were analysed on an ABI 3730 capillary sequencer, at the James Hutton Institute, Dundee, UK.

3.2.6. Soil microbial properties

Microbial Biomass C, N and P: Soil from each composite sample was sieved through a 2-mm diameter mesh and stored at 4°C prior to analysis. Soil microbial biomass C (MBC), N (MBN) and P (MBP) were determined by the chloroform-fumigation extraction method (Joergensen, 1995). 10 gram aliquots of field-moist soil were fumigated with ethanol-free chloroform for 24 h, and non-fumigated soils were incubated at the same room temperature. Both fumigated and non-fumigated soils were extracted with the ratio 1:4 of soil: 0.5 M K_2SO_4 (for MBC and MBN) or the ratio 1:20 of soil: 0.5 M $NaHCO_3$ for MBP by shaking for 30 min, and then filtrated to cleaned bottles. Both fumigated and non-fumigated filtrated suspensions extracted by K_2SO_4 were measured by TOC-V_{CPH} and TNM-1 (Shimadzu Scientific Instruments, USA) for MBC and MBN. The P in the extracts was measured by a modified ammonium molybdate–ascorbic method (Murphy and Riley, 1962). The k-factors used for converting extractable C, N and P flush to microbial C, N and P were respectively 0.41 (Sparling *et al.*, 2000), 0.45 (Jenkinson *et al.*, 1988) and 0.40 (Brookes *et al.*, 1985). Also all un-fumigated data was used as soil extractable C, N and P in the results.

Bacterial biomass: Soils were sieved (2-mm mesh) immediately after sampling, and were stored at -80°C in plastic bags for soil DNA extraction and PLFA analysis. Soil 16S rDNA was extracted by UltraClean® Soil DNA Isolation Kit (Mumy and Findlay, 2004), and then diluted to 1/100 for quantitative real-time PCR assessment. A bacterial strain, *Escherichia coli* K12 (DSM 4304), was used to construct the standard curves. Genomic DNA was extracted from the pure standard strain culture (Yu *et al.*, 2006). The target 16S rRNA gene sequence was amplified from each genomic DNA sample by conventional PCR with the corresponding primers. The resulting PCR products were purified and cloned into pGEM-T Easy vectors (Promega, Mannheim, Germany). The concentrations of the recombinant plasmids were measured in duplicate using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) with the PicoGreen dsDNA Quantification Reagent (Molecular Probes,

Eugene, OR), and the corresponding copy concentrations were calculated using the equation (Whelan *et al.*, 2003). For each plasmid, a 10-fold serial dilution series ranging from 10^0 to 10^9 copies ml^{-1} was generated and amplified in triplicate using real-time PCR with the corresponding primer and probe sets. Each reaction well contained 2 μl template DNA, 2 μl distilled water, 1 μl primers BCT 338F (ACTCCTACGGGAGGCAG) and BAC (GACTACCAGGGTATCTAATCC) and 10 μl LightCycler 480 probes Master (Roche Applied Science, Germany). The following general real-time PCR protocol was used: preincubation program (94°C for 10 min), a two-segment amplification and quantification program repeated 45 times (94° for 10 s, 60°C for 30 s), and finally a cooling down program to 40°C for 30 s. We used at least three non-zero standard concentrations per assay, and the plasmid DNA concentrations ranged from 1×10^9 to 1×10^3 ng of final reaction. Target copy numbers for each reaction were calculated from the standard curves, assuming that the average molecular mass of a double-stranded DNA molecule is 660 g mol^{-1}.

Bacterial biomass and fungal biomass assessed by phospholipid fatty acid (PLFA) analysis: Subsamples of 10 g soil from each sample were frozen and then freeze-dried for PLFA analysis. PLFAs were extracted from soil according to described methodology (Bossio and Scow, 1998), 5 g freeze-dry soils were extracted with 20 ml a one-phase mixture (1:2:0.8 v/v/v) of chloroform, methanol, and citrate buffer (0.15 M, pH 4.0), and fractionated into neutral, glyco-, and phospholipids on columns containing silicic acid (Frostegård *et al.*, 1991). After centrifugation for 10 min at 2000 rpm, the supernatant was decanted to a clean glass media bottle. In order to separate the organic layer into 2 phases, a further 4ml chloroform and 4ml citrate buffer were added, and then left overnight to allow a clear interface between the two phases. The organic lower layer was dried under a stream of N$_2$ at 36°C, re-suspended with 1ml chloroform, and phospholipids separated from neutral and glycolipids on solid phase extraction columns, (0.50 g Sep-Pak Vas Silica 3cc, Waters Corporation, Milford, Massachusetts, USA) containing 0.5 g sodium sulphate. The column was conditioned with 2ml methanol, acetone and chloroform firstly; lipids were transferred to the column and eluted with 5 ml chloroform, followed by 12 ml acetone; polar lipids were eluted with 8 ml methanol and dried under N$_2$ at 37°C. 200 μl methanol containing Nonadecanoic acid Methyl Ester (19:0) as an internal standard was added to the dried samples and dried again under N$_2$ at 37°C. After that, samples were then subjected to mild alkaline methanolysis by dissolving in 1 ml 1:1 toluene: methanol and 1 ml 0.2 M KOH, and heating at 37°C for 30 min. The reaction was stopped by adding 0.25 ml of 1 M acetic

acid. Resulting fatty acid methyl esters were extracted with 5 ml aliquots of 4:1 v/v hexane: chloroform and 3 ml deionised water, sonicated for 30 minutes, and left overnight to separate two phases. Sodium hydroxide was used here as a base wash to clean the samples and remove any underrivatised fatty acids or other acidic components. The top layer was added with 3 ml of 0.3 M NaOH and filtered the top layer through sodium sulphate to a clean glass media bottle, and evaporated to dryness under N_2 at 22°C. Samples were then redissolved in 200 μl hexane and were ready to inject into G.C., and measured by gas chromatography. The weights of individual PLFAs were measured as ng g^{-1} dry soil. Total weight of PLFAs was used as a measure of total microbial biomass.

A total of 26 different PLFA were detected and identified. The biomass of bacteria was determined using the combined weights of fatty acids i15:0, a15:0, 15:0, i16:0, i17:0, cy17:0, 17:0 and cy19:0. That of fungi was determined as the sum of 18:2ω6c, 18:1ω9c and 18:1ω9t (Frostegård and Bååth, 1996; Bossio and Scow, 1998; Mikola and Setälä, 1998).

3.2.7. Statistical analysis

All results were expressed on the basis of the oven-dry soil (12 h at 105°C). Means (n = 6) and their standard error (SE) of the measured variables are presented. The significant difference among the fertilization treatments at each sampling time was tested by one-way ANOVA followed by the Fisher LSD test.

To define the nematode community/assemblage morphologically nematodes were assigned to five main trophic groups (bacterial feeders (Ba), fungal feeders (Fu), plant-feeders (Pl), omnivores (Om) and predators (Pr) after Yeates *et al.* (1993). Additionally, the ratio of fungivore decomposers to bacterivore (Fu/Ba), was calculated to reflect the main pathways in the soil decomposition processes (Yeates, 2003). From directed T-RFLP analysis, the relative abundance of T-RFLP peaks corresponding to nematode families, and thus the five main trophic groups (Donn *et al.*, 2011) were analysed. Peaks in the directed T-RFLP profiles were assigned to nematode taxa on the basis of sequencing from nematode assemblages and design of the enzyme digest. A number of small peaks (representing < 5 % of the total peak area) were assigned to taxa on the basis of in *silico* digest of database sequences (Table 3.2).

Principal component analysis (PCA) was used to determine differences in nematode community composition and PLFA community. Data were tested by one-way ANOVA followed by the Fisher LSD test ($P < 0.05$).

All statistical analyses were performed using the Statistica software package (StatSoft Inc.,

Tulsa, OK, USA). Analysis of variance (ANOVA) was used to quantify the variations of soil test, which included parameters of soil nutrient, nematode community, microbial biomass, bacterial and fungal biomass among the treatments.

Table 3.2. T-RFLP peaks were assigned to nematode taxa. Source indicates whether the peak was assigned by sequencing or in *silico* digest of database sequences.

T-RFLP peak size	Taxa	Source
132	Rhabditidae, *Panagrolaimus*	sequencing
137	Rhabditidae, *Mesorhabditis*	database
160	Monhysterida	database
226	Aphelenchida, *Aphelenchoides*	sequencing
296	Tylenchidae	sequencing
308	Dorylaimida	sequencing
325	Mononchida*	sequencing
356	Tylenchidae	database
366	Plectida	sequencing
466	Aphelenchoididae	database
470	*Rhabditoides*	database
579	*Mesorhabditis*	database
585	Tylenchidae	sequencing
588	*Pratylenchoides*	sequencing
592	*Helicotylenchus*	sequencing
598	*Rhabditis*	sequencing
605	Dorylaimida, *Longidorus*	sequencing
622	Nygolaimidae*	database
636	Aphelenchidae	database
639	Cephalobidae	sequencing
768	Cephalobidae	sequencing

*Mononchida: only found *Anatonchus* in morphological data; *Nygolaimidae: no this family found in morphological data, so calculated to family Dorylaimida.

3.3. Results

3.3.1. Soil nutrient characteristics

Soil nutrient data came from the third sampling time (2011) for the six P treatments of the Cowlands Trial are shown in Figure 3.3. Both P fractions (total and available) were affected by the rate of P application. Total soil P ranged from 393 g kg^{-1} in P0 to 930 g kg^{-1} in P30 (Figure 3.3A), and was positively correlated ($r = 0.97$, $P < 0.001$) with the sum total of P applied to the plots (Figure 3.4A). Soil available P measured by Morgan's P ranged from 2.7 g kg^{-1} in P0 to 18.6 g kg^{-1} in P30 (Figure 3.3B), and also was positively correlated ($r = 0.96$, $P < 0.001$) with the sum total of P applied to the plots (Figure 3.4B).

Soil available K did not differ significantly between P treatments (Figure 3.3C), while other data showed significant differences between P treatments. Morgan's Mg ranged from 138 g kg^{-1} in P0 to 280 g kg^{-1} in P15-5 (Figure 3.3E), and pH measured in water varied from pH 5.5 in P0 to pH 6.1 in P15-5 (Figure 3.3F), indicating the similar tendency that treatments

P15-5, P0-30 and P15 showed significantly higher than the values from other treatments. The data from soil lime requirement was different to other nutrient data, higher in P0 with 5.6 t ha^{-1}, while was lower in P15-5 with 1.8 t ha^{-1} (Figure 3.3D), and was negatively correlated (r = 0.98, $P < 0.001$) with pH (Figure 3.4C).

The effects on soil P fractions of P applied during the two time periods (1968-1998 and 1999-2011) were different. For soil total P, P15 and P15-5 were similar, while from P0 to P0-30, and from P30 to P30-0, while total P has decreased significantly where P application was stopped on the highest P treatment (from P30 to P30-0) and has increased significantly where P was applied on the zero P treatment (from P0 to P0-30). For the Morgan's P, the differences were not obvious, and available P was only increased significantly from P0 to P0-30 (Figure 3.4). From the PCA plot (Figure 3.5), it can be seen that soil nutrients varied between treatments, but the two P applied time periods showed differently.
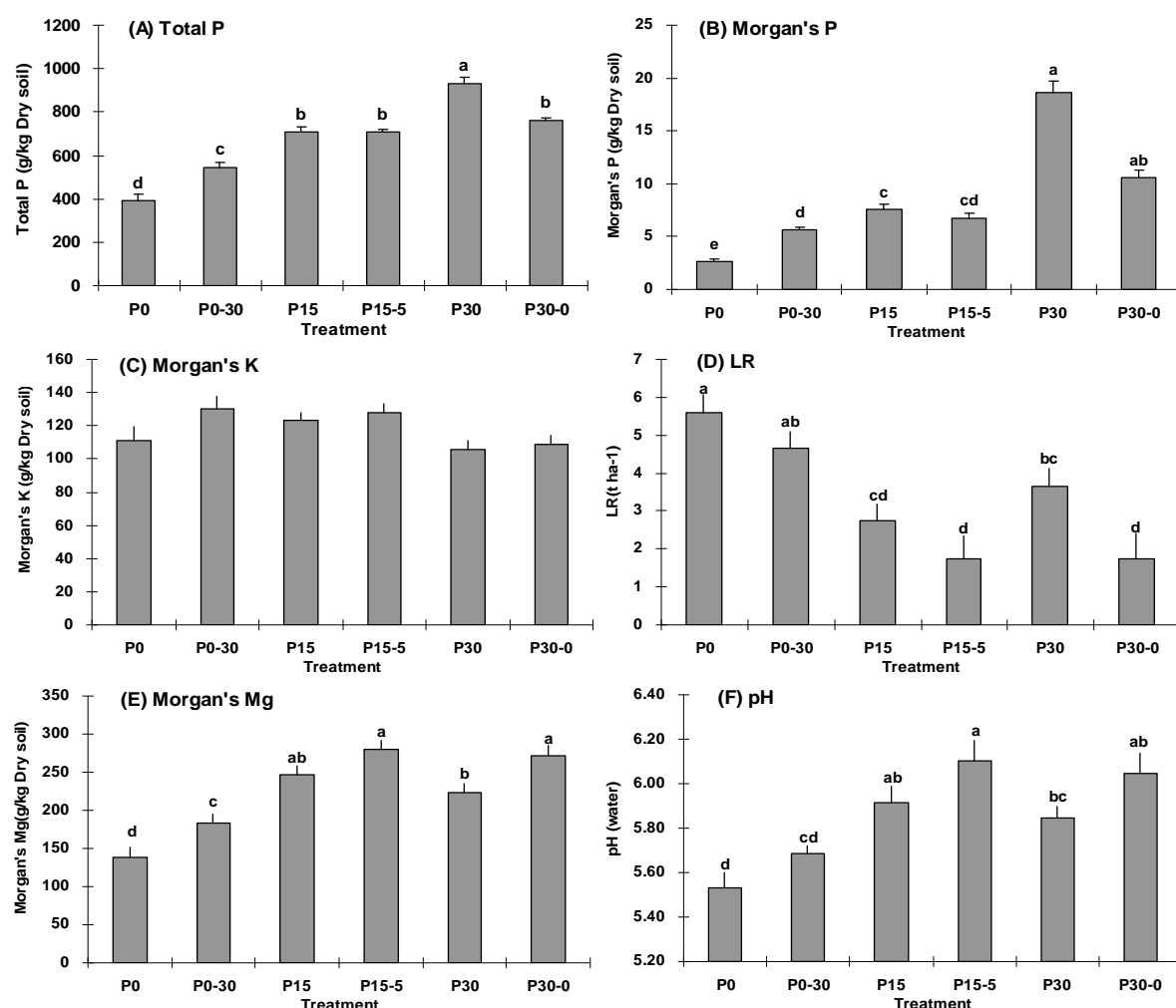
Figure 3.3. Background soil nutrient composition of the Cowlands Trial in 2011. Values were means of samples from each of six replicate plots of each P treatment. The soil analysis included total P (A), Morgan's P (B), Morgan's K (C), Lime (D), Morgan's Mg (E) and pH extracted from water (F). The different letter was significantly different ($P < 0.05$). Error bars represented standard error.

The K_2SO_4 extracted C, N and $NaHCO_3$ extracted (Olsen) P from unfumigated soil was used as an indicator of soil nutrient availability. The Olsen P values were significantly lower ($P < 0.01$) in P0, and the increasing application of P increased Olsen P values, but has no effect on extractable C and N (Figure 3.6).
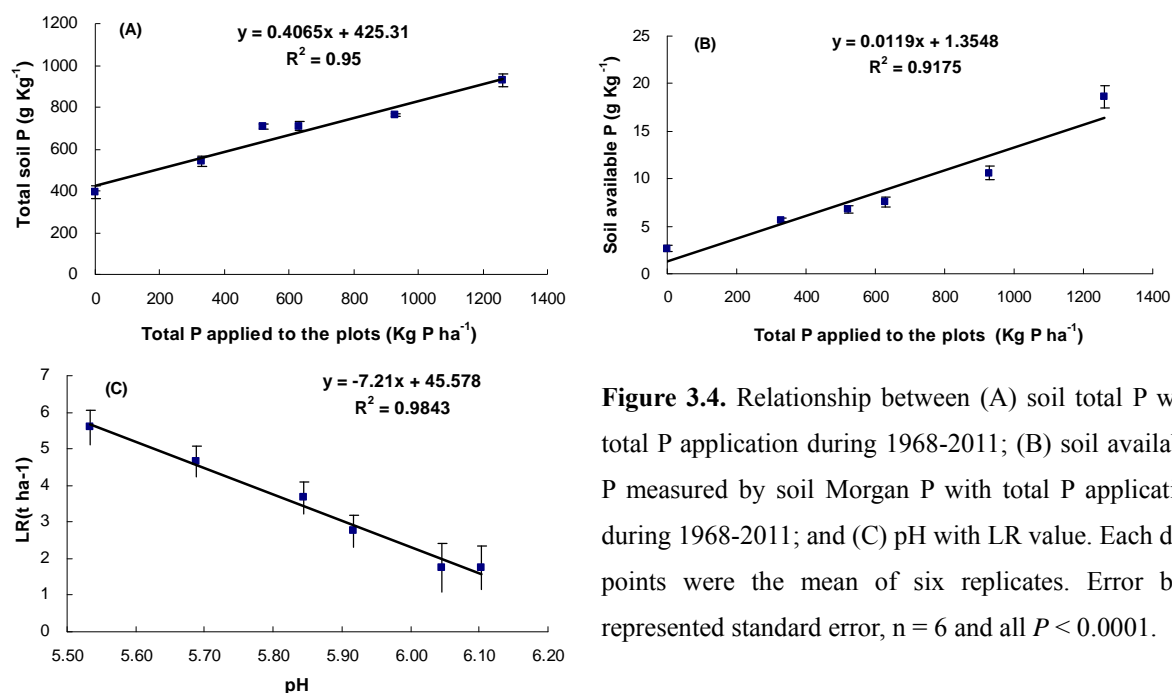
Figure 3.4. Relationship between (A) soil total P with total P application during 1968-2011; (B) soil available P measured by soil Morgan P with total P application during 1968-2011; and (C) pH with LR value. Each data points were the mean of six replicates. Error bars represented standard error, n = 6 and all $P < 0.0001$.
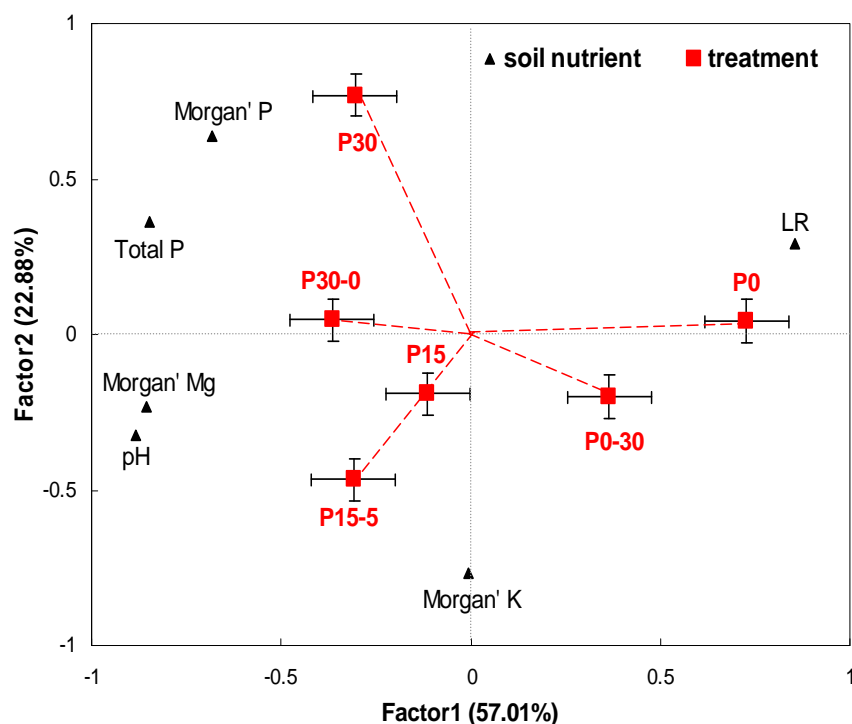
Figure 3.5. Principal Component analysis of the relationship between different treatments ■ and soil nutrients ▲ (n = 6). The factor1 and factor2 explained 79.89% of the variation, bars represented standard
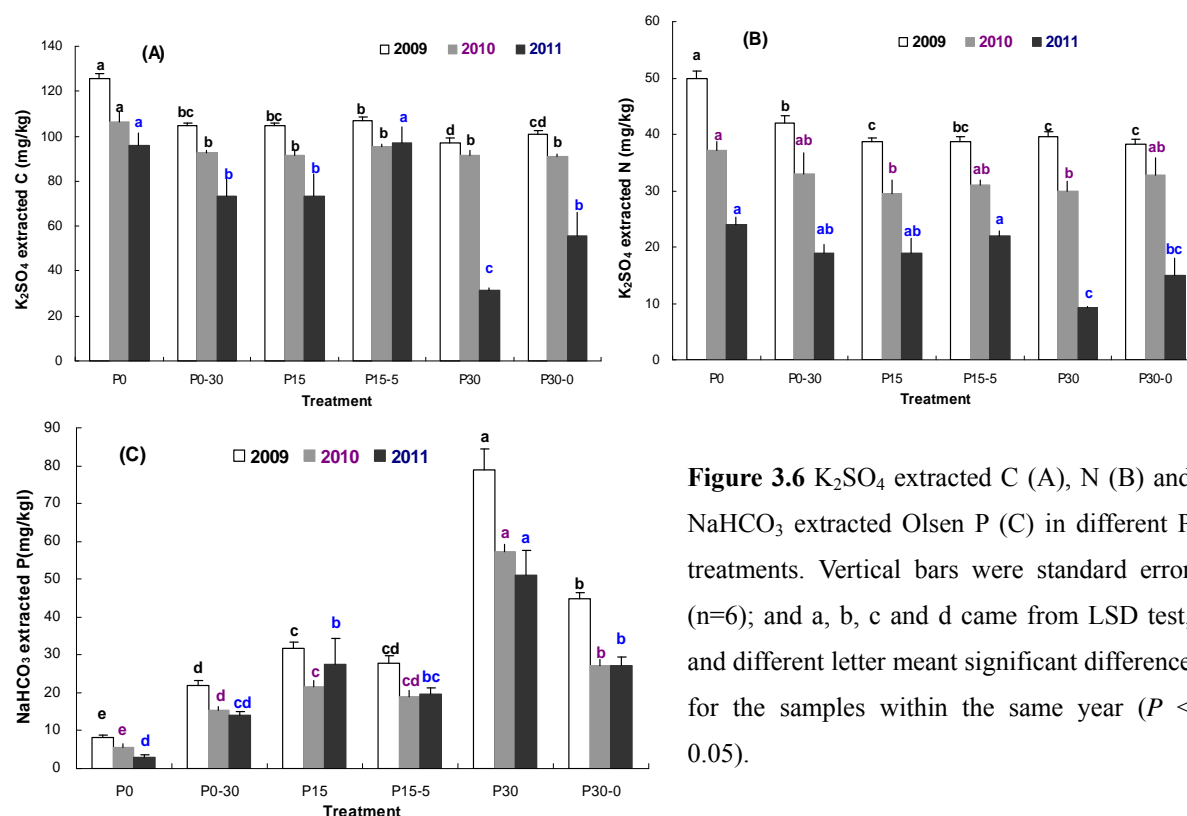
error.

Figure 3.6 K_2SO_4 extracted C (A), N (B) and NaHCO$_3$ extracted Olsen P (C) in different P treatments. Vertical bars were standard error (n=6); and a, b, c and d came from LSD test, and different letter meant significant difference for the samples within the same year ($P < 0.05$).

3.3.2. Impact of P fertilizer on nematodes community structure

3.3.2.1. Nematode morphological data

From the first sampling in 2009, 22 genera of nematodes, representing 17 families, were identified from all 36 plots (Table 3.3).

3.3.2.2. Total nematode abundance and nematode community structure from morphology

Total nematode abundance was affected significantly ($P < 0.001$) by P application and the sampling times, and their interaction (Table 3.4).

From 3 years' nematode data, the average of nematode abundance was 21.0 per gram soil in 2010, which was very close to 20.6 per gram soil in 2011, while was higher than nematodes in 2009 with average of 18.3 per gram soil. Nematodes were significantly more abundant in P30 treatment in 2009, with no significant differences between the other treatments. Nematode abundance differed on the samples of 2010 and 2011, with significantly more nematodes in P15 (Figure 3.7).

Table 3.3. Nematode species identified from the first sampling across all plots

Family	Genus	Group[a]	cp value[b]
Tylenchidae	Malenchus	Plant feeders	2
Hoplolaimidae	Helicotylenchus	Plant feeders	3
Paratylenchidae	Paratylenchus	Plant feeders	2
Leptonchidae	Tylenchorhynchus	Plant feeders	3
Longidoridae	Longidorus	Plant feeders	5
Tylenchidae	Filenchus	Fungal feeders	2
Aphelenchoididae	Aphelenchoides	Fungal feeders	2
Cephalobidae	Eucephalobus	Bacterial feeders	2
Cephalobidae	Acrobeloids	Bacterial feeders	2
Cephalobidae	Teratocephalus	Bacterial feeders	2
Panagrolaimidae	Panagrolaimus	Bacterial feeders	1
Rhabditidae	protorhabditis	Bacterial feeders	1
Plectidae	Anaplectus	Bacterial feeders	2
Alaimidae	Amphidelus	Bacterial feeders	4
Diploscapteridae	Diploscapter	Bacterial feeders	1
Qudsianematidae	Eudorylaimus	Omnivorous	4
Thornenematidae	Laimydorus	Omnivorous	4
Thornenematidae	Prodorylaimus	Omnivorous	4
Aporcelaimidae	Aporcelaimellus	Omnivorous	5
Tripylidae	Tripyla	Carnivorous	3
Anatonchidae	Anatonchus	Carnivorous	4

[a] Group designation is according to Yeates *et al.*,1993.

[b] Colonist-persister value (cp value) seee Bongers, 1990; Bongers and Bongers, 1998.

Table 3.4. Two-way ANOVA results of P treatments (T) and sampling time (S) on nematode abundance for the three year's samples.

	Nematode abundance			
	d.f.	**MS**	**F**	**P**
Treatment (T)	5	68.61	6.03	***
Sampling time (S)	2	76.49	6.72	***
T*S	10	41.61	3.66	***
Error	90	11.38		

Factor-ratios and degree of freedom (d.f.) for principal in the AVOVA; values labelled '***' were significant at $P < 0.001$.

Nematode morphological data in 2009 showed that every treatment had its own dominant species (Figure 3.8). PCA showed that P0 and P0-30 were in the same statistical group, and had more *Filenchus*, *Helicotylenchus*, *Dorylaimus*, and *Tripyla* than the other treatments. P15, P15-5 and P30 belonged to the same statistical group, with more *Anatonchus*, *Laimydorus*, and *Acrobeloides*. The P30-0 treatment was a different group again which contained the dominant species *Paratylenchus*, *Diploscapter*, *Prodorylaimus*, *Malenchus*, and *Longidorus*.
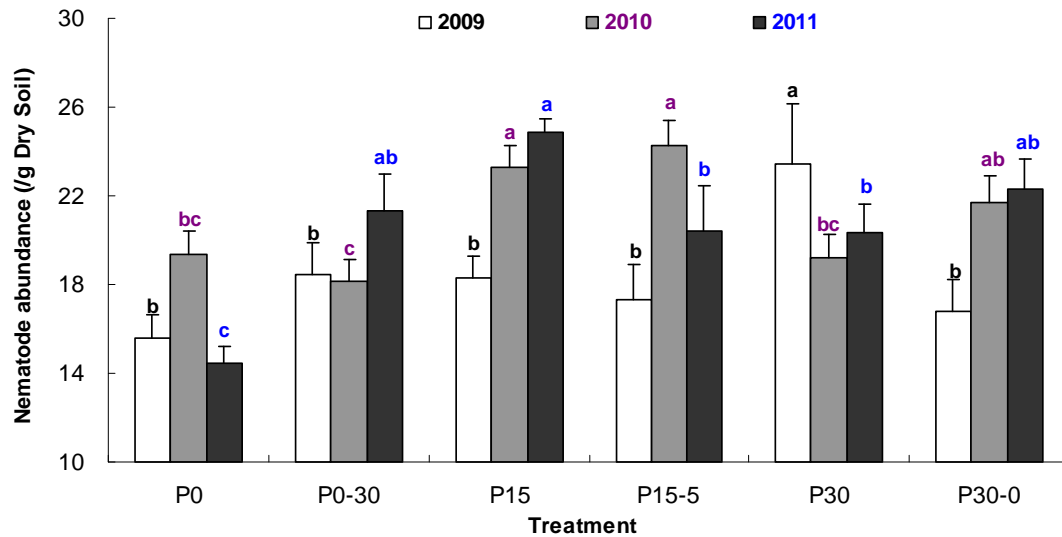
Figure 3.7. Mean abundance (+SE, n = 6) of nematodes in Cowlands grassland in the three sampling time. The letters a, b, c and d came from LSD test, which compared the data from the same sampling year, and different letters meant significantly difference for the samples within the same year ($P < 0.05$).
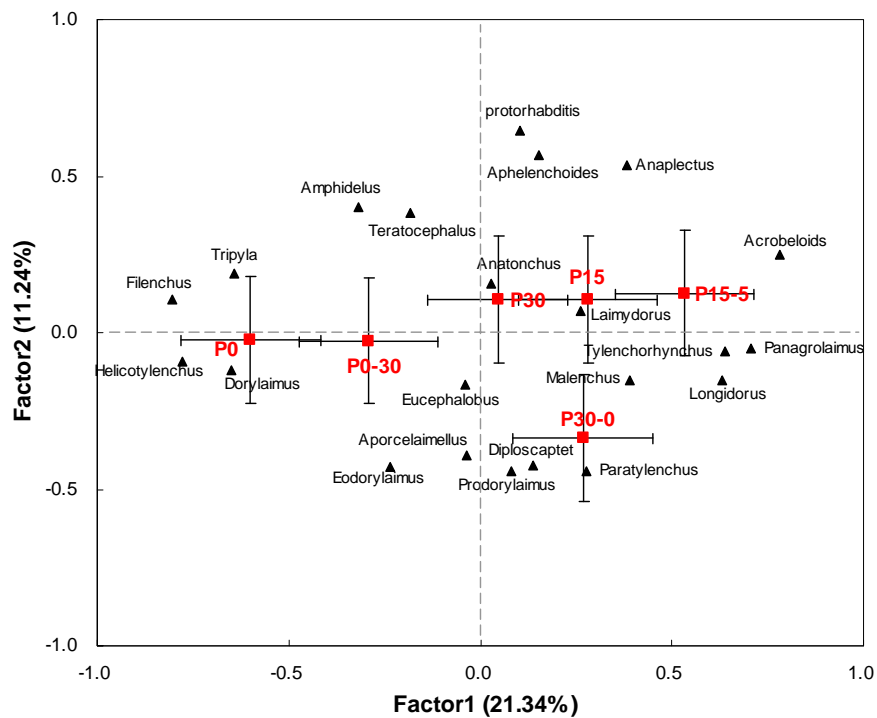
Figure 3.8. Principal component analysis of the relationship between different treatments and nematode community and composition. ▲ represented nematode species identified by morphology, ■ represented six P treatments. Bars represented standard error, n = 6.

Two-way ANOVA data showed that P application had significant effects on bacterial feeders and plant-feeding nematodes ($P < 0.001$), while sampling time affected fungal

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feeders, omnivore, and predator significantly ($P < 0.05$) (Table 3.5). Of the five main trophic groups, bacterial-feeders and plant-feeders were the most abundant in the Cowlands, averaging 38% and 34% of the community respectively, in 2009 across all plots (Table 3.6). Bacterial-feeding nematodes were a significantly greater percentage, while fungal-feeding nematodes were a significantly lower percentage in P0 and P0-30 than in the other P treatments. No significant differences were observed between P application on plant-feeding and omnivore nematodes. Also predators were scarce, 2.4% in P0, which is the highest in all plots.

For the samples in 2010 and 2011, nematodes in the P0, P15 and P30 treatments contained average of 30% and 33% of bacterial-feeders and plant-feeding nematodes, similar to the first samples (Table 3.6). P application increased significantly bacterial-feeders, while decreasing plant-feeding nematodes.

Table 3.6. Effects of P application on the percentage composition (%) of nematode trophic groups from morphological data.

