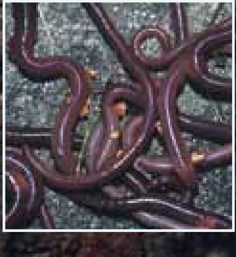
Grassland management, soil biota and ecosystem services in sandy soils

Nick van Eekeren



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Abstract

Recent legislative restrictions on the use of fertilizers and irrigation, and a quest for sustainable farming systems have drawn renewed attention to the functioning of the soil and the ecosystem services it provides. Soil biota play an important role in the provision of these ecosystem services, which may be influenced by grassland management. The two objectives of this thesis were 1) to gain insight into the effect of different grassland management measures on soil biota in sandy soils; and 2) to explore the effect of grassland management on the ecosystem services provided by soil biota.

In a long-term experiment comparing a ley-arable crop rotation with permanent grassland, most soil biota were found to recover quickly from declines during the arable phase of the crop rotation, reaching overall abundance and activity levels in the ley phase that were comparable to those observed in permanent grassland. However, anecic earthworm abundance was significantly reduced in the ley-arable crop rotation compared to permanent grassland, and this may impair the ecosystem service of water regulation.

The results of a field experiment suggest that grass-clover combines the positive effects of clover-only on the ecosystem service of nutrient supply with the positive effects of grass-only on soil structure maintenance. The grass-clover mixture had a higher soil mineralizable C than grass-only, and a higher percentage of soil crumbs than clover-only.

In an experiment comparing the effects of various inorganic and organic fertilizers, it was found that organic fertilizers significantly increased soil organic C, total N and the activity of decomposers. These effects did not significantly differ between the various organic fertilizers tested: there was no indication that soil nutrient supply through farm yard manure and adjusted manure slurries was better than through normal manure slurry.

To examine whether off-farm manure export has implications for the soil biological quality of grass-clover pastures on organic dairy farms, the effect of different N application rates of farm yard manure and slurry were compared. The results suggest that clover N fixation has a dominant influence on soil biological quality, and may compensate for reduced N application.

In an experiment comparing grass species, little significant differences were found with respect to soil microbiological and nematode parameters. However, root biomass was significantly higher in *L. perenne* and *F. arundinacea* compared to *D. glomerata*, which suggests that the ecosystem service of soil structure maintenance is better supported by the first two grass species.

In 20 grasslands, biotic and abiotic soil parameters were measured simultaneously with process parameters that reflect various ecosystem services. Grassland production without N fertilization was mainly explained by soil organic matter and soil moisture, and to a lesser extent by soil total N. The response of N yield to N fertilization was significantly explained by the abundance of enchytraeids, the underlying mechanism of which should be further investigated. The finding that the presently used fertilization recommendation model underestimated the soil nitrogen supply capacity of the majority of the soils in this study, taken together with the fact that these soils represented a broad range of N response to N fertilizer, legitimates reconsideration of the present fertilizer recommendations.

It is concluded that a semi-permanent grass-clover or a semi-permanent grass-only with moderate N fertilization, with grass species with a dense and deep root system, and fertilization with organic fertilizers, is the most favorable combination of management measures for sustaining the ecosystem services of soil structure maintenance, water regulation and nutrient supply. Four scenarios are described for which management choices can be specified.

Voorwoord

Dat je nooit, nooit moet zeggen, gaat zeker op voor mijn promotie. Het idee om te promoveren ontstond al werkend in verschillende projecten aan het effect van management maatregelen op bodemleven. De basis voor dit proefschrift vormen dan ook de gegevens verzameld in de jaren 2002 tot en met 2008 in de projecten Bodem Biologische Indicator (BoBi), 100% biologische mest (Bioconnect), Bodem Bedrijf en Biodiversiteit (BBB), Zorg voor Zand, en Boeren en Biodiversiteit (B&B). Na wat vertraging door een auto-ongeluk en fase II van de verbouwing van ons huis, werden ideeën in 2006 verder geconcretiseerd. In 2007 ben ik uiteindelijk met het schrijfwerk begonnen, tijdens en naast het normale werk. Gedurende de afgelopen jaren was de focus op het schrijven van artikelen een welkome afwisseling naast de hectiek van het dagelijkse projectenwerk. Zoals gewoonlijk wogen ook bij mij de laatste loodjes, met de deadline in zicht, het zwaarst. Voor u ligt nu het eindresultaat. De vele mensen die in de afgelopen periode hieraan hun bijdrage hebben geleverd wil ik graag bedanken.

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Contents

Chapter 1

General introduction

Based on: N. van Eekeren, P.J. Murray, F.W. Smeding, 2007. Soil biota in grassland, its ecosystem services and the impact of management. In: De Vliegher, A., Carlier, L. Permanent and Temporary Grassland: Plant, Environment and Economy. Grassland Science in Europe

1.1 Introduction

Production grassland is with one million hectares the main crop in the Netherlands. It is mainly used for dairy farming (CBS, 2000). Until recently, the soil quality of production grasslands was generally not a matter of great concern. Nutrients and irrigation could always be applied in abundance, which compensated for a possible deterioration in soil quality. However, recent legislative restrictions on the use of fertilizers (Vellinga, 2006) and irrigation, and a quest for sustainable farming systems have drawn renewed attention to the functioning of the soil and the ecosystem services it provides (MA, 2005), not only among policymakers and scientists but also the farmer community.

45 % of the total grassland area of The Netherlands is situated on sandy soils (CBS, 2000). In general, sandy soils have the lowest inherent quality for agricultural production, which necessitates external inputs such as fertilizers and irrigation. At the same time, sandy soils are the most vulnerable to nitrate leaching, hence legislative restrictions on the use of fertilization are the most severe for these soils (for grassland which is grazed, the annual N input from organic and inorganic fertilizers on sandy soils is restricted to 250 kg N ha^{-1}, compared to 320 kg N ha^{-1} for clayey soils). Therefore, managing the quality of sandy soils and the ecosystem services they provide, is of utmost importance for sustainable grassland production.

Next to soil chemical and physical quality, biological soil quality is one of the pillars of soil quality management. The soil biota play an important role in the provision of ecosystem services, including maintenance of soil structure, water regulation and nutrient supply (Brussaard et al., 1997; Swift et al., 2004; Mulder, 2006; Kibblewhite et al., 2008). At present farmers consciously and unconsciously use soil biota for the provision of ecosystem services. For example, by sowing white clover (*Trifolium repens* L.), farmers consciously use *Rhizobium* soil bacteria to fix atmospheric nitrogen. Unconsciously, with each grass harvest farmers stimulate the exudative response of grasses to defoliation, and they instinctively know that the first "weed" cut of newly sown grassland is important to get grass growth started (Hamilton and Frank, 2001; Patterson, 2003). In contrast, some farmers claim that slit injection of manure slurry is harmful to soil biota, but without scientific evidence on sandy soils (de Goede et al., 2003; van Vliet and de Goede, 2006). Developing and optimizing sustainable grassland systems requires more awareness of and insight into the mechanisms by which soil biota are influenced by management, and how this affects the functioning of the soil-plant system. This knowledge would allow to make better use of the ecosystem services associated with soil biota and could help to limit inadvertent negative effects of management measures on soil biota and ecosystem functioning.

This chapter is a general introduction to the soil biota and their associated ecosystem services in grasslands used for dairy farming on sandy soils, followed by an outline of this thesis.

1.2 Soil biota

The soil biota measured by the Biological Indicator System for Soil Quality in The Netherlands are presented in Table 1.1 (Rutgers et al., 2008, 2009). The average fresh biomass of soil biota on dairy farms on sandy soils is 2693 kg ha $^{-1}$. This amount exceeds the weight of the livestock aboveground. The live biomass consisted of 70 % bacteria, 11 % fungi and 18 % earthworms. The remainder was made up of enchytraeids, mites, collembola and nematodes.

Table 1.1 Average biomass and taxonomic diversity of soil biota in sandy soils of 87 dairy farms in The Netherlands (Rutgers et al., 2008, 2009).

Classification by body width	Biota	Soil layer	Fresh biomass (kg ha $^{-1}$)	Number of taxa
Macrofauna > 2mm[1]	Earthworms	0-20 cm	475	4.6
Mesofauna 100 µm-2mm[1]	Enchytraeids	0-15 cm	22.4	8.2
	Mites+Collembola[3]	0-7.5 cm	1.6	26
Microfauna <200 µm[2]	Nematodes[3]	0-10 cm	9.8	32
Microflora[2]	Bacteria	0-10 cm	1898	nd
	Fungi	0-10 cm	286	nd

[1] Samples collected in grassland only.

[2] Samples collected in both grassland and arable land of the same dairy farm

[3] Converted from abundance to fresh biomass according to Didden et al. (1994).

nd, not determined.

Organism size plays an important role in soil biological interactions, because the soil habitat is composed of differently sized pores (Brussaard et al., 1997). Based on body width, soil biota are classified into microfauna, mesofauna and macrofauna (Table 1.1). Soil biota can also be classified into trophic groups based on food preference, or into functional groups based on life strategies (indicating how they respond to food availability, stress and disturbance). The complex interactions between the trophic groups can be visualized in soil food webs (De Ruiter et al., 1993).

1.3 Ecosystem services of the soil biota

Agricultural grassland is a perennial cropping system without annual soil tillage, subject to soil compaction from animal trampling and heavy machinery. Therefore, soil structure maintenance and water regulation are, next to nutrient supply, the key ecosystem services of the soil biota for grassland production (Brussaard et al., 1997; Swift et al., 2004; Mulder, 2006; Kibblewhite et al., 2008). In this section, the contribution of soil biota to these ecosystem services in grassland is reviewed.

1.3.1 Soil structure maintenance

In regard to the ecosystem service of soil structure maintenance, there is evidence that the polysaccharides (slime) produced by bacteria bind aggregates together, and that fungal hyphae entangle soil particles and smaller aggregates into larger aggregates. For example, in arable land, Mäder et al. (2002) found a positive correlation between aggregate stability and microbial biomass. Under perennial ryegrass and white clover, Tisdall and Oades (1979) measured a positive relation between mycorrhizal hyphal length and water stable aggregates.

Cole et al. (2006) concluded that macrofauna, particularly earthworms, have a more profound effect on soil structure than microflora. Earthworms in particular affect soil structure through producing faecal casts, promoting humification and creating pores. The introduction of earthworms to grasslands on reclaimed land resulted in increased infiltration capacity, improved permeability and aeration of the upper soil layers, and decreased soil penetration resistance (Hoogerkamp et al., 1983). Similar effects were reported in an experiment by Clements et al. (1991) in which a 20 years' absence of earthworms and other soil invertebrates (due to pesticide treatment) had resulted in increased soil bulk density, and decreased penetrability, soil organic matter content, initial infiltration rate and soil moisture content.

1.3.2 Water regulation

The ecosystem service of water regulation is closely related to soil structure maintenance. The importance of soil biota for water regulation was shown by the increased water-logging of Scottish grassland soils where flatworm predation had decimated earthworm populations (Haria et al., 1998). Water infiltration through macropores and stable crumb formation are two key soil processes strongly affected by earthworms. For example, Bouché and Al-Addan (1997) calculated a mean water infiltration rate of 150 mm water m^{-2} h^{-1} per 100 g earthworms. Accordingly, the presence of earthworms has been shown to reduce surface runoff (Edwards and Bohlen, 1996; Pitkänen and Nuutinen, 1998). However, earthworm burrows can also lead to increased bypass flow and nutrient leaching (Edwards et al., 1992; Dominiguez et al., 2004).

Grass rooting depth is important particularly for the drought resistance of grassland. Earthworm burrows provide pathways for root penetration throughout the soil profile. The deep-burrowing, surface-feeding earthworm species *Lumbricus terrestris* Linnaeus and *Aporrectodea longa* Ude generally make vertical burrows and are able to penetrate hard pans. In several pot and field studies increased root growth and root depth has been measured in response to earthworm inoculation (Logsdon and Linden, 1992).

1.3.3 Supply of nutrients

All soil biota groups are involved in the ecosystem service of nutrient supply. Bacteria and fungi contribute to this service through nutrient mineralization and immobilization. De Ruiter et al. (1993) estimated that in arable soil the contribution of bacteria to N-mineralization is 20 to140 kg N ha^{-1} yr^{-1} and of fungi 1 to 24 kg N ha^{-1} yr^{-1}. Through symbioses with mycorrhizal fungi, plants can take up more nutrients and produce more biomass. For example, Van der Heijden et al. (1998) measured positive mycorrhizal effects on shoot phosphorus concentrations and shoot biomass of grasses such as *Bromus* spp. and *Festuca* spp. Moreover, the ability of legumes to fix atmospheric N_2 through symbiosis with *Rhizobium* bacteria plays an invaluable role in grassland production (Frame et al., 1998).

Nematodes and protozoa affect nutrient cycling processes indirectly through grazing on soil microbial biomass and nutrient excretion. Griffiths (1989) observed that the nitrogen content of ryegrass increased by 14% when nematodes or protozoa were added to microcosms with bacteria. Ingham et al. (1985) found increased grass shoot and root growth when nematodes were added to a microcosm with bacteria. Not only microbivorous nematodes are involved in nutrient cycling. In experiments with clover, low levels of root infestation by clover cyst nematodes *(Heterodera trifolii* Goffart) positively influenced the rhizosphere microbial community in the soil (Yeates et al., 1998a; Denton et al., 1999) and increased the root growth of white clover and perennial ryegrass with 141% and 217%, respectively (Bardgett et al., 1999a). Associated with this increased root production was a 322% increase in uptake of white clover-derived ^{15}N. Bardgett et al. (1999a) suggested that herbivory of white clover roots enhanced the flux of clover N into the soil, which was subsequently recycled and taken up by the neighboring ryegrass plants. Similar results have been demonstrated for larger invertebrates. For example, the larvae of the clover root weevil (*Sitona lepidus* Gyllenhal) have been shown to facilitate the transfer of nitrogen from clover to companion ryegrass (Murray and Hatch, 1994; Murray and Clements 1998).

Mesofauna and macrofauna affect nutrient cycling processes directly through fragmentation and transport of organic and mineral particles, and indirectly through regulating microbial communities and stimulating microbial activity. For instance, soil microflora is stimulated by earthworm mucus in casts and burrows (Brown, 1995). Particularly in grassland, earthworms contribute significantly to organic matter fragmentation through breaking down the sod. Hoogerkamp et al. (1983) observed that the introduction of earthworms to grasslands in reclaimed polders resulted in the development of dark-colored top soil within three years after earthworm introduction. In an experiment by Clements et al. (1991), the most apparent effect of a pesticide treatment that excluded earthworms was the accumulation of litter. Next to fragmentation, the transport and mixing of organic and mineral particles is an important function of earthworms. In a glasshouse study with perennial ryegrass and rock phosphate, the presence of earthworms resulted in a higher yield, not only through the better mixing of rock phosphate by earthworm activity but also due to increased availability of P in worm casts (Mackay et al., 1982).

1.3.4 Grass production

For dairy farmers, grass production (quantity and quality) is the ultimate ecosystem service, in which soil services such as soil structure maintenance, water regulation and particularly nutrient supply play prominent roles. The section on nutrient supply (1.3.3) provides several examples of positive effects of soil biota on root growth, shoot nitrogen and phosphorous content, and yield. It appears that the positive effects of soil biota on grass production under field circumstances are most obvious for earthworms, particularly when earthworms are introduced to soils where they were previously absent (Stockdill, 1982; Hoogerkamp et al., 1983; Baker, 1998).

1.4 Plant-soil interactions

The influence of grassland management on soil biota cannot be seen apart from their effects on the grass sward. There is an intimate link between soil organisms and the grass plant. In this section various plant-soil interactions are illustrated and elaborated in a conceptual framework.

Grasses supply nutrients to the soil food web through root exudates and litter from roots and aboveground parts. According to Whipps (1990), 35-80% of the net fixed C in perennial grasses is transferred belowground. Deinum (1985) calculated that in a perennial ryegrass sward 4500 kg ha^{-1} of organic root mass is decomposed on a yearly basis. In a cut and fertilized grass sward, 8000-9000 kg ha^{-1} of organic material from dead roots, dead leaves and stubble is transferred into the soil (Whitehead, 1995). The quantity and quality of litter and root exudates (including secondary metabolites) of various herb and grass species have been shown to be major determinants of soil food web structure and soil biodiversity (Bardgett, 2005). Various experiments have shown that grassland plants differ markedly in their impact on soil microbes (Bardgett et al., 1999b), nematodes (Wasilewska, 1995) and enchytraeids (Griffiths at al.,1992). Next to providing litter and root exudates, grass roots are a host for many soil organisms including root pathogens, root herbivores, and symbiotic soil biota. Therefore, the rhizosphere is recognized as a “hot spot” of biological activity (Beare, 1995). In turn, soil biota influence grass production and quality indirectly through ecosystem services and through antagonistic effects on root herbivores. By means of these processes, soil biota influence the composition of grassland plant communities. For example, Grime et al. (1987) showed that the introduction of mycorrhizal fungi led to a shift in plant species composition.

Thus, through the quantity and quality of roots, root exudates and litter, plants influence the abundance and diversity of soil biota. In turn, soil biota influence plant production, plant quality and species composition through ecosystem services and antagonistic effects on root herbivores. In regard to earthworms, Syers and Springett (1983) concluded that these plant-soil relationships are interactive, cyclical and complex. Bardgett and Wardle (2003) elaborated the interrelationships between grazers, plants and soil decomposer organisms. An illustrative example of these interrelationships is provided by an experiment of Hamilton and Frank (2001), in which grazing of *Poa pratenis* L. promoted root exudation. This stimulated microbial biomass//activity in the rhizosphere,which in turn increased soil N availability and plant N acquisition, which resulted in enhanced grass growth. Brussaard (1998) presented a diagram on soil-plant interrelationships including interactions between plants and “decomposers”, “root-biota” such as root herbivores, and “ecosystem engineers” such as earthworms. In this diagram Brussaard (1998) considered plant roots as ecosystem engineers, since roots create habitats for other organisms. Here, I propose a conceptual framework (Figure 1.1) in which this diagram is worked out for a grassland sward with a cycle based on Syers and Springett (1983).

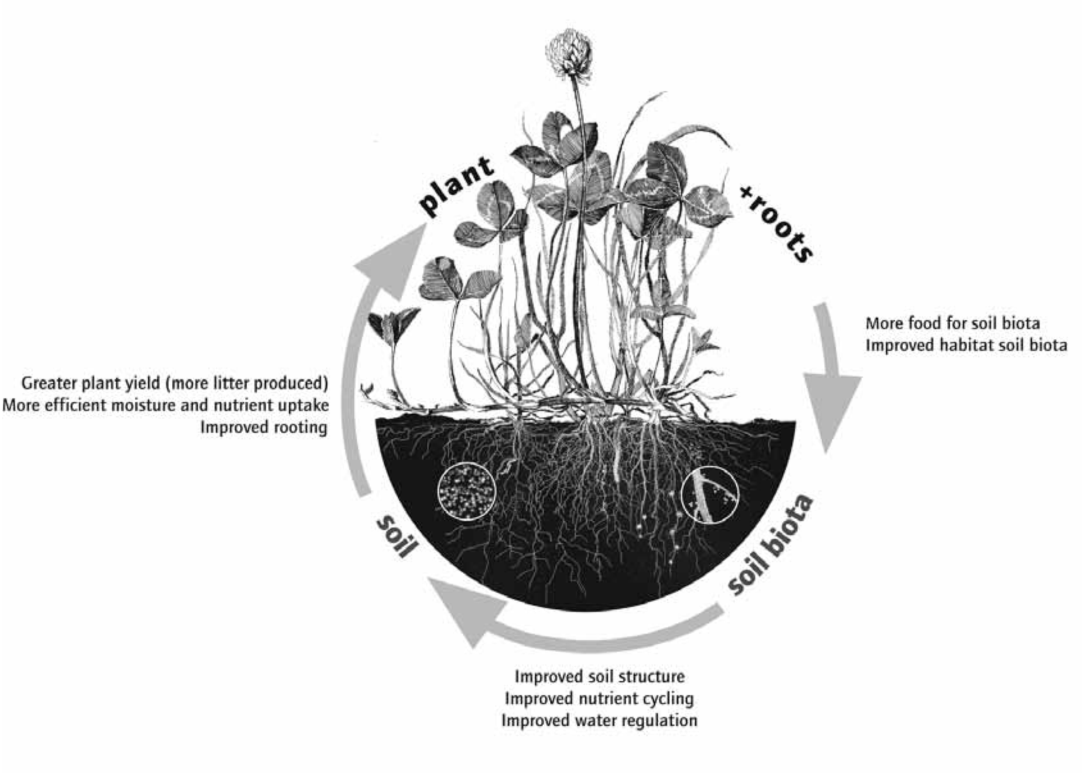

Figure 1.1 Cyclic interactions between plants/roots, soil biota (root biota, decomposers and ecosystem engineers) and soil properties (chemical and physical).

Through plants and roots and interactions with soil biota, plants deliver food and habitat to soil biota. In turn, soil biota improve soil structure, water regulation and nutrient cycling, resulting in increased root production and plant water and nutrient uptake. This results in a greater plant yield, which in turn increases the quantity and quality of litter and root exudates. The challenge for sustainable grassland management is to allow this cycle to function with an optimum use of inputs (nutrients and water). The roots are a major link between the aboveground and belowground system; when they fail to grow, the cycle shuts down.

We can make a distinction between fertile systems that support high levels of herbivory, and infertile systems that support low herbivory. Ecosystems dominated by plant species adapted to fertile conditions ('mull sites'), have high litter quantity and quality, and support soil food webs with a bacterial-based energy channel and a predominance of microfauna (nematodes and protozoa) and earthworms. In contrast, infertile soils (‘mor sites’) support plants with low litter quantity and quality, and tend to have soil food webs dominated by fungi and microarthropods such as mites and collembola (Swift et al., 1979; Wardle, 2002; Wardle et al. 2004). This distinction is apparent in the different land uses of sandy soils in The Netherlands (Table 1.2; Rutgers et al., 2008). Here, agricultural land use represents the most fertile soil conditions, whereas heathland and mixed forest represent the most infertile conditions. Bacterial biomass, and the abundance of nematodes and earthworms (bold-faced numbers in Table 1.2) are higher in agricultural and semi-natural grassland, whereas fungi and especially micro-arthropods are more abundant in heathland and mixed forests.

In fertile soils the activity of soil biota and their trophic interactions result in high bioturbation, rapid decomposition and nutrient mineralization, high nutrient supply rates, low soil C sequestration and possibly increased nutrient leaching. Under infertile conditions soil biotic effects on these soil processes are opposite. Whereas in fertile conditions the interactions between these soil processes and aboveground plant production are positive, under infertile conditions they are negative (Wardle et al., 2004). Within the proposed framework (Figure 1.1), sustainable grassland management would require the positive interactions between the belowground and aboveground sub-systems However, loss of soil C and leaching of nutrients should be minimized.

1.5 Impact of grassland management on soil biota

The majority of dairy farmers on sandy soils in The Netherlands have a minimum of 70% grassland, due to legislative prescriptions. The rest of their land is arable, mainly used for fodder maize production, either under continuous cultivation (conventional farms) or in a ley-arable crop rotation (organic farms). In 1999, 6% of the grasslands on sandy soils was used in a ley-crop rotation, with fodder maize or other crops. In the same year, 13% of the grasslands on sandy soils were plowed and re-sown directly with grass (CBS, 2000). On average, grasslands on sandy soils that are not used in crop rotation are plowed and re-sown every 6-8 years. Grass mixtures are dominated by perennial ryegrass (*Lolium perenne* L.) and sometimes mixed with white clover (*T. repens*). Conventional dairy farmers on sandy soils are allowed to fertilize their grasslands with 250 kg N total ha^{-1} yr^{-1} from animal urine, dung and manure. On top of the application of animal urine, dung and manure, they are allowed to apply 138 kg N ha^{-1} yr^{-1} from inorganic fertilizer on grassland for grazing, and 170 kg N ha^{-1} yr^{-1} on grassland for purely cutting. Organic dairy farmers on sandy soils depend for their N-supply on nitrogen fixation by clover, in addition to the allowed maximum of 170 kg N total

Table 1.2 Soil biota in sandy soils with different land uses in the Netherlands (Rutgers et al., 2008).

Land use	Unit (No. of locations)	Arable farms (34)	Dairy farms (87)	Semi-natural grassland (10)	Heath-land (10)	Mixed forest (20)
Soil chemical parameters						
SOM[2]	g kg dry soil^{-1}	75	64	93	73	57
C/N[2]		20	14	18	34	25
pH-KCl[2]		5.2	5.2	4.5	3.2	3.2
P-Al[2]	mg $P_2O_5$100g soil^{-1}	56	54	27	2	3
P-total[2]	mg $P_2O_5$100g soil^{-1}	105	149	144	41	19
Soil biological parameters						
Earthworm number[1]	n m^{-2}	38	**187**	**133**	0	9
Earthworm taxa[1]	n	2.0	4.6	6.8	0	0.7
Enchytraeids number[1]	n 10^3 m^{-2}	22	24	13	14	21
Enchytraeids taxa[1]	n	8.1	8.2	14	6.2	4.7
Micro-athropod number[1]	n 10^3 m^{-2}	23	46	101	**157**	**150**
Micro-athropod taxa[1]	n m^{-2}	22	26	24	22	59
Nematode number[2]	n 100g soil^{-1}	3717	**4926**	**5054**	2053	730
Nematode taxa[2]	n	27	32	36	22	25
Bacterial biomass[2]	µg C g dry soil^{-1}	88	**146**	**204**	75	47
Bacterial activity[2]	Thymidime incorp.	67	66	17	4	2
Bacterial diversity	n DNA bands	68	51	--	--	25
Fungal biomass[2]	µg C g dry soil^{-1}	--	22	24	**53**	--
CLPP ES50[2,3]	µg dry soil	1415	637	324	9293	39712
CLPP slope[2,4]		0.55	0.57	0.35	0.41	0.60

[1] Data for dairy farm land use were collected in grassland only.

[2] Data for dairy farm land use were collected in grassland as well as the arable land of the same dairy farms.

[3] CLPP ES50: The amount of soil extract needed to convert 50% of all substrates in ECO plates. This is a measure for the physiological activity of the bacterial community. A low ES50 indicates a high activity.

[4] CLPP slope: A measure of the physiological diversity of the bacterial community. A low slope indicates a high diversity.

ha^{-1} yr^{-1} (ecological farms) or 112 kg N total ha^{-1} yr^{-1} (bio-dynamic farms) from urine, dung and manure.

Land management on dairy farms affects soil biota through:

1. influencing their habitat through liming, drainage, irrigation etc.;
2. direct disturbance through soil compaction (animal trampling and heavy machinery) and plowing (for grassland renewal or crop rotation);
3. influencing the quantity and quality of plant litter and root exudates through crop rotation, fertilization, grazing versus cutting management, introduction of legumes and species mixtures;
4. introducing synthetic compounds and heavy metals into the soil, through the use of pesticides on crops, and the use of antibiotics and minerals for animal health (Wardle et al., 1999; Brussaard et al., 2007a).