		Bacterial-feeders			Fungal-feeders			Plant-feeders			Omnivorous			Carnivorous		
2009																
Treatment	N	Mean	SE		Mean	SE		Mean	SE		Mean	SE		Mean	SE	
P0	6	30.91	1.29	c	17.87	1.56	a	33.52	2.50	a	15.28	1.42	a	2.42	0.65	a
P0-30	6	35.23	2.24	bc	14.06	1.65	b	32.72	2.94	a	16.59	1.93	a	1.40	0.35	ab
P15	6	43.31	2.85	a	8.65	0.87	c	32.78	2.19	a	14.37	1.51	a	0.88	0.31	b
P15-5	6	41.96	2.96	ab	7.28	0.95	c	35.90	2.75	a	14.25	1.07	a	0.60	0.16	b
P30	6	40.69	2.22	ab	9.69	1.01	c	34.36	3.31	a	13.83	0.98	a	1.43	0.38	ab
P30-0	6	40.15	4.13	ab	8.99	1.15	c	35.11	1.65	a	15.20	1.64	a	0.55	0.26	b
2010																
P0	6	23.86	1.98	b	17.76	1.09	a	39.61	1.84	a	18.01	0.73	a	0.77	0.37	a
P15	6	33.57	2.55	a	21.96	3.07	a	29.52	2.50	b	13.43	1.65	a	1.52	0.20	a
P30	6	33.40	3.56	a	20.34	1.70	a	30.75	3.09	b	14.32	2.59	a	1.19	0.19	a
2011																
P0	6	23.64	1.47	b	14.54	1.25	a	38.56	0.65	a	20.49	2.45	a	2.76	0.36	a
P15	6	38.06	3.44	a	12.13	1.99	a	25.31	2.27	b	22.69	2.43	a	1.81	0.49	a
P30	6	42.26	4.86	a	10.58	0.95	a	25.18	3.69	b	19.59	2.31	a	2.38	0.44	a

The letters a, b, c and d came from LSD test, and the different letter meant significantly difference for the samples within the same year ($P < 0.05$).

3.3.2.3. Molecular analysis (T-RFLP) of nematode communities

Nine main nematode groups detected by T-RFLP were Rhabditida, Plectida, Aphelenchida, Tylenchidae, Dorylaimida, Longidorus, Mononchidae, Pratylenchoides, and Helicotylenchus. Similar to the morphological data, two-way ANOVA molecular data showed that P application had significant effects on bacterial feeders and plant-feeding nematodes ($P < 0.001$), while sampling time affected fungal feeders, omnivores, and predators significantly ($P < 0.001$) (Table 3.7). The molecular abundance data was

dominated by bacterial-feeders and plant-feeding nematodes (Figure 3.9). P15 had more (P < 0.05) bacterial feeders than the other treatments at all three sampling times, while fewer plant-feeding nematodes were detected in P15. P application did not significantly affect the other three trophic groups significantly.
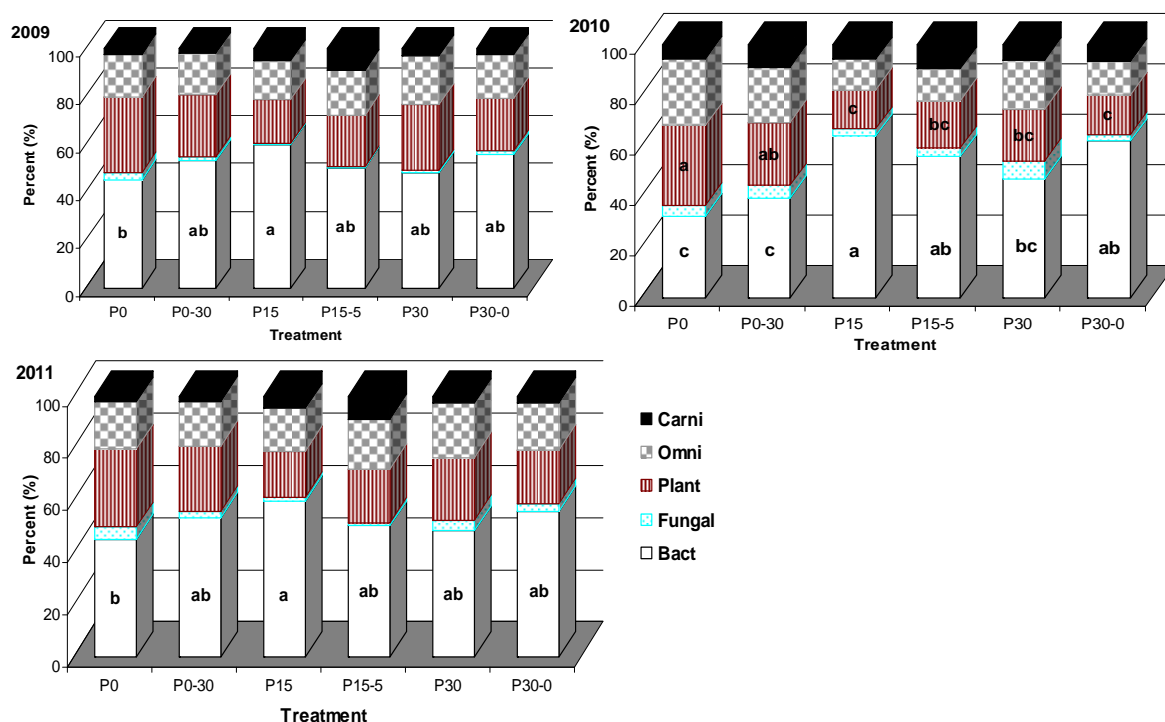
Figure 3.9. Mean percent of nematode functional groups for the three year's samples using T-RFLP method. The letters a, b, c and d came from LSD test, and the different letter meant significantly difference for the samples within the same year (P < 0.05).

T-RFLP profiles were used to assess the relationships between P treatments and nematode taxa, which came with different T-RFLP peaks (Table 3.2). PCA was performed on the composition data from three years (Figure 3.10A), showing that the P0 treatment was significantly separated from the other P application treatments. There was no clear separation of other treatments. But from T-RFLP data assessed from separated years (Figure 3.10B, C and D), it can be seen that P application and sampling time were both associated with different nematode taxa.
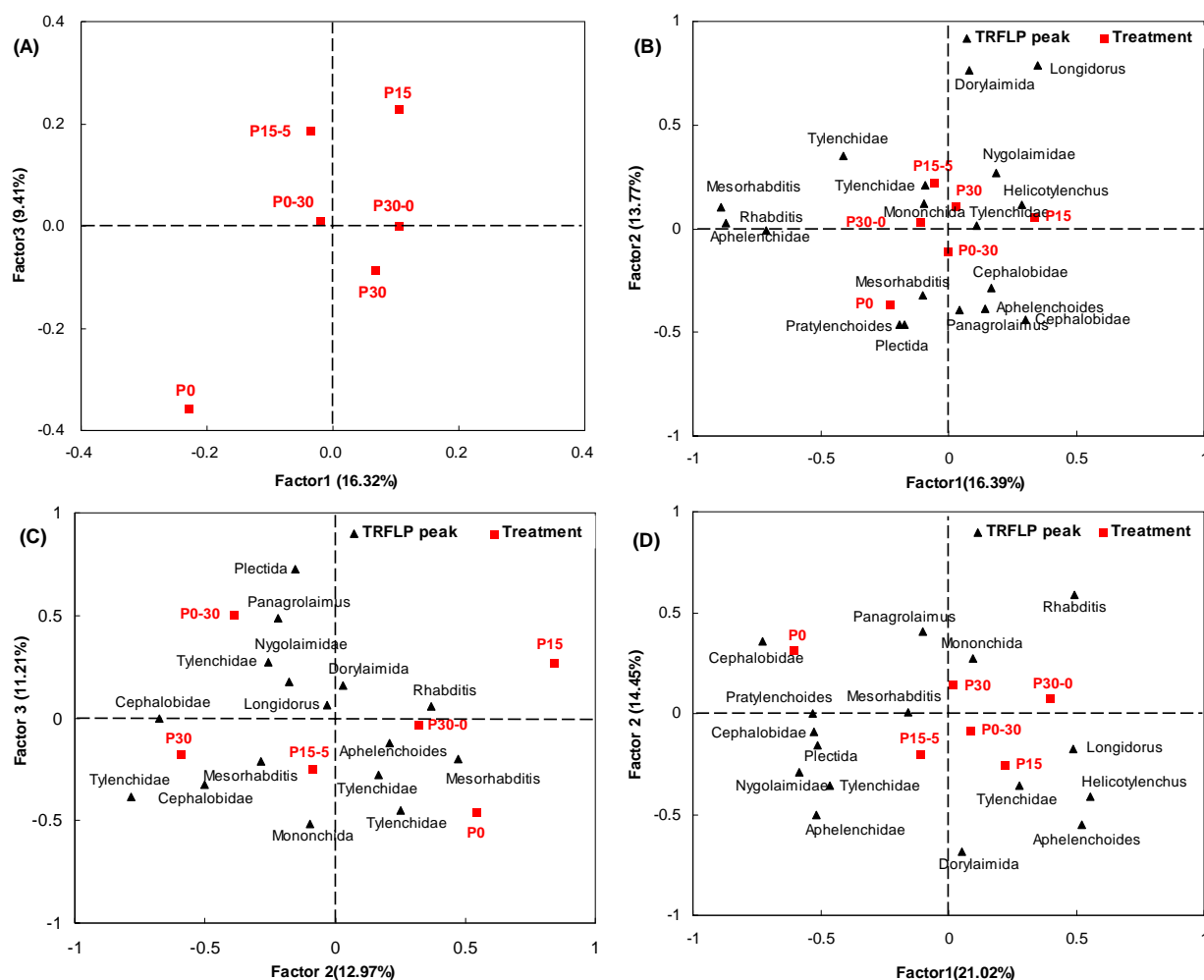
Figure 3.10. Principal component analysis of the relationship between different treatments and peaks, including (A) all years' data, (B) 2009, (C) 2010, and (D) 2011. ▲ represented peaks from T-RFLP, ■ represented six P treatments (n = 6).

3.3.2.4. Comparison of nematode community composition determined by molecular technique to that determined by morphological methods

All nematode samples in 2009, and those from P0, P15 and P30 treatment in 2010 and 2011 were analyzed using morphological and molecular methods (Figure 3.11). In both methods, P15 and P30 increased the percentage of Rhabditida significantly, especially P15 in the 2009 and 2010 samples. But comparing the two data sets, there was a significantly ($P <$ 0.05) greater percentage of Rhabditida in the T-RFLP than the morphological analysis.

For the plant-feeding Tylenchida, the percentage composition as determined by both methods did not differ significantly between P0, P15 and P30 during the three sampling times. However, the proportion from morphology was much higher than from the molecular data (Figure 3.11). The same tendency was seen with another plant-feeding nematode *Helicotylenchus*, but in this case the proportion of *Helicotylenchus* in P0 was similar to that

in P15 and P30 treatments.
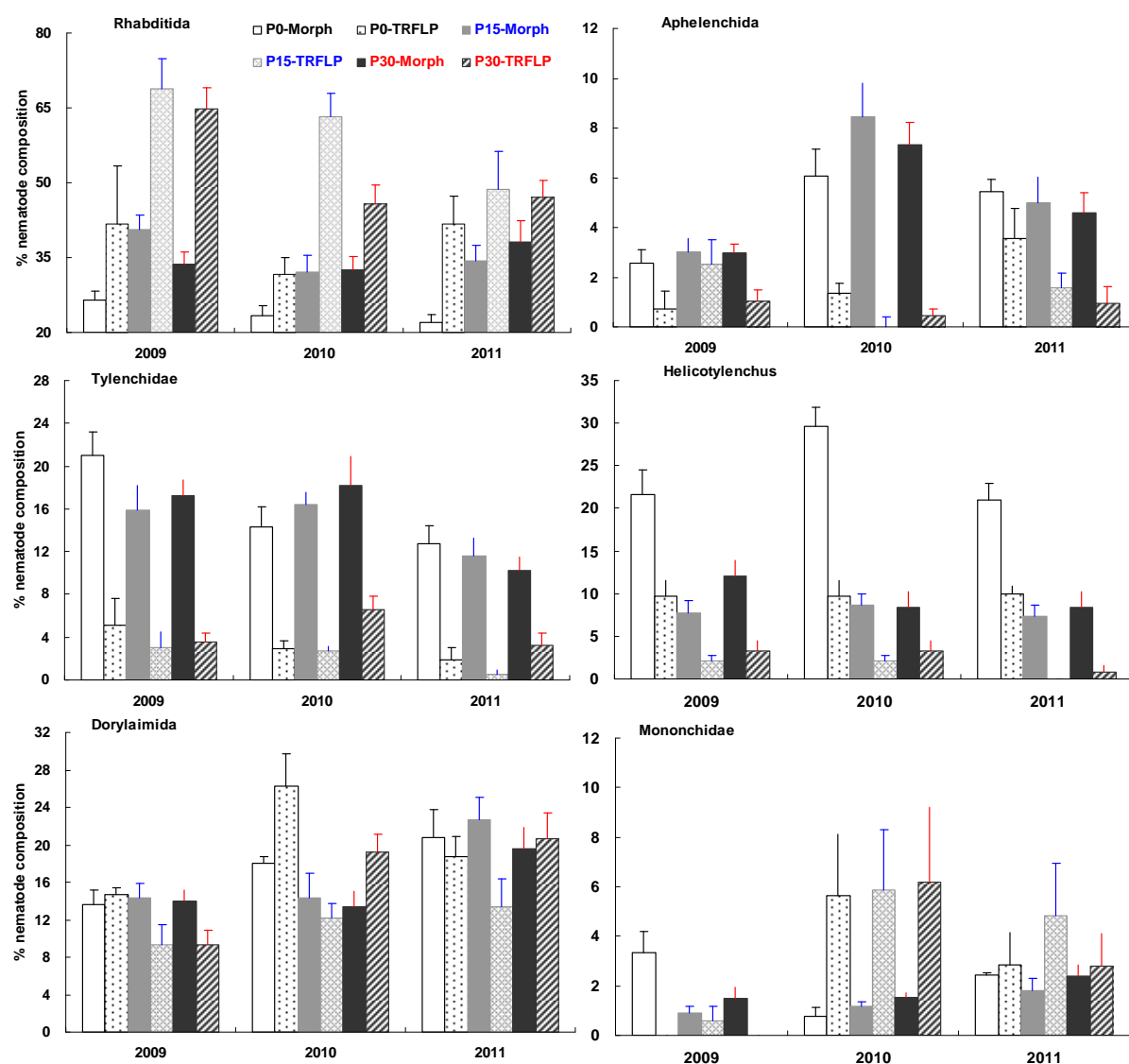
Figure 3.11. Percent of nematode taxa (%) for the three sampling times using morphological and T-RFLP method for different nematode families. Bars represented standard error, n = 6.

Dorylaimida showed few differences between the methods, indicating that the molecular and morphological methods matched most closed with Dorylaimida.

There was a lower composition of Aphelenchida (< 8%) and Mononchidae (< 6%) and differences in the composition of two methods.

The ratio of fungivore to bacterivore nematode (Fu/Ba), from all morphological data in 2009 and 2011, were significantly higher in the P0 treatment ($P < 0.05$), while because of big standard error showed in 2010, there was no significantly difference between P0, P15 and P30. From the data assessed by T-RFLP, we found that there was still the same

tendency that the Fu/Ba ratio was higher significantly in P0 treatment, but in different year, showed differences (Figure 3.12).
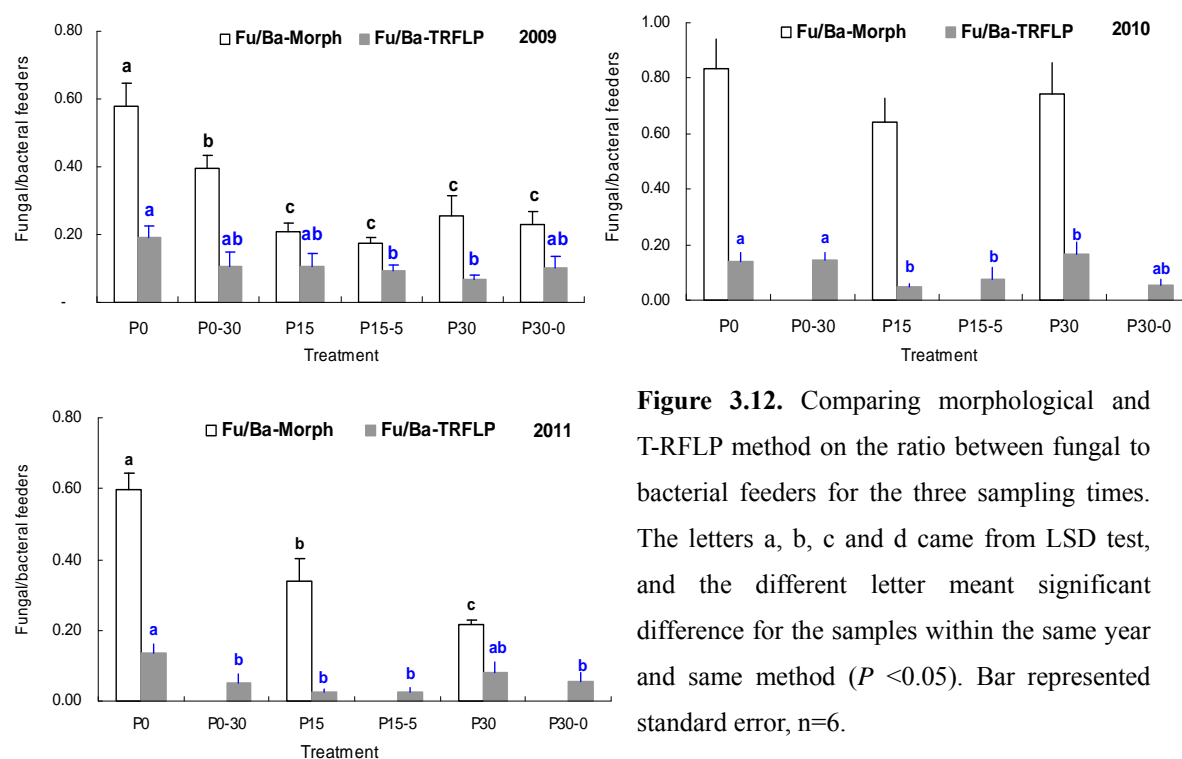
Figure 3.12. Comparing morphological and T-RFLP method on the ratio between fungal to bacterial feeders for the three sampling times. The letters a, b, c and d came from LSD test, and the different letter meant significant difference for the samples within the same year and same method ($P < 0.05$). Bar represented standard error, n=6.

3.3.3. Impact of P fertilizer on soil microbial biomass C, N and P

The two-way ANOVA (Table 3.8) showed that P application had significant effects on microbial biomass C, N and P, also affected the microbial C: N, C: P, N: P ratio ($P < 0.001$). Except microbial biomass P ($P < 0.05$), sampling time affected all the microbial indices significantly ($P < 0.001$).

Microbial biomass C was much lower in 2009 than in 2010 and 2011, and was significantly higher in the P30-0, P30 and P15 than other three treatments (Figure 3.13A). While just the P30 and P30-0 treatments had more microbial biomass C ($P < 0.05$) in 2010 and 2011, and also even higher microbial biomass C showed in P0 and P0-30 than in the treatments of P15 and P15-5 in 2010, while no differences was found in 2011 between the treatments P0, P0-30, P15 and P15-5.

Table 3.5. Two-way ANOVA results of P treatments and sampling time (S) on percent nematode functional groups assessed by morphology for the three year's samples.

	d.f.	Bacterial-feeder			Fungal-feeders			Plant-feeders			Omnivorous			Predator		
		MS	F	*P*	MS	F	*P*	MS	F	*P*	MS	F	*P*	MS	F	*P*
Treatment (T)	2	926	16	***	49	3		345	9	***	25	1		2	2	
Sampling time (S)	2	291	5	*	363	23	***	84	2		222	10	***	6	6	**
T*S	4	44	1		78	5	**	98	2		15	1		2	2	
Error	45	59			16			40			22			1		

F-ratios and degree of freedom (d.f.) for principal factors in the AVOVA; values labelled with '*', '**' and '***' were significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table 3.7. Two-way ANOVA results of P treatments (T) and sampling time (S) on percent nematode functional groups assessed by T-RFLP for the three year's samples.

	d.f.	Bacterial-feeder			Fungal-feeders			Plant-feeders			Omnivorous			Predator		
		MS	F	*P*	MS	F	*P*	MS	F	*P*	MS	F	*P*	MS	F	*P*
Treatment (T)	5	1695	15	***	11	1		905	18	***	127	3	*	30	1	
Sampling (S)	2	511	4	*	91	9	***	258	5	*	778	16	***	399	19	***
T*S	10	241	2	*	12	1		92	2		59	1		17	1	
Error	90	114			10			51			48			21		

F-ratios and degree of freedom (d.f.) for principal factors in the AVOVA; values labelled with '*', '**' and '***' were significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table 3.8. Two-way ANOVA results of P treatments (T) and sampling time (S) on soil microbial biomass C, N, P and microbial C:N, C:P and N:P ratio for the three year's samples.

	d.f.	Microbial biomass C			Microbial biomass N			Microbial biomass P			Microbial C:N ratio			Microbial C:P ratio			Microbial N:P ratio		
		MS	F	*P*	MS	F	*P*	MS	F	*P*	MS	F	*P*	MS	F	*P*	MS	F	*P*
Treatment (T)	5	1151651	16	***	16179	28	***	905	18	***	10	11	***	852	7	***	7	11	***
Sampling time (S)	2	8668237	122	***	17227	30	***	258	5	*	320	366	***	47448	387	***	354	519	***
T*S	10	172114	2	*	877	2		92	2		3	4	***	1014	8	***	9	13	***
Error	90	70832			575			51			1			123			1		

F-ratios and degree of freedom (d.f.) for principal factors in the AVOVA; values labelled with '*', '**' and '***' were significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
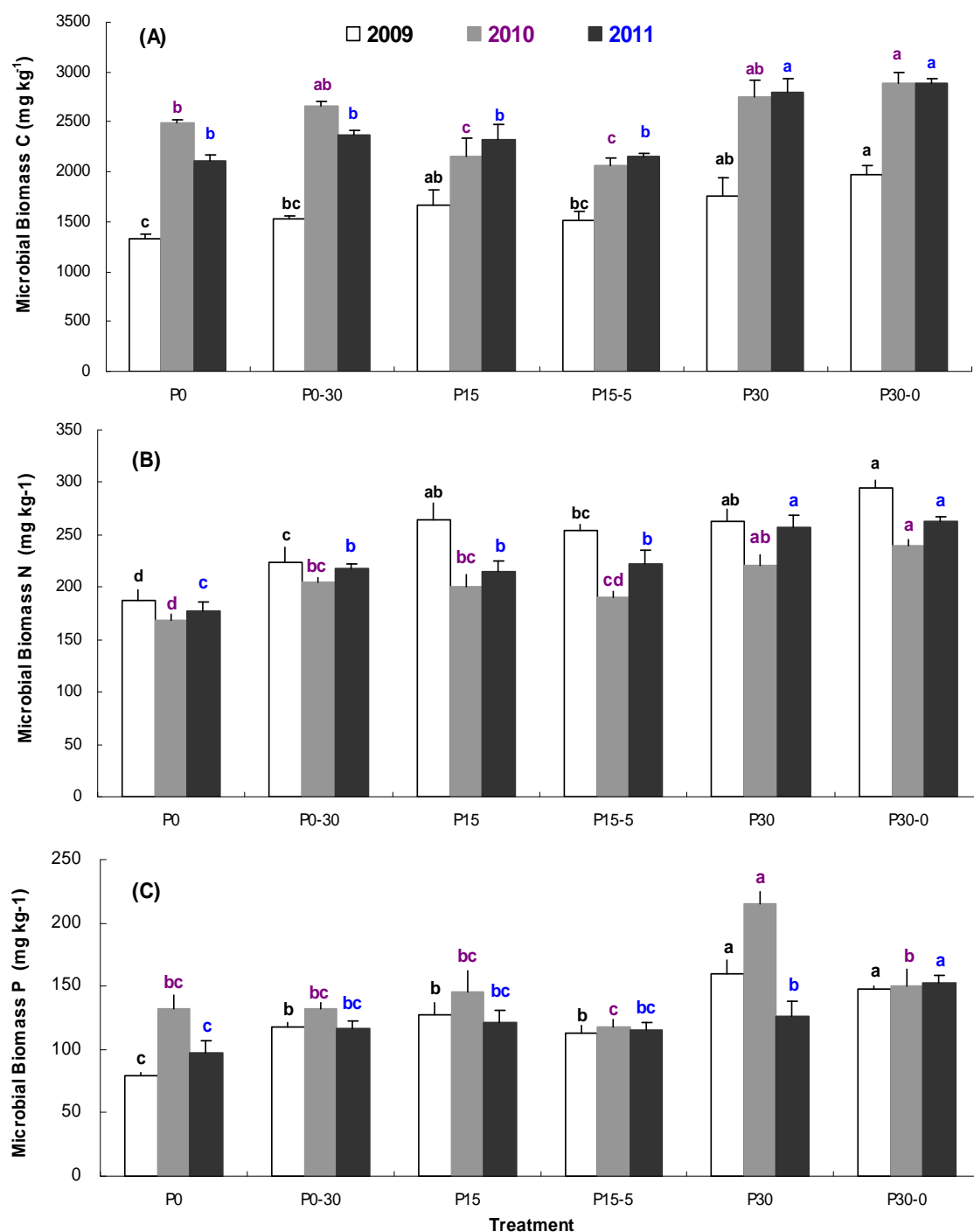
Figure 3.13. The effects of P fertilizer on microbial biomass C (A), N (B) and P (C). Means (+SE) based on n = 6, and the letters a, b, c and d came from LSD test, and the different letter meant significant difference for the samples within the same year ($P < 0.05$).

Reverse to microbial biomass C, microbial biomass N was higher in 2009 than in 2010 and 2011, and also except the P15-5 treatment in 2010 was no difference, P application increased significantly microbial biomass N in three years' soil samples ($P < 0.05$, Figure 3.13B). Microbial biomass P showed differences on 3 years' samples. In 2009, P application increased

significantly microbial biomass P ($P < 0.05$). In 2010, only the P30 was significant higher than other treatments, and also in 2011 only the P30 and P30-0 were significant higher than other treatments (Figure 3.13C), indicating seasonal changes on microbial biomass P.

Soil microbial C: N, C: P, N: P ratio also showed differences across sampling times (Figure 3.14). Microbial C:N ratios covered a low and narrow range (6.0-7.1) in 2009, as same tendency as in 2011 with the range (9.1-12.0), and only the P0 treatment was significantly higher than P15-5 ($P < 0.05$). While microbial C:N ratios ranged from 10.7 in P15 to 14.9 in P0 with the samples in 2010, and decreased significantly all P treatments.

The microbial N: P ratios in 2009 ranged from 1.7 in P30 to 2.4 in P0, and were significantly higher in P0 and P15-5 than P30 and P30-0. While in 2010, the microbial N: P ratios ranged from 1.7 in P30-0 to 1.0 in P30, meaning differences on the sampling time. But no differences were found in 2011.
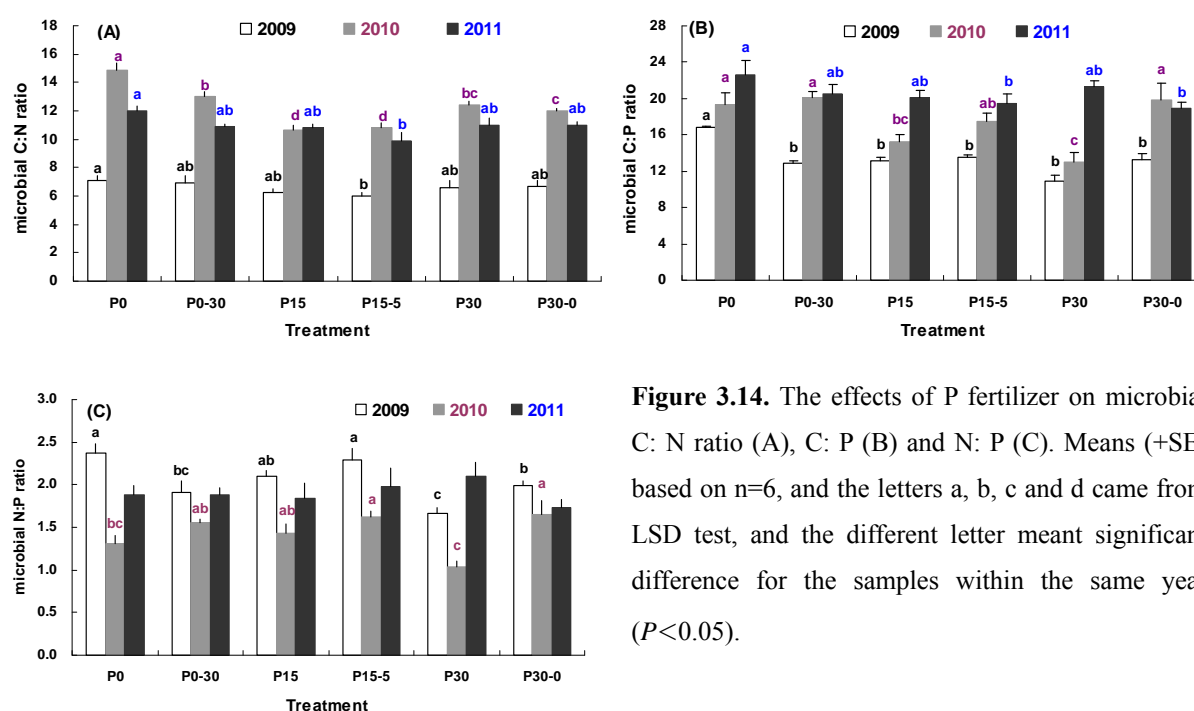
Figure 3.14. The effects of P fertilizer on microbial C: N ratio (A), C: P (B) and N: P (C). Means (+SE) based on n=6, and the letters a, b, c and d came from LSD test, and the different letter meant significant difference for the samples within the same year ($P<0.05$).

3.3.4. Impact of P fertilizer on bacterial biomass

Soil DNA analysis was included in this study because of its potential to provide information on soil biology that is not available using traditional technique. Two year's soil bacterial DNA was tested using the samples from 2010 and 2011. Of the soil DNA samples in 2010, the P0-30 treatment had a significantly greater bacterial biomass than P0 and other P treatments. While in 2011, P0 and P15-5 were significantly greater than the P30 and P30-0 treatments (Figure 3.15).
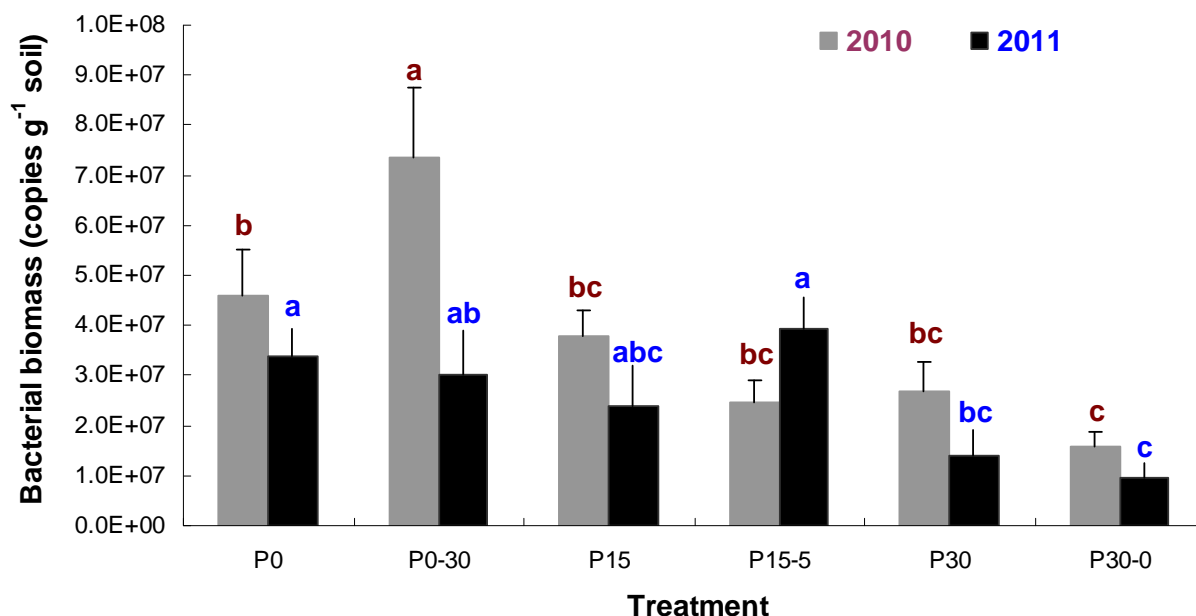
Figure 3.15. The effects of P fertilizer on soil bacterial biomass assessed by quantitative real-time PCR. The letters a, b, c and d came from LSD test, and the different letter meant significant difference for the samples within the same year ($P < 0.05$). Bars represented standard error, n = 6.

3.3.5. Impact of P fertilizer on PLFA profiles

The total amount of PLFAs is an indicator of the mass of microbial cell membranes, a measure of total microbial biomass. The abundances of total PLFAs from the samples of 2011 were similar, just significantly higher in P30-0 comparing to P0, while were greater in all P treatments than P0 for the samples of 2010 (Figure 3.16A).