Specific management measures can affect soil biota directly or indirectly, or through combined effects. For example, in an upland grassland, lime application had direct effects on the enchytraeid community structure through soil chemical changes, and indirect effects on the nematode community structure through increased plant production and root turnover (Cole et al., 2006; Murray et al. 2006). In the case of enchytraeids liming influenced their habitat, while in the case of nematodes liming influenced the quantity and quality of their food resource.

The main effects of grassland management on biological soil quality in different land uses can be inferred from Table 1.2: from dairy farms to mixed forest there is a gradient in habitat (e.g., pH), from arable farms to semi-natural grasslands there is a gradient of direct disturbance, while from dairy farms to heathland there is a gradient in resource quantity and quality. However, to gain more insight into how specific agricultural management measures affect soil biota, long-term agricultural field experiments are needed which simultaneously consider several groups of organisms and/or soil processes (Wardle et al., 2001).

1.6 Objectives and outline of this thesis

The main objectives of this thesis are:

- To gain insight into the effect of grassland management measures on the abundance, activity and species richness of the soil biota in sandy soils;
- To explore the effect of grassland management measures on the ecosystem services associated with soil biota, such as soil structure maintenance, water regulation, supply of nutrients, and ultimately, grass production.

Specific hypotheses are given in the pertinent chapters.

This thesis focuses on management measures that are presently considered sustainable by scientists as well as dairy farmers. Within this group, I looked specifically at management measures that are most likely to disturb soil biota, affect their habitat, and/or affect the quantity and quality of their food resources. In each of the chapters 2 to 6 the effect of a combination of grassland management measures on soil biota and ecosystem services are reported and discussed (Table 1.3). Chapter 2 compares the effects of 36 years of ley-arable cropping, permanent grassland and permanent arable land. Chapter 3 compares the effects of inorganic N fertilization and N-fixation by white clover. Chapter 4 compares the effect of five years' application of adjusted manure slurries to grassland with the effect of organic and inorganic fertilizer applications. In chapter 5 the effects of N-fixation by clover (chapter 3) and of fertilizer type (chapter 4) are combined in an analysis of the effects of different fertilizer types, applied at different N application rates, on grass-clover. The effect of different grass species and their mixtures is reported and discussed in chapter 6. Finally, in chapter 7 the relationships between soil biological quality and ecosystem services are analyzed based on data collected in 20 grasslands. The thesis is concluded by a general discussion of the results and their implications for grassland management (chapter 8).

Table 1.3 Management measures reported and discussed in chapters 2 to 6.

Chapter	Habitat		Disturbance		Resource quantity and quality			
	SOM	pH	Cropping system	Age of grassland	N input from fertilizer or N-fixation	Type of fertilizer	Plant species (grass, clover)	Grass species
2	x		x	x				
3					x		x	
4	x	x			x	x		
5	x	x			x	x		
6	x	x						x

Chapter 2

Soil biological quality after 36 years of ley-arable cropping, permanent grassland and permanent arable cropping

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Abstract

Insight is needed into how management influences soil biota when sustainable grassland systems are developed. A crop rotation of grass and maize can be sustainable in terms of efficient nutrient use. However, there is lack of information on the effect of such a crop rotation on soil biological quality. Earthworms, nematodes, bacteria and fungi were sampled over three years in a 36 years old experiment. Permanent arable land was compared with permanent grassland and with a ley-arable crop rotation. In the rotation, a period of three years of grassland (temporary grassland) was followed by a period of three years of arable land (temporary arable land) and vice versa. In the first year of arable cropping in the rotation, the number of earthworms was already low and not different from continuous cropping. In the three-year grass ley, the abundance of earthworms returned to the level of permanent grassland in the second year. However, the restoration of earthworm biomass took a minimum of three years. Furthermore, the anecic species did not recover the dominance they had in the permanent grassland. The numbers of herbivorous and microbivorous nematodes in the ley-crop rotation reached similar levels to those in the permanent treatments within one to two years. Although the same holds for the nematode genera composition, the Maturity Index and the proportion of omnivorous nematodes in the temporary treatments remained significantly lower than in their permanent counterparts. Differences in recovery were also found among microbial parameters. In the temporary treatments, bacterial growth rate and the capacity to degrade a suite of substrates recovered in the second year. However, the Community-Level Physiological Profiles in the permanent grassland remained different from the other treatments. Our results suggest that many functions of soil biota that are well established in permanent grassland, are restored in a ley-arable crop rotation. However, due to a reduction in certain species, specific functions of these soil biota could be reduced or lost. The ley-arable crop rotations were intermediate to permanent grassland and continuous arable land in terms of functioning of soil biota (e.g., N-mineralization). In terms of the functional aspects of the soil biota, permanent grassland might be preferable wherever possible. For maize cultivation, a ley-arable crop rotation is preferable to continuous arable land. However, a ley-arable crop rotation is only preferable to continuous arable cropping if it is not practiced at the expense of permanent grassland at farm level.

Keywords

Earthworms, nematodes, microbiology, soil biota, crop rotation, grassland

2.1 Introduction

Organic farming and reduced use of external inputs - such as fertilizers and pesticides - in conventional agriculture, implies a greater reliance on ecosystem self-regulating processes (Yeates et al., 1997). Soil biota play an important role in these processes and in the provision of various ecosystem services: supply of nutrients, maintenance of soil structure, water regulation and, more generally, in the resistance and resilience of the below-ground system (Brussaard et al., 1997; Mulder, 2006). Grassland management directly and indirectly influences the soil food web and its functions (Bardgett, 2005). To develop and optimise sustainable grassland systems, insight is needed into how management influences soil biota and how it affects the functioning of the soil-plant system.

On dairy farms in The Netherlands, the main crops are grass (mainly based on *Lolium perenne* L.) and maize (*Zea mays* L.). Due to legislative restrictions in The Netherlands, most dairy farms have a maximum of 30% of their land cultivated with maize. For economic reasons, both crops are mainly continuously cropped, which is only possible thanks to the addition of external inputs. However, recent legislative restrictions on the use of organic and artificial N fertilizers (Vellinga, 2006) and a quest for sustainable farming systems, have brought attention back to crop rotations with grass and maize. In an experiment with a three year grass and three year maize rotation, Nevens and Reheul (2002) found that maize yield was similar to that in continuous maize cultivation, while the input of 231 kg of mineral N fertilizer ha^{-1} was saved over the three-year period of maize in the rotation. In the same experiment the average feed energy yield of grass in the three-year ley phase was similar to that in permanent grassland (Nevens and Reheul, 2003). Thus, compared to continuous maize cropping and permanent grassland, a crop rotation of grass and maize can be sustainable in terms of efficient nutrient use. Furthermore, ley farming guarantees a high clover content and provides an opportunity to control perennial weeds on organic farms (Younie and Hermansen, 2000). However, farmers lack information on the effect of a ley-arable crop rotation on soil quality, especially about soil biological quality, compared with continuous maize cropping and permanent grassland. What are the consequences for soil biological quality at field and farm level if the 30% of maize now cultivated continuously is cultivated in rotation with grass? Furthermore, what does this mean for the functions of soil biota at field and farm level, in the short and long term?

Permanent grassland and continuous arable cropping represent two types of land use that have distinct effects on biological soil quality. Fromm et al. (1993) showed that the type of cultivation (arable versus pasture) had more influence on soil biota than different soil types. Yeates et al. (1998b) and Lamandé et al. (2003) found earthworms to be more abundant and populations to have greater biomass under long-term pasture than under long-term cropping. Similar trends have been found for collembola, nematodes and microbes (Fromm et al., 1993; Yeates et al., 1998b; Steenwerth et al., 2002).

Various studies have reported changes in the composition of soil biota on sites with an arable cropping history after which a perennial pasture was established or cultivation was abandoned. Yeates et al. (1998b) found that earthworm populations increased when perennial pasture was established on sites formerly under arable cropping. In the case of nematodes, Nombela et al. (1999) detected only a significant difference in the Plant Parasite Index (PPI) during the recovery time after temporary rye cultivation. Buckley and Schmidt (2001) found that the soil microbial community structure of an old field, abandoned seven years after cultivation, retained more similarities to cultivated sites nearby than to fields with a similar plant community which had never been cultivated.

None of the above mentioned studies compared a ley-arable crop rotation as a cropping system with permanent grass or arable land. In the present research project we analyzed the soil biota in a long-term crop rotation experiment established in 1966. Our objectives were (1) to determine the long term effects of a ley-arable crop rotation system on earthworms, nematodes, bacteria and fungi, in comparison with permanent grassland and continuous arable cropping, and (2) to assess the short-term recovery of soil biota in a ley-arable crop rotation. We explored the relevance of changes and/or differences in soil functioning in the short and long term. We hypothesized that three years of grass in a rotation leads to a significant recovery of earthworms, nematodes, bacteria, fungi and mineralization. Furthermore, we hypothesized that, in the long term, the soil biota in a ley-arable crop rotation would reach an intermediate position between permanent grassland and continuous arable cropping.

2.2 Materials and methods

2.2.1 Sampling site and experimental design

In 1966, a crop rotation experiment was established on a sandy loam soil at the experimental farm of Ghent University at Melle (50° 59’N, 03°49’E; 11 m above sea level). The clay (<2 µm), silt (2-20 µm), fine sand (20-200 µm) and coarse sand (200-2000 µm) contents of the soil were 86 g kg $^{-1}$, 116 g kg $^{-1}$, 758 g kg $^{-1}$ and 40 g kg $^{-1}$, respectively (Nevens and Reheul, 2001). Four treatments were established in a complete randomized block design with four blocks. The individual plot size was 750 m^2. The four treatments were:

PG: Permanent grassland since 1966;

TG: Temporary ley-arable crop rotation, started in 1966 with three years of grass ley followed by three years of arable land cropped with forage crops;

TA: Temporary arable crop-ley rotation. This treatment is comparable to TG but started in

1966 with three years of arable cropping followed by three years of grass ley;
PA: Permanent arable cropping since 1966.

The history of the permanent grassland and the temporary grassland is described in detail in Nevens and Reheul (2003), and the permanent and temporary arable cropping systems in Nevens and Reheul (2001). In the seventh rotation of the trial the TG treatment was established on 12 April 2002 after rotavating the maize stubble of the preceding three years' arable cropping. The seed mixture used was 40 kg *Lolium perenne* L. ha^{-1} (cvs. Plenty and Roy) and 4 kg *Trifolium repens* L. ha^{-1} (cv. Huia). In the seventh rotation the TA treatment was established in 2002 after rotavating the former grass ley on 9 April.

In addition to a nitrogen fertilizer (ammonium nitrate 27%), all plots received a basal fertilizer application of triple super phosphate (45% P_2O_5) and potassium chloride (40% K_2O). In the spring of 2003 and 2004 treatments were limed. Herbicides were used in the treatments with maize. No pesticides were used on the grassland plots.

2.2.2 Soil sampling, chemical and physical measurements

In the first three years of the seventh rotation, soil samples were collected for soil chemical, physical and biological parameters on 30 October 2002, 7 October 2003 and 15 October 2004. In two of the four blocks with the PG and TG treatments, subplots of 27 m^2 with a fertilizer application of 100 kg mineral N ha^{-1} were sampled, while in the other two blocks subplots with 300 kg mineral N ha^{-1} were sampled. In the PA and TA treatments, subplots of 45 m^2 with a fertilizer application of 75 kg mineral N ha^{-1} were sampled.

A bulk sample of 70 cores (0-10 cm, ø of 2.3 cm) per plot was collected, sieved through 1 cm mesh, homogenized and stored at field moisture content at 4°C before analysis. Sub-samples were taken and used for chemical analysis, nematode and microbiological analysis.

Prior to chemical analysis, sub-samples were oven-dried at 40 °C. Soil acidity of the oven-dried samples was measured in 1 M KCl (pH-KCl). Total soil N was determined by digestion with H_2SO_4, salicylic acid, H_2O_2 and selenium as described by Novozamsky et al. (1984) and measured by Segmented Flow Analysis (Skalar Breda). Soil organic matter was determined by loss-on-ignition (Ball, 1964).

In 2003 and 2004 soil bulk density was measured in the 5-10 cm layer below the soil surface, in three undisturbed ring samples containing 100 cm^{-3} soil. In 2004, the soil sampling was combined with other measurements on soil structure and physical processes. Penetration resistance was measured with an electronic penetrometer with a cone diameter of 1 cm^2 and a 60° apex angle. Cone resistance was recorded per cm of soil depth and expressed as an average value of 6 penetrations per plot in the soil layers of 0-10 cm, 10-20 cm etc. Soil structure was determined in 1 block (20 cm x 20 cm x 10 cm) per plot. Soil of this block was divided by visual observation into crumbs, sub-angular blocky elements and angular blocky elements. These were weighed and expressed as a percentage of total fresh soil weight. On horizontal surfaces (20 cm x 20 cm) exposed at 10 cm and 20 cm depth, the total number of roots was counted and expressed per m^2.

2.2.3 Soil biological parameters

2.2.3.1 Earthworms

Earthworms were sampled in 2 blocks (20 cm x 20 cm x 20 cm) per plot. The blocks were transferred to the laboratory where the earthworms were hand-sorted, counted, weighed and fixed in alcohol prior to identification. Numbers and biomass were expressed per m^2. Adults were identified according to species. A distinction was made between (1) epigeic species (pigmented, living superficially in the litter layer, little burrowing activity), (2) endogeic species (living in burrows at approximately 10-15 cm depth) and (3) anecic species (relatively large worms, living in vertical burrows from which they collect dead organic matter from the surface at night) (Bouché, 1977). In 2004, before the blocks were sorted, the earthworm burrows with a diameter >2 mm were counted on horizontal surfaces (20 cm x 20 cm) exposed at 10 cm and 20 cm depth. The horizontal surface was the same as used for counting the number of roots.

2.2.3.2 Nematodes

From the bulk soil sample a sub-sample of 100 ml soil was taken, from which the free-living nematodes were extracted, using the Oostenbrink elutriator (Oostenbrink, 1960). Total numbers were counted and expressed per 100 g fresh soil. Nematodes were fixed in hot formaldehyde 4%, and at least 150 randomly selected nematodes from each sample were identified to genus and, whenever possible, to species. Nematode genus and species were assigned to trophic groups following Yeates et al. (1993) and allocated to the colonizer-persister groups (cp-groups) following Bongers (1990) and Bongers et al. (1995). The Nematode Channel Ratio (NCR) was calculated to express the relative contributions of bacterivorous (B) and fungivorous (F) nematodes to the total nematode abundance (NCR=B/(B+F)) (Yeates, 2003). The Maturity Index is calculated as the weighted mean of the individual cp-values, in accordance with Bongers (1990). It is an ecological measure, which indicates the condition of an ecosystem based on nematode species composition.

2.2.3.3 Microbial parameters

Microbiological analyses were performed in 2003 and 2004. To avoid the effects of temperature and moisture fluctuations in the field and to stabilize soil conditions, a subsample of 200 g field moist soil was adjusted to 50% WHC (Water Holding Capacity) and pre-incubated at 12 °C for four weeks (Bloem et al., 2006). After pre-incubation, fungal and bacterial biomass, bacterial growth rate and Community-Level Physiological Profiles (CLPP) were measured. Potential N mineralization was measured without pre-incubation because these methods already include a soil incubation.

For each sample, 20 g of soil and 190 ml of dematerialized water were homogenized in a blender (Waring, New Hartford, Conn.) for 1 min at maximum speed (20,000 rev min^{-1}). A 9 ml sample was fixed by adding 1 ml of 37% formaldehyde. The soil suspension was resuspended and after 2 min of settling 10 µl of the soil suspension was evenly smeared in a circle of 12 mm diameter on a printed glass slide (Cel-line Associates Inc., Vineland, NJ, USA). Slides with soil suspension were air-dried (Bloem and Vos, 2004).

Slides for counting of fungi were stained for 1 hour with Differential Fluorescent Stain (DFS) solution. The stain solution consisted of 3.5 g l^{-1} europium chelate (Kodak cat no. 1305515, Eastman Fine Chemicals, Rochester NY, USA) and 50 mg l^{-1} fluorescent brightener, C40H42N120O10S2 Na2 (FW 960.9, Fluostain I, cat no. F0386, Sigma Chemical Co., St. Louis MD, USA) in 50% ethanol, filtered through a 0.2 µm pore-size membrane. Europium chelate stains DNA and RNA red, FB stains cellulose and polysaccharide (cell walls) blue. After staining the slides were rinsed three times in a bath of 50% ethanol. After air-drying a coverslip was mounted with immersion oil.

Fungi were counted under an epifluorescence microscope at 400x magnification. Blue hyphae are assumed to be inactive or dead, red hyphae are active. Unstained (melanin-forming) hyphae were also counted. Hyphal lengths are estimated by counting the number of intersections of hyphae with the lines of a counting grid. Hyphal length (µm grid^{-1}) was calculated as $H = I\pi A/2L$, where I = number of intersections per grid, A = grid area, L = total length of lines in the counting grid. The total length of fungal hyphae F (m g^{-1} soil) was calculated as $F = H\ 10^{-6}(A/B)(1/S)$, where H = hyphal length, A = area of the slide covered by sample, B= area of the grid and S = amount of soil on the filter. Biovolumes were calculated from length L and width W using the equation $V = (\pi/4)W^2(L-W/3)$. Fungal biomass was calculated assuming a mean hyphal diameter (width) of 2.5 µm and a specific carbon content of $1.3\text{x}10^{-13}$ g C µm^{-3}.

Slides for counting of bacteria were stained for 30 min. with the fluorescent protein dye 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF). This solution consisted of 2 mg DTAF dissolved in 10 ml buffer solution (0.05 M Na_2HPO_4 (7.8 g l^{-1}) and 0.85% NaCl (8.5 g l^{-1}), adjusted to pH 9), filtered through a 0.2 µm pore-size membrane. After staining the slides were rinsed three times with buffer. After air-drying a coverslip was mounted with immersion oil

(Bloem and Vos, 2004). On the stained slides, bacterial numbers and cell volumes were measured automatically with a confocal laser-scanning microscope (Leica TCS SP2) combined with image analysis software (Leica Qwin pro) as described by (Bloem et al., 1995). Bacterial biomass (C) was estimated from the biovolume using a specific carbon content of 3.1 x 10^{-13} g C μm^{-3} (Fry, 1990).

Bacterial growth rate was determined as the incorporation of [^{3}H]thymidine and [^{14}C] leucine into bacterial DNA and proteins (Bloem and Bolhuis, 2006; Michel and Bloem, 1993). [Methyl-^{3}H] Thymidine (925 GBq/mmol) and L-[U-^{14}C]leucine (11.5 GBq/mmol) were purchased from Amersham Ltd., Amersham, U.K.. Per sample (tube) we used 1.5 µl [^{14}C]leucine, 2.0 µl [^{3}H]thymidine and 16.5 µl unlabelled thymidine (2.35 mg/l). This corresponds with 2 µM and 2.78 kBq [^{14}C]leucine and 2 µM and 74 kBq [^{3}H]thymidine per tube. 20 g soil and 95 ml Prescott and James's mineral salt solution (P&J medium, Prescott and James, 1955) were shaken by hand in a bottle for 30 sec. 100 µl of soil suspension was added to 20 µl labelled thymidine and leucine in a 13 ml polypropylene centrifuge tube with screw cap. After 1 h incubation the incorporation was stopped by adding 5 ml of 0.3 N NaOH, 25 mM EDTA and 0.1% SDS. Blanks were prepared by adding the extraction mixture immediately after the start of the incubation. Macromolecules (DNA and proteins) were extracted at 30°C for 18-20 h (overnight). The suspension was mixed and centrifuged for 40 min at 5000 x g at 25°C in an MSE High Speed 18 centrifuge. The supernatant was aspired in a 13 ml tube and cooled on ice. After 5 min 1.3 ml ice-cold 1 N HCl and 1.3 ml ice-cold 29% TCA (w/v) were added. The suspension was cooled further for at least 15 min. The precipitated macromolecules (DNA and proteins) were collected on a 0.2 µm pore size cellulose nitrate filter (BA 83, Schleicher & Schuell). The filters were washed 3 times with 5 ml ice-cold 5% TCA. The filters were transferred to glass scintillation vials and 1 ml 0.1 N NaOH and 1 ml ethylacetate were added to dissolve macromolecules and filters. Fifteen ml Ready Safe scintillation cocktail (Beckman Instruments, Fullerton, Calif., U.S.A.) was added and radioactivity was counted in an LKB Wallac 1215 liquid scintillation counter (LKB Instruments, Turku, Finland). Blanks were subtracted and the counted dpm were multiplied by 0.0028378 to calculate pmol thymidine incorporated per gram soil per hour, and by 0.07587 to calculate pmol leucine incorporated per gram soil per hour.

The CLPPs of the bacterial communities in the soil extracts were determined with ECO-plates from BIOLOG Inc. (Hayward, USA). These plates contain a triplicate set of 31 different carbon substrates, a control, a freeze-dried mineral medium and a tetrazolium redox dye. 25 g of fresh soil, based on its dry weight, was mixed with 250 ml buffer (10 mM BisTris, pH 7), blended for 1 min at maximum speed and then centrifuged for 10 min at 500 x g. The homogeneous supernatant containing extracted bacteria was used for further analysis of CLPPs (Boivin et al., 2006). For each bacterial extract, a dilution series was made using 10 mM BisTris buffer at pH 7. Each dilution series (3^{-1} until 3^{-12}) was used to inoculate four ECO-plates with a volume of 100 µl per well. The colour formation in the plate was measured every 8 hours for 7 days with a plate reader spectrophotometer at 590 nm. The CLPPs were calculated from the colour formation in the wells, and corrected for inoculum density using a regression approach applied to the average well colour development (AWCD) as described by Rutgers et al. (2006). This produced CLPPs describing the relative abundance for substrate conversion (31 substrates; log scaled). To survey the bacterial community activity in the ECO-plate, the AWCD was calculated after 7 days of incubation.

Potential N mineralization was determined by incubating 200 g homogenized and sieved (< 2 mm) soil in 1.5 l airtight jars at 20°C and 50% WHC in the dark for six weeks (Bloem et al., 1994). Results of the first week were not used to avoid effects of soil homogenization. The increase in mineral N between week 1 and week 6 was used to calculate N mineralization rates. Sub-samples of 80 g soil were extracted with 200 ml of 1 M KCl. After 1 h shaking the extracts were filtered over a paper filter. Mineral N contents (ammonium and nitrate) were determined by Skalar Segmented Flow Analysis (Breda, The Netherlands). Potentially mineralizable C (potentially mineralizable C) was measured by incubation for seven days at 20°C and 50% WHC. During this period CO_2 was absorbed in alkali (1N KOH) followed by titration with 0.1 N HCl (Pell et al., 2006).

2.2.4 Statistical analyses

The data were analyzed with GENSTAT (8th Edition, VSN International, Hemel Hempstaed, UK) using a two-way ANOVA in randomized blocks with treatment (PG, TG, TA and PA), and year of sampling as factors. Where a relationship could be anticipated between parameters, a regression analysis was carried out on a model, in which year and treatment were taken into account where relevant. Data of nematode taxa and CLPPs were squareroot transformed and subjected to a redundancy analysis (RDA) (CANOCO 4.5, Biometris, Wageningen, The Netherlands). In the biplots the ‘species’ (nematode taxa and bacterial CLPPs) are represented by vectors and the treatments (crop, year) by centroids. The length and the slope of the vectors and the position of the centroids indicate the strength of the correlation with the ordination axes and with other variables. Perpendicular projection of a treatment centroid on a ‘species’ vector indicates the relative abundance of the species in that treatment, with an average abundance at the origin, a higher than average abundance in the direction of the arrow, and a less than average abundance in the opposite direction. A Monte Carlo permutation test (499 random permutations) was performed to test for statistically significant ($P=0.05$) relationships between community structure and environmental variables following a multivariate analysis of variance design (Van Dobben et al 1999).

2.3 Results

2.3.1 Soil chemical and physical parameters

The soil organic matter (SOM) in the permanent grassland (PG) plots was almost three times higher than in the permanent arable (PA) plots (Table 2.1). The crop rotation treatments (TG and TA) had intermediate SOM. The year effect and interaction with treatments was mainly caused by a significant increase of SOM in the PG from 2002 to 2003 and a decrease from 2003 to 2004. The same applies to soil total N. The variation in total N was explained by a regression model (R^2=0.94) with significant effects of treatment ($P<0.001$), year ($P<0.001$) and SOM ($P=0.004$). The pH-KCl was highest in the PA plots and lowest in the TG plots.

Table 2.1 Soil chemical characteristics, physical characteristics and number of roots of permanent grassland (PG), temporary grassland (TG), temporary arable land (TA) and permanent arable land (PA).

Chemical/ Physical	Units	Treatments					Year	Treat.*year
		PG	TG	TA	PA	P-value	P-value	P-value
SOM*	g kg dry soil^{-1}	60.7 a	33.2 b	34.9 b	21.1 c	<0.001	0.004	0.003
Total N*	g N kg dry soil^{-1}	2.95 a	1.52 b	1.61 b	0.95 c	<0.001	<0.001	NS
pH-KCl*		5.69 c	5.42 c	5.83 b	6.04 a	<0.001	<0.001	0.002
Bulk density**	g cm^{-3}	1.14 c	1.41 a	1.29 b	1.40 a	<0.001	<0.001	NS
Soil structure 0-10 cm***								
Crumb	%	33 a	32 a	8 b	8 b	<0.001	--	--
Sub-angular	%	43 a	32 a	11 b	12 b	<0.001	--	--
Angular	%	24 b	35 b	81 a	79 a	<0.001	--	--
Roots***								
10 cm depth	n m^{-2}	1888 b	3344 a	906 c	575 c	<0.001	--	--
20 cm depth	n m^{-2}	1081 b	1813 a	1269 b	963 b	<0.008	--	--

* Means of 2002, 2003 and 2004,

** Means of 2003 and 2004,

*** Measured in 2004 only.

Values followed by the same letter (a-c) within a row are not statistically different at the 5% error level for the main treatment effect.

PG showed the lowest bulk density while TG and PA showed the highest bulk density. TA bulk density was intermediate (Table 2.1). When SOM was included in a regression model with treatment, SOM did not significantly ($P=0.160$) explain the variance in bulk density.

Although the penetration resistance in soil layers below 10 cm was always lowest in the grass treatments (TG and PG), differences were not statistically significant. Average penetration resistance was 1.56 MPa in 0-10 cm, 2.70 MPa in 10-20 cm, 3.42 MPa in 20-30 cm, 3.54 MPa in 30-40 cm and 3.17 MPa in 40-50 cm. The grass treatments had more crumb and sub-angular blocky elements than the arable treatments. Numbers of roots at 10 cm and 20 cm were significantly higher in the TG treatment.

2.3.2 Soil biological parameters

2.3.2.1 Earthworms

The number of earthworms was highest in the PG treatment followed by the TG treatment (Table 2.2). On arable land (TA and PA) the number of earthworms was 12-24% of the PG. The significant interaction of treatment and year is mainly due to the recovery in the number of earthworms in the TG treatment (Fig. 2.1). In October 2003, the second year after the establishment of grass (TG) in the arable-ley crop rotation, the number of earthworms reached the same level as in the PG treatment.

The body biomass of the earthworms in the TG was significantly lower ($P<0.001$) than in the PG, and therefore the recovery of the total biomass was not as spectacular as the total numbers. Even in October 2004, the final year of the three-year period of grass ley, the earthworm biomass in the TG was different from the PG: 96 g m^{-2} compared to 163 g m^{-2} ($P=0.002$). In total numbers and biomass the TA plots resembled the PA plots. Numbers and especially biomass in the TA treatment had already been at a low level in the first year, suggesting a rapid decrease in earthworms after rotavating the grass ley.

Species of earthworms found in the trial were *Lumbricus rubellus*, *Aporrectodea caliginosa*, *Allolophora chlorotica*, *Aporrectodea rosea* and *Aporrectodea longa*. PG had the highest number of species and the arable treatments the lowest number. The interaction between treatment and year was mainly due to an increase of the number of species in the TG treatment from 2002 to 2003. As with the total numbers, the number of species in the TG almost recovered within two years in comparison with the PG treatment. Among the adult earthworms in the PG plots, the anecic species were dominant (52 % anecic species). In the TG and the arable treatments (TA and PA), the endogeic species were most common: 62 %, 88 % and 100 %, respectively. The epigeic species were mainly found in the grass treatments.

The number of earthworm burrows showed a clear decrease in the order

PG>TG>TA>PA (Table 2.2). The variation in the number of burrows at 10 cm depth, measured in October 2004, was explained by a regression model (R^2=0.93) with treatment and earthworm biomass measured in 2004 as fitted terms (Fig. 2.2). There was a significant positive relation between the biomass and the number of burrows for all treatments (slope=0.058, P=0.044). In the model all four individual treatment levels differed significantly (P<0.001).

Table 2.2 Earthworm numbers, biomass, species, functional groups and earthworm burrows in permanent grassland (PG), temporary grassland (TG), temporary arable land (TA) and permanent arable land (PA): averages from three consecutive years (2002-2004).

Earthworms	Units	Treatments					Year	Treat.* year
		PG	TG	TA	PA	P-value	P-value	P-value
Total number	n m^{-2}	256 a	187 b	62 c	30 c	<0.001	NS	0.008
Body biomass	g worm^{-1}	0.65 a	0.25 b	0.23 b	0.12 b	<0.001	0.033	NS
Total biomass	g m^{-2}	166 a	52 b	14 bc	5 c	<0.001	NS	NS
Number of species	n 20x20x-20cm^{-1}	2.0 a	1.3 b	0.5 c	0.2 c	<0.001	NS	<0.001
Epigeic adults	n m^{-2}	20 a	25 a	1 b	0 b	0.016	0.011	NS
Endogeic adults	n m^{-2}	46 ab	49 a	22 bc	7 cd	0.009	NS	0.031
Anecic adults	n m^{-2}	71 a	4 b	2 b	0 c	<0.001	NS	NS
Earthworm burrows*								
10 cm depth	n m^{-2}	388 a	238 b	106 c	6 d	<0.001	--	--
20 cm depth	n m^{-2}	356 a	206 b	100 c	6 d	<0.001	--	--

* Earthworm burrows were counted in 2004 only.

Values followed by the same letter (a-d) within a row are not statistically different at the 5% error level for the main treatment effect.

2.3.2.2 Nematodes

The abundance of nematodes in the grassland treatments (PG and TG) and arable treatments (TA and PA) was significantly different over the years (Table 2.3). The year 2003 showed significantly higher total numbers of nematodes than the years 2002 and 2004. This was mainly caused by higher numbers of nematodes in the PG and TG treatments (Fig. 2.3). Over the years, nematode numbers were stable in the PA treatment. The interaction between treatment and year resulted from a significant increase in the total number of nematodes in the

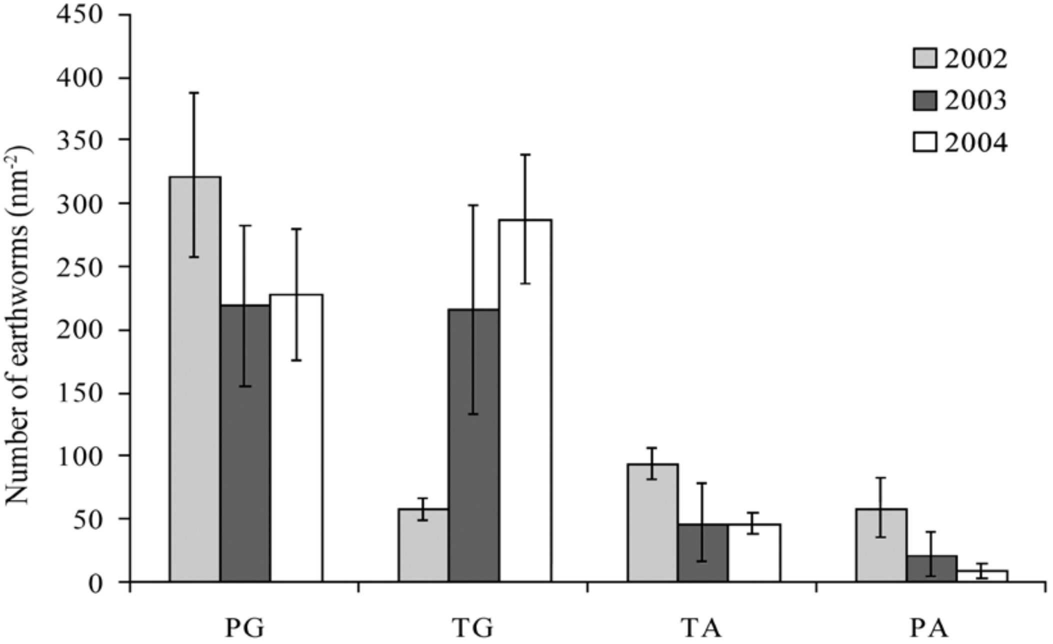

Fig. 2.1 Number of earthworms (n m^{-2}) in permanent grassland (PG), temporary grassland (TG), temporary arable land (TA) and permanent arable land (PA): mean values ± S.E .are shown.

TG plots and a significant decrease in the TA plots. In 2002, the first year of the 7th rotation in the TG and TA treatments, the number of nematodes was not significantly different between the two treatments: 5007 and 5248 nematodes per 100 g soil^{-1}, respectively. This could suggest a rapid increase in the number of nematodes after the establishment of the grass and a slow decrease after rotavating the grass. However, changes in trophic groups had already taken place (Fig. 2.3).

The two grassland treatments (PG and TG) were dominated by herbivorous nematodes. TG showed the highest proportion of herbivores (Table 2.3) and the highest number of roots in the 0-10 cm soil layer (Table 2.1). The arable treatments (PA and TA) had the lowest abundance and proportion of herbivorous nematodes and the lowest number of roots in the 0-10 cm soil layer. The two arable treatments were relatively dominated by bacterivorous nematodes (Table 2.3). The year effect and interaction for the proportion of bacterivorous and herbivorous nematodes was mainly due to a relative decrease in bacterivores and an

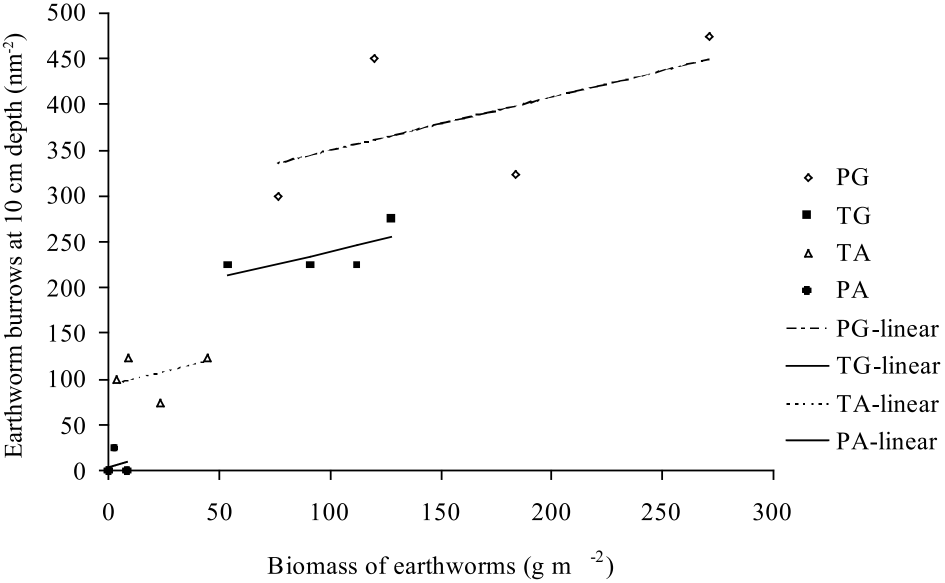

Fig. 2.2 Relation between earthworm biomass (g m^{-2}) and earthworm burrows at 10 cm depth (n m^{-2}) in 2004 for the four treatments ($R^2=0.93$). Earthworm burrows (n m^{-2} at 10 cm depth) = treatment ($P<0.001$) (intercept 5 for PA, 95 for TA, 182 for TG, 293 for PG) + 0.58 * earthworm biomass (g m^{-2}) ($P=0.044$). Permanent grassland (PG), temporary grassland (TG), temporary arable land (TA), permanent arable land (PA).

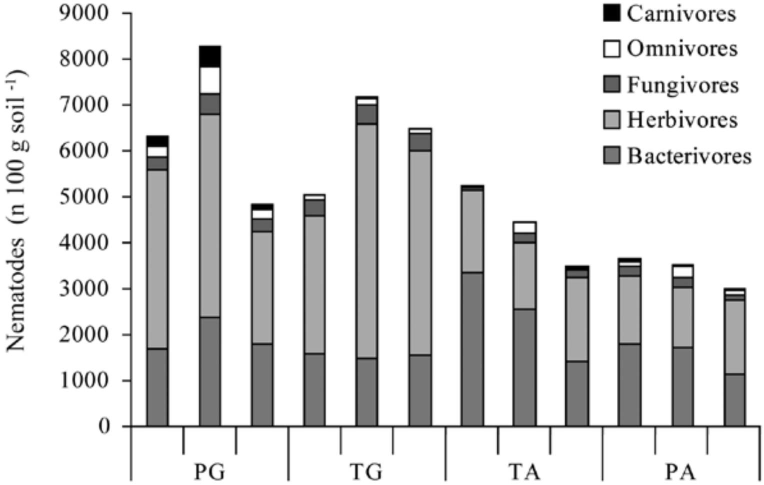

Fig. 2.3 Nematode abundance (n 100 g $soil^{-1}$) divided over the different feeding groups in permanent grassland (PG), temporary grassland (TG), temporary arable land (TA) and permanent arable land (PA).

increase in herbivores in 2004 in the TA and PA treatments. Moreover, the relative abundance of herbivorous nematodes increased in the years 2003 and 2004 in the TG treatment (Fig. 2.3). The relative abundance of the fungivorous nematodes did not show statistically significant differences between the treatments (Table 2.3). However, their absolute numbers were significantly ($P<0.001$) higher in the grassland plots than in the arable plots. The Nematode Channel

Table 2.3 Nematode abundance, throphic groups, life history groups (cp=colonizer-persister groups) and community structure indices in permanent grassland (PG), temporary grassland (TG), temporary arable land (TA) and permanent arable land (PA): averages from three consecutive years (2002-2004).

Nematodes	Units	Treatments					Year	Treat.*year
		PG	TG	TA	PA	P-value	P-value	P-value
Total number	n 100g soil^{-1}	6463 a	6222 a	4400 b	3389 b	<0.001	0.002	0.004
Bacterivorous	n 100g soil^{-1}	1971 b	1548 b	2445 a	1571 b	<0.001	0.003	0.008
Herbivorous	n 100g soil^{-1}	3577 a	4186 a	1691 b	1455 b	<0.001	NS	0.003
Fungivorous	n 100g soil^{-1}	336 a	378 a	140 b	176 b	<0.001	NS	NS
Omnivorous	n 100g soil^{-1}	326 a	111 b	110 b	143 b	<0.001	<0.001	0.007
Carnivorous	n 100g soil^{-1}	265	13	14	44	NS	NS	NS
Bacterivorous	%	31.2 c	25.6 c	54.2 a	46.1 b	<0.001	0.014	0.002
Herbivorous	%	55.2 b	66.4 a	39.7 c	43.0 c	<0.001	0.015	0.004
Fungivorous	%	5.3	6.1	3.2	5.3	NS	NS	NS
Omnivorous	%	4.9 a	2.0 b	2.5 b	4.2 a	0.001	0.001	NS
Carnivorous	%	3.6 a	0.2 b	0.4 b	1.3ab	0.032	NS	NS
Cp-1	%	13.8 b	18.2 b	38.5 a	15.3 b	<0.001	<0.001	NS
Cp-2	%	61.6 bc	67.8 ab	54.9 c	72.3 a	0.002	<0.001	NS
Cp-3	%	3.4 b	6.3 a	0.9 b	2.6 b	0.003	NS	0.020
Cp-4	%	10.7 a	5.6 b	4.4 b	6.8 b	0.010	0.049	NS
Cp-5	%	10.7 a	2.2 b	1.5 b	3.2 b	0.010	NS	NS
Number of genera		21.6	19.4	18.4	20.3	NS	<0.001	NS
Maturity Index(cp1-5)		2.43 a	2.06 b	1.75 c	2.11 b	<0.001	0.002	NS
Nem. Channel Ratio		0.84 b	0.80 b	0.94 a	0.90 a	<0.001	NS	NS

Values followed by the same letter (a-c) within a row are not statistically different at the 5% error level for the main treatment effect.

Ratio (NCR) suggests that the two arable treatments were dominated to a larger extent by bacterial-based energy channels of decomposition than the two grassland treatments. The two permanent treatments (PG and PA) showed higher proportions of omnivorous and carnivorous nematodes than the temporary treatments (TG and TA).

The life-strategy group distribution showed a high percentage of cp-1 (enrichment opportunists) in TA. This is mainly due to the dominance of the typical colonizer family Rhabditidae in this treatment in the first year after rotavating the three-year temporary grassland. In the second year, species of the bacterivorous Rhabditidae were replaced by the genera *Eucephalobus* and *Acrobeloides* of the Cephalobidae family, which are classified as cp-2. The relatively high percentages of omnivorous and carnivorous nematodes in the permanent treatments (PG and PA) resulted in relatively high percentages of cp-4 and cp-5 groups. Consequently, the Maturity Index (MI) was highest in PG, followed by PA and TG, and lowest in TA. The MI increased in TA and TG with time. However, the MI of TA in 2004 remained statistically significantly lower than the MI of PA, whereas the MI of TG in 2004 was not statistically significantly different from the MI of PA.

In a simultaneous multivariate analysis (RDA) of all nematode taxa, statistically significant effects were found for the variables treatment ($P<0.001$), year ($P<.001$) and the interaction year x treatment ($P<0.001$). In the ordination plot (Fig. 2.4) the horizontal axis separates the grassland (right) and the arable (left) treatments, the vertical axis shows the time effect. This again shows that the high numbers of Rhabditidae distinguished the TA treatments in 2002 from the other treatments. In 2002, the first year after rotavation, the TG treatment had an intermediate position between arable and grassland, but from 2003 onwards the temporary treatments resembled their permanent counterparts in terms of nematode taxa. The carnivorous nematodes were not discriminating in the results of this RDA analysis.

2.3.2.3 Microbial parameters

Bacterial biomass was 52% higher in the PG than in the PA treatment (Table 2.4). In contrast, the bacterial growth rate (viz. thymidine incorporation or DNA synthesis) was highest in PA and 43% lower in PG. The bacterial growth rate in the TA treatment did not differ from PA, and the same was found for TG compared to PG. Leucine incorporation (i.e. bacterial protein synthesis) showed similar trends.

Fungal biomass, averaged over the years, also tended to be higher in grassland, but the differences were small (Table 2.4). The difference was caused by unusual results in 2003 when fungal biomass in the arable fields was as great as or even greater (PA) than in the permanent grassland (PG). The reason is not clear. The difference could not be related to wet or dry conditions. In 2004, a more common result was found with 4 times less fungal biomass in PA than in PG, and a clear decrease in the order PG>TG>TA> PA. In October 2004, after three years of grass on TG, fungal biomass was significantly higher than after three years of arable farming on TA (data not shown). Fungal activity (percentage of active hyphae) did not show a consistent pattern.

The CLPP-slope parameter (Table 2.4) was calculated from the colour development in the ECO-plates. This parameter indicates the rate at which the capacity of the soil to de-

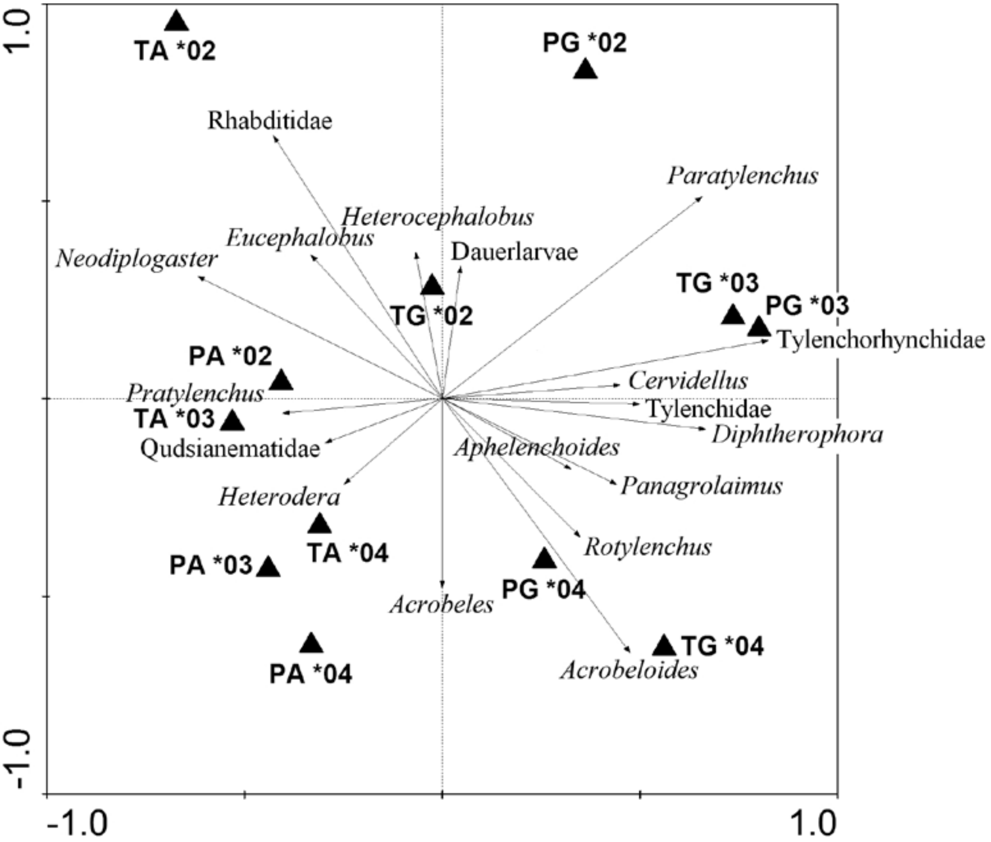

Fig. 2.4 Ordination diagram of the nematode taxa based on RDA with treatment (PG, TG, PA, TA) and year (2002, 2003, 2004) accounting for 38% of the variance in the abundances and 68% of the variance in the fitted abundances. Only taxa are shown of which ≥10% of the variance is accounted for. Permanent grassland (PG), temporary grassland (TG), temporary arable land (TA), permanent arable land (PA).

grade a set of carbon and energy substrates disappears upon dilution. A low slope parameter is indicative of a slow disappearance rate and can be considered as a measure of high physiological diversity (Gomez et al., 2004; Rutgers et al., 2006). The PG treatment had the lowest, and the arable treatments the highest slopes (Table 2.4). This result suggests that the PG treatment had a higher physiological diversity than all other treatments, and that the TG treatment had a slightly higher physiological diversity than the continuous arable treatment (PA). In addition, the capacity to degrade a set of different substrates was greatest in the PG and the TG treatments (Table 2.4), i.e. < 500 µg of dry weight (DW) soil was required for 50% conversion of the different substrates, whereas the arable treatments needed >2000 µg of DW soil.

In a multivariate analysis (RDA) of all CLPPs simultaneously, significant effects were found for treatment ($P<0.001$), year ($P<0.001$) and the interaction treatment x year ($P=0.003$). In the ordination plot (Fig. 2.5), the CLPPs of PG are clearly separated from the other treatments in both years and appeared to be different for both years ($P<0.002$). The CLPPs of TG seemed to be slightly separated from both arable treatments in the multivariate space,

Table 2.4 Microbial biomass, activity, Community-Level Physiological Profiles (CLPP), potential N-mineralization and potentially mineralizable C in permanent grassland (PG), temporary grassland (TG), temporary arable land (TA) and permanent arable land (PA): averages from two consecutive years (2003-2004).

Microbial	Units	Treatment					Year	Treat. *year
		PG	TG	TA	PA	P-value	P-value	P-value
Bact. biomass	μg C g dry soil^{-1}	105 a	78 b	82 b	69 b	<0.001	NS	NS
Thym. incorp.	pmol g dry soil^{-1} h^{-1}	23 b	26 b	36 a	40 a	<0.001	<0.001	NS
Leuc. incorp.	pmol g dry soil^{-1} h^{-1}	274	336	348	368	NS	<0.001	NS
Fung. biomass	μg C g dry soil^{-1}	29	23	18	23	NS	<0.001	0.021
Fung. activity	% of hyphal length	7.7	11.0	3.6	15.1	NS	0.004	0.046
CLPP								
slope		0.26 c	0.50 b	0.53 ab	0.63 a	<0.001	NS	NS
ES50*	µg dry soil	286 b	467 b	2391 a	2811 a	<0.001	NS	NS
Pot.N-mineral.	mg N kg dry soil^{-1}wk^{-1}	6.8 a	4.0 b	3.2 c	2.3 d	<0.001	<0.001	NS
Potentially mineralizable C	mg CO_2 kg dry soil^{-1} wk^{-1}	1356 a	844 b	639 c	445 d	<0.001	0.008	NS

* ES50=Effective soil needed for 50% substrate utilization.

Values followed by the same letter (a-d) within a row are not statistically different at the 5% error level for the main treatment effect.

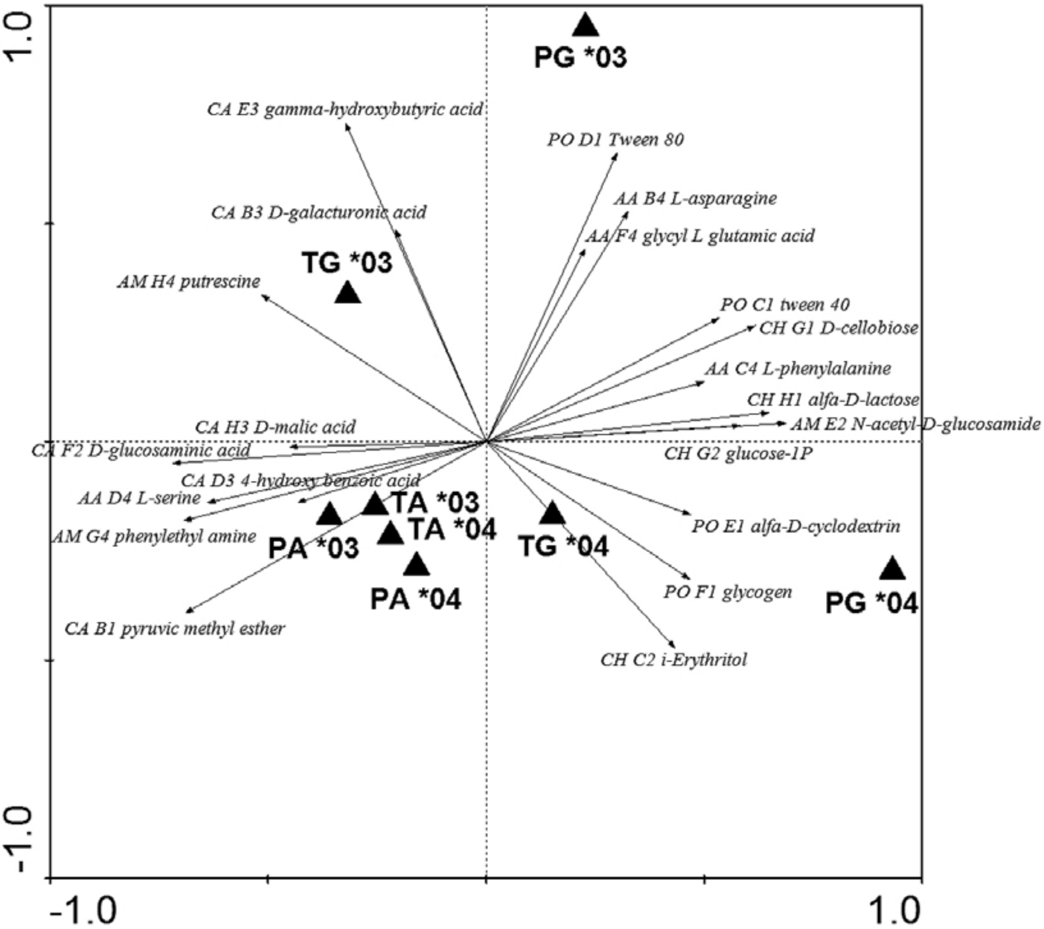

Fig. 2.5 Ordination diagram of CLPP's of bacterial communities based on RDA with treatment (PG, TG, PA, TA) and year (2003, 2004) accounting for 30% of the variance in the abundances and 72% of the variance in the fitted abundances. Only wells are shown of which ≥20% of the variance is accounted for by the diagram (20 out of the total 31 wells). Permanent grassland (PG), temporary grassland (TG), temporary arable land (TA), permanent arable land (PA).

CH=carbohydrate, PO=polymer, AA=amino acid, CA=carboxylic acid, AM=amine but proved only significantly different for the 2003 data (P = 0.010). Differences in CLPPs originate from differences in capability of the bacterial communities to degrade a suite of carbon and energy substrates. The PG treatment showed a predominant ability to decompose a distinct set of substrates in the ECO-plates: mainly the carbohydrates, amino acids and polymers, whereas the other treatments (TG, PA, TA) showed a predominant ability to decompose carboxylic acids and amines.