P application increased bacterial biomass, while decreased fungal biomass from the PLFA data (Figure 3.16C), which was very similar to nematode community. In addition, the ratio from fungal/bacterial biomass affirmed that P fertilizer favoured the bacterial pathway in soil decomposition processes (Figure 3.16D).

Principal components analysis showed that P application did significantly influence PLFA profiles (Figure 3.17A, and B), and that there were differences between the two samples in 2010 and 2011. P0 plots were significantly different from each other for the 2011's samples, and no obvious functional groups were close to any treatment. While P0 and P0-30 were different to other P treatments in 2011, and also they had positive relationships with fungal groups 18:2ω6c, 18:1ω9c and 18:1ω9t.
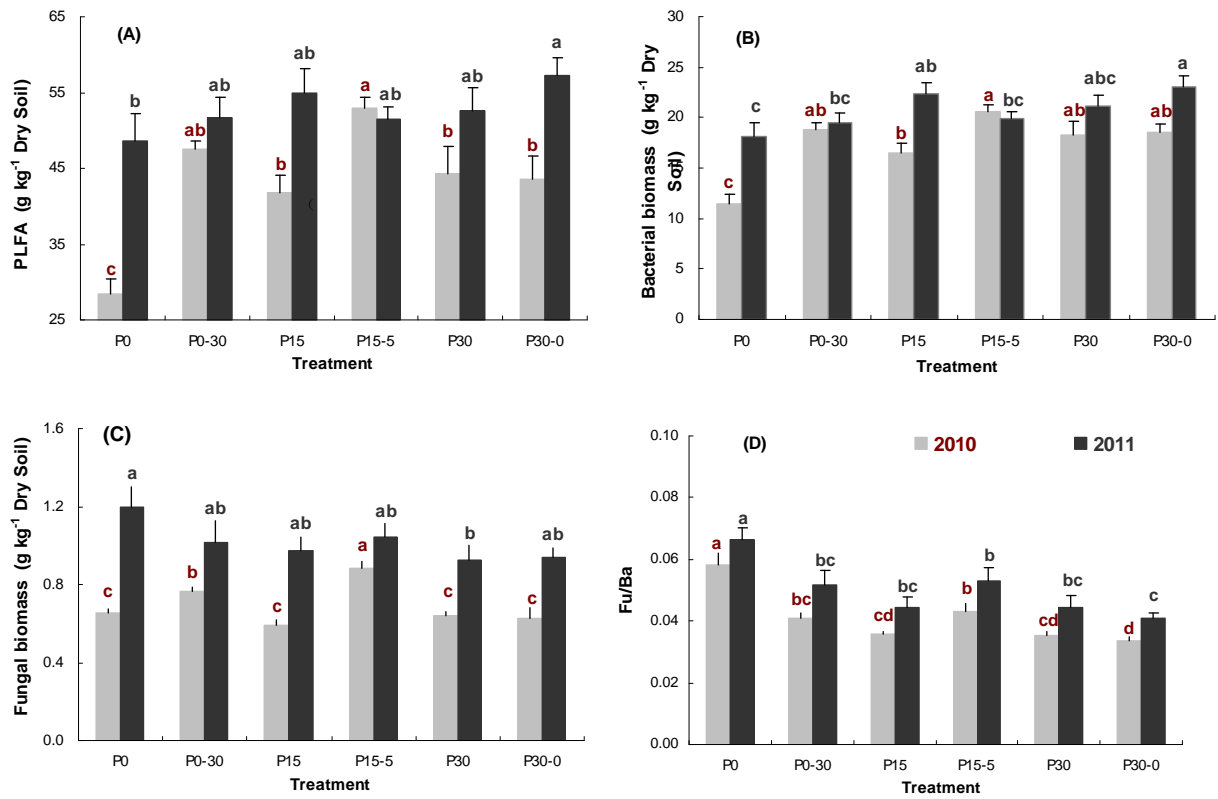
Figure 3.16. The effects of P fertilizer on PLFAs. (A) Total PFLAs from the soil in 2010 (■) and 2011(■); (B) Total bacterial biomass from PLFAs; (C) Total fungal biomass from PLFAs; (D) the ratio of fungal to bacterial biomass. The letters a, b, c and d came from LSD test, and the different letter meant significant difference for the samples within the same year ($P < 0.05$). Bars represented standard error, n = 6.
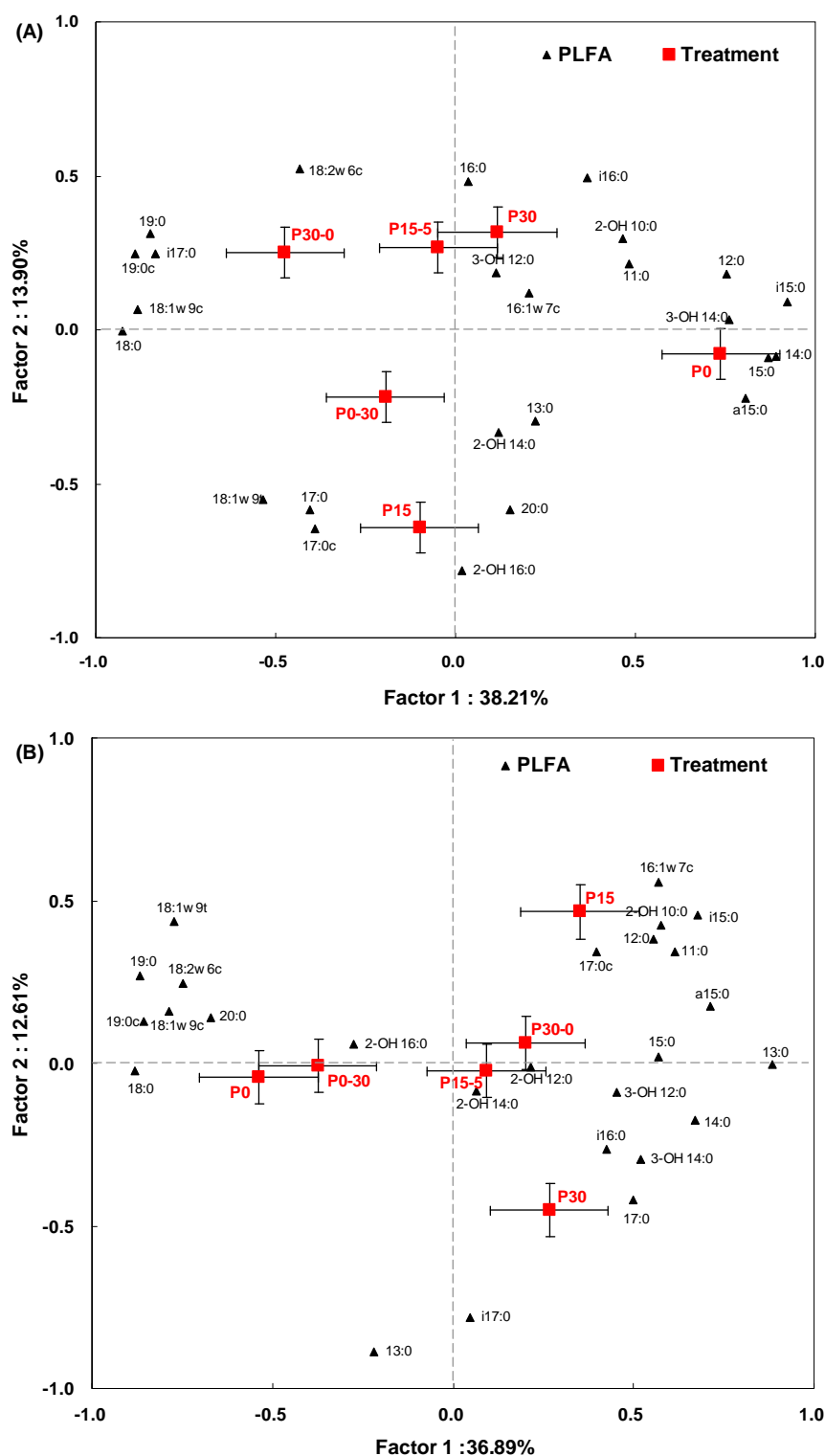
Figure 3.17. PCA plot of PLFA profiles from the sampling in 2010 (A) and 2011 (B) in relation to different P application treatments. ▲ represented PLFA profiles, ■ represented treatment. Bars represented standard error, n = 6.

3.4. Discussion and conclusion

3.4.1. Comparison among soil nutrients in different P treatments

Total soil P and soil inorganic P (Morgan's P) both have strong linear relationships with the total quantity of P applied, indicating substantial accumulation of inorganic P in the soil. In 1968, the mean soil phosphorus level was 6 g kg^{-1} of soil, over the entire site (Culleton, 2002). The P treatments had a dramatic influence on soil P status. Where no P (P0) was applied, soil P levels had dropped to 2.6 g kg^{-1}. On the other hand, when 15 and 30 kg P/ha was applied, soil P levels rose to 7.6 and 18.6 g kg^{-1}. Much of the applied fertiliser P has remained in the soil.

If we look at the labile nutrients in the soil, the P0 plots had very low levels of labile P, and relative high levels of labile C and N (Figure 3.6), suggesting P limitation was greater than N limitation, which may be due to the equal amount of N fertilizer applied to all the plots.

It is well known that soil pH plays a vital role in P solubility and absorption processes (Havlin *et al.*, 2005). Nevertheless P dynamics in soil are a complex function of soil chemical composition, particularly of the amount and reactivity of clay minerals, $CaCO_3$, and Fe oxides, and of the size, timing, and duration of P additions (Afif *et al.*, 1993). A major physico-chemical control of P availability in soil is pH. Even small pH changes strongly affect P concentrations in soil solution and thus plant uptake. P application increased pH significantly, but not significantly so for the treatment P0-30, which indicates that the change in pH is a longer-term processes requiring more than 10 years application to cause a significant increase. Treatments P15 and P15-5 belonged to the same statistical group (Figure 3.5). One reason may be that both treatments received the same amount of P fertilizer for first 30 years and after that, the treatment which P application reduced from 15 to 5 kg P ha^{-1} yr^{-1} was relatively small. On the other hand, another two groups, P0 and P0-30, P30 and P30-0 showed significant differences for the data analysis on total P, Morgan's Mg, while no significant differences on Morgan's P, pH and LR value. The main reason was that P application hugely increased from 0 to 30 kg ha^{-1} yr^{-1} and decreased hugely from 30 to 0 kg ha^{-1} yr^{-1}.

Generally, adsorption of phosphate decreases as pH is raised from 5.5 to 6.5, as this reduces the concentrations of soluble and exchangeable Fe and Al ions which could react with fertilizer phosphate to form the sparingly soluble Fe and Al phosphates (Haynes, 1982). Furthermore, liming can increase phosphate availability by stimulating mineralization of soil organic P. At high soil pH values, liming increases phosphate fixation by precipitation of relatively insoluble Ca phosphates and accordingly phosphate availability decreases (Sample *et al.*, 1980). In Ireland, the pH values of grassland soils are commonly lower than 6.5, which is not considered ideal for grass growth. Therefore lime is applied to maintain the soil pH

value. The lower the pH value, the more lime should be applied in the field. In the experimental plots, a significantly negative correction between pH and LR was found, which mean that P application not just changed soil acidity, but also soil acidity influenced a number of important aspects of soil fertility including phosphorus availability, and LR value.

3.4.2. The effects of P fertilizer on nematode community structure

In grasslands, the key management variables that strongly influence the soil physical and chemical properties are fertiliser application (organic and inorganic) and the activities of grazing ruminant livestock (Bardgett and Cook, 1998). It is widely reported that application of organic manures and mineral fertilizer applications impact the diversity and abundance of nematode trophic groups (Neher, 1999; Yeates *et al.*, 1999; de Goede *et al.*, 2003; Forge *et al.*, 2005), especially organic fertilizers which increased nematode numbers due to increased substrate availability, plant growth, and altered soil conditions (Griffiths and Bardgett, 2000). Bardgett and Cook (1998) mentioned that in temperate agricultural grassland, nematode populations are dominated by plant-feeders and bacterial-feeders, which matches the results found in the Cowlands soils. Omnivorous and fungal-feeders were a similar, though smaller proportion of the total nematode population and predators were the least abundant. P application increased nematode abundance, especially increasing bacterial-feeding nematodes, indicating a shift from fungal to bacterial dominated soil decomposition, which were proved from the F/B ratio in 2009 and 2011, but not in 2010. The ratio of fungal- to bacterial-feeding nematodes has been proposed and used as an indicator of decomposition pathways (Freckman and Ettema 1993, Todd, 1996). Wardle (2002) found at a non-fertilized site, the proportion of fungal-feeding nematodes to bacterial plus fungal feeders was 44%, and possibly the fungal channel was more active than bacterial channel, which was similar to our results. Intensively managed grasslands (P15 and P30) appear to correspond to the 'fast cycle' dominated by labile substrates and bacteria, while less productive grasslands related to the 'slow cycle' dominated by more resistant substrates and fungi (P0).

On the other hand, in Cowlands, although the stocking rate was the same (Cattle stocking rate 3300 Kg ha^{-1}), the animals grazed the different treatments for different times. So, P30 plots had plenty of grass so the cattle graze longer than the P0. Different above-ground grazing is known to affect soil biota through, for example, the consumption of phytomass (Bardgett and Wardle, 2003), microclimatic changes of soil temperature and moisture (Merrill *et al.*, 1994), deposition of dung (Bardgett *et al.*, 1998), and soil compaction (Petersen *et al.*, 2004). In the present plots, application of P and different grazing presumably changed the above-ground

plant diversity and even plant biomass (Figure 3.18, King-Salter, 2009), which might also affect nematode abundance and community structure. Plant species are also known to influence microbial and microfaunal diversity of the rhizosphere, and microbial biomass have been shown to be affected by temporal changes in the productivity of different grass species (Bardgett *et al.*, 1999).
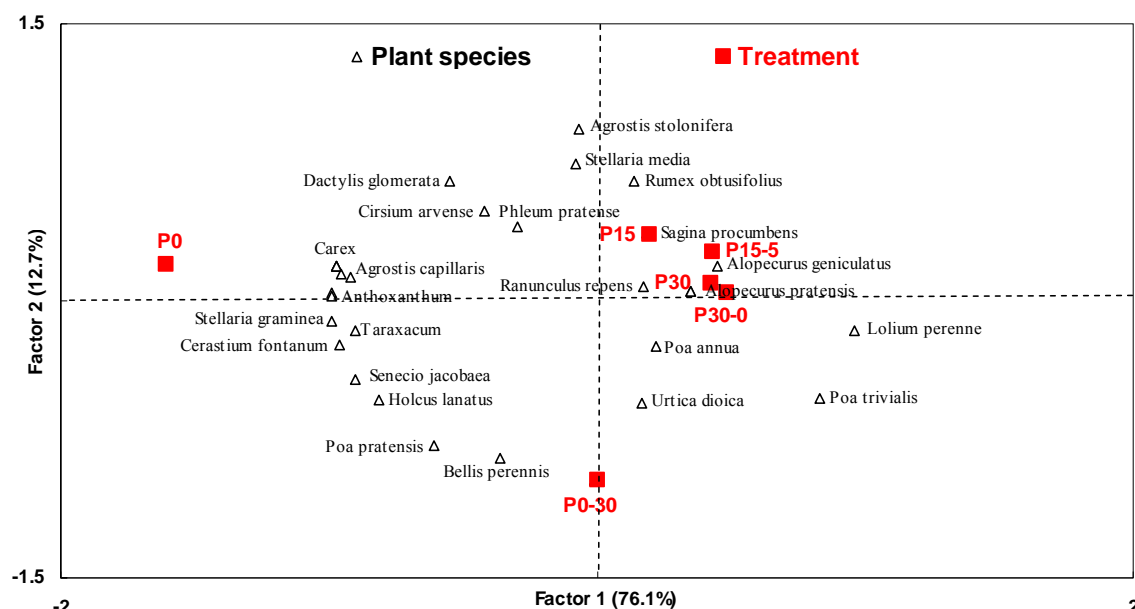
Figure 3.18. PCA plot of plant vegetation cover under different P treatment.

3.4.3. Comparison of molecular and morphological methods on nematode composition analysis

Nematode taxonomy has traditionally been based on morphological identification. Now under the development of molecular methods on soil microorganisms, the molecular approaches have employed as an alternative to morphological identification of soil nematode community (Floyd *et al.*, 2002; Waite *et al.*, 2003; Griffiths *et al.*, 2006; Jones *et al.*, 2006b; Okada and Oba, 2008). Advantages of molecular methods have been discussed previously in Chapter 2, but it is essential that the two methods are comparable with each other to explain the role of nematodes in soil biological processes.

There might be some reasons why big differences exist between nematode taxa, especially in the Rhabditida, *Helicotylenchus*, and Tylenchida, determined by the two methods. First, the analytical result obtained by the morphological method may not always reflect the 'true' community in question because in this case, just 100 individuals per sample, out of a total of approximately 1000 individuals, were examined due to the laborious work for morphological identification. Second, DNA extraction and PCR amplification might work with different

efficiencies for different nematode species; for examples here, Rhabditida, which are very susceptible to bead beating used in the extraction, might give more DNA compared to bigger nematodes such as Dorylaimda. Third, there are still some questions about whether the quantification of nematode types by T-RFLP is dependent on the amount of nematode DNA and thus the total volume (biomass) of each type, rather than the numerical abundance of each type. This latter point is the subject of Chapter 5.

3.4.4. The effects of P fertilizer on soil microbial biomass and microbial C, N and P ratios

In general, microbial C pools are not only related to the below-ground resource base (i.e. bottom-up control), but also to predators such as soil nematodes that consume them (i.e. top-down control) (Wardle *et al.*, 2002). For example, the pools of bacterial-feeding nematodes at our P plots were greater than those in no P treatments, possibly indicating more bacterial turnover and activity, and also appreciable grazing on the microbial biomass at those plots. In contrast, the No-P plots had the lowest number of bacterial-feeding nematodes. This would indicate a low turnover rate of microbial biomass at P0 plots, and consistent with its low soil net N mineralization. In the Cowlands experiment, the results showed P application increased soil microbial biomass C, N and P, especially in the higher P treatment (P30 and P30-0) during long-term fertilization. Over a period covering more than 50 years, the soil fertility had been improved for livestock production. This improved fertility has resulted from continued application of fertilizers and the introduction of high producing grasses and legumes. Analyses of microbial communities in plant diversity experiments indicate that fungal biomass in particular increases with plant diversity (Zak *et al.*, 2003; Chung *et al.*, 2007), pointing to increasing importance of the fungal energy channel in species-rich plant assemblages (Figure 3.18), which matched F:B ratio increase in the P0 (Figure 3.14).

Decomposition of organic matter may proceed through different channels in the soil food web. At one extreme, materials of high cellulose and lignin content and high C: N ratio are decomposed through fungal-dominated pathways; at the other extreme, moist, N-rich tissues are decomposed through bacterial-dominated pathways (Wardle and Yeates, 1993). In natural systems, a high C: N ratio of the organic material in soil may be necessary for long-term sustained production. In this case, decomposition channels tend to be fungal-mediated. Nematode faunal analysis is readily applied to such higher resolution food web diagnostics (Ferris *et al.*, 2001). The molar C: N ratios in soil microbial biomass averaged 8.6 ± 0.3 and ranged from 3 to 24, and molar N: P ratios averaged 6.9 ± 0.4, ranging from 1 to 55 (Cleveland and Liptzin, 2007). Higher C: N ratios in microbial biomass suggest relatively

high C: N ratios in fungal biomass, indicating higher fungal biomass in total microbial biomass. In the Cowlands, the microbial molar C: N ratios average 12.7±1.1, being significantly higher in P0 (14.0±0.5) than P treatments (12.2±0.8), indicating that in P0, more fungal biomass without P fertilization. Much higher than average soil microbial N: P ratios suggest P demand in the soils. But in our Cowlands, the microbial N: P ratios were very lower than the average, and in a narrow range (4.2±0.7), indicating that soil microbial biomass assimilates N and P in those proportions when these nutrients are moderately available. This situation could arise on farms that if no P was applied to soils, the microbial biomass probably has a P limitation, and therefore immobilizes and competes with plant for labile P (Saggar *et al.*, 1998; 2000).

3.4.5. The effects of P fertilizer on soil microbial community structure

Because of the limitations of traditional culture-dependent methods to characterize most of the microorganisms (Scully *et al.*, 2005), the culture independent method of real-time PCR was used to detect bacterial16S rRNA gene (rDNA) and PLFA analysis for bacteria and fungi. From the nematode and PFLA data, the implication was that P application increased bacterial biomass. Actually from the bacterial DNA data, it is evident that bacterial biomass was significantly higher in P0-30 in 2010, and significantly higher in P0 and P15-5 compared to P30-0, indicating that from bacteria direct data that P application didn't increasing bacterial biomass, the reason is easily understood. In P0 and P0-30 treatments, lower abundances of bacterial-feeders means less bacteria consumption by bacterial-feeding nematodes. Microbial feeding nematodes are known to be the most important grazers of the microflora in terrestrial ecosystems (Ingham *et al.*, 1985; 1986), concomitant with an increase in microbial turnover in the presence of microfauna grazing on microflora which may result in a decrease in bacterial numbers (Bonkowski *et al.*, 2000).

Griffiths (1994) discussed the importance of soil fauna in N mineralization, approximately 30% of total net nitrogen mineralization was due to the direct effects of soil fauna. de Ruiter *et al.* (1993) had modeled the possible indirect effects of fauna by deleting particular faunal groups from their food web model, and calculating the subsequent effects on N mineralization. They showed that bacterial-feeding nematodes could potentially account for up to 10% of total net nitrogen mineralization. So the soil available C and N in P0 were higher than P treatments, especially P30 and P30-0 treatments, possibly because of the contribution from bacterial-feeding nematodes in P0 treatment.

PLFA method was used for the analysis of the biomass and structure of microbial

communities in the soils. There are two approaches to analyzing PLFA data. One relies on using the whole PLFA pattern, filtered through a multivariate statistical technique, and gets the changes in the community due to different treatments. Another approach involves trying to elucidate the effects on specific groups of microorganisms (Frostegård *et al.*, 2011). In this study, different P fertilizer application significantly influenced PLFA profiles.

There were significant unidirectional changes across the long-term P fertilization for both the microbial community (assessed by microbial biomass and PLFA) and the nematode community. The most significant compositional changes observed were the relative importance of the fungal to bacterial energy channels of the soil food web. Across the P fertilization, there were substantial decreases in both the fungal to bacterial ratio (Figure 3.14D, PLFA data), and the fungal feeding to bacterial feeding nematode ratio (Figure 3.10). This indicates that as P fertilization managed grasslands and nutrient becoming richer, bacterial metabolism become increasingly favoured relative to fungal metabolism. The increasing role of the bacterial-based energy channel with increasing nutrient contribute to faster rates of mineralisation, the higher productivity , and at the same time, maybe cause some environmental problems, such as greenhouse gas emissions, and water pollution.

3.4.6. Conclusion

In the 1950's when soil P levels were low, animal productivity and health were serious national concerns. During the last 50 years Irish agriculture benefited greatly from the continuous use of fertiliser. As soil P levels rose, production increased steadily. Agronomically, a valuable capital asset was created in the form of a large pool of soil phosphorus. However, it becomes apparent that the large inputs of P fertiliser and the increasing soil P status contributed to increased loss of P to water (Tunney, 1990).

The shifts in many of the variables in this study from 'time 0' to the first 30 years of P fertilization, and then another 10 to 12 years' changes demonstrate that to effectively evaluate the effects of long-term P application on soil ecosystem function, it is essential to assess their effects when the agricultural ecosystem has attained different degree of equilibrium.

In grasslands, therefore, a shift from fungal to bacterial pathway happened in more intensively managed systems which were possibly related to the deterioration of ecosystem complexity following continuous P application. On the other hand, soil ecosystems contain many specific interactions at different spatial and temporal scales with food webs. Studies of soil food web complexity in grassland system have been conducted in relation to systems stability and nutrient cycling. In addition, above-ground plant diversity and grazing have strong effect on

the soil ecosystems, which we have not been mentioned in the Cowlands experiment.

Chapter 4. Responses of grassland soil nematodes to applications of cattle slurry and N fertilizer

4.1. Introduction

Nitrogen is an essential element for plant growth, and is often the key limiting nutrient in grassland soil systems (Haynes and Williams, 1993; Moir, 2007). Dairy cattle manure and slurry are a potentially important source of nitrogen (N), as well as phosphorus (P), potassium (K) and other nutrients, and can replace or supplement the use of chemical fertilizers in crop production (Bittman *et al.*, 1999). Slurry provides a rich source of labile inorganic nutrients, and increases microbial activity and the growth of soil organisms (Bardgett *et al.*, 1998; Wardle *et al.*, 2004). In addition to major plant nutrients, manures contain an array of organic compounds such as carbohydrates, fatty acids and peptides that are substrates for growth of heterotrophic soil microorganisms (Paul and Beauchamp, 1989), whereas chemical fertilizer does not (Bittman *et al.*, 2005). Although in more intensively managed pastures inorganic nutrients provide an increase in bacterial biomass in the short term, the physical increase in organic matter resulting from manure and urine deposition contribute to an overall increase in soil fungal biomass over the long term (de Vries *et al.*, 2007).

Fertilization with inorganic and organic fertilizer is common in grassland management, especially when initial levels of soil organic matter are low, so soil organic matter content and soil biological activity can be enhanced through inorganic as well as organic fertilizers (van Eekeren *et al.*, 2009). Inorganic N fertilizers (containing only mineral N) feed the plant and soil microorganisms directly and the entire soil biota both directly and indirectly by increased root biomass and exudates and plant litter and through increased microbial biomass. However, inorganic N fertilizers involve high fossil energy consumption and N is relatively easily lost from the soil by nitrate leaching and denitrification (van Eekeren *et al.*, 2009). Organic fertilizers (20–50% mineral N) on the other hand, such as cattle manure and leguminous green manure, rely upon microbial degradation and mineralization of organic matter, to supply nutrients for subsequent crop use (Bolton *et al.*, 1985), and feed the soil biota directly and the crop both directly (inorganic constituents) and indirectly following mineralization of organic constituents (Sonneveld and Bouma, 2003; Reijs *et al.*, 2007). When more N is organic and mineralized slowly, the risk of leaching is reduced as long as an actively growing crop is present that can take up the nutrients supplied. Nitrogen is mineralized by predators of bacteria and fungi, such as protozoa and microbivorous nematodes. Excess nitrogen generated

by microbial grazing is released to the soil and becomes available for plant uptake. Additions of organic matter to soil are, therefore, expected to increase numbers of bacterivorous and fungivorous nematodes and decrease numbers of plant-parasitic nematodes (Griffiths *et al.*, 1994).

Many previous studies have shown that even single applications of dairy manure under pasture would increase soil microbial biomass (Barkle *et al.*, 2001; Stenger *et al.*, 2001), whereas some research work showed that application of inorganic fertilizer generally does not increase soil microbial biomass (Biederbeck *et al.*, 1996; Hopkins and Shiel, 1996). Better knowledge of factors affecting turnover of the microbial biomass would improve current understanding of nutrient fluxes in manure-treated soil.

The soil micro-fauna, i.e. protozoa and nematodes, are important consumers of the microbial biomass (Hunt *et al.*, 1987; Forge *et al.*, 2003) and are therefore indicative of microbial turnover and fluxes of nutrients through the soil food web. Soil fauna affects the mineralization of nutrients from soil organic matter. Their contribution to N mineralization has been estimated at about 30% for a range of natural ecosystems and agro-ecosystems (Verhoef and Brussaard, 1990). Soil micro-fauna, such as nematodes, occupy several key functional groups in the soil food web, and consequently nematode community structure is responsive to changes to both the basal soil food resources as well as soil physico-chemical conditions (Ferris *et al.*, 2001). Most nematode indices have previously provided insight into the dynamics of the nematode community in the relation to the comparison of agricultural systems. Nematode excretion may contribute, at certain times, up to 27% of soluble nitrogen (Ekschmitt *et al.*, 1999).

The abundance of microbivorous nematodes and some indices of nematode community structure have been correlated with net N mineralization or potentially mineralizable N across sites or fertility treatments in field studies (Forge and Simard, 2001; Ferris and Matute, 2003). Opperman *et al.* (1993) correlated bacterivorous nematode population growth with soil nitrate levels after the application of cattle slurry to soil. Whereas Griffiths *et al.* (1998) related differences between cattle and pig slurries, in terms of dissolved organic carbon content and potential for denitrification, to differential population growth of bacterivorous nematodes and protozoa.

Most studies focused on 'disturbed' soil. After disturbance, microbivorous nematodes (bacterivores and fungivores, called 'colonizers') with high reproduction rates and short life cycles, are thought to respond rapidly to high nutrient availability. Omnivorous and predatory nematodes (called 'persisters'), with low reproduction rates and longer life cycles, are

believed to be more sensitive to soil disturbance (Bongers, 1990). Any soil disturbance can affect nematode trophic structure and total abundance. In agroecosystems, tillage is the major disturbance to soil and it causes the redistribution of plant residue and soil organic matter, subsequently changing microbial structure and nematode trophic structure. For examples, Fu *et al.* (2000) monitored the response of different trophic groups of soil nematodes to residue decomposition in conventional tillage (CT) and no-till (NT) regimes, which were two sources of soil disturbances in their experiment treatments: application of crop residue (resource enrichment) and plowing (physical disturbance). They found that bacterivorous nematodes responded much earlier and faster to residue application than fungivorous nematodes, and fungivore numbers started increasing 1 month after residue application at both layers in CT and at the surface layer, but not in the deep layer of the NT treatment. Dmowska and Kozlowska (1988) also pointed out that plowing on arable land stimulated mineralization and resulted in increase of nematode numbers and dominance of the opportunistic taxa. Yeates and King (1997), who compared intensively managed grasslands with adjacent sites on the same parent material, which had been managed under organic prescriptions for several years, found that nematode populations were larger in the organically managed grassland systems and fungal-feeders were twice as abundant as in conventionally managed soils. Coleman *et al.* (1983) concluded that intensively managed grasslands appear to correspond to the 'fast cycle' dominated by labile substrates and bacteria, while less productive, organically fertilized grasslands relate to the `slow cycle' dominated by more resistant substrates and fungi.

The effective and efficient use of livestock manures as sources of nutrients is an important component of sustainable crop production system. In Atlantic Europe, particularly in Ireland, North of France and the UK, ruminant livestock production is based on grazing of grass *in situ*. The seasonal variation in growth rates usually results in a requirement for animals to be housed during periods of low grass growth during which time the animal diet is based on conserved grass forages that have been harvested and stored during high growth periods. Developments in grazing management technologies are increasing the length of the grazing season, thereby reducing the housed period. Seasonal sampling therefore in this study may increase understanding of soil processes in grassland management.

In Chapter 3, differences were observed between the morphological identification and T-RFLP analysis on nematode community structure. To determine if the nematode DNA dilutions (1: 10 dilution used in chapter 4) affect the PCR products and the final T-RFLP results, two series of nematode DNA dilution (1: 10 and 1: 100) will be compared in this chapter.

The hypothesis here is that nematode abundance would be stimulated more by organic than inorganic fertilizers, thus the objective of this experiment is to (1) compare the effects of an inorganic N fertilizer and cattle slurry on soil nematode abundance and community structure, particularly over time; (2) identify nematode taxa stimulated by different resources of fertilization, using the terminal restriction fragment length polymorphism (T-RFLP) technique developed earlier to characterise the nematode community structure; (3) explore the links between below-ground nematode community and N-cycling from the herbage dry matter production and N uptake, in order to understand soil microbiological status and nitrogen flux.

4.2. Materials and methods

4.2.1. Site and experimental setup

The experimental sites were located at Johnstown Castle, County Wexford (52°18'; 6° 30'W) (JC), Ireland. The field plots sampled in this study were part of a multifaceted study of the effects of the nitrification inhibitor dicyandiamide (DCD) mixed with cattle slurry on herbage dry matter production and N uptake (Cahalan *et al.*, 2011). The study applied dairy cattle slurry and N fertilizer to grassland at variable rates and frequencies from 2008 - 2011. The sward at each site was predominately perennial ryegrass (*Lolium perenne*) and has been used for silage production. White clover (*Trifolium repens*) and broad-leaved docks (*Rumex obtusifolius*) were removed from the swards using MCPA AMINE 500 herbicide (Nufarm, UK Limited) at the start of the experiment.

The experiment consisted of a randomized complete block design (Figure 4.1). Soil physical and chemical properties are presented in Table 4.1. The total area of the site was divided into six paddocks (blocks), each approximately 10 m^2. Plot sizes were 5m × 2m, with an additional 0.5 m discard area around each plot to prevent edge effects. A basal dressing of P and K as 0-7-30 (N-P-K) compound fertilizer at 250 kg ha^{-1}, i.e. in excess of plant requirements, was applied in autumn 2007 (i.e. the year before the experiment began) to ensure any herbage response would be solely due to the addition of N fertilizer. Cattle slurry was applied at the rate of 33 m^3 ha^{-1} in March, June or October of each year. These are common application dates and a typical application rate for slurry in Ireland. October and March are either side of the closed period for slurry applications and in June it is common practise to apply cattle slurry onto grass stubble after the first cut silage has been removed. Separate plots were used for the March, June and October slurry treatments rather than reusing each plot after 4 months to avoid variability due to mineralization of the organic N fraction of the slurry from the previous slurry application.