Potential N-mineralization and potentially mineralizable C were three times higher in PG than in PA, and decreased significantly in the order PG>TG>TA>PA. Both parameters were higher in 2003 than in 2004, which coincided with the higher SOM and N-total in that year. The variation in potentially mineralizable C was explained by a regression model ($R^2=0.91$) with significant effects of treatment ($P<0.001$), year ($P=0.012$) and SOM ($P=0.048$). In this model, the intercepts for PA and TA were 0 and -2.3, respectively, and for PG and TG 22.3 and 18.6, respectively. This model suggests that the potentially mineralizable C of the grassland treatments is higher than that of the arable treatments with the same SOM. Apparently, the SOM of the grassland treatments is of a different quality to that of the arable treatments. In the case of both N-mineralization and potentially mineralizable C, no significant relationship was found with N-total when treatment and year were taken into account.

2.4 Discussion

2.4.1 Earthworms

It is well established that grassland usually contains more earthworms than arable land (Edwards and Bohlen, 1996). In this experiment, the number of earthworms in the PA treatment was as low as 12% of the number in the PG treatment. Low (1972) reported that after 25 years of regular cultivation, the numbers of earthworms were only 11-16% of those in old grassland. Edwards and Bohlen (1996) mention two reasons for a decreased number of earthworms, besides the mechanical damage and predation after cultivation, the loss of an insulating layer of vegetation and a decreased food supply. The small number of earthworms in the TA treatment, six months after rotavating the grass ley, suggests that the decrease in earthworm numbers in our experiment was rapid. Growing grass over several years favours the growth of earthworm populations and the best way of maintaining a large earthworm population in agricultural land is to include ley farming (Edwards and Bohlen, 1996). In fact, in the ley phase of this experiment, earthworm biomass increased from 8 g m^{-2} in the first year to 51 g m^{-2} in the second year and to 96 g m^{-2} in the third year. This is a biomass increase of 40-

45 g m^{-2} per year. If the grass ley were to last for 4 to 5 years, similar biomass levels to those in PG could be reached. It has to be noted that due to the plot size, migration of earthworms from other plots or the paths with permanent grassland could have positively affected the results in the TG. However, taking into account the development of the body mass in TG compared to PG, and the dominance of endogeic species in TG versus anecic species in PG, migration from outside our plots did not have a major effect. Another factor which could have played a role in the recovery of the earthworms in the TG treatment was the high white clover content in the TG (28% of the dry matter production over the years 2002-2004, compared to 5% of the dry matter production in the PG (unpublished results). Van Eekeren et al. (2005a) have shown that a typical ryegrass clover sward sustains twice as much earthworm biomass than pure ryegrass swards. The effectiveness of using a grass-clover mixture for restoring the earthworm biomass is confirmed by Yeates et al. (1998b). In a comparison with a continuous cropping treatment, they measured an earthworm biomass increase of only 13 g m^{-2} biomass after five years perennial ryegrass, whereas there was a 113 g m^{-2} increase in a mixture of perennial rye and clover.

It can be concluded that during the three-year grass ley, the abundance and the number of earthworm species can be restored to the level of the PG. However, to restore the earthworm biomass in the crop rotation to the level of the PG treatment, the ley period should be extended. Clover in the grass mixture could help to restore the earthworm biomass. A more lasting difference between the PG treatment and the remaining ley-arable crop rotation treatments, is the dominance of the anecic species in the PG.

2.4.2 Nematodes

Nematode abundance was greater in the grassland treatments than in the arable treatments. The Dutch Soil Quality Monitoring Network also found higher numbers of nematodes in pastures than in arable land (Schouten et al., 2004). However, various authors (Freckman and Ettema, 1993; Juma and Mishra, 1988; Sohlenius and Sandor, 1989) showed greater abundance in annual than perennial treatments. Part of this difference could be explained by the sampling date, which fell in October in a ripened maize crop. Boström and Sohlenius (1986) and Juma and Mishra (1988) found a sharper decline in nematode abundance after the harvest of a grain crop than after a perennial crop. In Boström and Sohlenius (1986) the number of herbivorous nematodes followed the development of the root system. The significantly lower number of roots to a depth of 10 cm in the arable compared to the grassland treatments, combined with less active roots in the ripened maize crop, probably did not provide enough food to sustain high numbers of herbivorous nematodes, and may have influenced the total abundance. In fact, the difference in nematode abundance between the grassland and arable treatments was mainly explained by the difference in abundance of herbivorous nematodes.

Both in percentage and in absolute numbers, the grassland treatments were dominated by herbivorous nematodes. Among the PG and TG treatments, the percentage of plant-feeding nematodes was significantly higher in TG, which was the treatment with the highest number of roots in the 0-10 cm soil layer. Bouwman and Arts (2000) also found a higher number and percentage of herbivorous nematodes in a treatment with a higher grass root density in the upper soil layers.

The nematode community in the arable treatments (TA and PA) was dominated by bacterivorous nematodes. The dominance of bacterivorous nematodes under arable land and herbivores under a perennial crop was also found by Juma and Mishra (1988). In absolute numbers, however, the TA treatment had the highest number of bacterivorous nematodes in the first two years, followed by the PG treatment. In the TA treatment this was caused by the large input of organic matter and the destruction of roots after rotavating the three-year-old grass ley. In the first year, the bacterivorous nematodes in this treatment were mainly Rhabidtitidae, which generally increase following a resource pulse (De Goede et al., 1993; Ettema and Bongers, 1993; Yeates, 2003).

The herbivorous and bacterivorous nematodes in the temporary treatments (TG and TA) recovered to the levels in the permanent treatments (PG and PA) within one to two years. The permanent systems (PG and PA) distinguished themselves from the temporary systems (TG and TA) with a higher percentage of carnivorous and omnivorous nematodes. Comparing annual cropping systems with pastures, Wasilewska (1979) found omnivorous and carnivorous nematodes to be the most sensitive trophic groups with respect to tillage. However, in our experiment the continuous cropping with tillage apparently offered a more stable environment than the crop rotation of three-year arable land with three-year grass ley. Yeates et al. (1998b), on two soil types, also found the highest number of carnivorous nematodes (mononchids) in soil with a cropping history of either permanent pasture or continuous cropping.

The higher percentage of carnivorous and omnivorous nematodes was reflected in the maturity of the system (Bongers, 1990). The Maturity Index (MI) was highest for the PG and lowest for the TA. The ley-crop rotation only reached the level of maturity of the PA treatment in the third year of the grass leys in the TG. In an investigation into the long-term dynamics of nematode populations, Sohlenius et al. (1987) found a rather stable faunal structure in fields that were continuously cropped with barley. Concerning the difference in maturity between the PG and TG treatment, Wasilewska (1994) found that nematode taxa known to be r-strategists or colonizers dominated in younger meadows while K-strategists or persisters dominated in older meadows. Villenave et al. (2001) showed in their study that the nematode community after 11 years of fallow was still different from that after 21 years of fallow. This clearly shows that it takes more than three years before the nematode community in the TG has completely restored to the level of PG. In the present experiment the levels of abundance of herbivorous and microbivorous nematodes in the temporary treatments resembled those of their permanent counterparts within one to two years. While the same holds true for the genera composition, the MI and the proportion of omnivorous nematodes of the temporary treatments remained significantly different to (i.e. lower than) their permanent counterparts.

2.4.3 Microbial parameters

Bacterial biomass was 50% higher in PG than in PA. In the Dutch Soil Quality Monitoring Network bacterial biomass was also 50-100% higher in grassland than in arable land (Bloem et al., 2006). Fromm et al. (1993) found a strong correlation between soil carbon content and microbial biomass. Wardle (2002) showed that the ratio of microbial biomass carbon to soil carbon content was different for temperate grassland and temperate arable land. In our experiment the SOM did not significantly explain the variation in bacterial biomass in a regression model in combination with treatment and year. In contrast to the higher biomass in grassland soil, the bacterial growth rate (thymidine incorporation) was lower in grassland than in arable land. At the Wildekamp field in Bennekom, The Netherlands (described by Garbeva et al., 2006), similar results were found in soil from grassland and arable land, stored for six months at 12 °C (Bloem et al., 2006). In the grassland soil the bacterial biomass remained stable and the growth rate was reduced to almost zero, whereas in the arable soil the bacterial biomass was reduced to almost zero, but the growth rate was stable. The authors concluded that the slow-growing grassland bacteria survived better than the fast-growing arable soil bacteria, and suggested that this may be related to different energetic strategies. Grassland may select for K-strategists (slow-growing bacteria) because it is a more stable environment with a relatively constant food supply from grass roots, whereas arable soils may favor r-strategists (fast-growing colonizers), because substrate inputs are highly seasonal. In the same soils Garbeva et al. (2006) found a higher percentage of K-strategists in the culturable bacterial community from grassland soil, whereas arable soil had a higher percentage of r-strategists. Both cultivation-based and cultivation-independent techniques indicated a higher bacterial diversity in permanent grassland. Grayston et al. (2004) found different microbial community structure in grasslands with different management intensities.

Since the bacterial growth in the temporary treatments (TG and TA) in the second year of the crop rotation already resembled the bacterial growth of the permanent counterparts (PG and PA), our results suggest that selection for “grassland soil bacteria” and “arable soil bacteria” is a fairly rapid process. This is confirmed by the capacity of the grassland and arable soil to degrade 50% of the substrates in ECO-plates (Table 2.4, ES50).

The Community-Level Physiological Profiles (CLPPs) and the slope parameter in samples of the PG treatment suggest a potentially active bacterial community, with a predominant decomposition of carbohydrates, amino acids and polymers. The other treatments showed different CLPPs and slope parameters, indicating lower potential activity, and a predominant decomposition of carboxylic acids and amines. Grayston et al. (2004) observed a predominance of decomposition of some sugars and neutral amino acids in improved grasslands relative to unimproved grasslands and hypothesized an effect of the vegetation on microbial community composition. In our study the predominance of degradation of some sugars and amino acids in grasslands is striking, and corresponded with these observations. The TG treatment showed a slightly different CLPP than the arable treatments, but they still had many characteristics in common. Apparently, three years of grass ley did not restore the CLPP profiles to the level of PG. Similarly, Steenwerth et al. (2002) found distinct differences in PLFA profiles between old permanent pastures and profiles of fallow grasslands. They suggested that the soil environment and the associated microbial community may take decades to recover from cultivation effects. Hatch et al. (2002) showed that the CLPP profiles of a ley already resembled those of arable land in the first year after cultivation.

From our experiment it can be concluded that in the temporary treatments (TG and TA) the bacterial growth rate and the capacity to degrade substrates already resembled those of the permanent counterparts (PG and PA) in the second year of the experiment. However, the CLPP profiles and the slope parameter from the ECO-plates showed that the physiological diversity in the permanent grassland remained different from the other treatments.

2.4.4 Functional aspects in the short and long term

Earthworms play a role in the supply of nutrients (e.g., fragmentation and transportation of plant residues and SOM, enhancing microbial activity), soil structure improvement (e.g., creation of biopores, producing excrements, mixing organic and mineral particles) and water infiltration. Examples of effects of earthworms are provided by field experiments in which earthworms were introduced or removed. When Hoogerkamp et al. (1983) introduced earthworms in recently reclaimed polders they found an increase in grass production of 9.7%, as well as an improved physical soil fertility. Clements et al. (1991) found that bulk density increased and the initial infiltration rate decreased after removal of earthworms. In our experiment no relationship could be found between bulk density, soil structure, and earthworms. However, a clear relationship was established between earthworm biomass and the number of earthworm burrows, whereby the number of earthworm burrows was lower in the ley-arable crop rotation than in the PG treatment. The deep, vertical burrows of anecic species can increase water infiltration and root growth, while the shallow burrows of endogeic species generally increase the porosity of the topsoil (Edwards and Shipitalo, 1998). Bouché and Al-Addan (1997) measured an average infiltration rate of 150 mm h^{-1} per 100 g of earthworms

m^{-2}, and more specifically 282 mm h^{-1} per 100 g m^{-2} of anecic species. To restore the functional capacity of earthworms for water infiltration in the ley-arable crop rotation in comparison with the PG treatment, it would be necessary to increase the biomass of earthworms on the one hand and restore the species composition on the other. An increase in biomass could probably be achieved by extending the period of grass ley and including clover in the mixture. A shorter arable period in the crop rotation may help to restore the dominance of anecic species in TG.

Several studies have shown that both herbivorous and microbivorous nematodes can have a profound influence on microbial processes, on available nutrients and on grass growth (Ingham et al., 1985; Bardgett et al., 1999a; Ekschmitt et al., 1999). In our experiment the absolute and relative abundance and the genera composition of the herbivorous and microbivorous nematodes in the temporary treatments were the same as in their permanent counterparts within one to two years. In this sense crop rotation has no negative effect on these trophic groups and their functional aspects. However, specifically in the ley-arable crop rotation, we may wonder whether the lower percentage of omnivorous and carnivorous nematodes might affect the functioning of the soil. For example, Bardgett et al. (1999a) concluded that the effects of herbivorous and bacterivorous nematodes on nutrient fluxes and grass growth appeared to be strongly influenced by complex interactions between different trophic groups of nematodes and other fauna. Moreover, Wardle et al. (1999) reported significant negative correlations between microbivorous nematodes and predatory nematodes, suggesting top-down regulation in the decomposer foodweb. Our results suggest that these complex interactions between different trophic groups and the possible top-down regulation are not fully restored within a three-year grass ley period as part of a crop rotation.

Bacteria play an important role in nutrient cycling (e.g., catabolize fresh organic matter, mineralize and immobilize nutrients) and soil structure improvement (e.g., soil aggregation through bacterial and fungal compounds). In the treatments with temporary grassland and arable land, bacterial growth rate and the capacity to degrade substrates recovered in the second year to the level of their permanent counterparts. However, the Community-Level Physiological Profiles showed that the physiological diversity in the PG remained different from the other treatments.

Both potential N-mineralization and potentially mineralizable C can be seen as a functional output of the soil ecosystem. Potential N mineralization has been shown to be a good indicator of soil nitrogen availability for plant growth (Curtin et al., 2006). Parfitt et al. (2005) determined relationships between net N-mineralization as a measure of soil biological activity and N availability in different pasture soils, and explained differences in crop production. In their experiment the potential N-mineralization was three times higher in PG than in continuous cropping. Also Saggar et al. (2001) reported up to five times greater potential N and C mineralization rates in permanent pastures compared to 34 years of arable cropping.

In our experiment, TG and TA showed intermediate values of potential N-mineralization and potentially mineralizable C. For potentially mineralizable C this pattern was partly accounted for by SOM. Anderson and Domsch (1990) showed that the microbial biomass in a crop rotation had a more efficient carbon utilization than the microbial biomass in continuous cropping.

Our results suggest that major functions of the soil biota in PG are restored in a ley-arable crop rotation. However, due to a reduction in certain species groups (anecic earthworms and omnivorous and carnivorous nematodes) in a ley-arable crop rotation, specific functions of these soil biota are reduced or lost. Furthermore, restoration of soil biota and its functions in the ley phase is only temporary, due to the following arable phase of the crop rotation. As a result, a ley-arable crop rotation takes an intermediate position between permanent grassland and continuous arable land in terms of functioning of the soil biota (e.g., N-mineralization). In order to make better use of the functional aspects of the soil biota and to conserve as much biodiversity as possible, permanent grassland might be preferable wherever possible. For maize cultivation, a ley-arable crop rotation is preferable to continuous arable land. However, since 30% of the land on a dairy farm is generally cultivated with maize, a ley-arable crop rotation is only preferable to continuous arable cropping if it is not practiced at the expense of permanent grassland at farm level.

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Chapter 3

A mixture of grass and clover combines the positive effects of both plant species on selected soil biota

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Abstract

The introduction of N_2-fixing white clover (*Trifolium repens*) in grassland is a management measure that may contribute to sustainable grassland systems by making them less dependent on inorganic fertilizers. However, little is known about the impact of this measure on soil biota and ecosystem services. We investigated earthworms, nematodes, bacteria and fungi in an experiment in which white clover-only and a mixture of grass and white clover without fertilization were compared with grass-only with and without fertilization.

In comparison with grass-only, white clover-only had a lower total root biomass and a lower C/N ratio in the above- and below-ground plant biomass. These plant characteristics resulted in a lower bacterial biomass, a lower fungal biomass, a higher proportion of bacterivorous nematode dauerlarvae, a lesser proportion of herbivorous nematodes and a greater abundance of earthworms in clover-only.

The quantity and quality (C/N ratio) of the above- and below-ground plant biomass in the mixture of grass and white clover (20-30% clover in the DM) was comparable with grass fertilized with 150 kg N ha^{-1} of inorganic fertilizer. Differences between these treatments might show specific clover effects in the grass-clover mixture on soil biota other than quantity and C/N ratio of the litter. However, the only differences were a higher proportion of bacterivorous nematode dauerlarvae and a different nematode community composition in grass-clover.

The soil structure in white clover-only showed a higher proportion of angular blocky elements, a lower penetration resistance, a higher number of earthworm burrows, a higher potential N-mineralization and potentially mineralizable C than the soil in grass-only. This suggests that clover stimulates the ecosystem services of water infiltration and supply of nutrients, but is less conducive to soil structure maintenance. The grass-clover mixture differed from grass-only in a higher potentially mineralizable C and from clover-only in a higher percentage of soil crumbs. We suggest that when clover is introduced in grassland to reduce the reliance on inorganic fertilizer, the mixture of grass and clover maintains the positive impact of grass roots on soil structure and increases the supply of nutrients via the soil food web. Thus, a grass-clover mixture combines the agronomic benefits of the two plant types.

Keywords

Clover, earthworms, ecosystem services, grassland, microbial community, nematodes, soil biota

3.1 Introduction

Organic farming and reduced use of external inputs, such as fertilizers and pesticides implies a greater reliance on self-regulating ecosystem processes (Brussaard et al., 2007a). Soil biota (soil organisms and plant roots) play an important part in these processes and in the provision of various ecosystem services, such as supply of nutrients, maintenance of soil structure and water regulation (Brussaard et al., 1997; Swift et al., 2004; Mulder, 2006; Kibblewhite et al., 2008). Grassland management directly and indirectly influences the soil biota and their functions (Bardgett, 2005). To develop and optimize sustainable grassland systems, insight is needed into how grassland management influences the soil biota and how it affects the functioning of the soil-plant system.

One of the management measures that may contribute to sustainable grassland systems is the introduction of white clover (*Trifolium repens*). The primary motive for using clover in grasslands is its ability to fix atmospheric N_2 in symbiosis with *Rhizobium* bacteria. Although the timing of mineral N availability with atmospheric nitrogen fixation is not under management control, it could reduce or even end the reliance on inorganic N fertilizer. However, little is still known about the extent to which the introduction of clover affects soil biota under grassland and the functioning of the soil-plant system. For example, the root density of white clover is considerably lower than that of grass (Robinson and Jacques, 1958; Young et al., 1958; Evans, 1977; Tisdall and Oades, 1979; Schortemeyer et al., 1997), which could result in less food for soil organisms that contribute to soil structure. Robinson and Jacques (1958) measured a lower percentage of stable soil aggregates in white clover than in perennial ryegrass. This could mean that the introduction of clover for nitrogen fixation is at the cost of soil structure. It is important to understand such effects before sustainable grassland systems can be designed.

Biodiversity studies have shown that legumes can play a key role in the composition of the soil community under species-rich grasslands, by increasing above-ground plant production and affecting litter quality (Spehn et al., 2000; Salomon et al., 2004). Leaf litter from a clover species had a higher active microbial biomass than leaf litter from a grass (Beare et al., 1990). Elgersma and Hassink (1997) reported a higher amount of active soil microbial biomass in grass-clover than in grass-only swards. In a microcosm study, with comparable root weights for grass and white clover, a higher microbial biomass was found in clover (Mawdsley and Bardgett, 1997), whereas in a field experiment De Vries et al. (2006) measured a higher fungal and bacterial biomass in grass-only swards than in grass-clover. Tisdall and Oades (1979) and Ryan et al. (2000) reported that white clover roots had a higher infection with mycorrhizal fungi than ryegrass roots. Various researchers reported more bacterivorous nematodes under legumes (Sohlenius et al., 1987; Viketoft et al., 2005) *versus* more herbivorous nematodes under grass (Sarathchandra et al., 2001; Baars, 2002; Viketoft et al., 2005). Moreover, in field experiments a higher biomass of earthworms was found in grass-clover mixtures than in grass-only swards (Sears, 1950; Yeates et al., 1998b; Van Eekeren et al., 2005a).

In addition to the lower percentage of stable aggregates under white clover compared to perennial ryegrass (Robinson and Jacques, 1958), the introduction of clover can affect other soil ecosystem services. Mytton et al. (1993) found higher drainage rates in white clover than in perennial ryegrass. Elgersma and Hassink (1997) measured a higher N-mineralization in grass-clover without fertilization than in grass-only swards without fertilization. In most of the above-mentioned field studies, a mixture of grass and clover was compared with grass-only without nitrogen fertilization, and the treatment of clover-only was not incorporated. This makes it difficult to disentangle any specific clover effects on the soil biota and those of the quantity and the quality (C/N ratio) of the above- and below-ground biomass, as related to the ability of nitrogen fixation.

In the present field study, we compared the abundance, activity and taxonomic richness of earthworms, nematodes, bacteria and fungi in white clover-only, a grass-clover mixture, and grass-only with and without inorganic N fertilizer. Our objectives were (1) to measure the effect of white clover and perennial ryegrass on the soil biota, and (2) to disentangle any specific clover effects on the soil biota in a grass-clover mixture and those related to the quantity and C/N ratio of the above- and below-ground biomass. We hypothesized a food web with a relative dominance of bacteria and a higher abundance of bacterivorous nematodes and earthworms in clover. In grass, we hypothesized a food web with a relative dominance of fungi and a higher abundance of fungivorous and herbivorous nematodes. We interpret our results in terms of the provision of ecosystem services: soil structure maintenance, water regulation and supply of nutrients.

3.2 Materials and methods

3.2.1 Sampling site and experimental design

The experiment was established in spring 2004 on a free draining sandy loam soil (7.2-7.5 % clay (< 2 µm)) in the East of the Netherlands (52º26´N, 6º08´E). Four treatments were established in a completely randomized block design of six blocks. The individual plot size was 56 m^2 (4 m x 14 m). The four treatments were:
GN1: Grass with inorganic N fertilizer;
GN0: Grass without N fertilizer;

GCN0: Grass clover without N fertilizer;
CN0: Clover without N fertilizer.

The former production pasture (mainly based on *Lolium perenne*) was killed in March 2004 with 3 l ha^{-1} Roundup® Max (Monsanto Company, St. Louis, USA), after which the sward was ploughed and prepared for sowing. On 26 April, the different treatments were sown. The seed used was 35 kg *L. perenne* L. ha^{-1} (cvs. Plenty and Roy) for the grass-only treatments (GN1 and GN0), 30 kg *L. perenne* L. ha^{-1} and 5 kg *T. repens* L. ha^{-1} (cv. Alice) for the treatment GCN0 and 10 kg *T. repens* L. ha^{-1} for the treatment CN0. In order to get approximately the same quantity and quality (C/N ratio) in the above- and below-ground biomass in the GN1 and GCN0, inorganic fertilizer (calcium ammonium nitrate 27%) application on GN1 was 150 kg N ha^{-1} year^{-1}. The plots were mowed four times in 2004 and five times in 2005.

3.2.2 Soil sampling, chemical and physical measurements

On 16 December 2005, two growing seasons after the start of the experiment, soil samples for determination of chemical, physical and biological soil quality parameters were taken. A bulk sample of 70 cores (0-10 cm, ø of 2.3 cm) per plot was collected, sieved through 1 cm mesh, homogenized and stored at field moisture content at 4°C before analysis. Subsamples were taken and used for chemical analyses, nematode and microbiological analyses.

Soil dry matter content was determined after oven-drying of approximately 30 g of the bulk sample (in duplicate) at 105 ºC. Prior to further chemical analysis, samples were oven-dried at 40 °C. Soil acidity of the oven-dried samples was measured in 1 M KCl (pH-KCl). Organic C was measured by incineration of dry material at 1150 °C, after which the produced CO_2 was determined by an infrared detector (LECO Corporation, St. Joseph, MI, USA). For determination of total N, gasses after incineration were reduced to N_2 and detected with a thermal-conductivity detector (LECO Corporation, St. Joseph, MI, USA).

Bulk density was measured in the 5-10 cm layer below the soil surface, in three undisturbed ring samples containing 100 cm^3 soil. Penetration resistance was measured with a penetrometer (Eijkelkamp, Giesbeek, The Netherlands) with a cone diameter of 2 cm^2 and a 60° apex angle. Cone resistance was recorded per cm of soil depth and expressed as an average value of 6 penetrations per plot in the soil layers of 0-10 cm, 10-20 cm and 20-30 cm. Soil structure was determined in 1 block (20 cm x 20 cm x 20 cm) per plot. Soil of this block was divided by visual observation into crumbs, sub-angular blocky elements and angular blocky elements (FAO, 2006). These were weighed and expressed as a percentage of total fresh soil weight. Three soil cores (0-10 cm, ø of 8.5 cm) per plot were taken to determine the root biomass. The soil in the samples was thoroughly washed out with water, after which the roots were oven-dried at 70 °C and the dry matter of the roots was measured. After drying, the individual samples of roots were bulked together per treatment and analyzed for ash content and total N. Root biomass was expressed as grams of ash-free dry matter (AFDM).

Grass dry matter yield in the 1st and 5th harvest was determined in three of the six blocks by cutting a strip of 0.84 m x 5 m with a two-wheel tractor. After weighing the fresh biomass, a sub-sample was dried for 48 hours at 70 ° and analyzed for dry matter (DM), ash and total N. In a second sub-sample, grass and clover were hand-sorted and dried for 24 hours at 10 °C in order to determine the clover content in the dry matter.

3.2.3 Soil biological parameters

3.2.3.1 Earthworms and moles

Earthworms were sampled in two blocks (20 cm x 20 cm x 20 cm) per plot. The blocks were transferred to the laboratory where the earthworms were hand-sorted, counted, weighed and fixed in alcohol prior to identification. Numbers and biomass were expressed per m^2. Adults were identified to species. Furthermore, a distinction was made between (1) epigeic species (pigmented, living superficially in the litter layer, little burrowing activity), (2) endogeic species (living in burrows at approximately 10-15 cm depth) and (3) anecic species (relatively large worms, living in vertical burrows from which they collect dead organic matter from the surface at night) (Bouché, 1977). Before the blocks were sorted, in one block per plot the earthworm burrows with a diameter >2 mm were counted on horizontal surfaces (20 cm x 20 cm) exposed at 10 cm and 20 cm depth. The data for moles (*Talpa europaea*) presented here were not part of the experimental design, but resulted from a spontaneous invasion from October 2005 onwards. The abundance of moles per plot was estimated by the number of molehills counted on 13 December 2005.