The 5 treatments sampled in this study were: 1) Control, no fertilizer or slurry application (CON); 2) Fertilizer, 40 kg N ha^{-1} (Fer40); 3) Fertilizer, 120 Kg N Ha^{-1} (Fer120); 4) Cattle slurry (CS), and 5) Cattle slurry applied only in October (Oct CS). Fertilizer (Fer40, Fer120) and cattle slurry (CS) were applied to the plots in March, June and October of 2010 and next February of 2011, while Oct CS was only applied in October in 2010. Cattle slurry was applied manually using modified watering cans to simulate splash plate spreading.

Table 4.1. Soil chemical and physical properties in the study site.

Property	Sand	Silt	Clay	N	C	C/N	pH
Unit	%	%	%	%	%	ratio	
	47.2	37.7	15.1	0.27	3.1	11.3	6.3

4.2.2. Slurry analysis

Slurry NH_4^+-N contents were determined by extracting each slurry liquid sample with 0.1 *M* HCl acid and diluted to 1:10 in 250 ml flask, shaking thoroughly by hand hardly for 3 min to make sure the suspension was well-disturbed. Then the filtered suspension was followed by analysis on an Aquakem 600 discrete analyser (Thermo Electron OY, Vantaa, Finland).

4.2.3. Herbage yields and N uptake analysis

Herbage was harvested 4 months (cut 1, from 3rd March to 30th June 2010) after treatment applications in March and June allowing sufficient time for uptake of nutrients applied in the slurry. A residual harvest (cut 2, 2nd Oct. 2010) was taken from each plot 3 months after cut 1 to take into account N becoming available from the organic fraction of the slurry. A final herbage sample was taken at the end of the experiment (Cut 3, 15th of March 2011), the herbage harvest dates are detailed in Table 3.2. Harvesting was carried out using a Haldrup plot harvester with a cutting width of 1.5 m. A central strip was cut through each plot ruling out the influence of edge effects. The fresh weight of the harvested herbage from each plot was recorded and sub-samples (*c.* 100 g) of herbage were taken for herbage dry matter (DM) determination for each individual plot. Each herbage sample was dried at 40°C for at least 16h before being milled to 2mm. Chemical analysis was then carried out on the milled samples for % N by a LECO-FP 200 combustion analyser and P, K, S, Mg, Ca and Na were determined using a nitric/perchloric acid microwave digestion followed by ICP analysis using a Varian Liberty ICP spectrometer. Here only herbage from Cut 1 was used for P, K, S, Mg, Ca and Na analysis, to know the basic grass nutrient conditions.

Nitrogen uptake was calculated by multiplying the N concentration in the herbage by the DM

yield and expressed as g N uptake per plot. Herbage growth rates were calculated by dividing the herbage mass after cutting by the number of days between harvest and treatment application.

Block1	Block2	Block3	Block4	Block5	Block6
		Fer40	Fer120		CS
				Fer120	
	CON				
	Fer120				
					Oct CS
Oct CS	Oct CS		Fer40	CS	
		CS			
CS					Fer40
Fer120					
		Fer120		CON	
	CS			Fer40	
	Fer40		CS		
		Oct CS			
			Oct CS		
CON				Oct CS	
Fer40		CON		Oct CS	CON
			CON		Fer120

Figure 4.1. The detailed information on the N fertilizer and cattle slurry application plots in Johnstown Castle, Wexford sampled in this experiment. Treatments are listed as described in Section 4.2.1. Blank plots contain treatments not sampled for nematodes.

4.2.4. Soil sampling and nematode extraction

In the early spring of 2010 (Feb.), soil samples were taken from the 5 treatments before N fertilizer and cattle slurry application, then one week and one month after N fertilizer and cattle slurry application in spring, summer, autumn of 2010 and early spring 2011 (Table 4.2),. Twenty cores in a stratified random design were taken using an auger to a depth of 10cm from each plot and mixed together. Prior to nematode extraction, all soil samples were stored at 4°C. The moisture content of fresh soil was determined by oven drying at 105°C for 12 h. Soil samples for nutrient analysis were oven-dried at 40°C for 24 h.

4.2.5. Nematode DNA extraction, DNA dilution, PCR amplification and digestion

Nematode DNA extraction, PCR amplification and dilution are described in Section 3.2.4. Considering Fer40 and Fer120 were both N fertilizer treatments, the Fer120 treatment was chosen to represent the N application treatment. Nematode samples, analyzed with T-RFLP, were collected from four treatments CON, Fer120, CS and Oct CS.

To determine the amount of DNA necessary in the digestion to allow detection of soil nematode types with the T-RFLP method, two DNA dilution series were made to 1: 10 and 1: 100 from the samples in Autumn 2010. After comparison, the 1: 10 dilution was selected for T-RFLP assessment of extracted nematode DNA.

Table 4.2. Dates for N fertilizer and slurry application, grass cut and soil sampling.

| Sample date | Operation | | Sampling |
	fertilizer and slurry	cut	
10-Feb-10			√
03-Mar-10	√		
10-Mar-10			√
14-Apr-10			√
30-Jun-10		√	
05-Jul-10	√		
12-Jul-10			√
10-Aug-10			√
02-Oct-10		√	
03-Oct-10	√		
10-Oct-10			√
18-Dec-10			√
02-Feb-10			√
08-Feb-10	√		
15-Feb-11			√
08-Mar-11			√
15-Mar-11		√	

4.2.6. Molecular identification of nematode taxa

Nematode community data analyzed from T-RFLP peaks were assigned to the following trophic groups characterized by feeding habits: bacterial feeders (Ba), fungal feeders (Fu), plant-feeders (Pl), omnivores (Om) and predators (Pr) (Donn, 2008), as detailed in Table 3.2.

4.2.7. Statistical analysis

All soil and herbage results were expressed on the basis of the oven-dry weight. Data is shown as means (n = 6) and standard errors (SE) of the measured variables. The significant difference among the fertilization treatments at each sampling time was tested by one-way ANOVA followed by the Fisher's least significant difference (LSD) test. In addition, two-way ANOVA was used to analyze the treatment effect, sampling effect and their interaction on herbage yield, N uptake, soil nematode abundance, and nematode community analysis. Principal components analysis (PCA) was performed to explore the nematode community in relation to N fertilizer and cattle slurry manure management. All statistical analysis was done using the Statistica software package (StatSoft Inc., Tulsa, OK, USA).

4.3. Results

4.3.1. Cattle slurry and N fertilizer application

The N applied to each plot from Fer40 and Fer120 were 20 g and 60 g for every application, while the N from cattle slurry was around 16.5 g (Table 4.3). The total N applied to the plots was 240 g N for Fer120, 80 g for Fer40 66.2 g, CS, 17.1 g N in total Oct CS treatment.

Table 4.3. The information from slurry analysis applied into the plots. DM means slurry dry matter, and TN means slurry total nitrogen content.

Date	DM	pH	NH$_4^+$-N	Slurry TN	Total N applied to plots
	%		mg/kg	%	g
Mar-2011	7.56	-	1554	0.25	16.2
Jun-2011	7.12	7.97	775	0.25	15.8
Oct- 2011	5.7	7.36	1711	0.31	17.1
Feb-2012	6.04	7.13	1537	0.31	17.1

4.3.2. Effects of N fertilizer and cattle slurry on herbage yields and herbage N uptake

The two-way ANOVA data (Table 4.4) showed that both fertilizer application and sampling time had significant effects on herbage DM yield and N uptake ($P < 0.001$), and also their interaction had a significant effect on herbage DM yield ($P < 0.01$), but not on N uptake.

There was a seasonal variation in the herbage DM yield, N content and N uptake of the herbage (Figure 4.2a, b and c). There were higher DM yields with a high N uptake in herbage

in Cut1 while N content was lower.

Herbage N content showed no difference in Cut 2, while cattle slurry application increased herbage N absorbed in summer harvest. In Cut 3 N content in Fer40, Fer120 and CS treatments were significantly higher than CON and Oct CS treatments.

Compared to the control treatment CON, both medium Fer40 and higher Fer120 N application significantly increased total herbage DM yields over the entire experiment by 34.5% and 85.7%, respectively (Figure 4.3). While slurry application (CS and Oct CS) did not affect total herbage DM significantly.
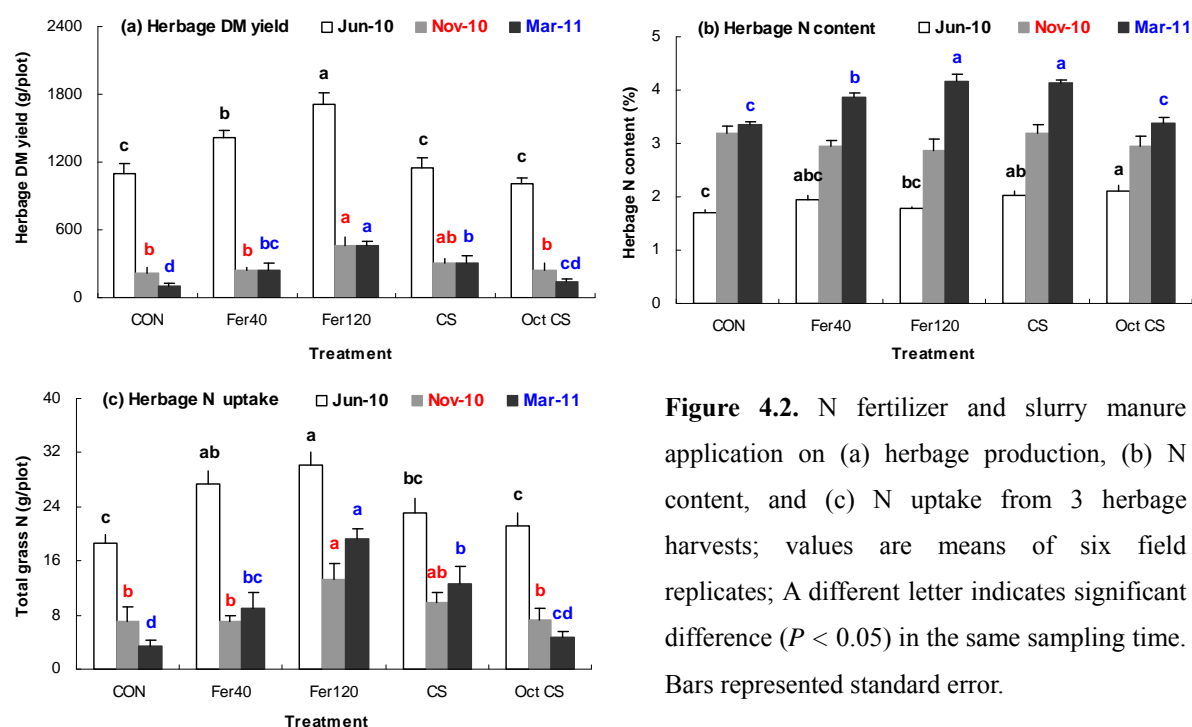
Figure 4.2. N fertilizer and slurry manure application on (a) herbage production, (b) N content, and (c) N uptake from 3 herbage harvests; values are means of six field replicates; A different letter indicates significant difference ($P < 0.05$) in the same sampling time. Bars represented standard error.
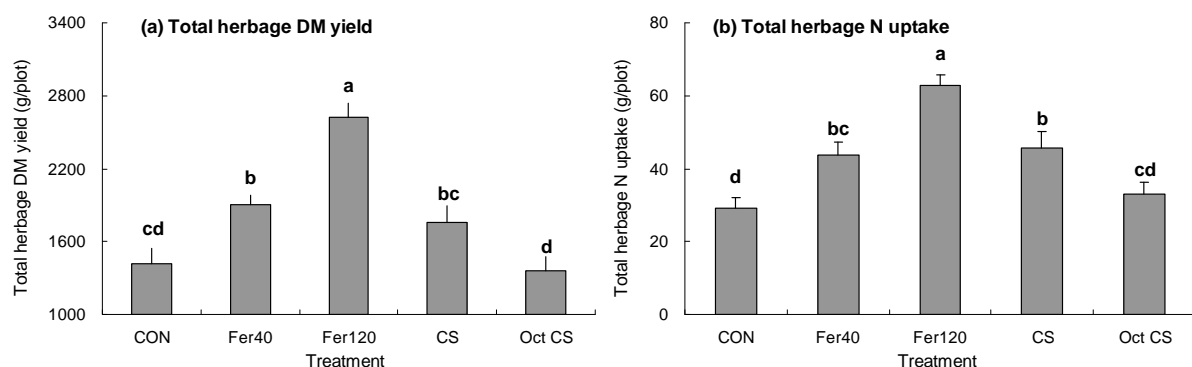
Figure 4.3. N fertilizer and slurry manure application on (a) total herbage yield and (b) total N uptake during the whole sampling time (from Feb-2010 until Mar-2011); values were means of six field replicates; Different letter indicates significant difference ($P < 0.05$). Bars represented standard error.

Table 4.4. Two-way ANOVA results of N fertilizer and slurry treatments (T) and sampling time (S) on herbage DM yield and total N uptake.

	d.f.	Herbage DM yield		
		MS	F	*P*
Treatment (T)	4	499099	21.25	***
Sampling time (S)	2	10021221	426.60	***
T*S	8	82776	3.52	**
Error	74	23491		

	d.f.	Total N uptake		
		MS	F	*P*
Treatment (T)	4	339.11	17.58	***
Sampling time (S)	2	2,171.97	112.57	***
T*S	8	38.91	2.02	ns
Error	74	19.30		

	d.f.	N content		
		MS	F	*P*
Treatment (T)	4	0.36	4.34	**
Sampling time (S)	2	26.53	319.13	***
T*S	8	0.44	5.26	***
Error	74	0.08		

Values labelled '**' and '***' were significant at $P < 0.01$ and $P < 0.001$, respectively; 'ns' was not significant.

In general, fertilisation increased total N uptake during the whole sampling time (Figure 4.3). Significantly higher levels were observed in the Fer120 treatment which increased N uptake 115.4% over the control treatment. CS and Fer40 treatments had a significantly higher effect (54.3% and 49.8, respectively) than CON, while no differences were found between CON and Oct CS.

The herbage nutrient parameters from Cut 1, showed that N fertilizer (Fer40 and Fer120) increased plant P and Mg absorption from soils, Fer120 had a favourable effect, not only on total P and Mg, but also on total K, Ca and Mn uptake. Oct CS application did not affect the plant nutrient uptake, while CS only significantly increased the plant Mg uptake (Figure 4.4).

4.3.3. Effects of N fertilizer and cattle slurry on nematode abundance

Total nematode abundance was affected significantly ($P < 0.001$) by the treatments, sampling times and their interaction (Table 4.5).

Compared with the control, CS treatment increases abundance significantly from mid-summer,

while the Oct CS caused a spike immediately after autumn slurry application (Table 4.6). N Fertilizer did not increase nematode abundance.
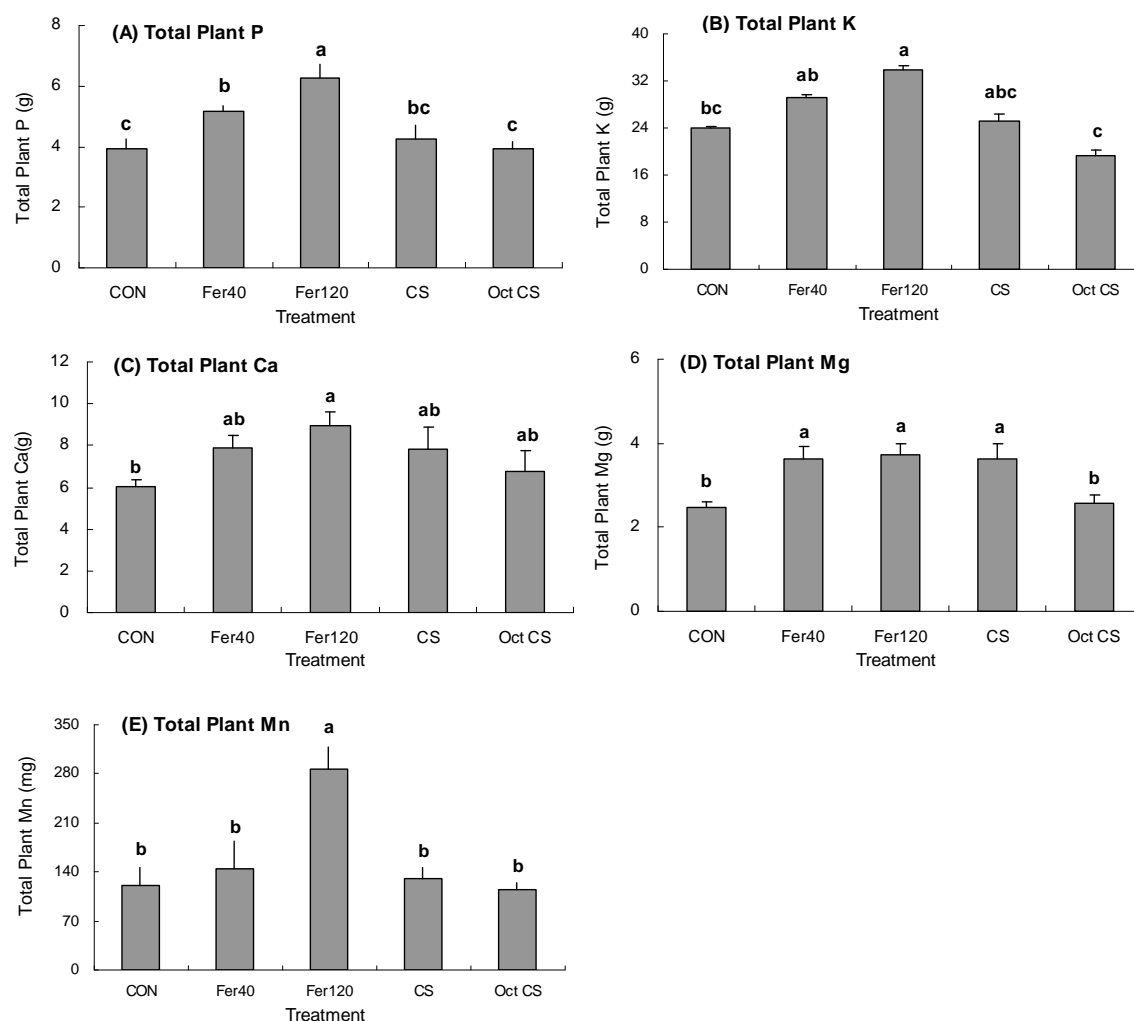
Figure 4.4. N fertilizer and slurry application effects on herbage nutrient composition from the grass samples in June-2010 (cut 1), (A) total P, (B) total K, (C) total Ca, (D) total Mg, and (D) total Mn. Values were means of six field replicates; A different letter indicates significant difference ($P < 0.05$). Bars represented standard error.

Table 4.5. Two-way ANOVA results of N fertilizer and slurry treatments (T) and sampling time (S) on nematode abundance for all samples.

	d.f.	Nematode abundance		
		MS	**F**	**_P_**
Treatment (T)	4	163.42	15.48	***
Sampling time (S)	8	132.05	12.50	***
T*S	32	28.97	2.74	***
Error	222	10.56		

Values labelled '***' were significant at $P < 0.001$.

4.3.4. Comparison of 1/10 and 1/100 DNA dilution on nematode community analysis

Table 4.7, comparing the effects of nematode DNA dilutions on nematode functional groups with T-test, revealed that the 1: 10 and 1: 100 dilutions gave very similar results for the nematode community analysis using T-RFLP ($P > 0.05$).

Comparing the different nematode functional groups determined with the two DNA dilutions from T-RFLP analysis (Figure 4.5), there were slight different mean values between the two DNA dilutions. From the whole data analysis, no significant differences between 1: 10 and 1: 100 DNA dilutions were determined. The 1: 10 DNA dilution was therefore used for all subsequent nematode analyses.

Table 4.6. Effect of N fertilizer and cattle slurry application on nematode abundance (nematode number/g dry soil)

Year	Sampling date	Application	Mean ± SE				
			CON	Fer40	Fer120	CS	Oct CS
2010	10th Feb	before	13.9±1.4 a	12.6±1.5 abc	9.9±1.3 bc	9.2±1. 5 c	13.2±1.0 ab
2010	10th Mar	after 1 week	14.3±1.1	17.0±0.7	17.6±2.0	18.1±0.9	16.4±1.3
2010	14th Apr	after 1 month	19.9±1.2 a	21.0±0.5 a	19.9±0.7 a	19.8±0.5 a	15.8±0.8 b
2010	12th Jul	after 1 week	20.9±1.3 bc	19.3±1.1 bc	24.1±2.7 ab	27. 8±1.7 a	18.3±2.0 c
2010	10th Aug	after 1 month	19.2±0.9 ab	16.7±1.0 bc	14.3±2.1 c	21.6±1.1 a	15.9±0. 8 bc
2010	10th Oct	after 1 week	15.0±1.1 b	15.6±1.5 b	16.5±1.4 b	22.8±2.3 a	22.4±1.5 a
2011	2nd Feb	before	15.4±1.4 b	15.9±2.0 b	13.2±0.2 b	22.4±1.6 a	16.3±0.9 b
2011	15th Feb	after 1 week	15.8±1.6 ab	15.6±0.3 b	15.0±1.4 b	20.3±2.0 a	15.2±1.5 b

Values are means of six field replicates; A different letter indicates significant difference ($P < 0.05$) within the same sampling date.

Table 4.7. Comparison of T-test on nematode DNA dilution 1: 10 and 1: 100 on nematode functional groups assessed by T-RFLP. Nematode functional groups included (a) bacterial feeders (Ba); (b) fungal feeders (Fu); (c) plant-feeders (Pl); (d) omnivores (Om) and (e) predators (Pr) (Yeates *et al.*, 1993).

		1:10 Dilution	1:100 Dilution	t-value	d.f.	p	F-ratio
Ba	1:10 vs. 1:100	40.45	38.58	1.01	94	0.313	1.117
Fu	1:10 vs. 1:100	0.75	0.57	0.60	94	0.548	1.570
Pl	1:10 vs. 1:100	24.14	24.82	-0.45	94	0.651	1.158
Om	1:10 vs. 1:100	26.38	26.54	-0.10	94	0.922	1.310
Pr	1: 10 vs. 1:100	8.28	9.49	-0.87	94	0.389	1.047

4.3.5. Effects of N fertilizer and cattle slurry on nematode community structure

Nematode communities and functional groups assessed by T-RFLP were affected significantly ($P < 0.001$) by treatments and the sampling times, and their interaction across time (Table 4.8).

Nine main nematode groups detected by T-RFLP were Rhabditida (*Panagrolaimus*,

Mesorhabditis, *Rhabditis*, and *Cephalobidae*), Plectida, Aphelenchida, Tylenchidae, Dorylaimida (Dorylaimida and Nygolaimidae), *Longidorus*, Mononchidae, Pratylenchoides, and *Helicotylenchus*. From the T-RFLP data, the most abundant nematode taxa were *Longidorus*, *Rhabditis* and Pratylenchoides. Populations of plant-feeding nematodes (average 37.1%) and bacterial-feeding nematodes (average 37.6%) were significantly higher in all treatments than the other three functional groups.

Table 4.8. Two-way ANOVA results of N fertilizer and slurry treatments (T) and sampling time (S) on (a) nematode taxa and (b) nematode trophic groups assessed by T-RFLP.

(a) nematode taxa	Value	F	Effect d.f.	Error d.f.	p
Treatment (T)	0.1985	7.26	48	482.62	***
Sampling time (S)	0.0041	10.53	128	1180.92	***
T × S	0.0065	2.65	384	2219.18	***
(b) nematode trophic groups	**Value**	**F**	**Effect d.f.**	**Error d.f.**	**p**
Treatment (T)	0.4692	12.71	12	460.65	***
Sampling time (S)	0.0273	33.29	32	643.28	***
T × S	0.1646	4.16	96	691.82	***

Values labelled '***' were significant at $P < 0.001$.

PCA from all sampling data was performed on the composition data from T-RFLP profiles (Figure 4.6). CON was associated with 620, 582, 226 and 137, which were mainly fungal-feeding nematodes; inorganic N was associated with 132, 137 and 593, which were bacterial-feeding nematodes; CS and Oct CS were associated with 326, 599, 356 and 298, which represented different trophic groups. PCA performed a separation of the 4 treatments, while just CON had a clear difference to other three treatments, indicating that N fertilizer and cattle slurry application changed the nematode community composition compared to the CON treatment.

From the first sampling time before spring N fertilizer and cattle slurry application, there were more bacterial feeders in the CS and Oct CS treatments, more plant feeders in Fer120 than the control (Figure 4.7), but a greater proportion of omnivorous nematodes in the CON treatment. After slurry and fertilizer application, the nematode community in all treatments underwent complicated changes. N fertilization tended to significantly increase bacterial-feeding nematodes, while, there were no obvious differences in CS and Oct CS after slurry application.

From Figure 4.7, it was apparent that the proportion of fungal-feeding nematodes in the community was very small (average 1.21%), and fungal-feeding nematodes mainly occurred in CON treatment rather than in other three treatments.
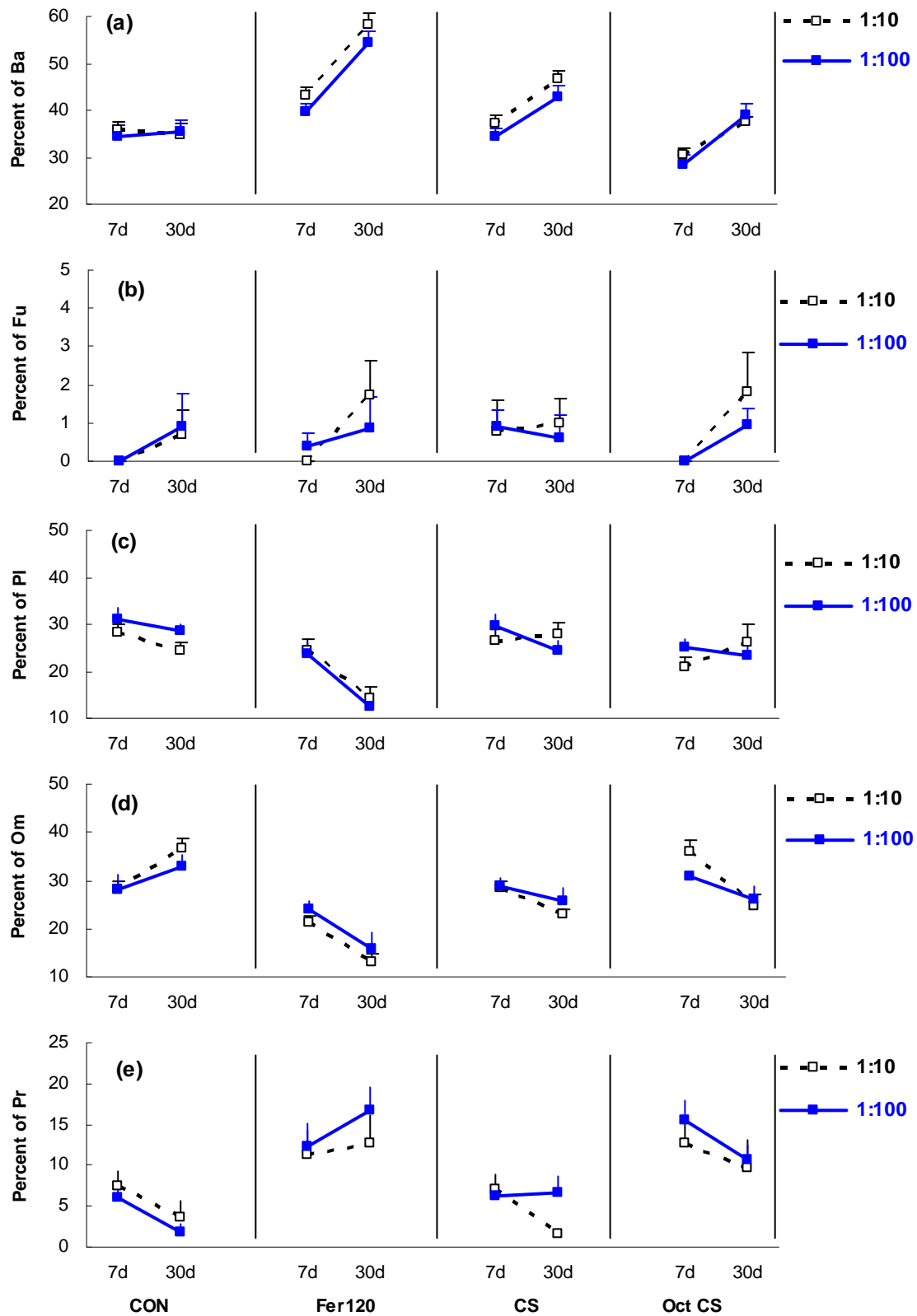
Figure 4.5. Comparison of nematode DNA 1: 10 and 1: 100 dilutions on nematode functional groups analysis using T-RFLP. Nematode trophic groups included (a) bacterial feeders (Ba); (b) fungal feeders (Fu); (c) plant-feeders (Pl); (d) omnivores (Om) and (e) predators (Pr) (Yeates *et al.*, 1993).
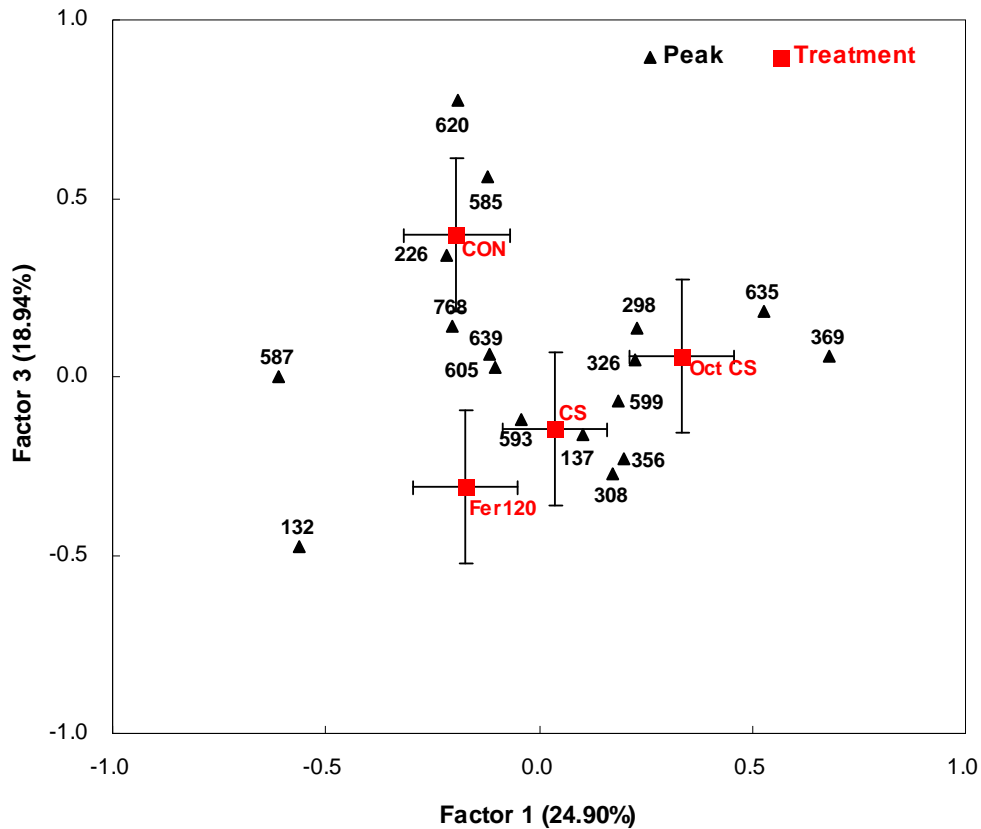
Figure 4.6. Principal component analysis of the relationship between different treatments ■ and nematode community and composition (TRFLP – peaks, see Table 3.2) ▲ treatments. N = 6, bars represented standard errors.
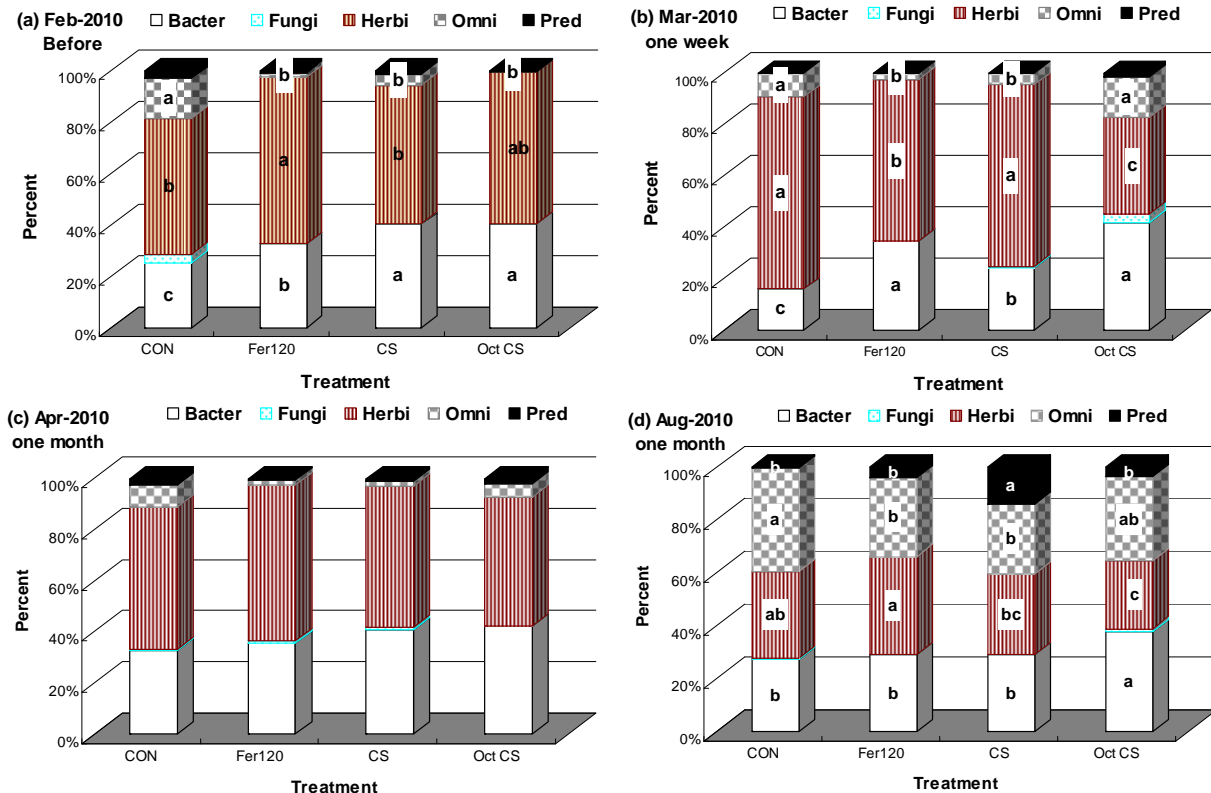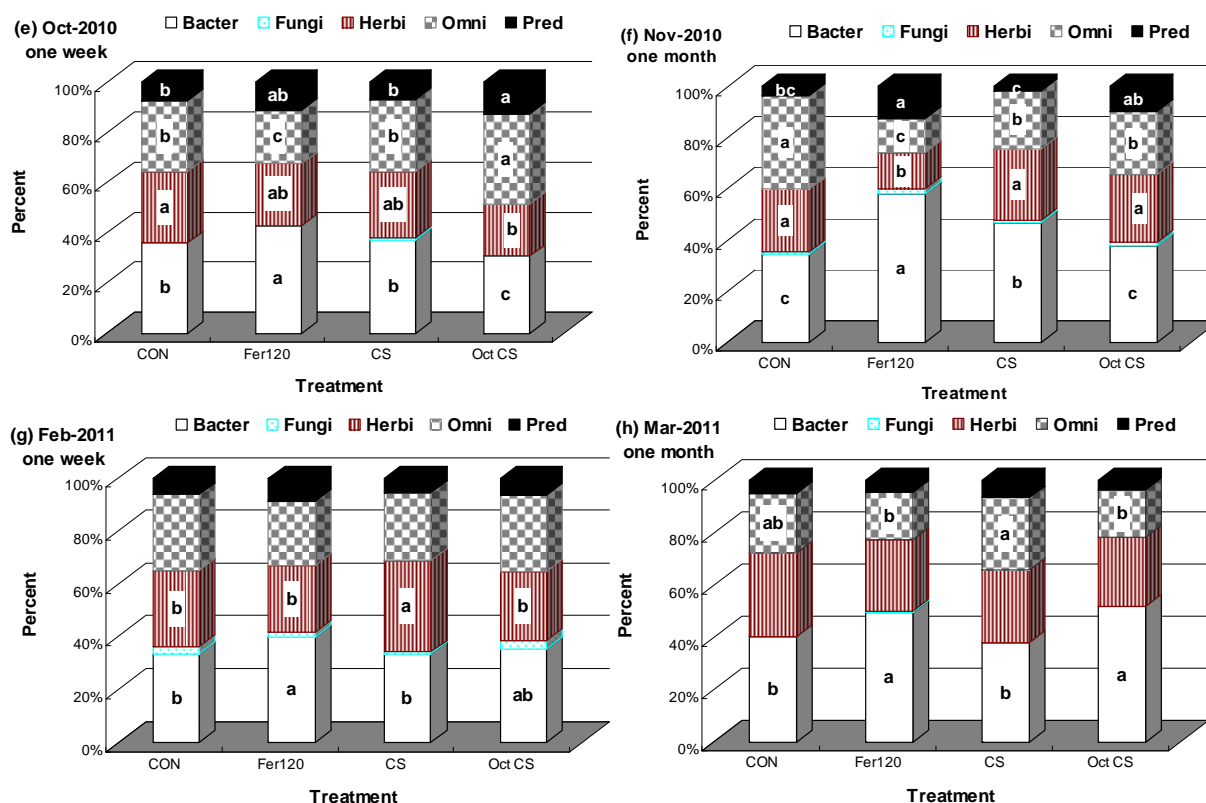
Figure 4.7. Mean percentage of nematode trophic groups for the every time's samples using T-RFLP method. The letters a, b, c and d came from LSD test, and a different letter meant significant difference for the samples within the same sampling time ($P < 0.05$).

4.4. Discussion and conclusion

The objectives of this experiment were to evaluate: (1) the effects of N fertilizer and cattle slurry application on soil nematode abundance and community structure over time (assessed using T-RFLP); (2) combining the herbage DM yield and N uptake, to find the links between above-ground herbage growth and the below-ground nematode community; and (3) to understand short-term effects of N application on soil microbiological status and nitrogen flux.

4.4.1. Effects of N fertilizer and cattle slurry on nematode abundance and nematode community structure

The hypothesis, that the abundance of nematodes would be stimulated more by organic than inorganic fertilizers was confirmed. The experimental data showed that slurry application increased nematode abundance on most sampling occasions compared to N fertilizer treatments and the control. The inorganic N fertilizer treatments Fer40 and Fer120 did not affect nematode abundance on most sampling occasions. Yeates (1987) observed a positive

relationship between total nematode abundance and increased herbage production in New Zealand grasslands. However the data from this study showed that whilst a statistically significant increase in herbage production was observed due to inorganic N fertilizer, there was no significant effect on nematode abundance. There was a significant increase in nematode abundance due to cattle slurry application, but no increase in herbage production. The main reason may be that in this plots, slurry N application (CS, 16 g N for each N applied time) is much lower than the highest level of inorganic N (Fert120) but much close to the lower inorganic N level Fert40 (60 g N for each N applied time). The differential availability of the inorganic and organic N probably account for the observed differences in herbage yield. While the inorganic fertiliser N, is readily available for absorption by the grass, the slurry is spatially separated, in that the solid component remains on the soil surface leaving ammonia readily and temporally volatisable, as the organic fractions will only release N slowly as decomposition progresses.

When averaged over all sample dates, bacterivorous nematodes were more abundant in the high fertilizer treatment Fer120 and in the two different slurry manure treatments CS and Oct CS than in the control treatment CON. This is inconsistent with previous findings that bacterivorous nematodes were more prevalent under organic than conventional chemical fertilizer treatment (Ferris et al., 1996; Neher, 1999; Berkelmans et al., 2003). But their studies focused on managed agricultural soils rather than grassland soils, which might be the reason why my results differed with others.

Combining the consistence on the increase in bacterial-feeding nematodes and above-ground herbage yield, N uptake, suggesting that inorganic N application is undergoing a bacteria-dominated decomposition pathway (Ferris et al., 2001). Griffiths et al. (1998) observed that the number of protozoa responded more quickly to the application of pig manure slurry than cattle manure slurry and explained this by the greater proportion of readily available C in pig slurry compared to cattle slurry. So it explained that the slurry was higher C/N manure, and N releases were slower to provide the sufficient N to grass growth.

Generally, slurry manure is surface applied to grassland, leading to environmentally unacceptable N emissions through ammonia volatilization (Huijsmans et al., 2001). To reduce ammonia emissions, alternative slurry manure application methods were developed (Wouters, 1995), which led to a range of so called low-emission techniques, from injection at a depth of 15 cm to band spreading. Schils and Kok (2003) found that slit injection had a positive effect on N utilization of grassland. De Goede et al. (2003) studied slit injection on grassland, and found that in the summer slit injection had a negative effect on the earthworm population. A

reduced earthworm population signifies a potentially lower N mineralization. In the experimental plots, slurry is applied to the surface and not incorporated, so available nutrients would be slowly leached into the soil and would not be immediately available for microbes and nematodes. Perhaps there are indirect effects through increased root production. The main effects are in CS, which had a continued slurry application, rather than Oct CS so something might build up in the soil to encourage nematodes.

4.4.2. T-RFLP method on nematode community analysis

T-RFLP has several key advantages over morphological methods for studying nematode diversity, particularly the considerable saving in time. In addition, morphological identification of soil nematodes is a skilled task that requires training and much practice to achieve rapid results, whereas the T-RFLP technique requires only rudimentary skills in molecular biology.

Plant-parasitic and bacterivorous nematodes were the most abundant trophic groups present in the soils given inorganic and organic fertilizer. Although numbers of both trophic groups were greater in fertilized plots, these results contrast with other reports that suggest that numbers of plant-parasitic nematodes decrease after additions of organic amendments (Bohlen and Edwards, 1994; Griffiths *et al.*, 1994; Clark *et al.*, 1998). This may be due to the incorporation of organic matter in the former studies whereas the slurry was not incorporated in this study.

Fungivore: bacterivore ratios observed in this study were relatively small (median = 0.10), indicating a predominance of bacterivorous nematodes in both management systems. This study contrasts with that of Bohlen and Edwards (1994), who found smaller bacterivore: fungivore ratios in soils amended with leguminous green manures (1.45) and ammonium nitrate (1.66) than with cow manure (1.83). The T-RFLP indicated a very small percent of fungal feeders in my plots. This may be because T-RFLP is not so sensitive at detecting the fungal-feeding nematodes. For example, from the Cowlands study (Chapter 3), the average percentage of fungal-feeding nematodes was 13.6% with morphological identification, but only 4.2% with T-RFLP identification. This might arise if: (a) DNA was extracted less efficiently from fungal-feeders; (b) DNA from fungal-feeders was discriminated against during PCR amplification, or (c) the small size of fungal-feeders, for example, Aphelenchida in this study, with average body length 530 μm, was smallest nematodes in all nematode samples, and could have led to a reduced TRFLP signal. This latter point is explored in Chapter 5.

Commonly agricultural managements such as fertilizer application lead to disturbance and

changes in the composition of nematode fauna (Yeates and King, 1997), and the effects of inorganic and organic fertilization may be different. From the results of this experiment nematode abundance in cut grassland benefits was more responsive to cattle slurry than N fertilizer application.

4.4.3. Effects of N fertilizer and cattle slurry on herbage yields and herbage N uptake

Herbage dry matter (DM) yields followed local seasonal patterns, i.e. it was generally greatest in spring, declined during the dry summer months, and then decreased in the winter. DM production was highest in Cut 1 (Figure 4.2, spring season), and then decreased in the Cut 2 (Nov-2010, i.e. summer growth) and Cut 3 (Mar-2011, i.e. autumn and winter growth). Grassland systems in temperate climates are typically associated with a potential to produce high annual herbage dry matter yields, with seasonally variable grass growth rates (Creamer *et al.*, 2010).

Comparing the different seasons on herbage N content, the N concentration in the herbage was lowest in the summer harvest, while the herbage DM yield was highest. This was due to the dilution effect on available N during rapid herbage growth. A more complete analysis of grass growth in the main experiment at the site showed similar results (Cahalan, 2012).

Many studies, mainly in arable soils, have shown that organic fertilizers can increase organic matter content, soil biological activity, and potential N mineralization (Bittman *et al.*, 2005; Fliessbach *et al.*, 2007; Birkhofer *et al.*, 2008). However, in grassland soils with a year-round crop, dense roots, and no soil tillage, the effects of the crop on the build-up and maintenance of soil organic matter and biological activity may be larger than in arable soils, and the added effects of organic fertilizers may be less clear (Van Eekeren *et al.*, 2009). The results reported here showed that higher inorganic N fertilizer application strongly affected the herbage DM production and total herbage N uptake. For example, by increasing the N fertilizer rate, from Fer40 to Fer120, increased herbage yields and N uptake. The cattle slurry was slightly less than the Fert40 in terms of the amount of N applied but gave significantly lower responses of herbage production, which probably reflected the differences in availability between the organic and inorganic forms of N.

4.4.4. Conclusion

In this study, the T-RFLP methodology proved successful in revealing changing nematode assemblage composition over season and with different N fertilization strategies in an Irish grassland. Inorganic N and cattle slurry applications had different effects on nematode

abundance and community structure. Nematode abundance increased significantly with slurry application, but not with inorganic N application. Both cattle slurry and inorganic N fertiliser induced similar changes in the soil nematode community structure, indicating increased involvement of the bacterial decomposition channel. The physical distribution of slurry may have prevented the nematode population from responding in a more extensive manner as hypothesized.

Chapter 5. Are all nematodes eutelic?

-The correlation between rDNA copy number and body size in four species of nematode

5.1. Introduction

Nematodes constitute one of the largest and most widely distributed groups of animals in marine, freshwater, and terrestrial habitats (Holterman *et al.*, 2006). PCR amplification of the nematode small subunit ribosomal RNA gene (SSU rDNA) has generated a more complete understanding of nematode ecology and evolution (Blaxter *et al.*, 2005). In recent years, molecular techniques have been increasingly applied to nematode community analysis due to the specialist and time-consuming nature of morphological identification examples. While much of the early work was directed towards plant and animal parasites, the recognition of the important roles free-living soil and marine nematodes play in ecosystems has led to a wider interest in the phylum. Blaxter *et al*. (1998) and Aleshin *et al*. (1998) published the first phylum-wide molecular analyses of the Nematoda and more recently, phylogenetics projects based on hundreds of sequences covering the entire phylum (Holterman *et al*., 2006; Meldal *et al*., 2007) have greatly increased the knowledge base which is necessary for molecular analyses. Molecular analysis of nematode communities relies on an existing framework to match morphological identifications supplied with nucleic acid sequences generated from accurately identified individuals.

However, the majority of previous studies have seen some differences between morphological and molecular assessment of nematode community analysis. For example, several studies have examined the potential of denaturing gradient gel electrophoresis (DGGE) in estimating nematode species diversity, and have found that when DGGE band patterns were compared with morphological taxonomic identification, DGGE tended to underestimate nematode diversity (Foucher *et al.*, 2004; Bhadury *et al.*, 2006). Information gained from Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis has also been used to assess nematode community structure. This method has enabled an assessment of nematode taxonomy at the family level across an entire community (Griffiths *et al*., 2012, Donn *et al*., 2011). The relative area of the T-RFLP peaks could be used quantitatively if applied in combination with a measure of abundance or biomass. From previous work it was suggested, from a comparison of T-RFLP to morphological identification, that the quantification of

nematode types by T-RFLP is dependent on the amount of nematode DNA and thus potentially, at least approximately, the total volume (biomass) of each type, rather than the numerical abundance of each type may be reflected in T-RFLP analysis (Griffiths *et al.*, 2006).

Nematodes are widely considered to be eutelic, *i.e.* to have an adult cell number that does not vary among wild-type individuals irrespective of size as a consequence of invariant cell lineages (Malakov, 1994). Free-living nematodes, which usually have restricted nuclear division, will undergo six to ten fold increase in body length after hatching. Thus, if there is an absence of extensive nuclear division, nematode growth must be due to an increase in cell size and not to an increase in cell number (Sin and Pasternak, 1970). This eutelic assumption of nematode development probably results from the extrapolation of the remarkable constancy of *Caenorhabditis elegans* development to the rest of the phylum (Sachs 1994), because for *C. elegans*, it is well known that a constant and defined number of cells are present in all individuals (Sulston *et al.*, 1983). However, recent studies suggest that most nematodes show considerable amounts of variation for cell number in at least some somatic tissues (Cunha *et al.*, 1999; Azevedo *et al.*, 2000). Sin and Pasternak (1970) found that the number of nuclei in the hypodermis, nerve, and intestine, from the free-living nematode *Panagrellus silusiae*, remains fairly constant during maturation, but there was a slight increase (~57%) in the number of muscle nuclei. Thus, this organism is not stringently eutelie. Cunha *et al*. (1999) reported that eight of thirteen species showed intraspecific variance in the number of epidermal nuclei, with higher variability for species with more nuclei. Azevedo *et al*. (2000) tested 13 free-living nematode species from three families (Cephalobidae, Panagrolaimidae and Rhabditidae), and found that the adult epidermis of most species contained variable numbers of nuclei.

C. elegans, one of the most-used and best-understood model organisms in science, possesses the same number of cells in all individuals, and so differs from mammals and other invertebrates in which body size changes are caused by increases in cell number rather than by increased cell volume (Azevedo *et al*., 2000, 2001). *C. elegans* is therefore eutelic, but some preliminary results showed some epidermal cell number variation which indicates that the idea of eutely should rather be a statistical one (Cunha and Leroi, 1998).

While ecologists assess microbial abundance in soils in terms of biomass, assemblages of other soil organisms usually are expressed as abundance of individuals (Ferris *et al.*, 1996). Yeates (1988) suggested calculation of biovolume as a measure of the importance of nematodes in soil systems but that approach has not been widely adopted. Clearly, the average

data of nematode abundance from the family level is not a good predictor of the weight of many of the genera and species within that family. If the dominant taxon in a sample is of a size lower than the mean, biomass calculations based on average-sized individuals will be inflated; if greater, they will be depressed (Ferris, 2010).

The majority of applications of real time PCR method have applied absolute methods where a standard curve is generated from a serial dilution of a clone of the target amplicon allowing estimation of copy number in reactions using the same conditions. Specific primers designed for nematode T-RFLP analysis Nem_SSU_F74 and Nem_18S_R (Chapter 3 and Chapter 4) produce more than 930 bp of PCR products, and thus the product was considered to be too long to be suitable for qPCR. Therefore other primer pairs, creating a PCR product of shorter length, were assessed for suitability.

To fully interpret the nematode directed T-RFLP pattern used throughout this thesis, a better understanding of the 18S copy numbers within and between species is required. In order to fully evaluate the relationship between nematode body size and amount of SSU rDNA, we used a real-time PCR assessment of gene copy numbers was carried out from four target nematode species, two were cultured bacterial-feeding nematodes and two were the most common predatory and omnivorous nematodes in the experimental plots used for field studies elsewhere in this thesis. This information may help interpret the T-RFLP patterns in terms of nematode abundance or biomass.

5.2. Materials and methods

5.2.1. Nematode culture and isolation

Bacterial-feeding nematodes *Caenorhabditis elegans* Maupas, 1900 and *Panagrolaimus detritophagus* Fuchs, 1930, were cultured on nematode growth media (NGM) seeded with *Escherichia coli* strain OP50 at 15°C (Wood, 1988).

Predatory and omnivorous nematodes were isolated from Cowlands soil samples (see Chapter 3, Section 3.3.1) by a standard Oostenbrink-elutriator procedure (Verschoor and de Goede, 2000). After 48 h at room temperature, nematodes that had migrated into the bottom of the funnel were collected. Individual nematodes of one characteristic genus from each of the Mononchidae and the Dorylaimidae were then hand-picked under a dissecting microscope and placed in individual eppendorfs.

5.2.2. Optimisation of single nematode DNA extraction

In previous chapters Bead beating and PureLink PCR purification kit methods were applied

for the extraction of nematode communities. Prior to the analysis of a large dataset of single nematodes, several methods of DNA extraction were tested to check DNA extraction from individual nematode.

5.2.2.1. NaOH method

DNA from single nematodes was extracted using a modification of the method described by Stanton *et al*. (1998). Briefly, individual nematodes were put into 5 μl of 0.25 M NaOH in 1.5 ml tubes, and kept at room temperature (20-25°C) overnight. Thereafter, samples were incubated at 99°C for 3 min and 2.5 μl 0.25 M HC1, 1.25 μl 0.5 M Tris-HC1(pH 8.0) and 1.25 μl 2% Triton X-100 were added to each tube. Samples were incubated at 99°C for a further 3 min, cooled and stored at -20°C.

5.2.2.2. NaOH method + purification

Individual nematode DNA was extracted with the NaOH method as described above and then purified using a PureLink PCR purification kit (Invitrogen, Paisley, UK) following manufacters instructions. DNA was eluted as described in section 3.2.2.1 in 20 μl PCR elution buffer and then stored at -20°C.

5.2.2.3. Bead-beating

This extraction method was exactly the same method used for the nematode community DNA extraction in Chapter 3, Section 3.2.4. The only differences were that individual nematodes were used and single nematode DNA was eluted in 20 μl PCR elution buffer and then stored at -20°C.

5.2.2.4. MOBIO kit

Individual nematodes were added to the 2ml Bead Solution tubes provided in an UltraClean Soil DNA Isolation Kit (MOBIO Laboratories, Inc.), and then extracted according to the instructions listed by the manufacturer, with DNA eluted in 20 μl elution buffer, and stored at -20°C.

5.2.2.5. Lysis buffer+ Proteinase-K

Single nematodes were transferred to a 0.5-ml micro-tube with lysis buffer (25 μl of 0.4 M NaCl, 0.4 M Tris-HCl (pH 8.0), 2% (v/v) b-mercaptoethanol, and 800 μg/ml Proteinase-K). Lysis took place in a shaker at 65°C and 200 rpm for 2 h, following by 5 min incubation at

100°C. Lysate was stored at -20°C (Holterman *et al.*, 2006).

The five methods were tested for the extraction of DNA from individual nematodes and its suitability for subsequent PCR.

5.2.3. Testing different nematode primers for PCR amplification

Primers used in the following steps for the amplification of small subunit (18S) rDNA are listed in Table 5.1. All primers were synthesised by Eurofins MWG Operon (Westway Estate, London, UK).

Single nematode DNA extracted using the NaOH method was amplified using the different pairs of primers (Table 5.1).

Table 5.1. Primer pairs tested for nematode DNA amplification.

Group	Forward primer	Reverse primer	Length	Source
1	Nem_SSU_F74	Nem_18S_R	890	www.nematode.org[1]
2	SSU_F_22	SSU_R_23	878.5	www.nematode.org
3	Nem_SSU_F74	SSU_R_09	475.5	www.nematode.org
4	SSU_F_22	SSU_R_09	151.5	www.nematode.org
5	SSU_F_22	Nem_18S_R	566	www.nematode.org
6	Nem_F1	Nem_896R		Waite *et al.*, 2003
7	Euk–A	Euk–B		Countway *et al.*, 2005

[1]Full URL: http://www.nematodes.org/barcoding/sourhope/nemoprimers.html.

PCR was performed in a 25 µl final volume containing 2 µl template DNA, 2.5 µl 10× PCR buffer (Bioline, London, UK), 2 µl 50 mM $MgCl_2$, 2 µl 10 pM of each PCR primer, 0.5 µl 10 mM dNTP Mix, 13.8 µl distilled water and 0.2 µl 4 units Start *Taq* polymerase (Bioline, London, UK). All PCRs were performed on a G-STORM Thermal Cycler (Gene Technologies Ltd., Braintree, Essex, UK) with the following run parameters: one initial denaturation cycle at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 51°C for 30 s, and 68°C for 1 min. A final elongation step was run at 68°C for further 10 min. Positive (nematode DNA, confirmed from chapter 3) and negative controls (DNase-free water) were included for each amplification series. Success of PCR and size of product was determined by agarose gel electrophoresis. An aliquot of PCR product was mixed 5:1 with gel loading buffer (Bioline, London, UK), and separated on a 1% agarose gel in 1% TAE buffer strained with GelRed Nucleic Acid Gel Stain (Cambridge Bioscience, UK), and HyperLadder 1 (Bioline, London, UK) was used as a size marker. Stained nucleic acid was visualised on a Gel Doc XR (Biorad)

to check the position of DNA.

5.2.4. Plasmid DNA preparation, cloning and sequencing from the four nematode species
PCR products, which came from the primers Nem_SSU_F74 and Nem_18S_R, from four
individual nematodes of each of the four nematode types were purified using S.N.A.P.™ Gel
purification Kit (Invitrogen, Paisley, UK), and then cloned into TOPO XL PCR Cloning Kit
(Invitrogen, UK) following the manufactures' protocols. After growth on LB (Invitrogen, UK)
/kanamycin (50 µg /ml) plates at 37°C overnight, 96 recombinant colonies from each species
were picked randomly into 200 µl of LB broth with 50µg/ml kanamycin in 96-well microtitre
plates and grown overnight at 37°C. Inserts in the recombinant plasmids were amplified from
1 µl of overnight liquid culture as template in a PCR reaction using the primers M13_F
(CTGGCCGTCGTTTAC) and M13_R (CAGGAAACAGCTATA) (Blaxter *et al.*, 2005), and
amplification assessed by gel electrophoresis as described above. Four clones containing PCR
product of the correct size of each species were selected and grown again in 5 ml LB broth
with kanamycin at 37°C overnight. The plasmid DNA from these cultures was extracted using
the QIAprep Spin Miniprep Kit (Qiagen, Cologne, Germany); and an aliquot used for
sequencing on a 3730 capillary sequencer (Applied Biosystems) by the sequencing service at
the James Hutton Institute, Dundee, UK and another portion was stored at -20°C for the
preparation of standard curves for qPCR.

The sequences were organised in Fasta format, and the cloning vectors were identified and
removed from the sequence using the VecScreen Tool
http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html. The orientation of sequences was
checked in relation to the primers present and all sequence were arranged from the 5'→3'
direction with an online orientation checker
http://www.bioinformatics-toolkit.org/Web-ReverseComplement/. All database sequences
from each clone used in alignments were imported to BioEdit (Hall, 1999), and a consensus
sequence was created, by the introduction of degenerate bases, encompassing the entire target
region. Sequences were then compared to those in the Genbank database BLAST
(http://www.ncbi.nlm.nih.gov/BLAST) for identification purposes.

Sequences were aligned in MEGA 4 and a Neighbour Joining tree was constructed with
bootstrap support calculated over 500 replications. Sequence groups were defined by
separating at 0.02 substitutions per base. Sequence groups were then assigned to order on the
basis of clustering and bootstrap support on the Neighbour Joining tree. A phylogenetic tree
for the four species was made using MEGA 4.

5.2.5. Preparation of reference spike

The reference spike was generated by PCR in situ mutagenesis (Vallette *et al.*, 1989) to form a product in which the four 3' terminal recognition bases of both PRIMER-1 (CCTACGGGAGGCAGCAG) and PRIMER-2 (ATTACCGCGGCTGCTGG) were altered to the requisite complementary bases providing a template suitable for amplification with the Mut-F (CCTACGGGAGGCAGGTC) and Mut-R primers (ATTACCGCGGCTGCACC) (Daniell *et al.*, unpublished). This was performed in a two stage PCR process using 342FMut (CCTACGGGAGGCACGTCTGGGGAATAT) and R534 (ATTACCGCGGCTGGACCC ACGGAGTTA) in the first reaction and Mut-F and 534RMut in the second using PCR conditions given above and 1 µl *E. coli* genomic DNA used as template in round one and 1 µl of 100 fold diluted round one product as template in the second reaction. PCR success was assessed by 1.5% agarose gel electrophoresis.

Products was cloned into pGEM t easy (Promega) following the manufacturer's instructions and transformed into *E. coli* DH10B electrocompetent cells prepared following the method of Tung and Chow (1995). Colonies were screened for mutation success by PCR with relevant PCR primers and conditions outlined above. Sequence identity of selected clones for both the mutated and wild-type products was confirmed by sequencing in a total volume of 10 µl using 1:8 dilution of BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Warrington, UK) with vector primers directed against the SP6 or T7 promoter regions and following manufacturer's instructions. Sequencing reactions were purified by ethanol precipitation and run on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Warrington, UK). Transformants with the correct insert were placed into long term storage at -80°C in 20% glycerol and plasmid generated from a single selected colony by large scale plasmid prep using Plasmid Midiprep System (Promega) following manufacturer's instructions. Plasmid was quantified by absorption at 260nm to give a mass concentration and converted to copies using the following formula:

(Total number of A/T bases in the insert + plasmid x 601) + (Total number of G/C bases in the insert + plasmid x 618) = molecular weight (g/mole) of the plasmid + insert.

The number of copies of plasmid/µl =

(µg plasmid DNA calculated from 260nm absorption/molecular weight of plasmid+insert) X Avagadros number

5.2.6. Morphological examination, DNA extraction and inhibition test of single nematodes

Forty-five specimens from each of the four nematode species were picked individually into drops of sterile distilled water on a microscope slide. Their body length (L) and width (W) was determined using a light microscope (Olympus BX51) equipped with differential interference contrast optics and a camera (Retiga Exi High resolution Cam) to take digital images of each nematode. Nematode volume (V) was calculated using the formula $V = \pi (W/2)^2 L$ (Browning *et al.*, 2004).

Individual nematode DNA was extracted using the NaOH method (See 5.2). In order to evaluate the potential presence of PCR inhibitors in the nematode DNA extract, a known quantity of a reference spike (equivalent to 1.98×10^6 copies in the final qPCR reaction) was added to the 5μl 0.25M NaOH solution prior to each DNA extraction (Daniell *et al.*, unpublished).

5.2.7. Quantitative real-time PCR assay

5.2.7.1. SSU rDNA copy number from individual nematodes

A LightCycler 480 (Roche Applied Science, Germany) was used for real-time PCR amplification and detection. Real-time PCR was performed in 20 μl reaction mixture in LightCycler® 480 white Multiwell 96-well Plates and sealed with LightCycler® 480 Sealing Foil. Each reaction well contained 1 μl template DNA (10 times diluted template DNA from an individual nematode) and 10 μl LightCycler 480 Green 1 Master (Roche Applied Science, Germany). The following general real-time PCR protocol was used for all pairs of primers: denaturation program (95°C for 10 min), a three-segment amplification and quantification program repeated 40 times (95°C for 10 s, 65°C for 10 s and 72°C for 40 s), melting curve program (95-65°C with a heating rate of 0.06°C/s and continuous fluorescence measurements), and finally a cooling program down to 40°C.

5.2.7.2. Standard curve for qPCR quantification

Plasmid DNA from each of the four species was purified with phenol chloroform and ethanol precipitation (Donn *et al.*, 2008), and was linearised with restriction enzyme Sac1 (GAGCT/C, Biolabs, UK), incubated for 1 hour at 37°C, and inactivated for 20 minutes at 65°C (Daniell *et al.*, unpublished). Thereafter it was quantified using Magic Blue High sensitivity dsDNA Quantitation Assay (Biotium, U.S.A.) on a Fluorescence Microplate reader (ModulusTM Microplate, Turner Biosystem, US). The plasmid DNA copy number (PlCN) can be calculated from the formula of Pushnova *et al.* (2000).

$$\text{PlCN per genome} = \frac{\text{Size of chromosomal DNA (bp)} \times \text{Amount of plasmid DNA (pg)}}{\text{Size of plasmid DNA (bp)} \times \text{Amount of genomic DNA (pg)}}$$

The 10^9-10^1 serial ten fold dilutions of plasmid DNA were prepared in triplicate to establish the standard curves. The standard curves were the plots of the threshold cycle (Ct) values versus log concentration (Co), which got through real time PCR and the Roche software. For any unknown total DNA sample, gene copy numbers were obtained by interpolating its Ct value against the standard curve.

5.2.8. Data analysis

In this chapter, 18S rDNA was used as a phylogenetic marker for determination of lineage relationships within the data. The correlation between biovolume and copy number, among the same nematode species was tested by General Linear Model (GLM), while the relationship between biovolume and copy number between the different nematode species were assessed using a GLM analysis of covariance. The significance of correlation coefficients and differences among the relationships were analyzed using an Ellipse test ($P < 0.05$). All statistical analysis and graphics were carried out using Excel 2002 and STATISTICA 6.0 softwares. The non-normal distribution of points in the residual plot suggested that log transformations of the data would be useful to create constant variance. Unless stated otherwise, all analyses were done on log-transformed data.