3.2.3.2 Nematodes

Free-living nematodes were extracted from a sub-sample of about 100 g field-moist soil, using the Oostenbrink elutriator (Oostenbrink, 1960). Total numbers were counted and expressed per 100 g fresh soil. Nematodes were fixed in hot formaldehyde 4%, and at least 150 randomly selected nematodes from each sample were identified to genus and, whenever possible, to species. Nematode genera and species were assigned to trophic groups, following Yeates et al. (1993), and allocated to the colonizer-persister groups (cp-groups), according to Bongers (1990) and Bongers et al. (1995). The Nematode Channel Ratio (NCR) was calculated to express the relative contributions of bacterivorous (B) and fungivorous (F) nematodes to the total microbivorous nematode abundance (NCR=B/(B+F)) (Yeates, 2003). The Maturity Index was calculated as the weighted mean of the individual cp-values, in accordance with

Bongers (1990) and Korthals et al. (1996). The Maturity Index is an ecological measure which indicates the condition of an ecosystem based on nematode species composition.

3.2.3.3 Microbial parameters

From the bulk soil sample, a sub-sample of 200 g field moist soil was adjusted to 50% WHC (Water Holding Capacity) and pre-incubated at 12 °C for 4 weeks, to avoid the effects of temperature and moisture fluctuations in the field and to stabilize soil conditions (Bloem et al., 2006). After pre-incubation, fungal and bacterial biomass, and Community-Level Physiological Profiles (CLPP) were measured. Microscopic soil smears were prepared and measured as described by Bloem and Vos (2004). Fungal hyphae were measured using the grid intersection method. Bacterial numbers and cell volumes were measured by confocal laser scanning microscopy and automatic image analysis (Bloem et al., 1995). Biomass was calculated from biovolume.

The CLPPs of the bacterial communities in the soil extracts were determined with ECO plates described by Insam (1997) and manufactured by BIOLOG Inc. (Hayward, USA). These plates contain a triplicate set of 31 different carbon substrates, a control, a freeze-dried mineral medium and a tetrazolium redox dye. For each bacterial extract, a dilution series was made using 10 mM BisTris buffer at pH 7. Each dilution series (3^{-1} until 3^{-12}) was used to inoculate four ECO plates with a volume of 100 µl per well. The colour formation in the plate was measured every 8 hours for 7 days with a plate reader spectrophotometer at 590 nm. The CLPPs were calculated from the colour formation in the wells, and corrected for inoculum density using a regression approach applied to the average well colour development (AWCD) as described by Rutgers et al. (2006). This produced CLPPs describing the relative abundance for substrate conversion (31 substrates; log scaled). To survey the bacterial community activity in the ECO plate, the AWCD was calculated after 7 days of incubation. The CLPP-slope parameter was calculated from the color development in the ECO plates. This parameter indicates the rate at which the capacity of the soil to degrade a set of carbon and energy substrates disappears upon dilution. A low slope parameter indicates a slow disappearance rate and can be considered a measure of high physiological diversity (Gomez et al., 2004; Rutgers et al., 2006).

The potential N mineralization rate was determined as the increase in mineral N (ammonium plus nitrate) between week 6 and week 1, during 6 weeks' incubation of 100 g soil at 20 °C and 50% WHC. The first week was not used to avoid effects of soil homogenization. Sub-samples of 80 g soil were extracted with 200 ml of 1 M KCl. After shaking for 1 h, the extracts were filtered over a paper filter. Mineral N contents (ammonium and nitrate) were determined by Skalar Segmented Flow Analysis (Skalar Analytical B.V., Breda, the Netherlands). Potentially mineralizable C was measured by incubation for 7 days at 20°C and 50% WHC. During this period, CO_2 was absorbed in alkali (1*N* KOH) followed by titration with 0.1 *N* HCl (Pell et al., 2006).

3.2.4 Statistical analyses

The effects of grass-only and fertilization, clover-only, and the mixture of grass-clover on the measured parameters were tested using one-way ANOVAs in randomized blocks with treatment (GN1, GN0, GCN0, CN0) as factor. When relations between parameters were expected, the Pearson correlations between parameters were determined. Data of nematode taxa (numbers (log(x+1) per 100 g soil) and CLPPs were square-root transformed and subjected to a redundancy analysis (RDA). A Monte Carlo permutation test (499 random permutations) was performed to test for statistically significant (P=0.05) relationships between community structure and treatment effects following multivariate analysis of variance (Van Dobben et al., 1999). ANOVAs and correlation analyses were carried out using the GENSTAT statistical software (8th Edition, VSN International, Hemel Hempstead, UK). Multivariate analyses were carried out using CANOCO 4.5 (Biometris, Wageningen, The Netherlands)

3.3 Results

3.3.1 Soil chemical, soil physical and crop parameters

Organic C, total N and pH-KCl did not significantly differ between the treatments (Table 3.1).

The penetration resistance in all soil layers was lower in clover-only (CN0) than in the grass-only with inorganic N fertilizer (GN1) (Table 3.1), but this was only statistically significant in the soil layer of 20-30 cm. The penetration resistance at 20-30 cm was negatively correlated with earthworm biomass (r=-0.47, P=0.02). The proportion of crumbs was significantly higher in GN0 than CN0. GN1 and GCN0 took an intermediate position. The CN0 had the highest proportion of angular blocky elements. The proportion of crumbs was negatively correlated with clover root biomass (r=-0.53, P=0.008) but no significant correlation was present with grass or total root biomass.

CN0 had significantly lower grass root biomass and significantly higher clover root biomass than the other treatments (Table 3.1). The ranking of treatments in terms of total root biomass was comparable to that of grass root biomass. In terms of the total N in the root biomass, CN0 was significant lower than the other treatments. The C/N ratio in the total root biomass was lowest for CN0 and highest for GN0. GN1 and GCN0 were intermediate.

The percentage clover dry matter in 2005 was on average 26% for GCN0 and 75% for CN0 (Table 3.1). Above-ground total dry matter yield was highest in GN1 and GCN0. The trend in above-ground N-yield was similar to that in dry matter yield, except for a higher N-yield of CN0 compared to GN0.

Table 3.1 Soil chemical, soil physical and crop parameters in grassland with inorganic N fertilizer (GN1), grassland without N fertilizer (GN0), grass-clover without N fertilizer (GCN0) and clover without N fertilizer (CN0).

Chemical/ Physical	Unit	Treatments				
		GN1	GN0	GCN0	CN0	P-value
Organic C	g C kg dry soil^{-1}	19.5	20.2	21.2	19.2	0.051
Total N	g N kg dry soil^{-1}	1.76	1.88	1.79	1.76	0.888
pH-KCl		7.42	7.28	7.37	7.30	0.202
Bulk density	g cm^{-3}	1.47	1.42	1.49	1.47	0.098
Penetration resistance						
0-10 cm	mPa	1.48	1.44	1.46	1.39	0.776
10-20 cm	mPa	1.46	1.45	1.40	1.34	0.368
20-30 cm	mPa	2.51 a	2.39 ab	2.45 ab	2.13 b	0.036
Soil structure 0-10 cm						
Crumb	%	39 bc	53 a	50 ab	32 c	0.006
Sub-angular	%	13	9	12	5	0.094
Angular	%	47 b	38 b	38 b	62 a	0.009
Roots biomass 0-10 cm						
Grass	g AFDM m^{-2}	169 a	217 a	177 a	12 b	<0.001
Clover	g AFDM m^{-2}	0 c	1 c	16 b	62 a	<0.001
Total	g AFDM m^{-2}	169 a	218 a	193 a	73 b	<0.001
Total N	g N m^{-2}	4.0 a	4.1 a	4.5 a	2.6 b	0.043
C/N		21.0 b	26.3 a	21.3 b	14.2 c	<0.001
Grass yield 1st+5th harvest						
Clover	%	0 c	0 c	26 b	75 a	<0.001
Total biomass	g DM m^{-2}	405 a	270 b	352 a	232 b	<0.001
Total N	g N m^{-2}	14.4 a	6.8 c	12.3 ab	11.1 b	<0.001
C/N 1st		15.5 b	32.3 a	15.1 b	10.2 c	<0.001
C/N 5th		10.2	11.0	10.2	8.1	0.278

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

3.3.2 Soil biological parameters

3.3.2.1 Earthworms

Earthworm abundance was significantly higher in CN0 than in the other treatments (Table 3.2). CN0 had the highest earthworm biomass, GN1 and GN0 the lowest. Earthworm numbers and biomass were negatively correlated with the C/N ratio of the root biomass ($r=-0.59$, $P=0.002$ and $r=-0.52$, $P=0.01$, respectively). The adult earthworms were identified as *Allolobophora chlorotica*, *Aporrectodea caliginosa, Aporrectodea rosea, Dendrobaena hortensis, Lumbricus castaneus, Lumbricus festivus, Lumbricus rubellus* and *Lumbricus terrestris*.

The number of earthworm burrows at 10 cm depth was significantly higher in CN0 than in the other treatments. At 20 cm depth, the number of earthworm burrows was highest in the two treatments with clover (GCN0 and CN0) but not significant different from GN1. The number of burrows at 10 cm and 20 cm depth was positively correlated with the earthworm biomass ($r=+0.50$, $P=0.012$ and $r=+0.49$, $P=0.015$, respectively) and negatively correlated with the C/N ratio of the root biomass ($r=-0.69$, $P<0.001$ and $r=-0.55$, $P=0.005$, respectively).

Table 3.2 Earthworm and mole parameters in grassland with inorganic N fertilizer (GN1), grassland without N fertilizer (GN0), grass-clover without N fertilizer (GCN0) and clover without N fertilizer (CN0).

Earthworm	Unit	Treatments				
		GN1	GN0	GCN0	CN0	P-value
Total number	n m^{-2}	322 b	326 b	359 b	480 a	*0.002*
Body biomass	g worm^{-1}	0.26	0.24	0.31	0.28	0.236
Total biomass	g m^{-2}	82 b	76 b	110 ab	135 a	*0.009*
Number of species	n 20x20x20 cm^{-1}	3.0	2.2	2.8	3.3	0.063
Epigeic adults	n m^{-2}	21	4	6	19	0.169
Endogeic adults	n m^{-2}	65	48	79	92	0.083
Anecic adults	n m^{-2}	2	2	2	0	0.766
Earthworm burrows						
10 cm depth	n m^{-2}	58 b	67 b	138 b	225 a	*0.002*
20 cm depth	n m^{-2}	50 ab	8 b	113 a	121 a	*0.023*
Number of molehills	n m^{-2}	0.02 b	0.01 b	0.07 b	0.21 a	*0.017*

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

The number of molehills was significantly higher in CN0 than in the other treatments and was positively correlated with earthworm biomass (r=+0.44, P=0.032), although the percentage of variation explained was moderate.

3.3.2.2 Nematodes

Although not statistically significant, the treatments with clover (GCN0 and CN0) had the highest abundance of nematodes (Table 3.3), mainly due to a higher abundance of dauerlarvae. The proportion of dauerlarvae was significantly higher in CN0 than in GN1. The numbers in the other treatments were intermediate, but the two grass-only treatments (GN1 and GN0) had the lowest proportion of dauerlarvae. The proportion of herbivorous nematodes was significantly higher in GN0 than in CN0 and GCN0, while the proportion in GN1 was intermediate. Although marginally significant, the number of herbivorous nematodes was positively correlated with total root biomass (r=+0.43, P=0.038). The tendency towards a higher proportion of fungivorous nematodes in the two grass-only treatments was associated with a significantly lower NCR than in clover-only (CN0). Bacterivorous and fungivorous nematodes were not significantly correlated with bacterial and fungal biomass.

The life-strategy group distribution showed a high percentage of cp-1 taxa (enrichment opportunists) and was not affected by treatment. The number of nematode taxa was highest in GN0 and significantly different from GN1 and GCN0, which in turn were significantly higher in the number of genera than CN0. The number of taxa was positively correlated with the C/N ratio of the root biomass (r=+0.78, P<0.001). Multivariate analysis (RDA) showed a significant treatment effect on nematodes (P<0.001; Fig. 3.1). The nematode taxa *Pratylenchus*, Rhabditidae and *Eucephalobus* were most abundant in CN0, *Discolaimus* and *Prismatolaimus* in GCN0, Criconematidae, *Meloidogyne*, *Filenchus* and *Paratylenchus* in GNO and *Ditylenchus* and *Panagrolaimus* in GN1.

3.3.2.3 Microbial parameters

Bacterial biomass was significantly higher in GN0 and GCN0 than in CN0 (Table 3.4), with GN1 in an intermediate position. Fungal biomass was significantly higher in GN0 than in GCN0 and CN0, with GN1 again in an intermediate position. Fungal activity (measured as a percentage of active hyphae) did not differ significantly between treatments. There was a tendency (P=0.097) towards a lower fungal/bacterial ratio in GCN0 than in the other treatments. Although marginally significant, bacterial and fungal biomass were positively correlated with the C/N ratio of the root biomass (r=+0.44, P=0.033 and r=+0.42, P=0.039, respectively). Bacterial biomass was also positively correlated with total root biomass (r=+0.47, P=0.022).

The CLPP slope parameter did not differ significantly between treatments. The amount of extracted soil necessary for conversion of 50% of all substrates in ECO plates (ES50%; Table 3.4) tended to be lower in GN1 and CN0 than in GN0 and GCN0 (P=0.069).

Table 3.3 Nematode parameters in grassland with inorganic N fertilizer (GN1), grassland without N fertilizer (GN0), grass-clover without N fertilizer (GCN0) and clover without N fertilizer (CN0).

Nematode	Unit	Treatments				
		GN1	GN0	GCN0	CN0	P-value
Total number	n 100g soil^{-1}	6595	6971	7808	9465	0.146
Bacterivorous	n 100g soil^{-1}	3784	3074	3962	4613	0.163
Dauerlarvae	n 100g soil^{-1}	1176	1149	2314	3578	0.053
Herbivorous	n 100g soil^{-1}	1313	1360	1220	1046	0.439
Fungivorous	n 100g soil^{-1}	172	164	116	66	0.395
Omnivorous	n 100g soil^{-1}	98	126	96	91	0.674
Carnivorous	n 100g soil^{-1}	52	99	100	73	0.546
Bacterivorous	%	57	51	52	51	0.258
Dauerlarvae	%	18 c	20 bc	29 ab	34 a	*0.009*
Herbivorous	%	20 ab	23 a	15 bc	13 c	*0.008*
Fungivorous	%	3	3	2	1	0.056
Omnivorous	%	2	2	1	1	0.344
Carnivorous	%	1	2	1	1	0.265
Cp-1	%	54	51	55	59	0.577
Cp-2	%	37	36	36	34	0.929
Cp-3	%	3	5	3	2	0.454
Cp-4	%	3	5	3	3	0.158
Cp-5	%	2	4	3	2	0.287
Number of taxa		21 b	24 a	21 b	16 c	*<0.001*
Maturity Index(cp1-5)		1.62	1.76	1.63	1.56	0.141
Maturity Index(cp2-5)		2.34	2.54	2.42	2.38	0.260
Nematode Channel Ratio		0.96 b	0.96 b	0.97 ab	0.99 a	*0.042*

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

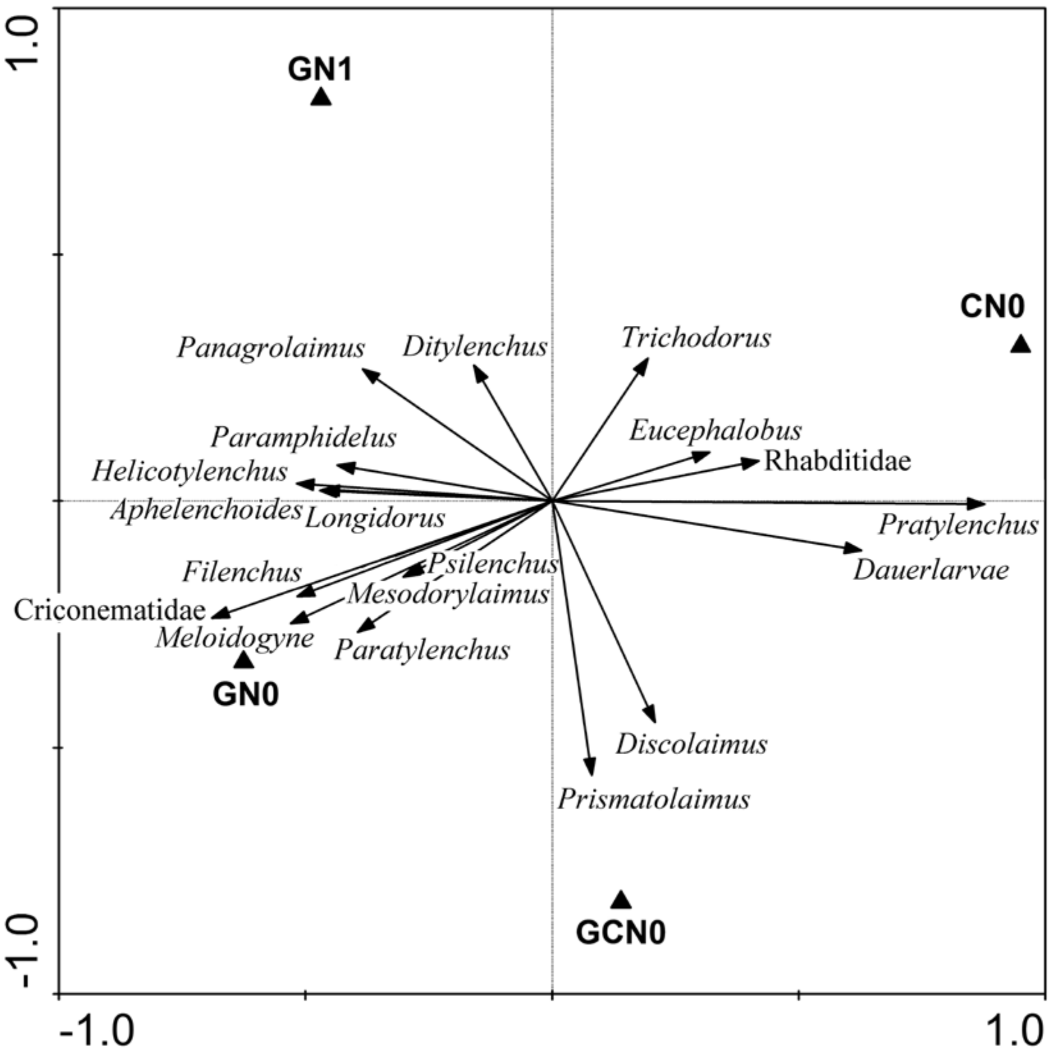

Fig. 3.1 RDA biplot of the nematode numbers belonging to different taxa according to the treatments grassland with inorganic N fertilizer (GN1), grassland without N fertilizer (GN0), grass-clover without N fertilizer (GCN0) and clover without N fertilizer (CN0) accounting for 20.4 % of the variance. Only taxa are shown of which ≥10% of the variance is accounted for.

Table 3.4 Microbial parameters in grassland with inorganic N fertilizer (GN1), grassland without N fertilizer (GN0), grass-clover without N fertilizer (GCN0) and clover without N fertilizer (CN0).

Microbial	Unit	Treatment				
		GN1	GN0	GCN0	CN0	P-value
Bacterial biomass	μg C g dry soil^{-1}	96 ab	119 a	111 a	80 b	*0.004*
Fungal biomass	μg C g dry soil^{-1}	63 ab	77 a	54 b	55 b	*0.024*
Active fungal hyphae	percentage length	6.2	8.6	5.2	5.1	0.390
Fungal/bacterial ratio		0.74	0.74	0.54	0.80	0.097
CLPP						
Slope		0.51	0.62	0.55	0.60	0.335
ES50%[*]	µg dry soil	406	930	720	469	0.069
Potential N mineralization	mg N kg dry soil^{-1}wk^{-1}	7.2 bc	6.7 c	8.6 ab	10.0 a	*0.002*
Potentially mineralizable C	mg CO_2 kg dry soil^{-1} wk^{-1}	1177 c	1203 c	1312 b	1382 a	*<0.001*

[*] ES50%= amount of soil needed for 50% substrate decomposition.

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

Redundancy analysis of all CLPPs showed significant differences between GN1 and all other treatments (P=0.002) (Fig. 3.2). Pair-wise redundancy analysis showed a significant difference between CN0 and GCN0 (P=0.024), a marginal difference between CN0 and GN0 (P=0.074), and no difference between GN0 and GCN0 (P>0.1). The GN1 treatment demonstrated a strong preference of the microbial community for carbohydrates, whereas there appeared to be a slight preference for nitrogen-containing compounds in GN0 and GCN0.

Potential N-mineralization was highest in the clover-only treatment (CN0) and decreased in the order CN0>GCN0>GN1>GN0 (Table 3.4). Potentially mineralizable C was also highest in CN0, followed by GCN0 and was lowest in the grass-only treatments (GN0 and GN1). Potential N-mineralization and potentially mineralizable C were strongly correlated (r=+0.60, P=0.002). Both parameters were correlated with the C/N ratio of the root biomass (r=+0.68, P<0.001 and r=+0.68, P<0.001, respectively), bacterial biomass (r=-0.44, P=0.031 and NS, respectively), fungal biomass (NS and r=-0.42, P=0.041, respectively), proportion of dauerlarvae (r=+0.56, P=0.004 and r=+0.67, P<0.001, respectively), proportion of herbivorous nematodes (r=-0.58, P=0.003 and r=-0.78, P<0.001, respectively), proportion of fungivorous nematodes (r=-0.44, P=0.033 and r=-0.40, P=0.05, respectively) and earthworm biomass (r=+0.62, P<0.001 and r=+0.44, P=0.034, respectively).

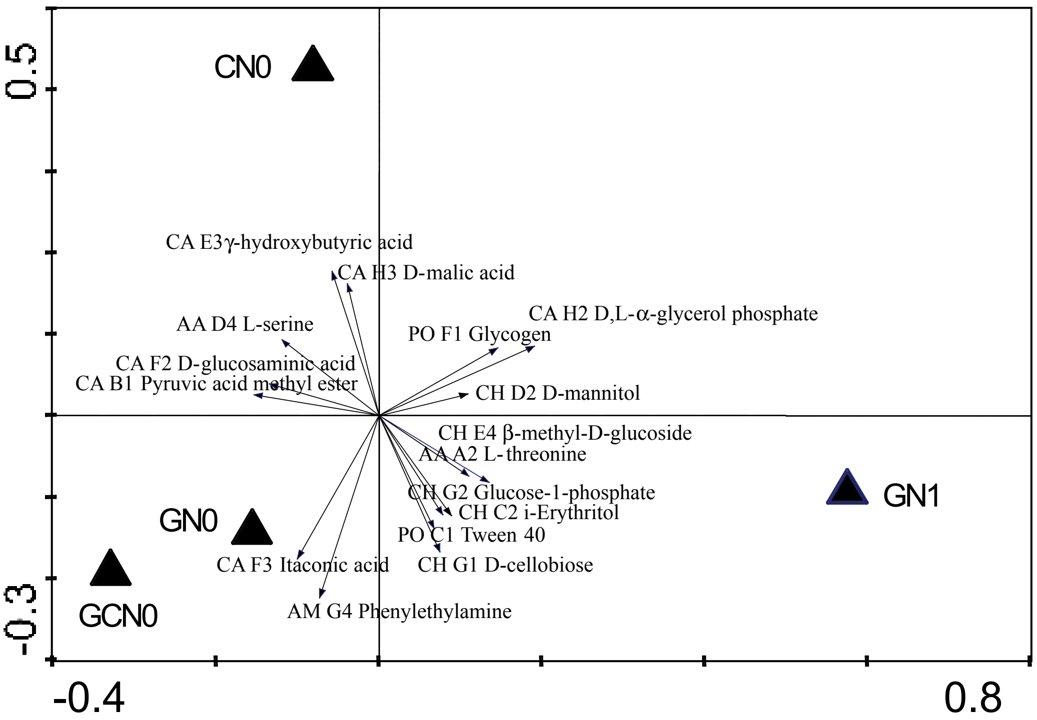

Fig.3.2 RDA-biplot of CLPPs of bacterial communities with the treatments grass with inorganic N fertilizer (GN1), grass without N fertilizer (GN0), grass-clover without N fertilizer (GCN0) and clover without N fertilizer (CN0), accounting for 21.9 % of the variance. Only wells are shown of which ≥15% of the variance is accounted for by the diagram (16 out of the total 31 wells).

CH=carbohydrate, PO=polymer, AA=amino acid, CA=carboxylic acid, AM=amine

3.4 Discussion

3.4.1 Earthworms

As hypothesized, the earthworm biomass was higher (70%) in clover-only (CN0) than in grass-only (GN1 and GN0), with the mixture of grass and clover in an intermediate position. Gastine et al. (2003) also found a higher earthworm biomass in legumes-only than in grass-only. Sears (1950) and Van Eekeren et al. (2005a) found a higher earthworm biomass in a grass-clover mixture than in grass-only swards. Thus, introduction of clover in a grass sward results in higher earthworm population densities. Sears (1950) explained such higher earthworm biomass by a higher above-ground dry matter production of grass-clover than in grass-only swards with a low N-fertilization. This is in accordance with Van Eekeren et al. (2005a), who found the highest earthworm biomass in plots with the highest above-ground dry matter production. However, in the present experiment, the treatment with the highest earthworm biomass, CN0, did not have the highest dry matter yield or the highest root biomass. Furthermore, we found a negative relationship between the C/N ratio of the root biomass and the total abundance of earthworms. This suggests that the quality of the litter (below- and above-ground) rather than the quantity played a prominent role in the higher abundance of earthworms. Shipitalo et al. (1988) reported large weight gains in earthworms on diets of Alfalfa or Red clover (C/N ratios' of 13.7 and 10.9, respectively), low weight gains on corn leaves (C/N ratio of 21.1) and a loss of weight on Brome grass (C/N ratio of 26.2) or no food. Van Vliet et al. (2007) estimated the preferred C/N ratio of the food for the different ecological groups of earthworms as 8.8-12.4, which suggests that the higher abundance and biomass of earthworms in the clover-only treatment is mainly explained by a lower C/N ratio of the available food.

The quantity and C/N ratio of the above- and below-ground biomass was similar in GCN0 and GN1. Therefore, differences between these treatments could possibly show specific clover effects in the grass-clover mixture on earthworms other than quantity and C/N ratio of the litter. In comparison to GN1, GCN0 had a higher earthworm biomass and a higher number of earthworm burrows at 10 cm and 20 cm, but none of these differences was significant. Negative effects of inorganic N fertilizers on earthworms in grassland are mainly related to changes in soil acidity (Ma et al., 1990). Our data suggest that, in the absence of a negative effect of inorganic fertilizer on soil acidity, both clover and inorganic fertilizer have a similar positive effect on earthworm parameters by improving the litter quality (lower C/N ratio) and quantity. Hence, the available data do not suggest an extra, specific, clover effect on earthworms.

3.4.2 Nematodes

We hypothesized a more bacterial-dominated food web under clover and a more fungal and herbivore-dominated food web under grass. Indeed, the proportion of bacterivorous nematode dauerlarvae was highest in the CN0 treatment. There was a tendency for a higher proportion of fungivorous nematodes and a significantly lower Nematode Channel ratio (NCR) in the grass treatments, and the proportion of herbivorous nematodes was highest in the GN0 treatment. The higher proportion of herbivorous nematodes in the grass treatments was probably related to the higher root biomass of grasses than white clover, a result also found by

Robinson and Jacques (1958), Young et al. (1958) and Evans (1977). The higher proportion of dauerlarvae in white clover-only was probably related to the easily decomposable litter in the rhizosphere to which bacterivorous nematodes with a short generation time can quickly respond. This is also reflected in the dominance of Rhabditidae in CN0 (Fig. 3.1). Rhabditidae commonly increase after a resource input (De Goede et al., 1993; Ettema and Bongers, 1993; Yeates, 2003) and are promoted by the presence of legumes (Viketoft et al., 2005). The in general higher abundance of dauerlarvae could be a sign of a less biological active period and could be caused by the time of sampling in December. However, in 2005 we had a very mild autumn without any frost. The date of sampling did not have a major effect on the other soil biological parameters measured when compared to measurements done in October, like in Van Eekeren et al. (2008).

The number of nematode taxa increased with a higher C/N ratio of roots. The number of nematode taxa was the highest in GN0 and the lowest in CN0. Likewise, Viketoft (2008) found a lower number of nematode taxa in white clover than in other legumes, forbs and grasses. Since the number of taxa in the treatments GN1 and GCN0 was in between CN0 and GN0, the number of nematode taxa seems more related to litter quality (C/N ratio) than to specific clover effects or inorganic fertilizer effects.

In terms of the proportion of herbivorous nematodes and the Nematode Channel Ratio (NCR), the mixture of grass-clover (GCN0) was intermediate between GN1 and CN0. Only in the proportion of dauerlarvae (Table 3.3) and the composition of the nematode community (Fig. 3.1), the differences between GCN0 and GN1 were significant. Hence, these two parameters suggest that there was a specific clover effect in the grass-clover mixture other than the quantity and the C/N ratio of the above- and below-ground biomass or an inorganic fertilizer effect in GN1. For larvae in the dauer stage, a particular reaction on the availability of nitrogen from litter or root exudates in the rhizosphere of clover could be an explanation. For the composition of the nematode community, both the availability of nitrogen in the rhizosphere and the cyanogenic potential of white clover could have played a role.

3.4.3 Microbial parameters

We hypothesized a higher bacterial biomass with clover, but it was significantly lower in CN0 than in GCN0 and GN0 (with GN1 in an intermediate position). Differences in bacterial biomass between perennial ryegrass and white clover may be explained from the denser root system of perennial ryegrass. Bacteria reach higher abundances in the rhizosphere than in the bulk soil and thus higher abundances in the soil when the rhizosphere/bulk soil ratio is higher (Schortemeyer et al., 1997). Wardle and Nicholson (1996) found a positive relationship between root biomass carbon and microbial biomass carbon. In a microcosm experiment, Mawdsley and Bardgett (1997) found, with the same root biomass, a significantly greater microbial biomass (fumigation extraction method) with clover-only than with ryegrass. In our experiment, the ratio between bacterial biomass and root biomass in the 0-10 cm layer was also highest in clover-only. This suggests that the bacterial biomass in the rhizosphere of clover is higher than in grass, but since the root mass of grass is larger, the total bacterial biomass in the entire soil is also higher.

As was the case for bacteria, the ratio between fungal biomass and root biomass in the 0-10 cm layer was also highest in clover-only. A higher concentration of fungi in the rhizosphere of clover and differences in root density between grass and clover cannot, however, explain the lower fungal biomass in CN0 and GCN0 than in GN0 (with GN1 in an intermediate position), as in the case of bacteria. De Vries et al. (2006) suggested that the amount of fungi in clover could be suppressed by nitrogen in root exudates or litter. A negative nitrogen effect on fungal biomass either by nitrogen fixation of clover or nitrogen from inorganic fertilizer is confirmed by the lower fungal biomass in the treatments GCN0, CN0 and, to a lesser extent, GN1 compared to GN0. Furthermore, it is in line with the positive relationship between fungal biomass and the C/N ratio of root biomass. However, if the fungal biomass were fully explained by nitrogen from nitrogen fixation or inorganic fertilizer and/or the C/N ratio from root litter, the fungal biomass in the treatment with clover-only (CN0) should have been lower than measured in our experiment. The latter could possibly be explained by the higher colonization of mycorrhizal fungi on roots of clover than of grass (Tisdall and Oades, 1979; Ryan et al., 2000). Depending on the root/bulk soil ratio of clover and grass, clover could have had a positive effect on the mycorrhizal fungi in the soil while the saprophytic fungi could have been suppressed from nitrogen in root exudates or litter.

The higher bacterial biomass in the grass-clover mixture in combination with a lower fungal biomass caused a tendency towards a lower fungal/bacterial ratio in the mixture compared to the other treatments. The effect was not significant due to the variation in the data. De Vries et al. (2006) also found a lower fungal/bacterial ratio in grass-clover mixtures compared to grass-only swards.

The CLPPs of GN1 and CN0 were significantly different from those of GN0 and GCN0 (Fig. 3.2), and indicated a capability to break down a wider range of substrates. Here it has to be mentioned that the Biolog approach has the advantage of combining both functional diversity and degradation rates, but it is culture dependent and will be biased towards a small fraction of the community (Ros et al., 2008). This is not a serious problem as long as the limited information from a small fraction of the community, like any other microbial technique, is still reflecting effectively community differences (Winding et al. 2005). Thus the culture dependent part of the microbial community can be effectively studied when replicates contain a similar inoculation density (Nannipieri et al. 2003) or when dilution series have been used (this study; Rutgers et al. 2006). The difference of the CLPPs of GN1 and CNO form the other treatments is in accordance with the tendency of a lower ES50% (Table 3.4) for GN1 and CN0 which indicates that less soil is needed to break down 50% of the substrates. The GN1 community had a preference for carbohydrate metabolization. This is in agreement with the observation of Grayston et al. (2004), who found a positive correlation of the greater utilization of sugars in the CLPP of improved grasslands with the soil nitrogen concentration. In our study, the differences between CLPPs of GN1 and GCN0 demonstrate that the stimulating effect of high nitrogen is positively correlated to the inorganic fertilizer and not an effect of clover. The only other difference between GCN0 and GN1 remaining was a tendency towards a lower fungal/bacterial ratio for the grass-clover mixture. However, this is a combination of the higher bacterial biomass in the bulk soil, probably caused by a higher root density, with the lower fungal biomass that is characteristic of clover-only, of which the underlying mechanisms remain to be investigated.

3.4.4 Relevance of changes in soil biota for ecosystem services

The soil biota are key for a range of ecosystem services, including soil structure maintenance, water retention and supply of nutrients (Swift et al., 2004; Mulder, 2006; Brussaard et al., 2007b; Kibblewhite et al., 2008). In our study, the soil structure in clover-only (measured as a proportion of crumbs) was less well developed than in grass-only and the mixture of grass-clover (Table 3.1). This is in line with our hypothesis and with other research (Robinson and Jacques, 1958; Tisdall and Oades, 1979) in which perennial ryegrass had a higher soil aggregate stability than white clover-only. However, Mytton et al. (1993) could not measure a difference in aggregate stability between grass-only, grass-clover and clover-only, while Yeates et al. (1998b) even measured a higher aggregate stability in grass-only swards than in a grass-clover mixture.

A general explanation for a higher aggregate stability under grass compared to legumes is the extensive fine root system under grass. Organic material released by living or decomposing roots stabilizes aggregates directly or indirectly by providing nutrients to microorganisms in the rhizosphere, which may in turn excrete soil-stabilizing substances. Robinson and Jacques (1958) found that the percentage of stable aggregates per gram root biomass was larger in clover than in grass. They explained their finding from the quality of clover roots, which decay more rapidly than the roots of perennial ryegrass (Robinson and Jacques, 1958). However, Tisdall and Oades (1979) found a larger hyphal length of mycorrhizal fungi per gram of soil in perennial ryegrass because of the denser root system of grass, although the mycorrhizal infection per root length was higher in white clover. Because mycorrhizal hyphae bind small aggregates into bigger aggregates, they found that ryegrass was more effective than white clover in stabilizing aggregates. In our experiment, the root system of grass-only was up to three times denser than that of clover-only. The better developed soil structure in grass-only than in clover-only (Table 3.1), the denser root system of grass (Table 3.1), the higher abundance of mycorrhizal fungi they support (Tisdall and Oades, 1979; Ryan et al., 2000), and inference from the cited studies on aggregate stability, all suggest that the ecosystem service of soil structure maintenance was better maintained in grass-only than in white clover-only. Since the grass root mass and the soil structure in the grass-clover mixture was comparable with the grass-only treatments (Table 3.1), we suggest that the soil structure in the mixture was maintained at the same level. Further research on soil aggregate stability is needed for confirmation.

Water regulation as an ecosystem service in grasslands is greatly influenced by earthworms (Clements et al., 1991; Bouché and Al-Addan, 1997). Especially earthworm burrows can increase water infiltration (Edwards and Shipitalo, 1998). In our experiment, the numbers of earthworm burrows at 10 cm and 20 cm depth were highest in clover-only. Furthermore, clover-only showed the lowest penetration resistance at 20-30 cm, also suggesting improved water infiltration. These data are consistent with results of Mytton et al. (1993), who found that white clover-only drained more rapidly than grass-only. In their research, soil moisture curves indicated a more free-draining structure in clover than in grass due to a higher ratio of macro- to micro-pores (Mytton et al, 1993). For both drainage and soil moisture characteristics, Mytton et al. (1993) found that the mixture of grass-clover (> 50% clover in the DM) took an intermediate position between the monocultures of grass and clover. In our research, the mixture of grass-clover (GCN0), with 20-30% clover in the DM, showed a higher number of earthworm burrows and a lower penetration resistance than grass-only with fertilization (GN1), but differences were not significant. This suggests that a positive effect of clover on water infiltration was not apparent in our grass-clover mixture. With a higher clover percentage in the dry matter this could be different.

Clover can enhance the ecosystem service of nutrient supply through the soil food web, which in our study was confirmed by the significantly higher potential N-mineralization (aerobic incubation) and potentially mineralizable C in clover. The potential N-mineralization of the grass-clover mixture was significantly higher than in GN0, but not different to GN1. Elgersma and Hassink (1997) also found a higher N-mineralization in a grass-clover mixture than in grass-only, both without fertilization. They explained the higher N mineralization in grass-clover from the lower C/N ratio of the organic matter fractions that are decomposed by the soil biota (light fraction of the macro-organic matter). In our study, the mixture of grass-clover showed a higher potentially mineralizable C than grass-only, which suggests that the positive effect of clover on the supply of nutrients through the soil food web is maintained in the mixture.

3.5 Conclusions

In comparison with grass-only, white clover-only had a lower total root biomass and a lower C/N ratio in the above- and below-ground plant biomass. These plant characteristics of clover were reflected in a lower bacterial biomass, a lower fungal biomass, a higher proportion of bacterivorous nematode dauerlarvae, a lower proportion of herbivorous nematodes, and a greater abundance of earthworms.

Grass-clover (20-30% clover in the DM) was comparable with grass fertilized with 150 kg N ha^{-1} of inorganic fertilizer in terms of quantity and quality (C/N ratio) of the above- and below-ground plant biomass, and its effect on soil biota. Hence, most effects of clover on the soil biota could be explained from the quantity and C/N ratio of the root and plant litter, although grass-clover had a higher proportion of nematode dauerlarvae and a different composition of the nematode community to GN1. Only for these two parameters, the availability of easily decomposable material in the rhizosphere of clover and specific litter quality aspects such as plant defensive compounds in clover may have modified the influence on the soil biota.

The soil in white clover-only had a higher proportion of angular blocky elements than in grass-only, a lower penetration resistance, a higher number of earthworm burrows, a higher potential N-mineralization and potentially mineralizable C. This suggests that clover stimulates the ecosystem services of water infiltration and supply of nutrients but reduces the soil structure maintenance. The grass-clover mixture differed from the grass-only treatments in a higher potentially mineralizable C, and differed from clover-only in a higher percentage of soil crumbs. We infer that, when clover is introduced in grassland to reduce the reliance on inorganic fertilizer, the mixture of grass and clover maintains the positive impact of grass roots on soil structure and increases the supply of nutrients via the soil food web. Thus, a grass-clover mixture combines the agronomic benefits of the two plant types.

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Chapter 4

Soil biological quality of grassland fertilized with adjusted cattle manure slurries in comparison with organic and inorganic fertilizers

N. van Eekeren, H. de Boer, J. Bloem, T. Schouten, M. Rutgers, R. de Goede, L. Brussaard, 2009. Biology and Fertility of Soils 45, 595-608.

Abstract

We studied the effect of five fertilizers (including two adjusted manure slurries) and an untreated control on soil biota and explored the effect on the ecosystem services they provided. Our results suggest that the available N (NO_3^- and NH_4^+) in the soil plays a central role in the effect of fertilizers on nematodes and micro-organisms. For micro-organisms this effect is both via a direct nutrient availability and indirectly via the grass root mass and for nematodes this is indirect via the microbial biomass and the grass root mass. A lower amount of available N in the treatment with inorganic fertilizer was linked to a higher root mass and a higher abundance and proportion of herbivorous nematodes. A higher amount of available N in the organic fertilizer treatments resulted in a twofold higher bacterial activity (measured as bacterial growth rate, viz. thymidine incorporation), a higher proportion of bacterivorous nematodes, a 30% higher potential N-mineralization (aerobic incubation), and 25-50% more potentially mineralizable N (anaerobic incubation). Thus, compared to inorganic fertilizer, organic fertilization increased the organic C, the total N, the activity of decomposers and the supply of nutrients via the soil food web. Within the group of organic fertilizers there was no significant difference in organic C, in abundances of soil biota and the potential N- mineralization rate. Thus, we have no indications that farmyard manure or the adjusted manure slurries provided the ecosystem service ‘supply of nutrients’ better than normal manure slurry. Normal manure slurry provided the highest bacterial activity and the highest amount of mineralizable N and it was the only fertilizer resulting in a positive trend in grass yield over the years 2000-2005. The number of earthworm burrows was higher in the treatments with organic fertilizers compared to the one with the inorganic fertilizer, which suggests that organic fertilizers stimulate the ecosystem service of water regulation more than inorganic fertilizer. The trend towards higher epigeic earthworm numbers with application of farmyard manure and one of the adjusted manure slurries, combined with the negative relation between epigeic earthworms and bulk density, and a significantly lower penetration resistance in the same fertilizer types, is preliminary evidence that these two organic fertilizer types contribute more to the service of soil structure maintenance than inorganic fertilizer.

Keywords

Earthworms, nematodes, microbiology, slurry, fertilizer, ecosystem services, grassland

4.1 Introduction

Soil biota play an important role in ecological processes in the soil and in the provision of various ecosystem services, such as maintenance of soil structure, water regulation, and supply of nutrients (Brussaard et al., 1997; Swift et al., 2004; Mulder 2006; Kibblewhite et al., 2008). In the search for sustainable agricultural grassland systems, these processes and ecosystem services, provided by soil biota, become more important (Brussaard et al., 2007a). Therefore, insight is needed into the mechanisms by which soil biota are influenced by grassland management, and what changes in soil biota mean for the functioning of the plant-soil system and the provision of ecosystem services.

Fertilization with inorganic and organic fertilizers is common in grassland management. Especially when initial levels are low, soil organic matter content and soil biological activity can be enhanced through inorganic as well as organic fertilizers. Inorganic fertilizers (containing only mineral N) feed the plant and soil micro-organisms directly and the entire soil biota indirectly by increased root biomass and exudates, and plant litter. However, inorganic fertilizers involve a high fossil energy consumption and are easily lost from the soil by nitrate leaching and denitrification. Organic fertilizers (20-50% mineral N) on the other hand feed the soil biota directly and the crop both directly (inorganic constituents) and indirectly following mineralization of organic constituents. When more N is organic and mineralized slowly, the risk of leaching is low as long as an actively growing crop is present that can take up the nutrients supplied. In older grasslands with relatively high soil organic matter levels, lower inputs of N may result in crop yields equal to those in grasslands with relatively low soil organic matter levels (Sonneveld and Bouma, 2003; Reijs et al., 2007). Many studies, mainly in arable soils, have shown that organic fertilizers can increase organic matter content, soil biological activity, and potential N mineralization (De la Lande Cremer, 1976; Mäder et al., 2002; Bittman et al., 2005; Fliessbach et al., 2007; Birkhofer et al., 2008). However, in grassland soils with a crop year-round, 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.

Due to legislative restrictions on the use of organic and inorganic fertilizers (Vellinga, 2006), animal manure is being re-valued, not only as a nutrient source but also as a means to improve soil quality (Velthof et al., 2000). Whereas animal manure was applied as farmyard manure until the 1970s, today it is slit-injected as manure slurry. Some researchers have stated that manure slurry with a low C/N ratio could have adverse effects on soil biological quality through significant amounts of phytotoxic components (Van Bruchem et al., 2000). Also, there is a general feeling among many dairy farmers that farmyard manure is the best organic fertilizer to improve soil quality. Although there is a renewed interest in deep-litter stables with a straw bedding, most dairy herds in The Netherlands are kept in a cubicle housing in which animal manure is produced in the form of slurry (faeces mixed with urine). To achieve the supposed benefits of farmyard manure, farmers are searching for methods to adjust manure slurry quality to that of farmyard manure by increasing the C/N ratio and achieving a lower mineral N/total N ratio (Verhoeven et al., 2003). This is either done by dietary adjustments (lower crude protein content and/or higher fiber content in the ration) or by mixing and composting manure slurry with other organic materials, like hay. However, little is known about the effects of these adjusted manure slurries on soil quality, especially on soil biological quality. It is also not known whether farmyard manure is really the best organic fertilizer for optimizing soil biological quality in grassland and what differences exist with other organic and inorganic N fertilizers.

Studies on the effect of specific quality aspects of organic fertilizers (higher C/N ratio and lower mineral N/total N ratio) on soil biota are rare. Cotton and Curry (1980) measured little difference in the effect of cattle (relatively high C/N) or pig (relatively low C/N) manure slurry application on earthworms. Griffiths et al. (1998) observed that the number of protozoa responded more quickly to 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. Van Den Pol-van Dasselaar et al. (2006) showed a positive correlation between organic N in cattle manure slurry and bacterial feeding nematodes in the soil, and a negative correlation between the mineral N content of manure slurry and predaceous nematodes in the soil. Sørensen (1998) found that the addition of straw to slurry stimulated soil microbial activity (measured as soil respiration). De Vries et al. (2006) measured no difference in bacterial and fungal biomass between manure slurry and farmyard manure applied on grass-clover. De Goede et al. (2003) measured higher numbers of earthworms in slurry amended with the additive Euromestmix®. Soil organic matter and organic N accumulation are determined by the input of C rather than the input of N (Ryden, 1984; Hassink, 1994). Soil organic C increases with the amount of C added to the soil (Van Den Pol-van Dasselaar and Lantinga, 1995). Fauci and Dick (1994) concluded that long-term organic soil amendments increase biological activity in proportion to the amount of C added. Therefore, we expect that, in the long-term, organic fertilizer with a relatively high C/N ratio (such as farmyard manure) will lead to a higher organic matter content, a higher N mineralization (Reijs et al., 2007) and a higher biomass of soil biota than organic fertilizers with a relatively low C/N ratio (such as normal manure slurry). In none of the above studies, manure slurries with adjusted qualities were compared with farmyard manure.

We carried out a five-year fertilization experiment with four different organic fertilizers, one inorganic N fertilizer and an untreated control (i.e., no N fertilizer) to assess differences in species richness, abundance and activity of earthworms, nematodes, bacteria, and fungi.

We measured bacteria and fungi as primary consumers in the soil food web. Nematodes were measured as representatives of the secondary consumers in the soil food web, while earthworms were measured as representatives of the ecosystem engineers. Our objectives were (1) to compare the effects of an inorganic N fertilizer and four organic fertilizers on soil biota, and (2) to explore the relevance of changes and/or differences in soil biota for the provision of ecosystem services (maintenance of soil structure, water regulation and supply of nutrients) in the plant-soil system. We hypothesized that, compared to inorganic N fertilizer, organic fertilizers would increase the species richness of earthworms, nematodes and micro-organisms and enhance the microbial activity. Furthermore, we hypothesized that, among the organic fertilizers, farmyard manure would have the most positive effect on the species richness of the soil biota, while normal manure slurry would stimulate the activity of the soil biota. Adjusted manure slurries would take an intermediate place between normal manure slurry and farmyard manure.

4.2 Materials and methods

4.2.1 Sampling site and experimental design

The experiment was established on a sandy soil (Gleyic podzol) in the southern part of The Netherlands (51º31´N, 5º42´E) in a permanent grassland sown in the autumn of 1997 with a seed mixture of *Lolium perenne* cultivars. In the years before the experiment started, the grassland had been fertilized with animal excreta during grazing (approximately150 kg total N ha^{-1} year^{-1}), manure slurry (approximately150 kg total N ha^{-1} year^{-1}) and inorganic fertilizer (approximately150 kg N ha^{-1} year^{-1}). In the spring of 2000 the treatments were installed comprising an untreated control (CON) and five different fertilizer types: inorganic N fertilizer (calcium ammonium nitrate 27%, CAN), "normal" cattle manure slurry (NMS), manure slurry of cattle fed a ration low in protein (LMS), manure slurry mixed and composted with hay from roadsides (CYM) and farmyard manure from a deep-litter stable with dairy cows (FYM). For details, see Table 4.1. Plot size was 8 m x 2.80 m; the samples were taken from the centre 6 m x 1.5 m of the plots. Two thirds of the total amount of fertilizers was applied before the first harvest of the grass and one third before the second harvest. The applied effective N with the fertilizers in 2004 was calculated according to the Dutch official advice on grassland fertilization as (Working coefficient mineral N * mineral N) + (Working coefficient organic N * organic N) http://www.bemestingsadvies.nl).The two manure slurries (NMS and LMS) were slit-injected, whereas the solid manures (CYM and FYM) were surface-applied. All treatments were replicated four times in four randomized blocks. The plots were harvested with a mower four to five times a year and not grazed. All plots received a basal fertilizer application of triple super phosphate (19.6 % P) and muriate of potash (33.2 % K).

Table 4.1 Average application rate (kg ha^{-1} $year^{-1}$) per treatment and characteristics of the fertilizer types.

Kg ha^{-1}	Treatments					
	CON	CAN	NMS	LMS	CYM	FYM
Total organic matter	0	0	3361	3718	4161	6347
Mineral N	0	150	104	102	13	46
Organic N	0	0	98	104	170	171
C/total N	-	-	7.5	8.1	10.2	13.2

Treatments: unfertilized (CON) or fertilized with inorganic N fertilizer (calcium ammonium nitrate 27%, CAN), “normal” cattle manure slurry (NMS), manure slurry of cattle fed a ration low in protein (LMS), manure slurry mixed and composted with hay from roadsides (CYM) and farmyard manure from a deep-litter stable with dairy cows (FYM).

4.2.2 Soil sampling, chemical and physical measurements and grass production

On the 4th of October 2004, five growing seasons after the start of the experiment, soil samples for analysis of chemical, physical and biological soil quality were taken. Per plot, a bulk sample of 70 cores (0-10 cm, ø 2.3 cm) was collected, sieved through 1 cm mesh, homogenized and stored at field moisture content at 4°C before analysis. Sub-samples were taken for chemical analysis, nematode analysis, and microbiological analysis.

Prior to chemical analysis, sub-samples were oven-dried at 40 °C. Soil acidity of the oven-dried samples was measured in 1 M KCl (pH-KCl) with a standard electrode. Organic C was measured by incineration of dry material at 1150 °C, after which the produced CO_2 was determined by an infrared detector (LECO Corporation, St. Joseph, Mich., USA). For determination of total N, evolved gases after incineration were reduced to N_2 and detected with a thermal-conductivity detector (LECO Corporation, St. Joseph, MI, USA). The N supply capacity of the soil was calculated from Total N in the soil, according to the Dutch official advice on grassland fertilization (http://www.bemestingsadvies.nl).

Soil bulk density was measured in the 5-10 cm layer, in three undisturbed ring samples containing 100 cm^3 soil each (Eijkelkamp, Giesbeek, The Netherlands). Soil penetration resistance was measured with an electronic penetrometer (Eijkelkamp, Giesbeek, The Netherlands) with a cone diameter of 1 cm^2 and a 60° apex angle. Cone resistance was recorded per cm of soil depth and expressed as the average value of 10 penetrations per plot in the soil layers of 0-10 cm, 10-20 cm, 20-30 cm, 30-40 cm, 40-50 cm. Soil structure was determined in one block (20 cm x 20 cm x 10 cm) per plot. Soil of this block was assessed by visual observation as crumbs, sub-angular blocky and angular blocky elements (FAO 2006). Amounts were weighed per type and expressed as a percentage of total fresh soil weight. To determine root biomass, three soil cores (0-10 cm, ø 8.5 cm) per plot were taken. After the soil in the samples was thoroughly washed out with water, root mass was sieved (mesh size 2 mm) and root dry matter was determined after oven-drying at 70 °C.

In 2004, grass was cut four times (14th of May, 22nd of June, 3rd of August, and 28th of September), using a 'Haldrup' small-plot harvester. The harvested area was 8 m x 1.5 m. Grass was cut at a stubble height of 6 cm, weighed, and sampled for dry matter analysis. Dry matter was determined after drying at 70 °C.

4.2.3 Soil biological parameters

4.2.3.1 Earthworms

To determine earthworm parameters, one soil block of 20 cm x 20 cm x 20 cm was sampled per plot. Only one sample per plot could be taken to prevent disruption of the experiment. This limited the statistical analysis of the earthworm data. Immediately after sampling, earthworm burrows with a diameter >2 mm were counted on the horizontal surface (20 cm x 20 cm) exposed at 10 cm depth. Hereafter, the blocks were transferred to the laboratory where the earthworms were hand-sorted, counted, weighed, and fixed in 70 % ethanol prior to identification. Numbers and biomass were expressed per m^2. Adults were identified to species, with a distinction between (1) epigeic species (pigmented, living superficially in the litter layer, little burrowing activity), (2) endogeic species (living in predominantly horizontal burrows at approximately 10-15 cm depth) and (3) anecic species (relatively large worms, living in vertical burrows from which they collect dead organic matter from the surface at night) (Bouché, 1977).