5.3. Results

5.3.1. Nematode species

Four species of free-living nematodes from four families: Rhabditidae, Panagrolaimidae, Anatonchidae, and Aporcelaimidae. Sequence groups were defined by separating at 0.05 substitutions per base (Figure 5.1). From the sequence tree, we confirmed Rhabditidae (*Caenorhabditis elegans*) and Panagrolaimidae (*Panagrolaimus detritophagus*), which came from agar culture. All individuals selected by handpicking from soil extraction tightly cluster to known species of Anatonchidae (*Anatonchus tridentatus*), and Aporcelaimdae (*Aporcelaimellus obtusicaudatus*), known to be common in grassland soils.

5.3.2. Nematode DNA extraction

Different extraction methods were tested to determine which one was the best to extract DNA

from single nematodes (Table 5.2). According to the extracted efficiency from DNA quantification, the NaOH and lysis/proteinase K methods provided the most consistent extraction of DNA from individual nematodes, with the NaOH method giving a consistently greater yield, which was then used for all subsequent analysis.

5.3.3. Primer testing for PCR amplification

Conventional PCR reactions and quantitative real-time PCR were performed to evaluate the efficiency of primers for the SSU rDNA target region. Primers in real-time PCR are ideally designed to produce 100-200 bp products (Toyota *et al.*, 2008). The primer pair SSU_F_22 and SSU_R_09, which produced 151 bp of PCR product, was found to be suitable for quantitative detection using real-time PCR. While other pairs of primers were also tested, primers pair SSU_F_22 and SSU_R_09 were found to be the best after considering amplification using both conventional PCR reactions and quantitative real-time PCR (Table 5.3).

5.3.4. Standard curve from quantitative real-time PCR

The standard curves, from plasmid DNA of 4 nematode species, from real-time PCR were established to build the relationship between nematode DNA copy number and Ct values.

Before determining the linear dynamic range, the qPCR amplification efficiency was evaluated from the absolute gradient value of Ct versus log Copy number curve. As shown in Figure 5.2, the absolute gradients of the curves for targeted plasmid DNA PCR product of *Caenorhabditis*, *Panagrolaimus*, *Anatonchus* and *Aporcelaimellus* were 3.348, 3.311, 3.344 and 3.518, respectively, resulting in a small percentage difference, 0.79%, 0.34%, 0.66% and 5.91% from the theoretical value. Standard curves indicate only slight differences, but non significant ($P > 0.05$) between the four species. Each standard curve was used to calculate its own species copy number.

Figure 5.1. Neighbour joining tree showing sequence groups from the four individuals of A (*Caenorhabditis elegans*), B (*Panagrolaimus detritophagus*), C (*Anatonchus tridentatus*) and D (*Aporcelaimellus obtusicaudatus*)

clustered with database sequences, indicating phylogenetic relationships between the four species of free-living nematodes based on SSU and 18S ribosomal DNA sequences (sample C4 was lost). The sequences of other nematodes were acquired from Gene Bank (NCBI/ BLAST).

Table 5.2. Different methods to extract single nematode DNA, and then checked by a combination of PCR and agarose gel electrophoresis. '+' and '−' represent a positive or negative signal on the agarose gel, '+ + +' represents a stronger signal than '+' (n = 4).

DNA extracted Methods	*Caenorhabditis elegans*	*Panagrolaimus detritophagus*	*Mononchidae*	*Dorylaimidae*
NaOH method	+ +	+ +	+ + +	+ +
NaOH method + purification	–	–	–	–
PureLink Kit	–	–	–	–
MoBio Kit	–	–	–	–
Lysis buffer+ Proteinase-K	+	+	+ +	+

5.3.4. Standard curve from quantitative real-time PCR

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Table 5.3. Results from primer pairs used in PCR and qPCR for single nematode DNA. PCR determined by agarose gel electrophoresis; qPCR determined by Ct values from LightCycler 480; and plasmid determined by cp values of nematode plasmid DNA from LightCycler 480 '+' = positive, and '−' = negative.

Groups	Forward primer	Reverse primer	Length	PCR	qPCR Bacterial feeders	qPCR Others	plasmid
1	Nem_SSU_F74	Nem_18S_R	890	+	–	+	+
2	SSU_F_22	SSU_R_23	878.5	+	–	+	–
3	Nem_SSU_F74	SSU_R_09	475.5	+	+	+	–
4	SSU_F_22	SSU_R_09	151.5	+	+	+	+
5	SSU_F_22	Nem_18S_R	566	+	+	+	+
6	Nem_F1	Nem_896R		–	–	–	–
7	Euk–A	Euk–B		–	–	–	–
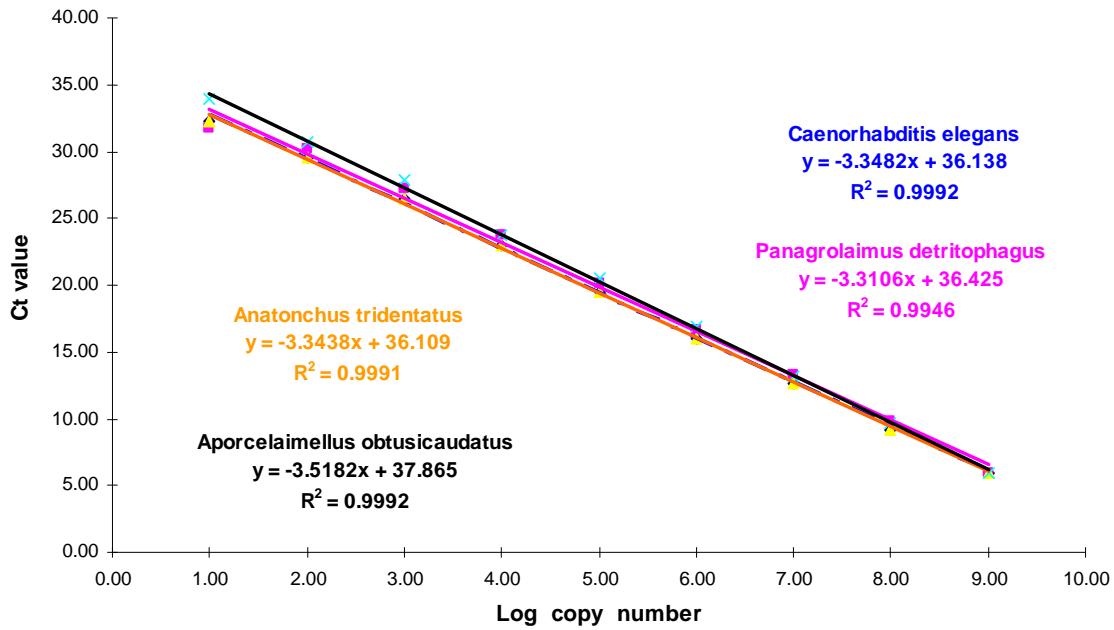
Figure 5.2. Standard curve establishment for quantification of the four nematode species. Plasmid DNA, ranging from 10^9 to 10^1 (copies μl^{-1}), was used as the template for real-time qPCR analysis. Threshold cycle versus log concentration for replicates of each concentration determined the linear dynamic range of plasmid DNA for four nematode species. Bars (which were negligible) represented standard errors.

5.3.5. Inhibition test for quantitative real-time PCR

An assessment of the presence of any substances inhibitory to PCR in DNA extracts is necessary for qPCR. In the regression plots of biovolume and spike copy number (Figure 5.3), a different trend was observed for each of the four species. For *C.elegans* and *Aporcelaimellus*, the relationship was not statistically significant. In contrast, inhibition was significantly decreased with nematode biovolume in *Panagrolaimus*, but increased significantly with biovolume in *Anatonchus*.
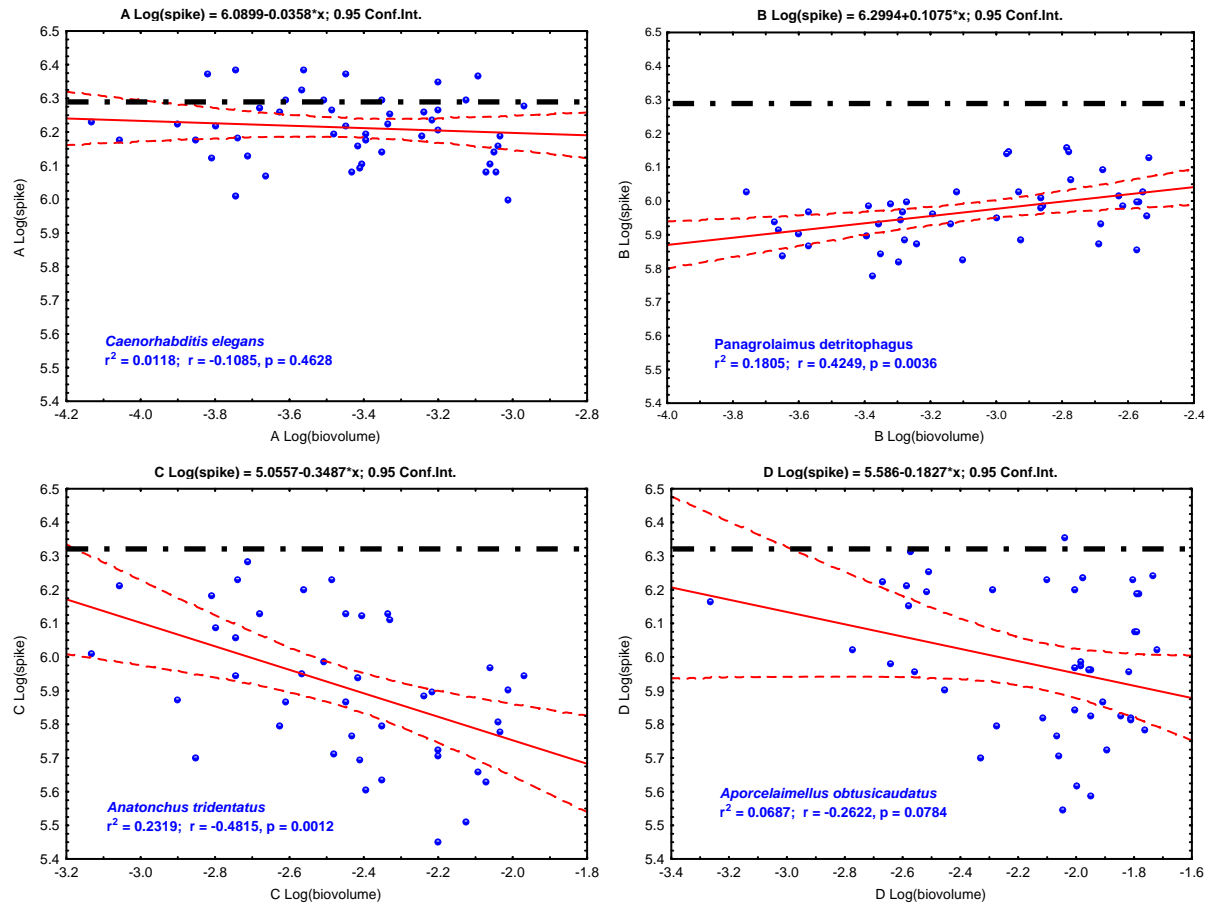
Figure 5.3. Quantification of standard DNA (spike) added to extracts of individual nematodes *C.elegans*, *Panagrolaimus*, *Anatonchus*, and *Aporcelaimellus*. Log spike versus log biovolume determined the linear dynamic range of relationship between the biovolume and the spike of four nematode species. Regression band meant confidence level < 0.05. ' **━** · **━** ' meant the concentration of standard spike tested by real-time PCR (1.98×10^6 copy numbers, and Log = 6.29).

5.3.6. Variance in nuclear 18S gene copy number with different nematode species

Relative quantification uses the spike to control for inhibition or losses during each individual extraction. Figure 5.4 showed the relationship between log copy number and log biovolume without any correction for inhibition and after the inhibition calculation. The relationship between biovolume and copy number were different for the four species. *C. elegans* showed no difference in copy number when the individual body size changed (Figure 5.4 a, $r^2 = 0.0003$ and $P = 0.9096$). While for the other three species, *Panagrolaimus*, *Anatonchus* and *Aporcelaimellus*, copy numbers increased slightly but significantly when the individual body sizes increased (Figure 5.4 b, c, d), and the values from r^2 were 0.2130, 0.3932 and 0.3025, respectively; while the values from *p* were 0.0014, 0.00009 and 0.0008, respectively.
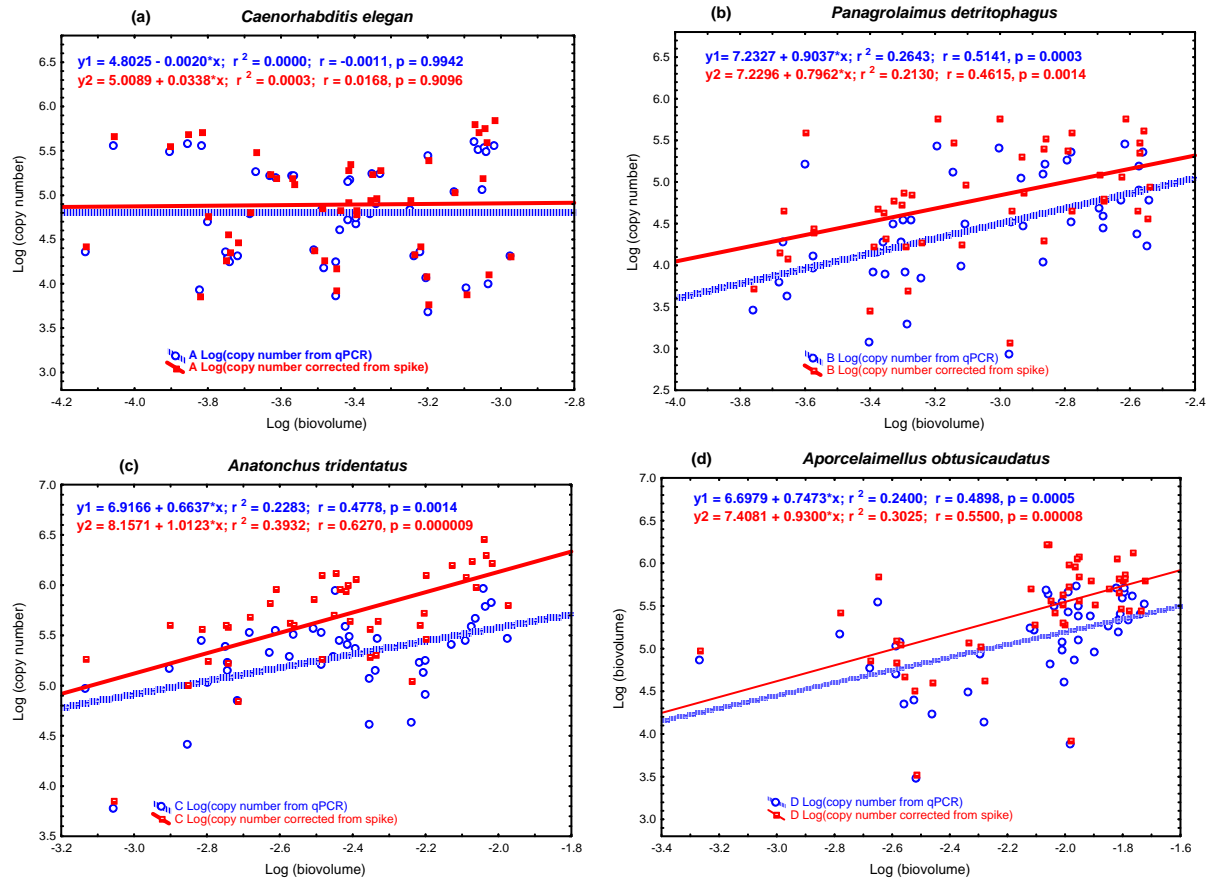
Figure 5.4. Correlations between log biovolume and log gene copy number estimated by relative real-time PCR for 4 nematode species. A, B, C and D indicated the 4 species, *C.elegans*, *Panagrolaimus detritophagus*, *Anatonchus tridentatus* and *Aporcelaimellus obtusicaudatus*, respectively. Regression band meant confidence level > 95%. The blue, coloured circles and the dotted line '••••••••••' presented raw qPCR data, while the red symbols and solid line '————' represented data whose copy number had been modified to account for inhibition.

No transformation on the data for the regression was calculated, from the relationship (trend line) between copy number and biovolume (Table 5.4). Assuming nematode biovolumes are 0.00063, 0.001, 0.00158 mm^3 for all four species, 0.00398 and 0.01mm^3 only for *Anatonchus* and *Aporcelaimellus* (Table 5.5), different copy numbers are calculated from the different trend line. Combining the copy number data from *Panagrolaimu*s, *Anatonchus* and *Aporcelaimellus*, there was a similar tendency for the relationship between log-copy number and log-biovolume, while *C. elegans* was significantly different. Analysis of covariance between the three species (Figure 5.5), suggested that copy number was increased significantly by body size within the three species, indicating that copy number, and therefore, cell number were not invariant, at least for these three nematode species. And also the slope in the Figure 5.5 was 0.8254, less than 1, which meant that if nematode body size doubled, the

copy number increased to a lower degree indicating lower than expected cell division.

Table 5.4. Results from relationships between nematode copy number and biovolume: copy number = a × biovolume + b.

	a	b	r^2
Caenorhabditis	3.00E+07	133677	0.0017
Panagrolaimus	6.00E+07	71198	0.092
Anatonchus	1.00E+08	154340	0.3801
Aporcelaimellus	4.00E+07	154340	0.3796

Table 5.5. The copy number per individual calculated from the correlation of four species, assuming that the nematode biovolumes are 0.00063, 0.00100, 0.00158, 0.00398, and 0.01mm^3, which were within the biovolumes of every species.

Biovolume (mm^3)	Copy number/individual			
	Caenorhabditis	*Panagrolaimus*	*Anatonchus*	*Aporcelaimellus*
0.00063	1.53E+05	1.09E+05	2.17E+05	1.80E+05
0.00100	1.64E+05	1.31E+05	2.54E+05	1.94E+05
0.00158	1.81E+05	1.66E+05	3.12E+05	2.18E+05
0.00398	n/a	n/a	5.52E+05	3.14E+05
0.01000	n/a	n/a	1.15E+06	5.54E+05
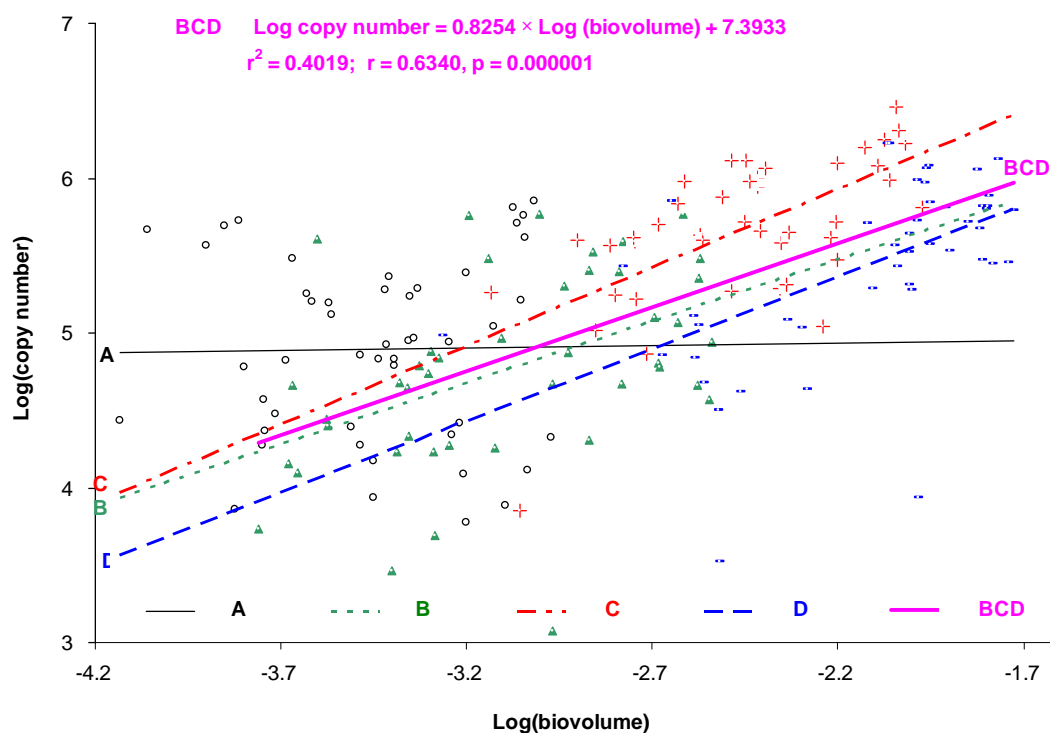
Figure 5.5. Correlation between log biovolume and log gene copy number estimated by relative real-time PCR for four nematode species. A, B, C and D indicated, *Caenorhabditis*, *Panagrolaimus*, *Anatonchus* and *Aporcelaimellus*, respectively. BCD is the analysis of covariance of *Panagrolaimus* (B), *Anatonchus* (C) and *Aporcelaimellus* (D) together.

5.4. Discussion and conclusion

In this chapter, the relationship between nematode body size and SSU rDNA copy number was investigated using single nematode DNA extraction with relative qPCR and comparing to nematode biovolume for four different nematode species.

To date, there has been no study to test the efficiency of DNA extraction techniques for single nematode. So we also tested a series of DNA extracted methods selecting the NaOH lysis method as it consistently generated greater yield of PCR product. Also a pair of primers, SSU_F_22 and SSU_R_09, suited the real-time PCR assessment was used in the real-time PCR experiment.

5.4.1 Different methods on single nematode DNA extraction

Hübschen *et al*. (2004) successfully used a sodium hydroxide extraction for DNA extraction from the whole community, while Donn *et al*. (2008) found this method was not successful in nematode community DNA extraction. The NaOH method was originally designed for the extraction of DNA from single nematodes, appearing to be more favourable for this type of extraction than for extraction from community sample (Stanton *et al*., 1998). NaOH method in this experiment gave better yields and quality of recovered DNA from individual nematodes than the other extraction methods tested.

Proteinase K lysis followed by phenol chloroform extraction produced the highest DNA yield from nematode community DNA extraction (Donn *et al*., 2008), and has also been used successfully to detect single nematode DNA (Holterman *et al*., 2008). Here, however, the DNA signal from agarose gel electrophoresis of the PCR amplification product was weaker than that of the NaOH method.

The PureLink Kit with bead-beating (Chapter 3, Section 3.2.4) and MoBio Kit (data not shown) were successful to extract DNA from soil nematode community, but failed to extract single nematode DNA. Both these methods incorporate a bead-beating step and dynamics of the beads and nematodes may not be favourable with only a single individual.

5.4.2. qPCR for single nematode DNA assessment

In theory, PCR efficiency or the slope of the standard curve should be computed as absolute gradient = $1/(\lg^2)$ = 3.322. In other words, theoretically, for a ten fold difference in template amount, a Ct value of 3.322 cycles should be expected. The absolute gradients of the curves from targeted plasmid DNA PCR product of *C.elegans*, *Panagrolaimus*, *Anatonchus* and *Aporcelaimellus* were 3.348, 3.311, 3.344 and 3.518, respectively, were very close to the

theoretical value. In addition, the four standard curves were perfectly linear (all $R^2 > 0.99$, Figure 5.2) suggesting an efficient estimation range from 10^1 to 10^9 copies of plasmid DNA.

5.4.3. Inhibition in quantitative real-time PCR

Some, or all, of the biological samples may contain inhibitors, which are not present in the nucleic acid samples used to construct that calibration curve, leading to an underestimation of the DNA levels in the test samples (Stahlberg *et al.*, 2005). Various methods can be used to assess the presence of inhibitors within biological samples. The PCR efficiency in a test sample can be assessed by serial dilution of the sample (Stahlberg *et al.*, 2003), although this is impossible when using very small amount of DNA extracted, as was the case with single nematode in this study. We instead placed a known concentration of spike into the extraction, and assessed the inhibition of single nematode DNA extraction, and amplification after qPCR. From our inhibition test, two bacterial-feeding nematodes showed no inhibition, while *Anatonchus* and *Aporcelaimellus* had more inhibition; probably because of habitat and what they eat. The bacterial-feeding nematodes came from agar-cultured plates, while another two nematodes came from soil samples, so inhibitions may be different for those two kinds of nematodes. Considering the huge differences in body size of these species, which *Anatonchus* and *Aporcelaimellus* have 10 and 20 times greater average body sizes than *C.elegans* and *Panagrolaimus*, the small nematode species may yield lower amount of DNA, so less inhibition showed in bacterial-feeding nematodes than predatory and omnivorous nematodes. This would have to be further tested, because the DNA concentration was below the level of detection from some samples with the Qubit™ dsDNA HS Assay Kits.

5.4.4. The relationships between biovolume and copy number from four nematode species

Although molecular methods have been widely used to analyze soil nematode communities (Foucher *et al.*, 2004; Griffiths *et al.*, 2006; Holterman *et al.*, 2008), most advances in quantitative PCR detection of nematodes have occurred based on studies of important species of plant parasitic nematodes (Bates *et al.*, 2002). To our knowledge the use of qPCR to assess the 18S copy number of individual soil nematodes has not previously been reported. Neither has the relationship between copy number and nematode species, or the relationship between copy number and body size within a species. The results presented here demonstrated that the nematode SSU rDNA copy number from four species changed with body volume dependent on nematode species.

5.4.5. Utilization of the relationships between biovolume and copy number from four nematode species to the nematode community analysis

The results presented here demonstrated that the relationship between biovolume and copy number from nematode species were variable. But the question remains as to how we can use the information from the relationship between nematode biovolume and copy number to analyse soil nematode communities, further releasing time from time-consuming laboratory morphological identification.

So if we look at the data from real-time PCR assessment about nematode DNA amplification from four species (Table 5.5), we can see there are differences of copy number per individual between species, and between different biovolumes within a species, with the exception of *C. elegans*, where copy number did not change significantly between individuals.

Table 5.6. Utilization of the relationships between biovolume and copy number to calculate the mixed nematodes community to compare the compositions of abundance, biovolume and copy number.

a) Same abundance and biovolume

Composition (%)	*Caenorhabditis*	*Panagrolaimus*	*Anatonchus*	*Aporcelaimellus*
abundance	25.00	25.00	25.00	25.00
biovolume	25.00	25.00	25.00	25.00
copy number	19.17	22.81	42.73	15.29

b) Same abundance and two biovolumes mixed (biovolumes were 0.00063 and 0.00158 mm^3)

Composition (%)	*Caenorhabditis*	*Panagrolaimus*	*Anatonchus*	*Aporcelaimellus*
abundance	25.00	25.00	25.00	25.00
biovolume	25.00	25.00	25.00	25.00
copy number	26.07	21.84	38.42	13.67

c) Same abundance and two biovolumes mixed (biovolumes were 0.001 and 0.00158 mm^3 for *Caenorhabditis* and *Panagrolaimus*; biovolumes were 0.001 and 0.01 mm^3 for *Anatonchus* and *Aporcelaimellus*)

Composition (%)	*Caenorhabditis*	*Panagrolaimus*	*Anatonchus*	*Aporcelaimellus*
abundance	25.00	25.00	25.00	25.00
biovolume	9.50	9.50	40.50	40.50
copy number	16.06	15.10	50.52	18.32

These findings suggest why differences are observed between morphological and molecular analyses of soil nematode communities. For example, only the mixed nematode communities from four species with same abundance (Table 5.6a), with same biovolumes (Table 5.6b) and different biovolumes (Table 5.6c), the composition of copy number from four species are changing. The predatory nematode *Anatonchus* occurs much in higher proportion than the other two bacterial-feeders and one omnivorous nematode. This indicates that if we use

molecular methods to analyze the much more complicated nematode community extracted from soil samples, we should consider about some factors here, such as different nematode size within the same species, and abundance composition, which yield different copy number. However, the effect of averaging in a large community assemblage, which reduces effects of division, remains to be measured.

5.4.6. Conclusion

Our results confirmed previous studies (Sulston *et al.*, 1983, Sachs, 1994, Malakov 1994, Cunha *et al.*, 1999) that for *C. elegans*, there was no correlation between body size and copy number showing that *C. elegans*, is eutelic. However, the three other species of soil free-living nematodes tested, *Panagrolaimus detritophagus*, *Anatonchus tridentatus* and *Aporcelaimellus obtusicaudatus*, showed a pattern where SSU rDNA copy number was increased significantly by the body size. This may provide support for the assertion of Cunha *et al.* (1999) that many, perhaps most, nematode species are not eutelic.

But the question is how we can use the data from real-time PCR to analyze the mixed nematode community, and find the connection between the real-time PCR data and T-RFLP. There are still more puzzles to be resolved in the future.

Chapter 6. Conclusion and future work

6.1. Conclusion

This study focused on the effects of grassland management, in terms of N and P fertilization, on the soil nematode community and used soil nematodes as an indicator of soil processes in grassland soil. The aim was to improve the understanding of grassland soil biodiversity and led to a better management of grassland soil food webs. The study also evaluated a high-throughput method for nematode community analysis, directed T-RFLP, for use as a potential bioindicator.

The main finding of the work reported here can be summarized as follows:

- **With long-term P fertilization (> 40 years) of grazed grassland**
    a) The soil nematode community showed significant changes with P management. P fertilization increased the proportion of bacterial-feeding nematodes and decreased that of fungal-feeding nematodes, indicating a shift from fungal to bacterial decomposition pathways in the more intensively fertilised systems which was possibly related to the deterioration of ecosystem complexity following continuous P application.
    b) Soil microbial biomass and community structure was also modified significantly. P fertilization increased soil microbial biomass C, N and P content, especially in the higher P treatments. P fertilization also significantly changed microbial PLFA profiles, confirming the reduced fungal biomass and fungal-bacterial ratio at high P fertilization.
    c) qPCR determination of the bacterial standing crop taken together with the nematode community analysis, indicated that bacterial turnover in grassland soils increased with P addition.

- **With short-term inorganic N and cattle slurry application (< 2 years)**
    a) Inorganic N and cattle slurry applications had different effects on nematode abundance and community structure. Nematode abundance increased significantly with slurry application, but not with inorganic N application. Inorganic N fertilization significantly increased the proportion of bacterial-feeding nematodes and decreased that of omnivorous nematodes, while no obvious differences were found following slurry application.
    b) Compared with slurry, higher inorganic N application increased the herbage

production and N uptake, indicating that inorganic N facilitated a faster N flux in this grassland system, as supported by the increasing proportion of bacterial-feeding nematodes. The discrepancy between plant performance and soil nematode community in response to slurry and inorganic N application revealed that the limiting factors for plants and soil nematodes were different.

c) Cattle slurry application stimulated nematode abundance, its lower N content applied to soil as well as slow N mineralization might mask its advantage on the herbage yields and N uptake, comparing with inorganic N fertilizer application.

- **T-RFLP for nematode community analysis**

    Directed T-RFLP was used as a molecular profiling approach to provide information regarding to trophic group composition of nematode community. Results obtained from both molecular and morphological analysis in the long-term P fertilization grassland trial were not identical but similarly indicated that P application resulted in a shift from fungi to bacteria dominated decomposition pathway. The rapid, easy and replicable T-RFLP approach is a robust tool for the routinely monitoring soil nematode community, which helps to advance the characterization of soil biodiversity and assessment of soil quality.

- **Real-time PCR assessment on nematode gene copy number**

    To account for the mismatch between molecular and morphological analysis of the nematode community, real-time PCR was adopted to determine the correlations between SSU rDNA copy number and body size in four species of soil free-living nematodes. For the bacterial-feeding *Caenorhabditis elegans*, there was no correlation between body size and copy number, showing that it was eutelic. However, for the three other species of soil nematodes tested, the bacterial-feeding nematode *Panagrolaimus detritophagus*, the predatory nematode *Anatonchus tridentatus* and the omnivorous nematode *Aporcelaimellus obtusicaudatus*, there were non-eutelic traits as the rDNA copy number increased significantly with body size.

6.2. Wider implications of the results

Because of the need to use grassland by sustainable way, there has been much recent interest in the characterization of soil biodiversity and its function in agricultural grasslands. Much of this interest has come from the need to develop grassland management strategies directed at manipulating the soil biota to encourage a greater reliance on ecosystem self-regulation than on artificial inputs such as fertilizers and pesticides (Yeates *et al.*, 1997).

How has the soil community reacted over the ten years since change the fertilization? This is exactly a question having been answered from the Cowlands experiment (Chapter 3). A more rapid respond was observed to P addition (from P0 to P0-30) than to stopping P addition (from P30 to P30-0). This means that available P can accumulate in the plant-soil system. This can be seen from the nematode, microbial and chemical data in Chapter 3. It might indicate that P fertilization can be more accurately managed by monitoring soil biological as well as chemical attributes and that P can be better used by not applying during those years when in excess. The response to P deficient long-term systems shifts to a more conserved P cycles, as indicated by the altered NCR and the microbial B: F ratio. The fungal dominated systems mean that organisms have long generation times and process matter at slower rates than those with the bacterial pathway (Moore *et al.*, 2005).