4.2.3.2 Nematodes

Free-living nematodes were extracted from a subsample of about 100 g field-moist soil, using the Oostenbrink elutriator (Oostenbrink, 1960). Total nematode numbers were counted and expressed per 100 g fresh soil. Nematodes were fixed in hot formaldehyde (4%), and at least 150 randomly selected nematodes from each sample were identified to genus and, whenever possible, to species. Nematode genera and species were assigned to trophic groups, following Yeates et al. (1993), and allocated to the colonizer-persister groups (cp-groups), following Bongers (1990) and Bongers et al. (1995). The Nematode Channel Ratio (NCR) was calculated to express the relative contributions of bacterivorous (B) and fungivorous (F) nematodes to the total nematode abundance (NCR=B/(B+F)) (Yeates 2003). The Maturity Index was calculated as the weighted mean of the individual cp-values, in accordance with Bongers (1990) and Korthals et al. (1996). The Maturity Index is an ecological measure which indicates the condition of an ecosystem based on non plant-parasitic nematode life-history group composition.

4.2.3.3 Microbial parameters

A sub-sample of 200 g field-moist soil was taken from the bulk soil sample, adjusted to 50% water holding capacity and pre-incubated at 12 °C for four weeks, to avoid the effects of temperature and moisture fluctuations in the field and to stabilize soil conditions (Bloem et al. 2006). After pre-incubation, fungal and bacterial biomass, bacterial growth rate and Community-Level Physiological Profiles (CLPP) were determined. Microscopic soil smears were prepared and measured as described by Bloem and Vos (2004). Fungal hyphae were measured using the grid intersection method. Bacterial numbers and cell volumes were measured by confocal laser scanning microscopy and automatic image analysis (Bloem et al., 1995). Bacterial biomass was calculated from biovolume. Bacterial growth rate was determined as the incorporation of [^{3}H]thymidine and [^{14}C]leucine into bacterial DNA and proteins respectively (Bloem and Bolhuis, 2006; Michel and Bloem, 1993).

The CLPP's of the bacterial communities in the soil extracts were determined with ECO-plates from Biolog Inc. (Hayward, USA). These plates contain a triplicate set of 31 different C substrates, a control, a freeze-dried mineral medium and a tetrazolium redox dye. For each bacterial extract, a dilution series was made using 10 mM BisTris buffer at pH 7. Each dilution series (3^{-1} down to 3^{-12}) was used to inoculate four ECO-plates with a volume of 100 µl per well. The colour formation in the plate was measured every 8 hours for 7 days with a plate reader spectrophotometer at 590 nm. The CLPP's were calculated from the colour formation in the wells, and corrected for inoculum density using a regression approach applied to the average well color development (AWCD) as described by Rutgers et al. (2006). This produced CLPPs describing the relative abundance from substrate conversion (31 substrates; log-scaled). To survey the bacterial community activity in the ECO-plate, the AWCD was calculated after 7 days of incubation. The CLPP-slope parameter was calculated from the color development in the ECO-plates. This parameter indicates the rate at which the capacity of the soil to degrade a set of carbon and energy substrates disappears upon dilution. A low slope parameter is indicative of a slow disappearance rate and can be considered a measure of high physiological diversity (Gomez et al., 2004; Rutgers et al., 2006).

Potential C mineralization (soil respiration) was measured by gas chromatography weekly as CO_2 evolution after a 6-week period of incubation. Soil was homogenized, sieved (5 mm mesh size) and brought to 50-60% of the water-holding capacity. Sub-samples of 200 g soil were incubated in the dark at 20 ºC in 1.5 l air-tight jars supplied with a gas septum. The gas chromatograph was a Carlo Erba 6000 with a column switching system, equipped with a 4-m Porapak q and a 2 mmol sieve 5A column. The detector (HWD) temperature was 180ºC, the column temperature was 50ºC, and the injection volume was 1 ml (Bloem et al., 1994). Potential C mineralization was expressed as mg C respired kg soil^{-1} week^{-1}. The results of the first week were not used to avoid effects of soil homogenization.

In the same jars in which potential C mineralization was measured, the potential N mineralization rate was determined as the increase in mineral N (ammonium plus nitrate) between week 1 and week 6. The first week was not used to avoid effects of soil homogenization. Sub-samples of 80 g soil were extracted with 200 ml M KCl. After 1 h shaking, the extracts were filtered over a paper filter and mineral-N contents (ammonium and nitrate) were determined by Skalar Segmented Flow Analysis (Breda, The Netherlands).

In addition to potential N mineralization by aerobic incubation of soil, potentially mineralizable N was measured by anaerobic incubation of a soil sample under water (in slurry) for 1 week at 40°C (Keeny and Nelson, 1982; Canali and Benedetti, 2006). These warm and anoxic conditions are optimal for a quick mineralization of organic matter by anaerobic bacteria. The lack of oxygen prevents conversion of released NH_4^+ to NO_3^- (nitrification), so N losses by denitrification can not occur. The amount of mineral N (NH_4^+-N) released is a measure of the quality (N-content and decomposability) of the organic matter, and thus for biological soil fertility (Sparling and Schipper, 2002).

4.2.4 Statistical analyses

The data were analyzed with GENSTAT (8th Edition, VSN International, Hemel Hempstead, UK). All parameters were analyzed using an one-way ANOVA in randomized blocks with treatments (CON, CAN, NMS, LMS, CYM, FYM) as factors. Treatment effects were apportioned on the basis of Least Significant Difference. P-values of the tests are presented in Tables 4.2-4.5. In case no significant ($P>0.05$) individual treatment effects were found, an unbalanced ANOVA in randomized blocks was carried out with CON, CAN and organic fertilizers combined (OFC) as factors. When relations between parameters were expected, a regression analysis was carried out. Data of nematode taxa and CLPP's were square root-transformed and subjected to redundancy analysis (RDA) (CANOCO 4.5, Biometris, Wageningen, The Netherlands). A Monte Carlo permutation test (499 random permutations) was performed to test for statistically significant ($P\leq0.05$) relationships between community structure and treatment effects following a multivariate analysis of variance design (Van Dobben et al., 1999).

4.3 Results

4.3.1 Soil chemical, physical parameters, and grass production

The application of organic fertilizers, considered separately, did not result in higher levels of organic C or total N than the application of inorganic fertilizer (CAN) and the untreated control (CON) (Table 4.2). However, when the organic fertilizers were considered as

Table 4.2 Soil chemical and physical characteristics in grassland (0-10 cm).

Chemical/ Physical	Units	Treatments						
		CON	CAN	NMS	LMS	CYM	FYM	P-value
Organic C	g C kg dry soil^{-1}	20.2	19.5	27.9	25.3	25.4	27.4	NS
Total N	g N kg dry soil^{-1}	1.40	1.41	1.97	2.03	2.05	2.05	NS
pH-KCl		5.30 b	5.10b	5.90a	5.90a	5.80a	5.88a	<0.001
Bulk density	g cm^{-3}	1.28	1.29	1.24	1.27	1.17	1.22	NS
Penetration resistance								
0-10 cm	mPa	1.22ab	1.29a	1.18ab	1.23ab	1.05c	1.13b	0.019
10-20 cm	mPa	2.11	2.21	2.05	2.03	1.79	2.00	NS
20-30 cm	mPa	2.62	2.54	2.96	2.66	2.36	2.56	NS
30-40 cm	mPa	2.75	2.60	3.36	2.82	2.85	2.77	NS
40-50 cm	mPa	2.39	2.18	3.18	2.14	2.63	2.24	NS
Soil structure								
Crumb	%	46	41	42	49	49	52	NS
Sub-angular	%	49	47	48	45	43	45	NS
Angular	%	5	13	10	7	9	3	NS
Root biomass	g DM m^{-2}	1056a	1035a	707b	696b	804b	813b	<0.001
Grass aboveground biomass								
1e harvest	g DM m^{-2}	190d	437ab	533a	421b	323c	431b	<0.001
2nd harvest	g DM m^{-2}	200d	339a	273b	214cd	251bc	226bcd	<0.001
3rd harvest	g DM m^{-2}	72c	133ab	173a	137ab	126b	165ab	<0.001
4th harvest	g DM m^{-2}	99	132	132	105	114	134	NS
Total	g DM m^{-2}	560c	1041a	1111a	878b	813b	956ab	<0.001

Values indicated by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

Treatments: unfertilized (CON) or fertilized with inorganic N fertilizer (calcium ammonium nitrate 27%, CAN), “normal” cattle manure slurry (NMS), manure slurry of cattle fed a ration low in protein (LMS), manure slurry mixed and composted with hay from roadsides (CYM) and farmyard manure from a deep-litter stable with dairy cows (FYM).

one overall treatment (OFC), this resulted in higher levels of organic C (P=0.01) and total N (P=0.004) in the soil treated with organic fertilizers. The pH-KCl of the individual organic fertilizers was significantly higher than CAN and CON (Table 4.2). The soil bulk density of the CYM treatment tended (P=0.081) to be lower compared to CAN and CON. In the soil layer 0-10 cm, the soil penetration resistance was significantly (P=0.019) lower in CYM and FYM than CAN. The soil penetration resistance in the 0-10 cm soil layer was positively related with the soil bulk density in the 5-10 cm soil layer (R^2=0.52, P<0.001). After five years, the treatments had no visible effect on soil structure. The root mass in the soil layer of 0-10

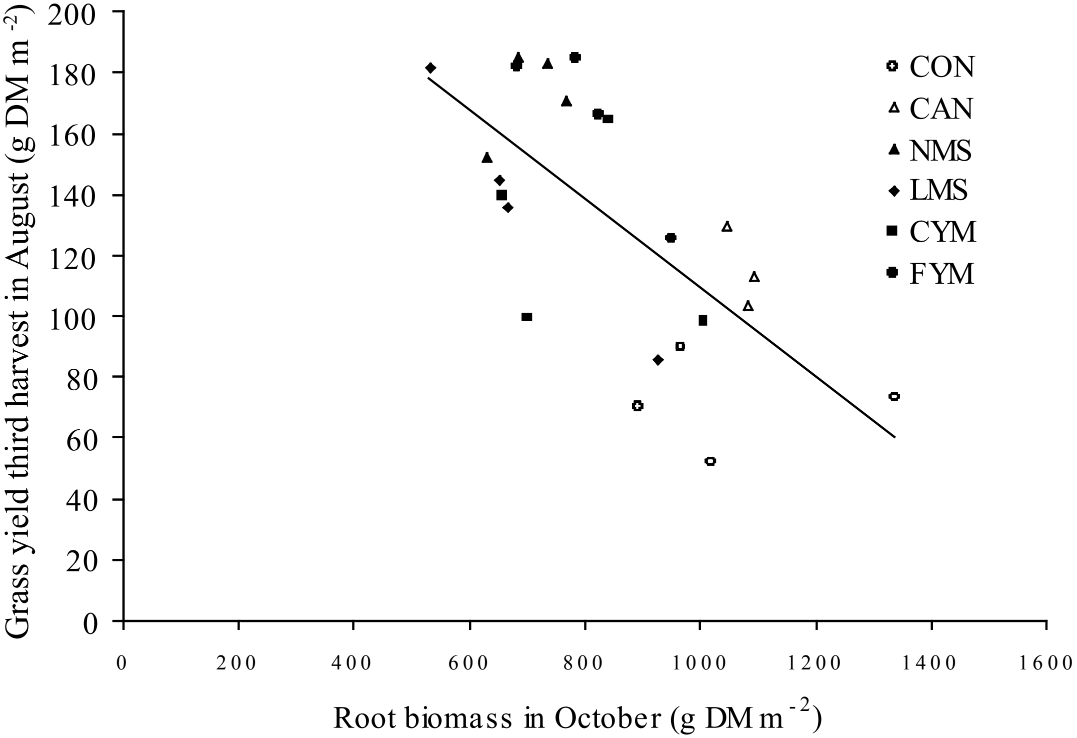

Fig. 4.1 Relationship between root biomass (g DM m^{-2}) in the 0-10 cm soil layer and the dry matter yield of grass (kg DM ha^{-1}) in the third harvest (R^2=0.43, P<0.001). Treatments: unfertilized (CON) or fertilized with inorganic N fertilizer (calcium ammonium nitrate 27%, CAN), "normal" cattle manure slurry (NMS), manure slurry of cattle fed a ration low in protein (LMS), manure slurry mixed and composted with hay from roadsides (CYM) and farmyard manure from a deep-litter stable with dairy cows (FYM).

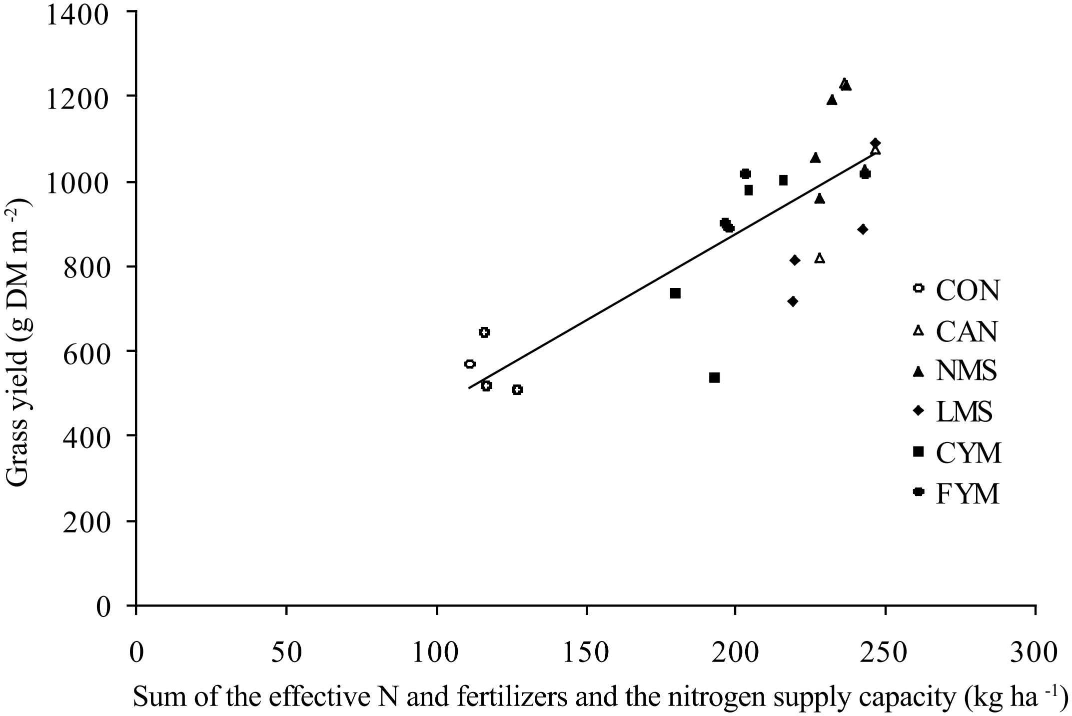

Fig. 4.2 Relationship between the sum of the effective N from the fertilizers (kg ha^{-1}) and the N supply capacity of the soil, and the dry matter yield of grass (g DM m^{-2}) (R^2=63, $P<0.001$). Treatments: unfertilized (CON) or fertilized with inorganic N fertilizer (calcium ammonium nitrate 27%, CAN), "normal" cattle manure slurry (NMS), manure slurry of cattle fed a ration low in protein (LMS), manure slurry mixed and composted with hay from roadsides (CYM) and farmyard manure from a deep-litter stable with dairy cows (FYM).

cm was lower in the organic fertilizer treatments than in CAN and CON. The root mass, measured in October, was negatively related with the dry matter production of the grass in the first (P=0.007), third ($P<0.001$) and fourth (P=0.047) harvest, but most of the variance was explained by the data from the third harvest, in August (R^2=0.43, $P<0.001$; Fig. 4.1). Root mass was also negatively related with the potentially mineralizable N (anaerobic incubation) (R^2=0.22, P=0.015). The total grass yield was highest for NMS and CAN, while CON had the lowest yield. Values for FYM, LMS and CYM were intermediate. Total grass yield was positively related with soil total N (R^2=0.14, P=0.043), bacterial growth rate (thymidine incorporation) (R^2=0.16, P=0.030), the proportion of bacterivorous nematodes (R^2=0.17, P=0.027) and the effective N from fertilizers (R^2=0.54, P<0.001). Variance of total grass yield was best explained with a regression model of the sum of the effective N from fertilizers and the N supply capacity of the soil (R^2=0.63, P<0.001; Fig. 4.2). The relationship between total grass yield and potentially mineralizable N (anaerobic incubation) was significant (R^2=0.24, P=0.037) when the CAN-treatment was left out of the analysis.

4.3.2 Soil biological parameters

4.3.2.1 Earthworms

Total earthworm biomass was not significantly different between the treatments since variance was high and only one sample per plot could be taken (Table 4.3). The total earthworm biomass was positively related with the proportion of crumbs in the 0-10 cm soil layer (R^2=0.26, P=0.007). The earthworm species richness in this experiment was limited; only *Lumbricus rubellus* (epigeic adults) and *Aporrectodea caliginosa* (endogeic adults) were found. No anecic species were found, not even after mustard extraction in a selection of the plots. *L.*

Table 4.3 Earthworm numbers, biomass, species, functional groups and earthworm burrows in grassland (0-20 cm).

Earthworms	Units	Treatments						
		CON	CAN	NMS	LMS	CYM	FYM	P-value
Total number	n m^{-2}	144	125	231	175	144	269	NS
Body biomass	g $worm^{-1}$	0.43	0.18	0.24	0.34	0.35	0.48	NS
Total biomass	g m^{-2}	58	24	63	67	49	82	NS
Ratio adult/juvenile		1.4	0.7	0.8	1.2	1.6	0.9	NS
Epigeic adults	%	40	43	38	43	70	72	NS
Endogeic adults	%	60	57	62	57	30	27	NS
Earthworm burrows								
10 cm depth	n m^{-2}	6	13	25	56	38	63	NS

Treatments: unfertilized (CON) or fertilized with inorganic N fertilizer (calcium ammonium nitrate 27%, CAN), "normal" cattle manure slurry (NMS), manure slurry of cattle fed a ration low in protein (LMS), manure slurry mixed and composted with hay from roadsides (CYM) and farmyard manure from a deep-litter stable with dairy cows (FYM).

rubellus dominated the earthworm adult population after five years of FYM and CYM application, while *A. caliginosa* was more dominant in the other treatments. The proportion of epigeic adults was negatively related with the soil bulk density in the 5-10 cm soil layer (R^2=0.41, P<0.001). The number of earthworm burrows was significantly higher in the organic fertilizer treatments (P=0.041) when compared as one group (OFC) with CAN and CON.

4.3.2.2 Nematodes

Nematode abundance was not affected by five years of fertilization with different fertilizer types (Table 4.4). However, CAN and CON were dominated by herbivorous nematodes (P<0.001), whereas the treatments with organic fertilizer were dominated by bacterivorous nematodes (P=0.002). No N fertilization (CON) for five years resulted in the highest proportion of herbivorous nematodes and the lowest proportion of bacterivorous nematodes, whereas applying NMS for five years resulted in the highest proportion of bacterivorous and the lowest proportion of herbivorous nematodes. The abundance of herbivorous nematodes was positively related with a higher root mass in the 0-10 cm layer (R^2=0.15, P=0.037). The proportion of bacterivorous nematodes increased with a higher bacterial growth rate (viz. thymidine incorporation or DNA synthesis) (R^2=0.27, P=0.006). The relatively low proportion of bacterivorous nematodes in CON resulted in a significantly (P=0.002) lower Nematode Channel Ratio (NCR) in CON compared to all individual fertilizer treatments. The life-history group distribution in CAN showed a high percentage of nematodes in the cp-2 group, where a high percentage of enrichment opportunists (cp-1) was expected. Differences between treatments in percentages of nematodes in the cp-2 to cp-5 group did not result in differences in the MI (cp1-5) and MI (cp2-5) (not shown). In a multivariate analysis (RDA) of all nematode taxa, no statistically significant effects of the different treatments were found.

4.3.2.3 Microbial parameters

Bacterial and fungal biomass, and the fungal/bacterial biomass ratio were not affected by five years of fertilization with different fertilizer types (Table 4.5). However, the bacterial growth rate (thymidine incorporation or DNA synthesis), was more than twofold higher in NMS than in CAN and CON (P<0.001). CYM, LMS and FYM were intermediate. A higher thymidine incorporation was negatively related with root mass (R^2=0.46, P<0.001). The fungal activity did not show significant differences between the treatments.

The CLPP-slope parameter and the amount of extracted soil necessary for conversion of 50% of all substrates in ECO plates were not significantly different between the treatments. In a multivariate analysis (RDA) of all CLPP's simultaneously (Fig. 4.3), all canonical axes together were statistically significant (P=0.012). A significant relationship was found with FYM (P=0.006) and a marginally significance relationship with CYM (P=0.054), i.e. both fer-

Table 4.4 Nematodes abundance, trophic groups, life-history groups (cp=colonizer-persister groups) and community structure indices in grassland (0-10 cm).

Nematodes	Units	Treatments						
		CON	CAN	NMS	LMS	CYM	FYM	P-value
Total number	n 10^3 m^{-2}	5381	4932	4788	4804	4146	5045	NS
Bacterivorous	n 10^3 m^{-2}	2082	1678	2642	2289	1965	2469	NS
Herbivorous	n 10^3 m^{-2}	2739ab	2920a	1772c	1999c	1863c	2039bc	0.017
Fungivorous	n 10^3 m^{-2}	227	138	98	123	139	96	NS
Omnivorous	n 10^3 m^{-2}	286	220	250	314	150	386	NS
Carnivorous	n 10^3 m^{-2}	0	21	11	66	28	54	NS
Bacterivorous	%	34c	39bc	57a	47ab	47ab	48a	<0.001
Herbivorous	%	56a	54ab	35d	42cd	45bc	41cd	0.002
Fungivorous	%	5	3	2	3	3	2	NS
Omnivorous	%	6	4	5	6	4	8	NS
Carnivorous	%	0	0	0	2	1	1	NS
Cp-1	%	31	26	29	22	27	30	NS
Cp-2	%	50bc	61a	57ab	57ab	51bc	48c	0.019
Cp-3	%	6b	4b	5b	7b	13a	6b	0.031
Cp-4	%	5b	2b	7ab	13a	6b	13a	0.007
Cp-5	%	9	8	2	1	2	3	NS
Number of taxa		22	23	23	25	24	23	NS
Maturity Index (cp1-5)		2.12	2.04	1.97	2.12	2.10	2.10	NS
Nematode Channel Ratio		0.89b	0.94a	0.96a	0.95a	0.94a	0.96a	0.002

Values indicated by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

Treatments: unfertilized (CON) or fertilized with inorganic N fertilizer (calcium ammonium nitrate 27%, CAN), "normal" cattle manure slurry (NMS), manure slurry of cattle fed a ration low in protein (LMS), manure slurry mixed and composted with hay from roadsides (CYM) and farmyard manure from a deep-litter stable with dairy cows (FYM).

tilizers with a high C/N ratio (Table 4.1). Differences in CLPP's originate from differences in capability of the bacterial communities to degrade a suite of carbon and energy substrates. In the treatments with FYM and CYM there was a preference for substrates with carbohydrates (CH; Fig. 4.3) whereas in all other treatments substrates like carboxylic acids appeared to be preferred (CA; Fig. 4.3).

Table 4.5 Microbial biomass, activity, Community-Level Physiological Profiles (CLPP), potential N mineralization, potentially mineralizable N, potential C mineralization, potentially C mineralization in grassland (0-10 cm).

Microbial	Units	Treatments						
		CON	CAN	NMS	LMS	CYM	FYM	P-value
Bact.biomass	μg C g dry soil^{-1}	51	41	56	57	53	52	NS
Fung. Biomass	μg C g dry soil^{-1}	15	14	15	16	14	17	NS
Thym. Incorp.	pmol g dry soil^{-1} h^{-1}	43c	41c	101a	65bc	74b	53bc	<0.001
Leuc. Incorp.	pmol g dry soil^{-1} h^{-1}	583	591	901	652	720	638	NS
Fung. Activity	% of hyphal length	5	5	1	6	10	1	NS
CLPP								
Slope		0.40	0.39	0.40	0.44	0.38	0.41	NS
ES50[*]	µg dry soil	827	877	941	872	806	928	NS
Pot.N mineral.	mg N kg dry soil^{-1}wk^{-1}	8.97a	7.22b	9.57a	9.45a	10.10a	9.65a	0.024
Pot.mineral. N	mg N kg dry soil^{-1}	65.1c	58.8c	88.9a	72.8bc	82.6ab	80.1ab	0.004
Pot.C mineral.	mg C kg dry soil^{-1}wk^{-1}	88.8	81.4	83.4	94.9	117.8	109.3	NS
Microbial metabolic quotient (qCO_2)		1.57	1.59	1.23	1.29	1.83	1.66	NS

[*] ES50=Effective weight of soil needed for 50% substrate utilization.

Values indicated by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

Treatments: unfertilized (CON) or fertilized with inorganic N fertilizer (calcium ammonium nitrate 27%, CAN), "normal" cattle manure slurry (NMS), manure slurry of cattle fed a ration low in protein (LMS), manure slurry mixed and composted with hay from roadsides (CYM) and farmyard manure from a deep-litter stable with dairy cows (FYM).