Interestingly a parallel study was undertaken at the same time on a cut, rather than grazed in grasslands, which showed no such response in microbial biomass or nematode community structure to a similar range of P fertilization (Massey, unpublished data). This may reflect the comparison between cutting, which no return of organic matter and nutrients back to soil system, and grazing. In grasslands, a large percentage of nutrients taken up by plants in grazing ecosystems is cycled directly through animal excreta, resulting in accelerated soil incorporation, particularly of nitrogen and phosphorus (Ruess and McNaughton, 1987). Therefore, soils of grazed grasslands tend to have small amounts of dead litter on the soil surface but have large amounts of organic nitrogen and carbon (Bardgett *et al.*, 1996), which are recycled by the grazing animals. These features combine to produce a soil environment that sustains an abundant and diverse faunal and microbial community. Grazing livestock increases incorporation of surface litter into the soil, which can increase total soil organic matter. Furthermore, herbivory can increase root exudation of labile C compounds, which can stimulate growth of the rhizosphere microbial community (Hamilton *et al.,* 2001). Bardgett and Wardle (2003) proposed that the positive effects from grazing on soil biota are most common in ecosystems of higher soil fertility, which exactly happened in the Cowlands. Grazing results in a clear increase in soil biomass C and N forms, and also significantly influenced soil nematode communities (Wang *et al.*, 2006).

Slurry application to grassland in this study (Chapter 4) did not cause such large change to the nematode community as in the P experiment, or as has been seen from other studies where slurry has been incorporated into soil. Forge *et al.* (2005) found long-term use of manure application increased microbial biomass and abundance of microbivorous nematodes, which were indicative of enhanced microbial turnover and flux of nutrient through the soil food web.

The relatively small effects on the nematodes might be related to spatial aspects of slurry applied to grassland in this study and to the shorter term of the study compared to the P experiment. Here slurry was applied on top of the grass, which effectively separated soil from substrate and could result in lower residue decomposition rate and slower distribution of organic matter at soil depth. This is similar to no- till versus conventional- till systems in arable soils, tillage causes the redistribution of organic matter subsequently changing microbial structure and nematode trophic structure. Fu *et al.* (2000) simulated the decomposition of organic matter in conventional and no-till systems and found the fungal feeders/bacterial-feeder ratio increased with time after residue application in the CT treatment and did not increase in the NT treatment until the end of the experiment, which suggested the added C at the surface was slower and different decomposed than when the C is mixed into the soil. Also they found that soil nematodes used carbon more efficiently under CT than NT system (Fu *et al.*, 2000).

The diversity of feeding habits and habitat preferences of soil nematodes in grasslands has helped gain a better understanding of grassland soil food webs. The use of nematodes in this way will be made easier by the use of molecular techniques.

Despite the change in copy number with body size of individual nematodes from the four species in Chapter 5, real-time PCR analysis offers a high potential to be further developed as a low-cost and high-throughput method for nematode diagnostics, also is useful for nematode DNA quantitative analysis (Derycke *et al.*, 2010; Holterman *et al.*, 2012). Because the body sizes of nematodes are totally different, for example, the range of biovolumes from the nematodes used in Chapter 5, 7.5×10^{-5}-1.1×10^{-3} mm^3 in *Caenorhabditis elegans*, 5.0×10^{-5}-2.9×10^{-3} mm^3 in *Panagrolaimus detritophagus*, 7.4×10^{-4}-1.1×10^{-2} mm^3 in *Anatonchus tridentatus* and 5.4×10^{-4}-1.9×10^{-2} mm^3 in *Aporcelaimellus obtusicaudatus*. The differences in body size will be averaged out over the whole nematode samples, which allow the quantitative approaches to work on analyzing the nematode community.

6.3. Future work

6.3.1. Developing nematode community analysis as a soil indicator

Although soil nematode community analysis is widely considered as a good candidate to indicate soil condition, there are still many more questions to be answered in order to fully explore its indicative value. In this study, the different responses of nematode community to inorganic N and cattle slurry applications, as well as the time-lag responses to P additions,

imply that future studies should be aware of much more functional information associated with the nematode community. On the one hand, the relations between nematode community and other soil properties should be verified, in particular the functional parameters characterizing soil process and ecosystem services. Correlational or investigative studies will provide sufficient knowledge on this respect. Comprehensively understanding such relationships will not only strengthen its indicative value, but also help us understand the functional implications and interpret the inherent mechanisms. On the other hand, it is also important to know the functional contributions of nematode community to soil process and soil ecosystem services. More manipulative studies are needed to clarify how nematode composition will affect various soil process and ecosystem services (Van Camp *et al*., 2004). Such information will not only form the bases of utilizing nematode community analysis as a bioindicator, but also contribute to clarifying the relations between soil biodiversity and ecological functions and to aid future sustainable soil management.

6.3.2. Developing molecular methods on nematode community analysis

Although the directed T-RFLP method for nematode community analysis showed high potential in determining nematode community, there were still some mismatches between the molecular and morphological results of nematode communities. Compared with the long history of morphological identification method via microscope, the new-born molecular analysis of whole nematode assemblage in soil is still in the developing stage. I provided some clues from real-time PCR assessment of SSU rDNA copy numbers within and between nematode trophic group, but more nematode species, such as those belonging to fungivorous and herbivorous nematodes, should also be included. More investigations combining both molecular and morphological methods in different habitats and ecosystem types are needed to corroborate the reliability of the molecular method and improve the molecular databases. The ecologically relevant information for interpreting nematode molecular results should also be strengthened. For this target, the relations between molecular results of nematode community and other soil parameters (as mentioned above) should be emphasized. There is no doubt that the development of nematode molecular analysis will also benefit from the fast development of molecular techniques in other research fields.

References

Afif, E., Matar, A., Torrent, J. 1993. Availability of phosphate applied to calcareous soils of West Asia and North Africa. Soil Science Society of America Journal 57, 756-760.

Aleshin, V.V., Kedrova, O.S., Milyutina, I.A., Vladychenskaya, N.S. and Petrov, N.B. 1998. Relationships among nematodes based on the analysis of 18S rRNA gene sequences: molecular evidence for monophyly of chromadorian and secernentian nematodes. Russian Journal of Nematology 6, 175-184.

Amann, R.I., Ludwig, W., Schleifer, K.H. 1995. Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. Microbiological Reviews 59, 143-169.

Andre, H., Ducarme, X., Anderson Jr., J., D.C., Koehler, H., Paoletti, M., Walter, D., Lebrun, P. 2001. Skilled eyes are needed to go on studying the richness of the soil. Nature 409, 761.

Andre, H.M., Ducarme, X., Lebrun, P. 2002. Soil biodiversity: myth, reality or conning? Oikos 96, 3-24.

Anon. 2006. S.I. 378 of 2006. European communities (Good Agricultural Practice for Protection of Waters) Regulation 2006. Publ: Government Publications Office, Dublin.

Azevedo, R.B.R., Cunha, A., Emmons, S.W., Leroi, A.M. 2000. The demise of the Platonic worm. Nematology 2, 71-79.

Azevedo, R.B.R., Leroi, A.M. 2001. A power law for cells. Proceedings of the National Academy of Sciences of the United States of America 98, 5699-5704.

Balmford, A., Bruner, A., Cooper, P., Costanza, R., Farber, S., Green, R.E., Jenkins, M., Jefferiss, P., Jessamy, V., Madden, J., Munro, K., Myers, N., Naeem, S., Paavola, J., Rayment, M., Rosendo, S., Roughgarden, J., Trumper, K., Turner, R.K. 2002. Ecology - Economic reasons for conserving wild nature. Science 297, 950-953.

Bardgett, R.D., Cook, R. 1998. Functional aspects of soil animal diversity in agricultural grasslands. Applied Soil Ecology 10, 263-276.

Bardgett, R.D., Denton, C.S., Cook, R. 1999. Below-ground herbivory promotes soil nutrient transfer and root growth in grassland. Ecology Letters 2, 357-360.

Bardgett, R.D., Hobbs, P.J., Frostegard, A. 1996. Changes in soil fungal: bacterial biomass ratios following reductions in the intensity of management of an upland grassland. Biology and Fertility of Soils 22, 261-264.

Bardgett, R.D., Leemans, D.K., Cook, R., Hobbs, P.J. 1997. Seasonality of the soil biota of grazed and ungrazed hill grasslands. Soil Biology & Biochemistry 29, 1285-1294.

Bardgett, R.D., Wardle, D.A. 2003. Herbivore mediated linkages between aboveground and belowground communities. Ecology 84, 2258-2268.

Bardgett, R.D., Wardle, D.A., Yeates, G.W. 1998. Linking above-ground and below-ground interactions: How plant responses to foliar herbivory influence soil organisms. Soil Biology & Biochemistry 30, 1867-1878.

Bates, J.A., Taylor, E.J.A., Gans, P.T., Thomas, J.E. 2002. Determination of relative proportions of Globodera species in mixed populations of potato cyst nematodes using PCR product melting peak analysis. Molecular Plant Pathology 3, 153-161.

Barkle, G.F., Stenger, R., Sparling, G.P., Painter, D.J. 2001. Immobilisation and mineralisation of carbon and nitrogen from dairy farm effluent during laboratory soil incubations. Australian Journal of Soil Research 39, 1407-1417.

Benizri, E., Amiaud, B. 2005. Relationship between plants and soil microbial communities in fertilized grasslands. Soil Biology & Biochemistry 37, 2055-2064.

Berkelmans, R., Ferris, H., Tenuta, M., van Bruggen, A.H.C. 2003. Effects of long-term crop management on nematode trophic levels other than plant feeders disappear after 1 year of disruptive soil management. Applied Soil Ecology 23, 223-235.

Bhadury, P., Austen, M.C., Bilton, D.T., Lambshead, P.J.D., Rogers, A.D., Smerdon, G.R. 2006. Molecular detection of marine nematodes from environmental samples: overcoming eukaryotic interference. Aquatic Microbial Ecology 44, 97-103.

Bhadury, P., Austen, M.C., Bilton, D.T., Lambshead, P.J.D., Rogers, A.D., Smerdon, G.R. 2008. Evaluation of combined morphological and molecular techniques for marine nematode (Terschellingia spp.) identification. Marine Biology 154, 509-518.

Biederbeck, V.O., Campbell, C.A., Ukrainetz, H., Curtin, D., Bouman, O.T. 1996. Soil microbial and biochemical properties after ten years of fertilization with urea and anhydrous ammonia. Canadian Journal of Soil Science 76, 7-14.

Birkhofer, K., Bezemer, T.M., Bloem, J., Bonkowski, M., Christensen, S., Dubois, D., Ekelund, F., Fliessbach, A., Gunst, L., Hedlund, K., Maeder, P., Mikola, J., Robin, C., Setala, H., Tatin-Froux, F., Van der Putten, W.H., Scheu, S. 2008. Long-term organic farming fosters below and aboveground biota: Implications for soil quality, biological control and productivity. Soil Biology & Biochemistry 40, 2297-2308.

Bittman, S., Forge, T.A., Kowalenko, C.G. 2005. Responses of the bacterial and fungal biomass in a grassland soil to multi-year applications of dairy manure slurry and fertilizer.

Soil Biology & Biochemistry 37, 613-623.

Bittman, S., Kowalenko, C.G., Hunt, D.E., Schmidt, O. 1999. Surface-banded and broadcast dairy manure effects on tall fescue yield and nitrogen uptake. Agronomy Journal 91, 826-833.

Blackshaw, R.P., Donovan, S.E., Hazarika, S., Bol, R., Dixon, E.R. 2006. Earthworm responses to long term agricultural management practices: Spatial relationships with soil properties. 8th International Symposium on Earthworm Ecology (ISEE8), Cracow, Poland, S171-S175.

Blaxter, M., Elsworth, B., Daub, J. 2004. DNA taxonomy of a neglected animal phylum: an unexpected diversity of tardigrades. Proceedings of the Royal Society of London Series B-Biological Sciences 271, S189-S192.

Blaxter, M.L., De Ley, P., Garey, J.R., Liu, L.X., Scheldeman, P., Vierstraete, A., Vanfleteren, J.R., Mackey, L.Y., Dorris, M., Frisse, L.M., Vida, J.T., Thomas, W.K. 1998. A molecular evolutionary framework for the phylum Nematoda. Nature 392, 71-75.

Blouin, M.S. 2001. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. Joint Meeting of the Australian-Society -for-Parasitology. 8th International Coccidiosis Conference, Palm Cove, Australia, 527-531.

Bohlen, P.J., Edwards, C.A. 1994. The response of nematode trophic groups to organic and inorganic nutrient inputs in agroecosystems. In *Defining soil quality for a sustainable environment,* Doran, J. W. (ed.), p. 235-244. SSSA Special Publication no. 34.

Bolton, H., Elliott, L.F., Papendick, R.I., Bezdicek, D.F. 1985. Soil microbial biomass and selected soil enzyme-activities - Effect of fertilization and cropping practices. Soil Biology & Biochemistry 17, 297-302.

Bongers, T. 1988. De Nematoden van Nederland. KNNV Pirola, Schoorl NL. 408 p.

Bongers, T. 1990. The Maturity Index - an ecological measure of environmental disturbance based on nematode species composition. Oecologia 83, 14-19.

Bongers, T., Bongers, M. 1998. Functional diversity of nematodes. Applied Soil Ecology 10, 239-251.

Bongers, T., Ferris, H. 1999. Nematode community structure as a bioindicator in environmental monitoring. Trends in Ecology & Evolution 14, 224-228.

Bongers, T., Yeates, G.W. 1988. Report on workshop - Nematodes in natural systems - the use of nematodes in environmental studies. Pedobiologia 32, 88-88.

Bonkowski, M., Cheng, W.X., Griffiths, B.S., Alphei, G., Scheu, S. 2000. Microbial-faunal

interactions in the rhizosphere and effects on plant growth. European Journal of Soil Biology 36, 135-147.

Bossio, D.A., Scow, K.M. 1998. Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. Microbial Ecology 35, 265-278.

Bossio, D.A., Scow, K.M., Gunapala, N., Graham, K.J. 1998. Determinants of soil microbial communities: Effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microbial Ecology 36, 1-12.

Brookes, P.C., Landman, A., Pruden, G., Jenkinson, D.S. 1985. Chloroform fumigation and the release of soil-nitrogen - a rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biology & Biochemistry 17, 837-842.

Browning, M., Dawson, C., Alm, S.R., Gorres, J.H., Amador, J.A. 2004. Differential effects of butyric acid on nematodes from four trophic groups. Applied Soil Ecology 27, 47-54.

Bulman, S.R., Marshall, J.W. 1997. Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). New Zealand Journal of Crop and Horticultural Science 25, 123-129.

Bustin, S.A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology 25, 169-193.

Bustin, S.A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology 29, 23-39.

Cahalan, E., Ernfors, E., Müller, C., Devaney, D., Khalil, M.I., Laughlin, R., Watson, C., McGeough, K., Hennessy, D., Richards, K.G. 2011. The effect of dicyandiamide (DCD) amended cattle slurry on herbage production at three Irish grassland sites. Agricultural Research Forum 2011, 33-33.

Chung, H., Zak, D., Reich, P.B., Ellsworth, D.S. 2007. Plant species richness, elevated CO2, and atmospheric N deposition alter soil microbial community composition and function. Global Change Biology 13, 980-989.

Clark, M.S., Ferris, H., Klonsky, K., Lanini, W.T., van Bruggen, A.H.C., Zalom, F.G. 1998. Agronomic, economic, and environmental comparison of pest management in conventional and alternative tomato and corn systems in northern California. Agriculture Ecosystems & Environment 68, 51-71.

Cleveland, C.C., Liptzin, D. 2007. C : N : P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? Biogeochemistry 85, 235-252.

Coleman, D., Crossley, D., Hendrix, P. 2004. Fundamentals of Soil Ecology. Elsevier Academic Press.

Coleman, D.C., Reid, C. P. P., Cole, C.V. 1983. Biological strategies of nutrient cycling in soil ecosystems. Advances in Ecological Research 13, 1-55.

Collins, W.B., Schwartz, C.C. 1998. Logging in Alaska's boreal forest: Creation of grasslands or enhancement of moose habitat. Alces 34, 355-374.

Condron, L.M., Davis, M.R., Newman, R.H., Cornforth, I.S. 1996. Influence of conifers on the forms of phosphorus in selected New Zealand grassland soils. Biology and Fertility of Soils 21, 37-42.

Countway, P.D, Gast, R.J, Savaia, P, Caron, D.A. 2005. Protistan diversity estimates based on 18S rDNA from seawater incubations in the Western North Atlantic. Journal of Eukaryote Microbiology, 52, 95–106.

Creamer, R.E., Brennan, F., Fenton, O., Healy, M.G., Lalor, S.T.J., Lanigan, G.J., Regan, J.T., Griffiths, B.S. 2010. Implications of the proposed Soil Framework Directive on agricultural systems in Atlantic Europe - a review. Soil Use and Management 26, 198-211.

Culleton, N., Liebhardt, B., Murphy, W.E. 2000a. The movement of phosphorus in soils over a 30-year period. Irish Journal of Agricultural and Food Research 39, 465-466.

Culleton, N., Liebhardt, B., Murphy, W.E. 2000b. An overview of the fate of phosphorus applied to grassland over a 30-year period. Irish Journal of Agricultural and Food Research 39, 476-476.

Culleton, N., Coulter, B., Liebhardt, W. C. 2002. The fate of phosphatic fertiliser applied to grassland. Irish Geography 35, 175-184.

De Deyn, G.B., Van der Putten, W.H. 2005. Linking aboveground and belowground diversity. Trends in Ecology & Evolution 20, 625-633.

De Goede, R.G.M., Brussaard, L., Akkermans, A.D.L. 2003. On-farm impact of cattle slurry manure management on biological soil quality. NJAS-Wageningen Journal of Life Sciences 51, 103-133.

De Ley, P., De Ley, I.T., Morris, K., Abebe, E., Mundo-Ocampo, M., Yoder, M., Heras, J., Waumann, D., Rocha-Olivares, A., Burr, A.H.J., Baldwin, J.G., Thomas, W.K. 2005. An integrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. Philosophical Transactions of the Royal Society B: Biological Sciences 360, 1945-1958.

de Ruiter, P.C., Neutel, A.M., Moore, J.C. 1994. Modelling food webs and nutrient cycling in agro-ecosystems. Trends in Ecology and Evolution 9, 378-383.

de Ruiter, P.C., Neutel, A.M., Moore, J.C. 1998. Biodiversity in soil ecosystems: the role of energy flow and community stability. Applied Soil Ecology 10, 217-228.

de Vries, F.T., Bloem, J., van Eekeren, N., Brusaard, L., Hoffland, E. 2007. Fungal biomass in pastures increases with age and reduced N input. Soil Biology & Biochemistry 39, 1620-1630.

Daniell, T., Davidson, J., Alexander, C., Caul, S., Roberts, D. (unpublished). Improved real time estimation of gene copy number in soil extracts using an artificial reference.

Decaëns, T. 2010. Macroecological patterns in soil communities. Global Ecology and Biogeography 19, 287-302.

Decaëns, T., Jimenez, J.J., Gioia, C., Measey, G.J., Lavelle, P. 2006. The values of soil animals for conservation biology. European Journal of Soil Biology 42, S23-S38.

Decaëns, T., Lavelle, P., Jiménez, J.J. 2006. Priorities for conservation of soil animals. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 3, S23-S38.

Delgado, C., Rosegrant, M., Steinfeld, H., Ehui, S., Courbois, V. 1999. Livestock to 2020. The next food revolution. Food, Agriculture, and the Environment Discussion Paper 28. International Food Policy Research Institute, Washington, D.C.

Derycke, S., Vanaverbeke, J., Rigaux, A., Backeljau, T., Moens, T. 2010. Exploring the Use of Cytochrome Oxidase c Subunit 1 (COI) for DNA Barcoding of Free-Living Marine Nematodes. Plos One 5.

Dmowska, E., Kozlowska, J. 1988. Communities of nematodes in soil treated with semi-liquid manure. Pedobiologia 32, 323-330.

Donn, S., Griffiths, B.S., Neilson, R., Daniell, T.J. 2008. DNA extraction from soil nematodes for multi-sample community studies. Applied Soil Ecology 38, 20-26.

Donn, S., Neilson, R., Griffiths, B.S., Daniell, T.J. 2011. Greater coverage of the phylum Nematoda in SSU rDNA studies. Biology and Fertility of Soils 47, 333-339.

Ekschmitt, K., Bakonyi, G., Bongers, M., Bongers, T., Bostrom, S., Dogan, H., Harrison, A., Kallimanis, A., Nagy, P., O'Donnell, A.G., Sohlenius, B., Stamou, G.P., Wolters, V. 1999. Effects of the nematofauna on microbial energy and matter transformation rates in European grassland soils. Plant and Soil 212, 45-61.

Ekschmitt, K., Klein, A., Pieper, B., Wolters, V. 2001. Biodiversity and functioning of ecological communities - why is diversity important in some cases and unimportant in others? Journal of Plant Nutrition and Soil Science-Zeitschrift Fur Pflanzenernahrung Und Bodenkunde 164, 239-246.

Elliott, E.T. 1997. Rationale for developing bioindicators of soil health. In: Pankhurst, C., Doube, B.M., Gupta, V.V.S.R. (Ed.), Biological Indicators of Soil Health.

Eriksson, A., Eriksson, O., Berglund, H. 1995. Species abundance patterns of plants in swedish seminatural pastures. Ecography 18, 310-317.

Ettema, C.H., Bongers, T. 1993. Characterization of nematode colonization and succession in disturbed soil using the maturity index. Biology and Fertility of Soils 16, 79-85.

FAO. 2001. FAOSTAT database collections. Food and Agriculture Organization of the United Nations, Rome.

Ferraro, P.J., Kiss, A. 2002. Ecology - Direct payments to conserve biodiversity. Science 298, 1718-1719.

Ferri, E., Barbuto, M., Bain, O., Galimberti, A., Uni, S., Guerrero, R., Ferte, H., Bandi, C., Martin, C., Casiraghi, M. 2009. Integrated taxonomy: traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). Frontiers in Zoology 6:1, doi:10.1186/1742-9994-6-1.

Ferris, H., Bongers, T. 2006. Nematode indicators of organic enrichment. Journal of Nematology 38, 3-12.

Ferris, H., Bongers, T., de Goede, R.G.M. 2001. A framework for soil food web diagnostics: extension of the nematode faunal analysis concept. Applied Soil Ecology 18, 13-29.

Ferris, H., Matute, M.M. 2003. Structural and functional succession in the nematode fauna of a.soil food web. Applied Soil Ecology 23, 93-110.

Ferris, H., Venette, R.C., Lau, S.S. 1996. Dynamics of nematode communities in tomatoes grown in conventional and organic farming systems, and their impact on soil fertility. Applied Soil Ecology 3, 161-175.

Fey, M. V. 1988. Digesting old bones: The interpretation of phosphate equilibria in soil. Proceedings Phosphorus Symposium, 79-82.

Fischer, M., Wipf, S. 2002. Effect of low-intensity grazing on the species-rich vegetation of traditionally mown subalpine meadows. Biological Conservation 104, 1-11.

Fiscus, D.A., Neher, D.A. 2002. Distinguishing sensitivity of free-living soil nematode genera to physical and chemical disturbances. Ecological Applications 12, 565-575.

Fliessbach, A., Oberholzer, H.-R., Gunst, L., Maeder, P. 2007. Soil organic matter and biological soil quality indicators after 21 years of organic and conventional farming. Agriculture Ecosystems & Environment 118, 273-284.

Floyd, R., Abebe, E., Papert, A., Blaxter, M. 2002. Molecular barcodes for soil nematode identification. Molecular Ecology 11, 839-850.

Forge, T.A., Bittman, S., Kowalenko, C.G. 2005. Responses of grassland soil nematodes and protozoa to multi-year and single-year applications of dairy manure slurry and fertilizer. Soil Biology & Biochemistry 37, 1751-1762.

Forge, T.A., Simard, S.W. 2001. Structure of nematode communities in forest soils of southern British Columbia: relationships to nitrogen mineralization and effects of clearcut harvesting and fertilization. Biology and Fertility of Soils 34, 170-178.

Forge, T.A., Hogue, E., Neilsen, G., Neilsen, D. 2003. Effects of organic mulches on soil microfauna in the root zone of apple: implications for nutrient fluxes and functional diversity of the soil food web. Applied Soil Ecology 22, 39-54.

Foucher, A., Bongers, T., Noble, L.R., Wilson, M.J. 2004. Assessment of nematode biodiversity using DGGE of 18S rDNA following extraction of nematodes from soil. Soil Biology & Biochemistry 36, 2027-2032.

Foy, R.H., Tunney, H., Carroll, M.J., Byrne, E., Gately, T., Bailey, J.S., Lennox, S.D. 1997. A comparison of Olsen and Morgan soil phosphorus test results from the cross-border region of Ireland. Irish Journal of Agricultural and Food Research 36, 185-193.

Freckman, D.W. 1988. Bacterivorous nematodes and organic-matter decomposition. Agriculture Ecosystems & Environment 24, 195-217.

Freckman, D.W., Ettema, C.H. 1993. Assessing nematode communities in agroecosystems of varying human intervention. Agriculture, Ecosystems & Environment 45, 239-261.

Frostegård, A., Bååth, E. 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22, 59-65.

Frostegård, A., Tunlid, A., Bååth, E. 1991. Microbial Biomass Measured as Total Lipid Phosphate in Soils of Different Organic Content. Journal of Microbiological Methods 14, 151-163.

Frostegård, A., Tunlid, A., Bååth, E. 2011. Use and misuse of PLFA measurements in soils. Soil Biology & Biochemistry 43, 1621-1625.

Fu, S.L., Coleman, D.C., Hendrix, P.F., Crossley, D.A. 2000. Responses of trophic groups of soil nematodes to residue application under conventional tillage and no-till regimes. Soil Biology & Biochemistry 32, 1731-1741.

Fynn, R.W.S., O'Connor, T.G. 2005. Determinants of community organization of a South African mesic grassland. Journal of Vegetation Science 16, 93-102.

Gachon, C., Mingam, A., Charrier, B. 2004. Real-time PCR: what relevance to plant studies? Journal of Experimental Botany 55, 1445-1454.

Gardi, C., Montanarella, L., Arrouays, D., Bispo, A., Lemanceau, P., Jolivet, C., Mulder, C., Ranjard, L., Rombke, J., Rutgers, M., Menta, C. 2009. Soil biodiversity monitoring in Europe: ongoing activities and challenges. European Journal of Soil Science 60, 807-819.

Gibb, K., Beard, J., O'Reagain, P., Christian, K., Torok, V., Ophel-Keller, K. 2008. Assessing the relationship between patch type and soil mites: A molecular approach. Pedobiologia 51, 445-461.

Goto, K., Sato, E., Toyota, K. 2009. A novel detection method for the soybean cyst nematode *Heterodera glycines* using soil compaction and real-time PCR. Nematological Research 39, 1-7.

Griffiths, B.S., Bardgett, R.D. 2000. Influence of resource unit distribution and quality on the activity of soil fungi in a particulate medium. New Phytologist 148, 143-151.

Griffiths, B.S., Bengough, A.G., Neilson, R., Trudgill, D.L. 2002. The extent to which nematode communities are affected by soil factors - a pot experiment. Nematology 4, 943-952.

Griffiths, B.S., Bonkowski, M., Dobson, G., Caul, S. 1999. Changes in soil microbial community structure in the presence of microbial-feeding nematodes and protozoa. Pedobiologia 43, 297-304.

Griffiths, B.S., Daniell, T.J., Donn, S., Neilson, R. 2012. Bioindication potential of using molecular characterisation of the nematode community: Response to soil tillage. European Journal of Soil Biology 49, 92-97.

Griffiths, B.S., Donn, S., Neilson, R., Daniell, T.J. 2006. Molecular sequencing and morphological analysis of a nematode community. Applied Soil Ecology 32, 325-337.

Griffiths, B.S., Ritz, K., Wheatley, R.E. 1994. Nematodes as indicators of enhanced microbiological activity in a Scottish organic farming system. Soil Use and Management 10, 20-24.

Griffiths, B.S., Wheatley, R.E., Olesen, T., Henriksen, K., Ekelund, F., Ronn, R. 1998. Dynamics of nematodes and protozoa following the experimental addition of cattle or pig slurry to soil. Soil Biology & Biochemistry 30, 1379-1387.

Haynes, R.J., Williams, P.H. 1993. Nutrient cycling and soil fertility in the grazed pasture ecosystem. In: Sparks, D.L. (Ed.), Advances in Agronomy 49, 119-199.

Grime, J.P. 1973. Control of species density in herbaceous vegetation. Journal of Environmental Management 1, 157-167.

Gupta, V.V.S.R., Yeates, G.W. 1997. Soil microfauna as bioindicators of soil health. In

Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R. (Eds.), Biological Indicators of Soil Health. CAB International, Wallingford, UK, 201-234.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95-98.

Hamilton, E.W., Frank, D.A. 2001. Can plants stimulate soil microbes and their own nutrient supply? Evidence from a grazing tolerant grass. Ecology 82, 2397-2402.

Hamilton, H.C., Strickland, M.S., Wickings, K., Bradford, M.A., Fierer, N. 2009. Surveying soil faunal communities using a direct molecular approach. Soil Biology & Biochemistry 41, 1311-1314.

Hansson, M., Fogelfors, H. 2000. Management of a semi-natural grassland; results from a 15-year-old experiment in southern Sweden. Journal of Vegetation Science 11, 31-38.

Havlin, J.L., Tisdale, S.L., Beaton, J.D. and Nelson, W.L. 2005. Phosphorus. *In* Soil fertility and fertilisers: An introduction to nutrient management. 7th Edition, Prentice Hall, Inc., New Delhi, India, 160-198.

Haynes R.J. 1982. Effects of liming on phosphate availability in acid soils. Plant and Soil 68, 289-308.

Hermann, E., Gautheron, N., Alabouvette, C., Steinberg, C. 2008. Fingerprinting methods to approach multitrophic interactions among microflora and microfauna communities in soil. Biology and Fertility of Soils 44, 975-984.

Hodgson, J.G., Montserrat-Marti, G., Tallowin, J., Thompson, K., Diaz, S., Cabido, M., Grime, J.P., Wilson, P.J., Band, S.R., Bogard, A., Cabido, R., Caceres, D., Castro-Diez, P., Ferrer, C., Maestro-Martinez, M., Perez-Rontome, M.C., Charles, M., Cornelissen, J.H.C., Dabbert, S., Perez-Harguindeguy, N., Krimly, T., Sijtsma, F.J., Strijker, D., Vendramini, F., Guerrero-Campo, J., Hynd, A., Jones, G., Romo-Diez, A., Espuny, L.D., Villar-Salvador, P., Zak, M.R. 2005. How much will it cost to save grassland diversity? Biological Conservation 122, 263-273.

Hoekstra, N.J., Lalor, S.T.J., Richards, K.G., O'Hea, N., Dungait, J.A.J., Schulte, R.P.O., Schmidt, O. 2011. The fate of slurry-N fractions in herbage and soil during two growing seasons following application. Plant and Soil 342, 83-96.

Holterman, M.H.M., Oggenfuss, M., Frey, J.E., Kiewnick, S. 2012. Evaluation of high-resolution melting curve analysis as a new tool for root-knot nematode diagnostics. Journal of Phytopathology. 160, 59-66.

Holterman, M., Rybarczyk, K., Van den Elsen, S., Van Megen, H., Mooyman, P., Santiago, R.P., Bongers, T., Bakker, J., Helder, J. 2008. A ribosomal DNA-based

framework for the detection and quantification of stress-sensitive nematode families in terrestrial habitats. Molecular Ecology Resources 8, 23-34.

Holterman, M., van der Wurff, A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J., Helder, J. 2006. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown clades. Molecular Biology and Evolution 23, 1792-1800.

Hopkins, D.W., Shiel, R.S. 1996. Size and activity of soil microbial communities in long-term experimental grassland plots treated with manure and inorganic fertilizers. Biology and Fertility of Soils 22, 66-70.

Hübschen, J., Kling, L., Ipach, U., Zinkernagel, V., Bosselut, N., Esmenjaud, D., Brown, D.J.F., Neilson, R. 2004. Validation of the specificity and sensitivity of species-specific primers that provide a reliable molecular diagnostic for xiphinema diversicaudatum, X. index and X-vuittenezi. European Journal of Plant Pathology 110, 779-788.

Huijsmans, J.F.M., Hol, J.M.G., Hendriks, M.M.W.B. 2001. Effect of application technique, manure characteristics, weather and field conditions on ammonia volatilization from manure applied to grassland. Netherlands Journal of Agricultural Science 49, 323-342.

Humphreys, J., Aarts, H.F.M., Watson, C.J., Wachendorf, M., Le Gall, A., Taube, F., Pflimlin, A. 2009. Sustainable options for grassland-based dairy production in the northwest of Europe. Tearmann 7, 175-194.

Humphreys, J., O'Connell, K., Casey, I.A. 2008. Nitrogen flows and balances in four grassland-based systems of dairy production on a clay-loam soil in a moist temperate climate. Grass and Forage Science 63, 467-480.

Hunt, H.W., Coleman, D.C., Ingham, E.R., Ingham, R.E., Elliott, E.T., Moore, J.C., Rose, S.L., Reid, C.P.P., Morley, C.R. 1987. The detrital food web in a short-grass prairie. Biology and Fertility of Soils 3, 393-413.

Hyde, B.P., Hawkins, M.J., Fanning, A.F., Noonan, D., Ryan, M., O'Toole, P., Carton, O.T. 2006. Nitrous oxide emissions from a fertilized and grazed grassland in the South East of Ireland. Nutrient Cycling in Agroecosystems 75, 187-200.

Ingham, E.R., Trofymow, J.A., Ames, R.N., Hunt, H.W., Morley, C.R., Moore, J.C., Coleman, D.C. 1986. Trophic interactions and nitrogen cycling in a semi-arid grassland soil. 1. Seasonal dynamics of the natural populations, their interactions and effects on nitrogen cycling, Journal of Applied Ecology 23, 597-614.

Ingham, R.E., Trofymow, J.A., Ingham, E.R., Coleman, D.C. 1985. Interactions of bacteria,

fungi, and their nematode grazers - effects on nutrient cycling and plant-growth. Ecological Monographs 55, 119-140.

Jacquemyn, H., Brys, R., Hermy, M. 2003. Short-term effects of different management regimes on the response of calcareous grassland vegetation to increased nitrogen. Biological Conservation 111, 137-147.

Janssens, F., Peeters, A., Tallowin, J.R.B., Bakker, J.P., Bekker, R.M., Fillat, F., Oomes, M.J.M. 1998. Relationship between soil chemical factors and grassland diversity. Plant and Soil 202, 69-78.

Jarvis, S.C. 1999. Nitrogen dynamics in natural and agricultural ecosytems. In: Wilson, W.S., Ball, A.S., Hinton, R.H. (Eds.), Managing Risks of Nitrates to Humans and the Environment, 2-20.

Jarvis, S.C., Hatch, D.J., Roberts, D.H. 1989. The effects of grassland management on nitrogen losses from grazed swards through ammonia volatilization - the relationship to excretal N returns from cattle. Journal of Agricultural Science 112, 205-216.

Jenkinson, D.S., Parry, L.C., Vance, E.D., Wu, J., Harkness, D.D. 1988. Modeling the turnover of organic matter in Soil. Journal of the Science of Food and Agriculture 45, 132-133.

Jobbagy, E.G., Jackson, R.B. 2000. The vertical distribution of soil organic carbon and its relation to climate and vegetation. Ecological Applications 10, 423-436.

Joergensen, R.G. 1995. The fumigation extraction method to estimate soil microbial biomass: Extraction with 0.01 ml CaCl2. Agribiological Research-Zeitschrift Fur Agrarbiologie Agrikulturchemie Okologie 48, 319-324.

Johnson, N.C., Rowland, D.L., Corkidi, L., Egerton-Warburton, L.M., Allen, E.B. 2003. Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. Ecology 84, 1895-1908.

Jones, K.L., Todd, T.C., Herman, M.A. 2006a. Development of taxon-specific markers for high-throughput screening of microbial-feeding nematodes. Molecular Ecology Notes 6, 712-714.

Jones, K.L., Todd, T.C., Wall-Beam, J.L., Coolon, J.D., Blair, J.M., Herman, M.A. 2006b. Molecular approach for assessing responses of microbial-feeding nematodes to burning and chronic nitrogen enrichment in a native grassland. Molecular Ecology 15, 2601-2609.

Kleijn, D., Berendse, F., Smit, R., Gilissen, N. 2001. Agri-environment schemes do not effectively protect biodiversity in Dutch agricultural landscapes. Nature 413, 723-725.

Kleijn, D., Sutherland, W.J. 2003. How effective are European agri-environment schemes in

conserving and promoting biodiversity? Journal of Applied Ecology 40, 947-969.

Knoepp, J.D., Coleman, D.C., Crossley, D.A., Clark, J.S. 1998. Biological indices of soil quality: an ecosystem case study of their use. 9th North American Forest Soils Conference, Tahoe City, Ca, 357-368.

Kurz, I., Coxon, C., Tunney, H., Ryan, D. 2005. Effects of grassland management practices and environmental conditions on nutrient concentrations in overland flow. Journal of Hydrology 304, 35-50.

Larson, W.E., Pierce, F.J. 1992. The dynamics of soil quality as a measure of sustainable management. In: Doran, J.W., Coleman, D.C., Bezdicek, D.F., Stewart, B.A. (Eds.), Symposium on Defining Soil Quality for a Sustainable Environment, at the 1992 American-Society-of-Agronomy Annual Meeting, Minneapolis, Mn, 37-51.

Lawton, J.H., Bignell, D.E., Bolton, B., Bloemers, G.F., Eggleton, P., Hammond, P.M., Hodda, M., Holt, R.D., Larsen, T.B., Mawdsley, N.A., Stork, N.E., Srivastava, D.S., Watt, A.D. 1998. Biodiversity inventories, indicator taxa and effects of habitat modification in tropical forest. Nature 391, 72-76.

Liu, W.T., Marsh, T.L., Cheng, H., Forney, L.J. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology 63, 4516-4522.

MacArthur, R.H., Wilson, E.O. 1967. The Theory of Island Biogeography. Princeton University Press, Princeton, NJ.

Malakhov, V.V. 1994. Nematodes. Structure, development, classification and phylogeny. Hope, W.D. (Ed.). Washington and London, Smithsonian Institution Press, 286 pp.

Marsh, T.L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Current Opinion in Microbiology 2, 323-327.

Martínez-Suller, L., Provolo, G., Carton, O.T., Brennan, D., Kirwan, L., Richards, K.G. 2010. The composition of dirty water on dairy farms in Ireland. Irish Journal of Agricultural and Food Research 49, 67-80.

Maupas, E. 1900. Modes et formes de reproduction des nematodes. Archives of Zoological Experimental Genetics 8, 463-624.

McGrath, D., Tunney, H. 2010. Accumulation of cadmium, fluorine, magnesium, and zinc in soil after application of phosphate fertilizer for 31 years in a grazing trial. Journal of Plant Nutrition and Soil Science 173, 548-553.

Maurer, K., Weyand, A., Fischer, M., Stocklin, J. 2006. Old cultural traditions, in addition

to land use and topography, are shaping plant diversity of grasslands in the Alps. Biological Conservation 130, 438-446.

Meldal, B.H.M., Debenham, N.J., De Ley, P., De Ley, I.T., Vanfleteren, J.R., Vierstraete, A.R., Bert, W., Borgonie, G., Moens, T., Tyler, P.A., Austen, M.C., Blaxter, M.L., Rogers, A.D. and Lambshead, P.J.D. 2007. An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. Molecular Phylogenetics and Evolution 42, 622-636.

Merrill, E.H., Stanton, N.L., Hak, J.C. 1994. Responses of bluebunch wheatgrass, idaho fescue, and nematodes to ungulate grazing in Yellowstone-National-Park. Oikos 69, 231-240.

Mikola, J., Setälä, H. 1998. No evidence of trophic cascades in an experimental microbial-based soil food web. Ecology 79, 153-164.

Mills, A.A.S., Adl, M.S. 2011. Changes in nematode abundances and body length in response to management intensive grazing in a low-input temperate pasture. Soil Biology & Biochemistry 43, 150-158.

Moeseneder, M.M., Arrieta, J.M., Muyzer, G., Winter, C., Herndl, G.J. 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 65, 3518-3525.

Moir, J.L. 2007. Effects of the nitrification inhibitor dicyandiamide on soil mineral N, pasture yield, nutrient uptake and pasture quality in a grazed pasture system. Soil Use and Management 23, 111-120.

Moore, K.J., Johnson, M.G., Morris, J.R. 1988. Indigenous Yeast Microflora on Arkansas White Riesling (Vitis-Vinifera) Grapes and in Model Must Systems. Journal of Food Science 53, 1725-1728.

Moore, J.C., McCann, K., de Ruiter, P.C. 2005. Modeling trophic pathways, nutrient cycling, and dynamic stability in soils. Pedobiologia 49, 499-510.

Mumy, K.L., Findlay, R.H. 2004. Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR. Journal of Microbiological Methods 57, 259-268.

Murphy, J., Riley, J.P. 1962. A modified single solution method for determination of phosphates in natural waters. Analytica Chimica Acta 27, 31-36.

Nahmani, J., Lavelle, P., Rossi, J.P. 2006. Does changing the taxonomical resolution alter the value of soil macroinvertebrates as bioindicators of metal pollution? Soil Biology &

Biochemistry 38, 385-396.

Nahmani, J., Rossi, J.P. 2003. Soil macroinvertebrates as indicators of pollution by heavy metals. Comptes Rendus Biologies 326, 295-303.

Nakićenović, N., J. Alcamo, G. Davis, B. de Vries, J. Fenhann, S. Gaffin, K. Gregory, A.Grübler, T.Y. Jung, T. Kram, E. Emilio la Rovere, L. Michaelis, S. Mori, T.Morita, W. Pepper, H. Pitcher, L. Price, K. Riahi, A. Roehrl, H. Rogner, A. Sankovski, M. Schlesinger, P. Shukla, S. Smith, R. Swart, S. van Rooyen, N. Victor Dadi, Z. 2000. Special Report on emissions scenarios. IPCC Special Reports.

Neher, D.A. 1999. Nematode communities in organically and conventionally managed agricultural soils. Journal of Nematology 31, 142-154.

Neilson, R., Donn, S., Griffiths, B.S., Daniell, T., Rybarczyk, K.D., Van Den Elsen, S., Mooyman, P., Helder, J. 2009. Molecular tools for analysing nematode assemblages. In: M.J. Wilson and T. Kakouli-Duarte (Eds.), Nematodes as environmental indicators, CAB International, Wallingford, 188-207.

Nowaczyk, K., Dobosz, R., Kornobis, S., Obrepalska-Steplowska, A. 2008. TaqMan REAL-Time PCR-based approach for differentiation between Globodera rostochiensis (golden nematode) and Globodera artemisiae species. Parasitology Research 103, 577-581.

Nunan, N., Daniell, T.J., Singh, B.K., Papert, A., McNicol, J.W., Prosser, J.I. 2005. Links between plant and rhizoplane bacterial communities in grassland soils, characterized using molecular techniques. Applied and Environmental Microbiology 71, 6784-6792.

Okada, H., Oba, H. 2008. Comparison of nematode community similarities assessed by polymerase chain reaction-denaturing gradient gel electrophoresis (DGGE) and by morphological identification. Nematology 10, 689-700.

Olff, H., Ritchie, M.E. 1998. Effects of herbivores on grassland plant diversity. Trends in Ecology & Evolution 13, 261-265.

Opik, M., Moora, M., Zobel, M., Saks, U., Wheatley, R., Wright, F., Daniell, T. 2008. High diversity of arbuscular mycorrhizal fungi in a boreal herb-rich coniferous forest. New Phytologist 179, 867-876.

Opperman, M.H., Wood, M., Harris, P.J., Cherrett, C.P. 1993. Nematode and Nitrate Dynamics in Soils Treated with Cattle Slurry. Soil Biology & Biochemistry 25, 19-24.

Osborn, A.M., Moore, E.R.B., Timmis, K.N. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology 2, 39-50.

Partel, M., Zobel, M. 1999. Small-scale plant species richness in calcareous grasslands

determined by the species pool, community age and shoot density. Ecography 22, 153-159.

Paul, J.W., Beauchamp, E.G. 1989. Relationship between volatile fatty-acids, total ammonia, and pH in manure slurries. Biological Wastes 29, 313-318.

Petersen, C.T., Hansen, S., Jensen, H.E., Holm, J., Koch, C.B. 2004. Movement of suspended matter and a bromide tracer to field drains in tilled and untilled soil. Soil Use and Management 20, 271-280.

Plantureux, S., Peeters, A., McCracken, D. 2005. Biodiversity in intensive grasslands: Effect of management, improvement and challenges. Agronomy Research 3, 153-164.

Poinar, H.N., Schwarz, C., Qi, J., Shapiro, B., MacPhee, R.D.E., Buigues, B., Tikhonov, A., Huson, D.H., Tomsho, L.P., Auch, A., Rampp, M., Miller, W., Schuster, S.C. 2006. Metagenomics to paleogenomics: Large-scale sequencing of mammoth DNA. Science 311, 392-394.

Porazinska, D.L., Duncan, L.W., McSorley, R., Graham, J.H. 1999. Nematode communities as indicators of status and processes of a soil ecosystem influenced by agricultural management practices. Applied Soil Ecology 13, 69-86.

Porazinska, D.L., Giblin-Davis, R.M., Faller, L., Farmerie, W., Kanzaki, N., Morris, K., Powers, T.O., Tucker, A.E., Sung, W., Thomas, W.K. 2009. Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. Molecular Ecology Resources 9, 1439-1450.

Porazinska, D.L., Giblin-Davis, R.M., Powers, T.O., Kanzaki, N., Ye, W., Morris, K., Thomas, W.K. 2007. 454 pyrosequencing for metagenomic analysis of nematode diversity. Journal of Nematology 39, 90-90.

Porazinska, D.L., Sung, W., Giblin-Davis, R.M., Thomas, W.K. 2010. Reproducibility of read numbers in high-throughput sequencing analysis of nematode community composition and structure. Molecular Ecology Resources 10, 666-676.

Powers, T. 2004. Nematode molecular diagnostics: From bands to barcodes. Annual Review of Phytopathology 42, 367-383.

Powers, T.O., Neher, D.A., Mullin, P., Esquivel, A., Giblin-Davis, R.M., Kanzaki, N., Stock, S.P., Mora, M.M., Uribe-Lorio, L. 2009. Tropical nematode diversity: vertical stratification of nematode communities in a Costa Rican humid lowland rainforest. Molecular Ecology 18, 985-996.

Pushnova, E.A., Geier, M., Zhu, Y.S. 2000. An easy and accurate agarose gel assay for quantitation of bacterial plasmid copy numbers. Analytical Biochemistry 284, 70-76.

Quader, M.A., Novotny, J., Flickinger, J.C., Huq, M.S., Gerszten, P.C. 2008. Evaluation

of patient positioning accuracy during stereotactic spinal radiosurgery using cone beam CT. International Journal of Radiation Oncology Biology Physics 72, S528-S528.

Raghothama, K.G. 1999. Phosphate acquisition. Annual Review of Plant Physiology and Plant Molecular Biology, 665-693.

Rath, M., Peel, S. 2005. Grassland in Ireland and the UK. In: McGilloway, D.A. (Ed.) Grassland: a global resource. The Netherlands: Wageningen Academic Publishers, 13–27.

Reijs, J.W., Sonneveld, M.P.W., Sorensen, P., Schils, R.L.M., Groot, J.C.J., Lantinga, E.A. 2007. Effects of different diets on utilization of nitrogen from cattle slurry applied to grassland on a sandy soil in The Netherlands. Agriculture Ecosystems & Environment 118, 65-79.

Ritz, K., Black, H.I.J., Campbell, C.D., Harris, J.A., Wood, C. 2009. Selecting biological indicators for monitoring soils: A framework for balancing scientific and technical opinion to assist policy development. Ecological Indicators 9, 1212-1221.

Ritz, K., Trudgill, D.L. 1998. Utility of nematode community analysis as an integrated measure of the functional state of soils: perspectives and challenges - Discussion paper. European-Society-of-Nematologists-Organises Biannual International Symposia on Soil Microbial Interactions and Communities, Dundee, Scotland, 1-11.

Ruess, R.W., McNaughton, S.J. 1987. Grazing and the dynamics of nutrient and energy regulated microbial processes in the serengeti grasslands. Oikos 49, 101-110.

Rutgers, M., Schouten, A.J., Bloem, J., van Eekeren, N., de Goede, R.G.M., Akkerhuis, G., van der Wal, A., Mulder, C., Brussaard, L., Breure, A.M. 2009. Biological measurements in a nationwide soil monitoring network. European Journal of Soil Science 60, 820-832.

Sachs, T. 1994. Variable development as a basis for robust pattern formation. Journal of Theoretical Biology 170, 423- 425.

Saggar, S., Hedley, C.B., Giddens, K.M., Salt, G.J. 2000. Influence of soil phosphorus status and nitrogen addition on carbon mineralization from C-14-labelled glucose in pasture soils. Biology and Fertility of Soils 32, 209-216.

Saggar, S., Parfitt, R.L., Salt, G., Skinner, M.F. 1998. Carbon and phosphorus transformations during decomposition of pine forest floor with different phosphorus status. Biology and Fertility of Soils 27, 197-204.

Sample, R.H., Farber, M.E., Glick, M.R. 1980. Urinary oxalate indirectly determined by continuous-flow analysis for calcium. Clinical Chemistry 26, 1105.

Sato, E., Min, Y.Y., Toyota, K., Takada, A. 2009. Relationships between the damage to

radish caused by the root-lesion nematode Pratylenchus penetrans, its density prior to cultivation and the soil nematode community structure evaluated by polymerase chain reaction-denaturing gradient gel electrophoresis. Soil Science and Plant Nutrition 55, 478-484.

Sato, E., Goto, K., Min, Y.Y., Toyota, K., Suzuki, C. 2010. Quantitative detection of Pratylenchus penetrans from soil using soil compaction and real-time PCR. Nematological Research 40, 1-6.

Sato, E., Suga, Y., Kisaki, C., Toyota, K., Miyake, K., Takada, A., Takeuchi, K., Matsuura, R. 2011. Quantification of Pratylenchus penetrans in radish fields using a combination method of soil compaction and real-time PCR to determine the economic threshold. Soil Science and Plant Nutrition 57, 213-220.

Schaad, N.W., Frederick, R.D. 2002. Real-time PCR and its application for rapid plant disease diagnostics. Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie 24, 250-258.

Schils, R.L.M., Kok, I. 2003. Effects of cattle slurry manure management on grass yield. NJAS 51, 41-65.

Schnepf, R., Hanrahan, C.E. 2011. U.S. and EU Agricultural Support: Overview and Comparison. CRS Report for Congress, Prepared for Members and Committees of Congress. Congressional Research Service.

Schouten, T., Breure, A.M., Mulder, C., Rutgers, M. 2002. Nematode diversity in Dutch soils, from Rio to a biological indicator for soil quality. In: Cook, R.C., Hunt, D.J. (Eds.), 4th International Nematology Congress, Tenerife, SPAIN, 469-482.

Scow, K.M. 1994. Effect of sorption on biodegradation of organic chemicals in soil. 15th World Congress of Soil Science, Vol 4a, Transactions: Commission : Symposia, 352-361.

Scully, C., Collins, G., O'Flaherty, V. 2005. Assessment of anaerobic wastewater treatment failure using terminal restriction fragment length polymorphism analysis. Journal of Applied Microbiology 99, 1463-1471.

Seifert, K.A., Samson, R.A., deWaard, J.R., Houbraken, J., Levesque, C.A., Moncalvo, J.-M., Louis-Seize, G., Hebert, P.D.N. 2007. Prospects for fungus identification using C01 DNA barcodes, with Penicillium as a test case. Proceedings of the National Academy of Sciences of the United States of America 104, 3901-3906.

Seré, C., Steinfeld, H. 1996. World livestock production systems. Current status, issues and trends. Animal Production and Health, Paper 127.

Sharpley, A.N., McDowell, R.W., Weld, J.L., Kleinman, P.J.A. 2001. Assessing site

vulnerability to phosphorus loss in an agricultural watershed. Journal of Environmental Quality 30, 2026-2036.

Shoemaker, H.E., McLean, E.O., and Pratt, P.F. 1961. Buffer method for determining lime requirement of soils with appreciable amounts of extractable aluminum. Soil Science Society of America Proceedings 25: 274–277.

Sin, W.C., Pasternak, J. 1970. Number and DNA content of nuclei in the free-living nematode Panagrellus silusiae at each stage during postembryonic development. Chromosoma 32, 191-204.

Sonneveld, M.P.W., Bouma, J. 2003. Effects of combinations of land use history and nitrogen application on nitrate concentration in the groundwater. Njas-Wageningen Journal of Life Sciences 51, 135-146.

Sparling, G.P., Shepherd, T.G., Schipper, L.A. 2000. Topsoil characteristics of three contrasting New Zealand soils under four long-term land uses. New Zealand Journal of Agricultural Research 43, 569-583.

Stahlberg, A., Aman, P., Ridell, B., Mostad, P., Kubista, M. 2003. Quantitative real-time PCR method for detection of B-lymphocyte monoclonality by comparison of kappa and lambda immunoglobulin light chain expression. Clinical Chemistry 49, 51-59.

Stahlberg, A., Zoric, N., Aman, P., Kubista, M. 2005. Quantitative real-time PCR for cancer detection: the lymphoma case. Expert Review of Molecular Diagnostics 5, 221-230.

Stanton, J.M., McNicol, C.G., Steele, V. 1998. Non-manual lysis of second-stage Meloidogyne juveniles for identification of pure and mixed samples based on the polymerase chain reaction. Australasian Plant Pathology 27, 112-115.

Stanton, J.M., McNicol, C.G., Steele, V. 1998. Non-manual lysis of second-stage Meloidogyne juveniles for identification of pure and mixed samples based on the polymerase chain reaction. Australasian Plant Pathology 27, 112-115.

Stenger, R., Barkle, G.F., Burgess, C.P. 2001. Mineralization and immobilization of C and N from dairy farm effluent (DFE) and glucose plus ammonium chloride solution in three grassland topsoils. Soil Biology & Biochemistry 33, 1037-1048.

Suding, K.N., Collins, S.L., Gough, L., Clark, C., Cleland, E.E., Gross, K.L., Milchunas, D.G., Pennings, S. 2005. Functional- and abundance-based mechanisms explain diversity loss due to N fertilization. Proceedings of the National Academy of Sciences of the United States of America 102, 4387-4392.

Sulston, J.E., Schierenberg, E., White, J.G., Thomson, J.N. 1983. The embryonic cell lineage of the nematode Caenorhabditis elegans. Developmental Biology 100, 64-119.

Thomas, G.W. 1996. Soil pH and soil acidity. Sparks, D.L. (Ed). Methos of Soil Analysis, Part 3, Chemical Methods. Soil Science Society of America Book Series No 5. Soil Science Society of America, Madison, WI, 475–490.

Tilman, D. 1982. Resource Competition and Community Structure. Princeton University Press, Princeton, NJ.

Todd, A. 1996. Health inequalities in urban areas: a guide to the literature. Environment and Urbanization 8, 141-152.

Torsvik, V., Ovreas, L. 2002. Microbial diversity and function in soil: from genes to ecosystems. Current Opinion in Microbiology 5, 240-245.

Treseder, K.K., Masiello, C.A., Lansing, J.L., Allen, M.F. 2004. Species-specific measurements of ectomycorrhizal turnover under N-fertilization: combining isotopic and genetic approaches. Oecologia 138, 419-425.

Tung, W.L., Chow, K.C. 1995. A modified medium for efficient electrotransformation of *E. coli.* Trends in Genetics 11, 128-129.

Tunney, H. 1990. A Note on a Balance-Sheet Approach to Estimating the Phosphorus -Fertilizer Needs of Agriculture. Irish Journal of Agricultural Research 29, 149-154.

Tunney, H. 2002. Phosphorus needs of grassland soils and loss to water. Agricultural Effects on Ground and Surface Waters: Research at the Edge of Science and Society, 63-69.

Tunney, H., Sikora, F.J., Kissel, D., Wolf, A., Sonon, L., Goulding, K. 2010. A comparison of lime requirements by five methods on grassland mineral soils in Ireland. Soil Use and Management 26, 126-132.

Tunney, H., Sims, J.T., Morton, J. 2000. Comparison of pH recommendations for four countries. Communications in Soil Science and Plant Analysis 31, 1465-1466.

Vallette, F., Mege, E., Reis, A., Adesnik, M. 1989. Construction of mutant and chimeric genes using the polymerase chain reaction. Nucleic Acids Research 17, 723-733.

van Eekeren, N., de Boer, H., Bloem, J., Schouten, T., Rutgers, M., de Goede, R., Brussaard, L. 2009. Soil biological quality of grassland fertilized with adjusted cattle manure slurries in comparison with organic and inorganic fertilizers. Biology and Fertility of Soils 45, 595-608.

van Gestel, C.A.M., Koolhaas, J.E., Hamers, T., van Hoppe, M., van Roovert, M., Korsman, C., Reineckec, S.A. 2009. Effects of metal pollution on earthworm communities in a contaminated floodplain area: Linking biomarker, community and functional responses. Environmental Pollution 157, 895-903.

van Megen, H., van den Elsen, S., Holterman, M., Karssen, G., Mooyman, P., Bongers,

T., Holovachov, O., Bakker, J., Helder, J. 2009. A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. Nematology 11, 927-950.

Vanderknaap, E., Rodriguez, R.J., Freckman, D.W. 1993. Differentiation of bacterial-feeding nematodes in soil ecological-studies by means of arbitrarily-primed PCR. Soil Biology & Biochemistry 25, 1141-1151.

Verhoef, H. A., Brussaard, L. 1990. Decomposition and nitrogen mineralization in natural and agro-ecosystems: the contribution of soil animals. Biogeochemistry l 1, 175-2ll.

Verschoor, B.C., de Goede, R.G.M. 2000. The nematode extraction efficiency of the Oostenbrink elutriator-cottonwool filter method with special reference to nematode body size and life strategy. Nematology 2, 325-342.

Waite, I.S., O'Donnell, A.G., Harrison, A., Davies, J.T., Colvan, S.R., Ekschmitt, K., Dogan, H., Wolters, V., Bongers, T., Bongers, M., Bakonyi, G., Nagy, P., Papatheodorou, E.M., Stamou, G.P., Bostrom, S. 2003. Design and evaluation of nematode 18S rDNA primers for PCR and denaturing gradient gel electrophoresis (DGGE) of soil community DNA. Soil Biology & Biochemistry 35, 1165-1173.

Wang, M., Ahrne, S., Antonsson, M., Molin, G. 2004. T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of fecal microbiota in infants of different ages. Journal of Microbiological Methods 59, 53-69.

Wang, S.B., Li, Q., Liang, W.J., Jiang, Y., Jiang, S.W. 2008. PCR-DGGE analysis of nematode diversity in Cu-contaminated soil. Pedosphere 18, 621-627.

Wardle, D.A. 2002. Islands as model systems for understanding how species affect ecosystem properties. Journal of Biogeography 29, 583-591.

Wardle, D.A. 2006. The influence of biotic interactions on soil biodiversity. Ecology Letters 9, 870-886.

Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setala, H., van der Putten, W.H., Wall, D.H. 2004. Ecological linkages between aboveground and belowground biota. Science 304, 1629-1633.

Wardle, D.A., Bonner, K.I., Barker, G.M. 2002. Linkages between plant litter decomposition, litter quality, and vegetation responses to herbivores. Functional Ecology 16, 585-595.

Wardle, D.A., Yeates, G.W. 1993. The dual importance of competition and predation as regulatory forces in terrestrial ecosystems - evidence from decomposer food-webs.

Oecologia 93, 303-306.

Wesolowski, P. 1999. Use of liquid manure on permanent grasslands. Agrochemia **10**: 20-21.

White, T.A., Campbell, B.D., Kemp, P.D., Hunt, C.L. 2000. Sensitivity of three grassland communities to simulated extreme temperature and rainfall events. Global Change Biology 6, 671-684.

Whelan, J.A., Russell, N.B., Whelan, M.A. 2003. A method for the absolute quantification of cDNA using real-time PCR. Journal of Immunological Methods 278, 261-269.

Wilson, J.R.U., Proches, S., Braschler, B., Dixon, E.S. Richardson, D.M. 2007. The biodiversity of science reflects the interests of society. Frontiers in Ecology and the Environment, 5, 409-414.

Wood,W.B. (Ed.) 1988. The Nematode *Caenorhabditis elegans*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Wouters, A.P. 1995. New application techniques make slurry again a valuable nutrient. In: Luten, W., Snoek, H., Schukking, S., Verboon, M. (Ed), Proceedings of the Symposium Applied Research for Sustainable Dairy Farming. Wageningen. Research Station for Cattle, Sheep and Horse Husbandry, Lelystad, 13-18.

Ye, W., Robin, M.G.D. Braasch, H., Morris, K., Thomas, W. K. 2007. Phylogenetic relationships among Bursaphelenchus species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. Molecular Phylogenetics and Evolution 43, 1185-1197.

Yeates, G.W. 1987. Nematode feeding and activity - the importance of development stages. Biology and Fertility of Soils 3, 143-146.

Yeates, G.W. 2003. Nematodes as soil indicators: functional and biodiversity aspects. Biology and Fertility of Soils 37, 199-210.

Yeates, G.W., Bongers, T. 1999. Nematode diversity in agroecosystems. Agriculture Ecosystems & Environment 74, 113-135.

Yeates, G.W., Bongers, T., Degoede, R.G.M., Freckman, D.W., Georgieva, S.S. 1993. Feeding habits in soil nematode families and genera - an outline for soil ecologists. Journal of Nematology 25, 315-331.

Yeates, G.W., King, K.L. 1997. Soil nematodes as indicators of the effect of management on grasslands in the New England Tablelands (NSW): comparison of native and improved grasslands. Pedobiologia 41, 526-536.

Yeates, G.W., Wardle, D.A., Watson, R.N. 1999. Responses of soil nematode populations, community structure, diversity and temporal variability to agricultural intensification over

a seven-year period. Soil Biology & Biochemistry 31, 1721-1733.

Yu, Y., Kim, J., Hwang, S. 2006. Use of real-time PCR for group-specific quantification of aceticlastic methanogens in anaerobic processes: Population dynamics and community structures. Biotechnology and Bioengineering 93, 424-433.

Zak, D.R., Holmes, W.E., White, D.C., Peacock, A.D., Tilman, D. 2003. Plant diversity, soil microbial communities, and ecosystem function: Are there any links? Ecology 84, 2042-2050.

Zvomuya, F., Rosen, C.J., Gupta, S.C. 2006. Phosphorus sequestration by chemical amendments to reduce leaching from wastewater applications. Journal of Environmental Quality 35, 207-215.

Zeppelini, D., Bellini, B.C., Creao-Duarte, A.J., Hernandez, M. 2009. Collembola as bioindicators of restoration in mined sand dunes of Northeastern Brazil. Biodiversity and Conservation 18, 1161-1170.

Publications

A comparison of molecular methods for monitoring soil nematodes and their use as biological indicators

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ABSTRACT

Soil fauna, especially soil nematode communities may be used as indicators for monitoring soil biodiversity and ecological processes. A major drawback facing ecologists is the specialised taxonomic knowledge and labour intensive nature of the work required for traditional morphological identification of soil fauna. We review rapid molecular methods, including: DNA Barcoding or sequencing, PCR-DGGE, PCR-TRFLP and real-time PCR, which could enable an empirical assessment of soil nematode assemblages, in relation to their use as monitoring tools. Based on advantages of: high-throughput; ease of comparison between samples; and rapid data analysis, we argue that PCR-TRFLP is well suited to monitoring purposes.

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1. Introduction

1.1. Nematodes as biological indicators for soils

In recent years, interest has been shown by soil scientists and ecologists in measuring soil quality, particularly since the drafting of the Soil Framework Directive and increased national requirements for soil monitoring [17]. Soil quality is a combination of the physical, chemical and biological properties that contribute to soil function. Indicators of soil quality should be responsive to manage practices, integrate ecosystem processes, and be components of existing, accessible databases [41]. Such indicators must be quantified to document the improvement, maintenance or degradation of soil quality [42], represent different aspects of soil quality in different ecosystems [21], and strive to monitor or measure three basic functions or parameters: 1). soil structure development; 2). nutrient storage; and 3). biological activity [21].

Soil invertebrates are recognised as useful indicators as most are highly sensitive to perturbations and disturbances, for example, earthworms have been used to indicate soil properties [7] and soil pollution [68]; nematodes for environmental monitoring [11,12]; macroinvertebrates for soil heavy metal pollution [47,48]; and collembola for the restoration of environmental conditions [78]. Nematodes have been used as indicators of overall ecological condition because of the wide range of feeding types and the fact that they seem to reflect the successional stages of the systems in which they occur [11,22,24,76]. Furthermore, nematodes are sensitive to environmental insults and changes in their distribution and activity are diagnostic of changes in soil health [12,20,24,25,35,49,62,75], and they are the most abundant of the soil metazoa [20]. Nematode species occurring in soils encompass a wide variety of feeding strategies [77], including many free-living species that feed on soil microbes (bacteria or fungi). Microbial-feeding nematodes are among the most important consumers of bacteria and fungi in many systems [33], and their interactions with microbial decomposers affect ecosystem processes such as decomposition and nutrient cycling [28].

1.2. Limitations to routine monitoring

The identification of soil fauna often requires a high degree of taxonomic expertise [3,16]. Furthermore the time spent on identification (with the corresponding costs) makes it difficult to have results over a relatively short period of time with affordability. This is particularly true for the nematodes, identification of all individuals to the species level is time-consuming [43], so the characterisation of nematode communities continues to be resolved more coarsely than

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