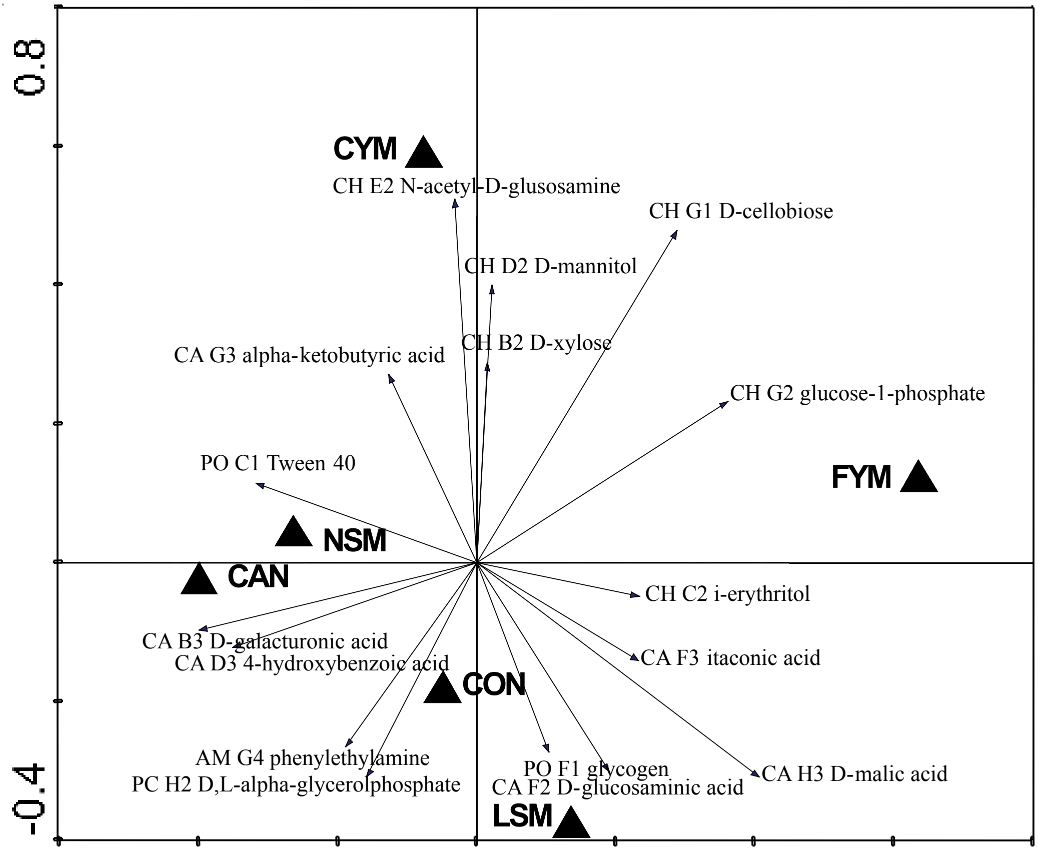

Fig. 4.3 Ordination diagram of CLPP's of bacterial communities based on RDA. Treatments: unfertilized (CON) or fertilized with inorganic N fertilizer (calcium ammonium nitrate 27%, CAN), "normal" cattle manure slurry (NMS), manure slurry of cattle fed a ration low in protein (LMS), manure slurry mixed and composted with hay from roadsides (CYM) and farmyard manure from a deep-litter stable with dairy cows (FYM).

CH=carbohydrate, PO=polymer, AA=amino acid, CA=carboxylic acid, AM=amine

The potential N-mineralization (aerobic incubation) was significantly (P=0.024) lower for CAN compared to the individual organic fertilizer treatments and CON (Table 4.5). The potentially mineralizable N (anaerobic incubation) was significantly (P=0.004) lower for the CON and CAN treatments. In contrast to the potential N-mineralization, the potentially mineralizable N did show differences among the organic fertilizers. NMS had a significantly higher value than LMS. CYM and FYM were intermediate in value. The potential N-mineralization (aerobic incubation) increased with a higher pH (R^2=0.30, P=0.003) and a higher percentage of crumbs (R^2=0.24, P=0.010). The potentially mineralizable N (anaerobic incubation) increased with a higher organic C (R^2=0.34, P=0.002), a higher total N (R^2=0.37, P<0.001), a higher soil pH (R^2=0.32, P=0.002), a higher bacterial growth rate (viz. thymidine incorporation or DNA synthesis)(R^2=0.43, P<0.001), a higher proportion of bacterivorous nematodes (R^2=0.25, P=0.008) and a higher earthworm abundance (R^2=0.22, P=0.011). The potential C mineralization was correlated with the number of adult epigeic earthworms (R^2=0.31, P=0.002).

4.4 Discussion

4.4.1 Effect of inorganic versus organic fertilizers on soil biota

We hypothesized that the abundance of earthworms, nematodes, and micro-organisms would be stimulated more by organic than inorganic fertilizers. The results on earthworm biomass, total number of nematodes, and microbial biomass did not confirm our hypothesis, since the differences were not significant. For earthworms, only the number of earthworm burrows was significantly higher in the group of organic fertilizers (OFC) than in CAN, which is an indirect indication that earthworms were stimulated more by organic than inorganic fertilizers. Negative effects of inorganic N fertilizers on earthworms in grassland are mainly related to changes in soil acidity (Ma et al., 1990). Since the pH-KCl was significantly lower in CAN than in the organic fertilizer treatments, this could be the reason for a lower number of earthworm burrows in CAN in our experiment.

For the total number of nematodes, the absence of treatment effects might be the result of differential effects of the inorganic and organic fertilizers on nutrient availability from the fertilizer, mineralization of soil organic matter, and the grass root mass. In turn, this resulted in differential effects on herbivorous and bacterivorous nematodes, respectively. At the end of the growing season, grass root biomass was highest in the CAN-treatment which coincided with increased densities (and proportion) of herbivorous nematodes. In contrast, organic fertilizer application resulted in an increased proportion of bacterivorous nematodes. Thus, while total abundance of nematodes was the same for the different treatments, the abundance and proportion of herbivorous nematodes was highest in the CAN-treatment and the proportion of bacterivorous nematodes was highest in the organic fertilizer treatments. A similar distribution of trophic groups over treatments with inorganic N and organic fertilizers was also found in a microcosm experiment by Van Den Pol-van Dasselaar et al. (2006). We conclude that the higher abundance and proportion of herbivorous nematodes in the CAN-treatment was probably a fertilizer effect via the increased root biomass. The abundance of herbivorous nematodes was positively related with a higher root mass. Bouwman and Arts (2000) also linked an increase in herbivorous nematodes to root mass, and Todd (1996) reported a significant correlation between the abundance of herbivorous nematodes and the root N content. The increased proportion of bacterivorous nematodes in the organic fertilization treatments was probably a fertilizer effect via the increased microbial biomass, consistent with previous laboratory and field studies with grassland soil (Dmwolska and Kozlowska, 1988; Griffiths et al., 1998; Bardgett et al., 1998; Forge et al., 2005).

The reason for no differences between the treatments for bacterial biomass was less clear than for the nematode abundance. As hypothesized, the bacterial activity, measured as bacterial growth rate (thymidine incorporation or DNA synthesis), was higher for the organic fertilizers compared to the CAN treatment. However, this higher bacterial growth rate (cell production) did not result in a larger bacterial biomass. Possibly, an increase in bacterial biomass was prevented by microbivores like protozoa and bacterivorous nematodes (Bloem et al., 1994). This was supported by the positive relationship between bacterial growth rate and the proportion of bacterivorous nematodes. However, in a Canadian experiment, an increased microbivore population in the organic fertilizer treatment was accompanied by a higher bacterial biomass (Forge et al., 2005; Bittman et al., 2005). A difference between the two experiments was that the fertilizer application and soil sampling in the Canadian experiment was more frequent over the season, whereas in our experiment, the fertilizer treatments were applied before the first and second harvest and sampling took place in October. As a result of this gap between fertilizer application and soil sampling, the direct nutrient availability for the microbial biomass via fertilizer application could have faded away, while the indirect nutrient availability via the plant effects (e.g., root biomass) of the fertilizer treatments could have brought the microbial biomass to the same level. Micro-organisms reach higher abundances in the rhizosphere than in bulk soil (Alphei et al., 1996) and thus a higher rhizosphere/bulk soil ratio goes with an increase in the microbial biomass in the soil. Similar results were found by Bardgett et al. (1999b) who measured strong positive relationships between root mass of grasses and total phospholid-derived fatty acids (PLFA), bacterial PLFA, and fungal PLFA.

Interesting in the whole discussion is the role of the grass root mass in the effect of the different fertilizer treatments on soil biota. The CAN-treatment had a higher root mass than the organic fertilizers. In first instance, this was not expected, since Ennik et al. (1980) found a negative relationship between N-supply and grass root mass. However, Ennik et al. (1980) also observed that even a short period of sub-optimal N-supply could result in a marked increase in root growth. In our experiment, grass root mass in CAN was similar to that in CON. Apparently, the N-supply before the date of sampling was low in the CAN-treatment. Although the available N was not measured over the year, the dry matter production of the grass could be used as an indirect estimation. There was a significant negative linear relationship between the grass DM production in the third harvest (August) and the root mass determined in October (Fig. 4.1). Furthermore, the lower available N in the CAN-treatment and its effect on root biomass could be seen from the potentially mineralizable N (Table 4.5) and its significantly negative relationship with root mass. A lower available N in CAN, from the third harvest onwards, could be explained by the application of the fertilizers only before the first and second grass harvest combined with the high direct availability of N from CAN immediately after application. Furthermore the lower total N in the soil of CAN reduced the mineralization of soil N. The outcomes suggest that the available N (NO_3^- and NH_4^+) in the soil plays a central role in the effect of fertilizers on nematodes and micro-organisms. For micro-organisms this effect is both via a direct nutrient availability and indirectly via the grass root mass and for nematodes this is indirect via the microbial biomass and the grass root mass In terms of species richness of soil biota, we hypothesized a higher number of species in the treatments with organic fertilizer compared to CAN. For earthworms and nematodes, no differences in species number were found. For nematodes only a shift in trophic groups was measured. The CLLP slope parameter did not indicate a difference in physiological diversity of the bacterial community. Our data suggest that the inorganic fertilizer maintains a similar species richness or community structure of soil biota as the group of organic fertilizers.

4.4.2 Effects of different organic fertilizers on soil biota

We hypothesized that from the four organic fertilizers tested, FYM would stimulate the abundance of the soil biota most. After five years, earthworm abundance was not significantly different between the organic fertilizers tested. Timmermans et al. (2006) measured higher earthworm abundance with farmyard manure compared to manure slurry, although the number of earthworms after application of manure slurry was already very high. Since in grassland large amounts of organic matter are present in the form of root litter, addition of extra organic matter with the different organic fertilizer was not expected to have a considerable effect on earthworm abundance and biomass (Cotton and Curry, 1980; Edwards and Lofty, 1982). Little or no effect of different organic fertilizers was found on the abundance of nematodes and micro-organisms. De Vries et al. (2006) also found no difference in bacterial and fungal biomass after three years of application of farmyard or manure slurry on a grass-clover mixture. However, for nematodes, Van Den Pol-van Dasselaar et al. (2006) found a positive relationship between the abundance of bacterivorous nematodes and the organic N content of eight different cattle manure slurries. Although Forge et al. (2005) have shown that the residual effect of organic fertilizer on bacterivorous nematodes can last for at least one year, in our experiment, only small differences in trophic group distribution were found. Altogether, the data suggest that, in explaining the effects of organic fertilizers on the abundance of nematodes, the interaction and leveling out of differential effects on various trophic groups via the microbial biomass and the grass root mass may have also played a role. As discussed in the previous paragraph we suggest that these effects are governed by the available N (NO_3^- and NH_4^+) in the soil and its effect on the microbial biomass and the grass root mass.

Since NMS had a higher labile organic matter content and a higher available N than FYM, we hypothesized that the bacterial activity would be highest in the treatment with NMS and lowest in FYM. The twofold higher bacterial activity, measured as bacterial growth rate (thymidine incorporation) in NMS compared to FYM confirmed this hypothesis.

The bacterial community and the species composition of earthworms was affected by the use of FYM and CYM. The effect of organic fertilizers on the bacterial community was demonstrated by a shift in the substrate preferences in the Biolog plates (Fig.4.3). For soils that had received organic fertilizer with a high C/N ratio (FYM and CYM), a preference for a suite of different carbohydrates was observed. We suppose that the bacterial community is more adapted to decomposition of carbohydrate-rich plant residues in the yard manures, compared to the manure slurry which is richer in proteins. Here it has to be mentioned that the Biolog approach has the advantage of combining both functional diversity and degradation rates, but it is culture dependent. Still, the culture dependent part of the microbial community can be effectively studied when replicates contain a similar inoculation density (Nannipieri et al., 2003) or when dilution series have been used (this study; Rutgers et al., 2006). The trend towards a higher proportion of epigeic adult earthworms in FYM and CYM could be explained by the application method and/or the food preference of earthworms. Both FYM and CYM were surface-applied, whereas both manure slurries (NMS and LMS) were slit-injected. De Goede et al. (2003) and Van Vliet and de Goede (2006) found a negative effect of slit-injection on the abundance of epigeic earthworms compared to surface application, depending on the weather conditions. Since the proportion of epigeic adults in the slit-injected treatments (NMS and LMS) was similar to that in CON and CAN, negative effects of injection did not probably occur. A more likely explanation is the food preference of epigeic earthworms. Edwards and Lofty (1982) and Whalen et al. (1998) showed an increase in the anecic species *L. terrestris,* when farmyard manure was applied. In our study, no anecic species were found, but the food preference of both anecic and epigeic earthworms is primarily plant litter and fresh organic residues (Lavelle 1998). Both FYM and CYM have the same consistency, have a higher C/N ratio than manure slurry, and contain fresh organic matter in the form of straw or grass particles.

4.4.3 Relevance of changes in soil biota for ecosystem services

Soil biota are key for a range of ecosystem services, including soil structure maintenance, water retention, and supply of nutrients (Swift et al., 2004; Mulder,, 2006; Brussaard et al., 2007b; Kibblewhite, et al. 2008). Soil structure maintenance is an ecosystem service provided by roots and soil biota like earthworms, bacteria, and fungi. In our experiment, the different fertilizers did not affect visual soil structure. In a field study on arable land, greater aggregate stability was demonstrated in a soil fertilized with farmyard manure compared to inorganic fertilizer only or no fertilization at all (Estevez et al., 1996). Probably, these effects could not be measured in our experiment, because the positive effects of grass and its roots on soil structure outweighed the added effects of fertilization on soil structure-forming biota. Williams and Cooke (1961) concluded from their study on arable land that a grass ley was much more effective than farmyard manure in improving soil structure. Although in our experiment soil structure was not significantly affected by any treatment, the percentage of soil crumbs significantly increased with a higher earthworm biomass. A similar relationship between earthworm abundance and soil structural stability was found by Estevez et al. (1996). A higher abundance and proportion of epigeic earthworms was in our experiment negatively related with the soil bulk density. Clements et al. (1991) and Hoogerkamp et al. (1983) found a higher bulk density when earthworms were absent. The positive trend towards more epigeic earthworms in CYM and FYM, the negative relation between epigeic earthworms and bulk density, and the significantly lower penetration resistance in CYM and FYM gave some evidence that both fertilizer types (CYM and FYM) contribute more to the service of soil structure maintenance than CAN, but this needs further study.

Water regulation is an ecosystem service greatly influenced by earthworms in grassland (Clements et al., 1991; Bouché and Al-Addan, 1997). Especially earthworm burrows can increase water infiltration (Edwards and Shipitalo, 1998). In our experiment, the number of earthworm burrows was significantly higher in the organic fertilizer treatments when compared as one group (OFC) with CAN. Therefore, it is likely that organic fertilization contributes to the ecosystem service of water regulation. Like for soil structure maintenance this warrants further study.

The ecosystem service ‘supply of nutrients’ is influenced, amongst others, by bacteria, fungi, nematodes, and earthworms. As discussed previously, the abundances of micro-organisms and nematodes were not significantly different between CAN and the organic fertilizers, most probably because of an interaction of direct and indirect fertilizer effects. However, after five years the soils with organic fertilizers had, in comparison with CAN, a higher organic C and total N (analyzed as one group of organic fertilizers), a twofold higher bacterial activity (measured as bacterial growth rate, i.e. thymidine incorporation), a 30% higher potential N mineralization (aerobic incubation), and 25-50% more potentially mineralizable N (anaerobic incubation). The bacterial activity was positively related with the proportion of bacterivorous nematodes and the potentially mineralizable N. The relation of the potentially mineralizable N with grass yield was significant when the CAN-treatment was left out of the analysis. This makes sense because, without chemical fertilizers, crop production is more dependent on mineralizable N. Parfitt et al. (2005) also found, in nine adjacent pastures spanning a wide range of soil fertility, a significant relationship between potential N mineralization and grass yield. In our experiment, with much smaller differences in soil fertility, the variance of total grass yield was best explained by the sum of the effective N provided by the fertilizer (calculated according to the Dutch official advice) and the N supply capacity of the soil (also calculated according to the Dutch official advice). Thus, compared to inorganic fertilizer, organic fertilization increased the organic C, the total N, the activity of decomposers, and the supply of nutrients via the soil food web. The processes behind the supply of nutrients (via mineralization of organic matter in soil and manures) translated well into dry matter yield of the grass by the formulas used in the Dutch official advice on grassland fertilization.

The differences between the organic fertilizer treatments for parameters related to the ecosystem service 'supply of nutrients' were small. N inputs were similar, but the FYM treatment received almost twice as much organic matter (6300 kg ha^{-1} yr^{-1}) as the NMS treatment (3400 kg ha^{-1} yr^{-1}). Still there was no significant (extra) increase in organic C and in abundances of soil biota. The potential C mineralization (soil respiration) and metabolic quotient (qCO_2) of FYM were 30% higher than NMS (but not significant), suggesting that part of the (extra) organic matter in FYM is quickly mineralized. There was no net effect on the potential N mineralization rate. There is no indication that NMS has negative effects on biological soil quality, as supposed by Van Bruchem et al. (2000). On the contrary, NMS provided the highest bacterial activity, the highest amount of mineralizable N, and was the only treatment positively associated with an increase in grass yield over the years 2000-2005 (De Boer et al. 2007). Thus, we have no indications that FYM or the other adjusted manure slurries provide the ecosystem service 'supply of nutrients' better than NMS, at least not within five years.

4.5 Conclusions

After five years, organic fertilization increased the organic C, the total N, the activity of decomposers, and the supply of nutrients via the soil food web in comparison with inorganic fertilization. Within the group of organic fertilizers, however, there was no significant difference in soil organic C, in abundances of soil biota, and in the potential N mineralization rate. Thus, we have no indications that farmyard manure or the adjusted manure slurries provided the ecosystem service ‘supply of nutrients’ better than normal manure slurry, at least not within five years. The higher number of earthworm burrows in the organic compared to the inorganic fertilizer treatments suggests that organic fertilizers might stimulate the ecosystem service of water regulation more than inorganic fertilizer. The trend towards higher epigeic earthworm numbers with farmyard manure application and one of the adjusted manure slurries, the negative relation between epigeic earthworms and the bulk density, and the significantly lower penetration resistance for these two organic fertilizer types suggest that they contribute more to the service of soil structure maintenance than inorganic fertilizer.

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Chapter 5

Manure export from organic dairy farms to organic arable farms: effects of reduced manure input on soil biological quality of grass-clover pastures

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Abstract

In order to meet the demand of organic arable farms for organic manure, organic dairy farms could reduce the amount of animal manure applied to grass-clover pastures and export part of it to specialized arable farms. However, for organic dairy farmers it is very important to know what the effect of a reduced input of animal manure will be on aspects of soil quality in general, and soil biological quality in particular. We conducted a field experiment in which the effect of normal slurry manure and farm yard manure at four N application rates were compared. Lime was applied following the recommendations for soil pH of grass-clover, and a basal dressing of potassium and phosphate was given. The abundance, activity and taxonomic richness of earthworms, nematodes, bacteria and fungi were investigated three and seven years after the start of the experiment. We found that under the reduced manure application, soil biological quality in the grass-clover sward was sustained if the clover persisted and clover N fixation compensated for reduced N application rates. However, even with organic manure application, SOM tended to decrease over the years. We suggest that one of the causes for this decrease of SOM was liming, which increased pH, bacterial activity and possibly mineralization rates. Over the years a reduction in clover content resulted in a reduced dry matter yield. The abundance and percentage of herbivorous nematodes increased, bacterivorous nematodes decreased and there was a raise in fungal biomass. The soil pH (due to liming) probably stimulated N mineralization which had a negative effect on the clover content. A higher mineralization rate did not compensate for the loss of N fixation by clover. We conclude that it is possible to sustain on-farm soil biological quality of grass-clover pastures despite off-farm export of animal manure, as long as the clover content of these pastures remains sufficient, and measures are taken to maintain SOM.

Keywords

Earthworms, nematodes, microbiology, soil biota, grass clover, fertilization, organic farming

5.1 Introduction

Organic farming implies a greater reliance on ecosystem self-regulating processes. Soil biota play an important role in these processes and in the provision of various ecosystem services like supply of nutrients, maintenance of soil structure and water regulation (Brussaard et al., 1997; Swift et al., 2004; Mulder et al, 2006; Kibblewhite et al., 2008). In the guidelines of the International Federation of Organic Agriculture Movements, the importance of soil biota is underscored by the use of notions like 'living soil', 'species diversity' and 'self-regulating processes' (IFOAM, 2006). Grassland management directly and indirectly influences the soil food web and its functions (Bardgett, 2005). To develop sustainable farming systems, insight is needed into the influence of management on soil biota and how it affects the functioning of the soil-plant system (Van Eekeren et al., 2007).

The organic farming system shows resemblance with the practices of the conventional farming system. In The Netherlands organic farming developed as a highly specialized and productive system (Baars, 2002). Nowadays Dutch organic farming consists mainly of arable farms without livestock, or dairy farms with grass-clover pastures and fodder crops, such as maize. Within these specialized arable farms there is only little opportunity for nitrogen-fixing crops. Therefore, such arable farms highly depend on animal manures from conventional farms for the fertilization of their crops (Nauta et al., 1999). A maximum of 170 kg N ha^{-1} from animal dung and urine is legally allowed on ecological farms and 112 kg N ha^{-1} on bio-dynamic farms. In the near future, organic arable farms are obliged to use only manure produced by animals kept and fed in an organic system. A concept of so-called partnership farming has been developed, in which specialized arable and dairy farms co-operate and exchange organic manure, straw and fodder between farms (Nauta et al., 1999). In order to close the nutrient cycle between organic arable and livestock farms, organic dairy farms could reduce the amount of animal manure applied to grass-clover pastures and export part of it to specialized arable farms. However, for organic dairy farmers it is very important to know what the effect of a lower or zero input of animal manure will be on aspects of soil quality in general and soil biological quality in particular.

It is known from research on grass-only pastures, that cessation of fertilization with organic manure results in many effects, like a higher root biomass, lower pH-KCl, lower bacterial activity, lower percentage of bacterivorous nematodes, higher percentage of herbivorous nematodes, lower number of earthworm burrows and lower potentially mineralizable N (Van Eekeren et al., 2009a). Reduction or cessation of N fertilization on grass-clover will lead to an increase in the clover content in the mixture (Baars, 2002; Schils et al., 1999) and an increased N fixation (Schils and Snijders, 2004), provided that the potassium and phosphorus availability in the soil is sufficient and maintained (Baars, 2002; Van Eekeren et al., 2005b). In research of Schils and Snijders (2004) the N yield did not decrease under a reduced input of fertilization on grass-clover (tested ≤190 kg N total ha^{-1}), due to increased N fixation. Moreover, comparison of the soil under grass and clover showed that white clover-only resulted in no effect on SOM, a lower total root biomass, a lower bacterial biomass, a lower fungal biomass, a higher percentage of bacterivorous nematode dauerlarvae, a lower percentage of herbivorous nematodes, more earthworms, a higher number of earthworm burrows, a higher percentage of angular blocky elements, a lower penetration resistance and a higher potential N mineralization and potentially mineralizable C (Van Eekeren, 2009b).

These results suggest that the potentially negative effects of organic manure reduction under grass-clover can be compensated for by a higher clover content and higher N fixation. In order to test this hypothesis and to explore the possibility of partnerships between organic arable and dairy farms on a regional basis, a field study was conducted with N application rate and manure type as treatments. We measured the abundance, activity, and taxonomic richness of earthworms, nematodes, bacteria, and fungi to evaluate the effect of reduced organic fertilizer application on the soil biological quality in a grass-clover sward. Our objectives were (1) to evaluate the effect of N application rate and manure type in grass-clover on soil biological quality, and (2) to explore the relevance of changes and/or differences in soil biota for the provision of ecosystem services for the soil-plant system.

5.2 Materials and methods

5.2.1 Experimental design and sampling site

The experiment was established on the experimental dairy farm “Aver Heino” in the east of the Netherlands (52º25´N, 6º15´E). The humid sandy soil was classified as a gleyey sand with a semi-permeable loam horizon at 70-80 cm. The pasture in which the trial was established was sown with a mixture of perennial rye grass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) in 1997. Prior to the trial this pasture had a mixed management with grazing and mowing and received dairy slurry manure till a level of 120 kg N-total ha^{-1} year^{-1}. In spring 2001, the treatments were installed. The trial consisted of seven rows, comprising an untreated control (CON) (one row) and two manure treatments: “normal” cattle manure slurry (NMS) (three rows) and farm yard manure from a deep-litter stable with dairy cows (FYM) (three rows). The three rows of NMS and FYM received manure at three N application rates equivalent to 40, 80 and 120 kg N ha^{-1}. Dimensions of the plots sampled were 2.7 m x 15 m, bordered by a 0.3 m buffer zone. For more details see De Vries et al. (2006). The manure slurry was slit-injected, whereas the farm yard manure was surface-applied. Eighty kg N-total ha^{-1} of the NSM and FYM was applied in the beginning of the growing season and the last 40 kg N-total was applied shortly after the first harvest. In addition to the manure all plots received Physalg 27 (Roullier) and Patent kali ($K_2SO_4.MgSO_4$) to an application rate of 107 kg P_2O_5 ha^{-1} and 372 kg K_2O ha^{-1} as (inclusive organic manure). For a detailed overview of treatments see Table 5.1. In February 2004 and 2006 Dolomitic lime was applied to all plots at a rate of 1163 and 1200 kg ha^{-1}, respectively. No herbicides were used except for the buffer zones.

Table 5.1 Average application rate of organic manure per treatment (kg ha^{-1} year^{-1} except for C/total N) per treatment. The application rate of P_2O_5 and K_2O is total for organic manure and additional application with Physalg and Patent kali.

Kg ha^{-1}	Treatments						
	CON	NMS			FYM		
N-total	0	40	80	120	40	80	120
Total organic matter	0	589	1178	1963	1079	2158	3238
Mineral N	0	20	40	60	4	8	12
Organic N	0	20	40	61	37	74	110
C/total N	0	7.0	7.0	7.0	11.4	11.4	11.4
P_2O_5 total	107	107	107	107	107	107	107
K_2O total	372	372	372	372	372	372	372

Treatments: unfertilized (CON) or fertilized with “normal” cattle manure slurry (NMS) and farmyard manure from a deep-litter stable with dairy cows (FYM).

5.2.2 Soil sampling, chemical and physical parameters

On 4 November 2003 and 8 October 2007, soil samples for determination of chemical, physical and biological soil quality parameters were taken.

A bulk sample of 70 cores (0-10 cm, ø of 2.3 cm) per plot was collected, sieved through 1 cm mesh, homogenized and stored at field moisture content at 4 °C before analysis. Subsamples were taken and used for chemical analysis, nematode and microbiological analysis.

Soil dry matter content was determined after oven-drying of approximately 30 g of the bulk sample (in duplicate) at 105 ºC. Prior to chemical analysis, samples were oven-dried at 40 °C. Soil acidity of the oven-dried samples was measured in 1 M KCl (pH-KCl). Soil Organic Matter (SOM) was determined by loss-on-ignition (Ball, 1964). Organic Carbon (C) was measured by incineration of dry material at 1150 °C, after which the produced CO_2 was determined by an infrared detector (LECO Corporation, St. Joseph, Mich., USA). For determination of total N, evolved gasses after incineration were reduced to N_2 and detected with a thermal-conductivity detector (LECO Corporation, St. Joseph, Mich., USA).

In 2007, the soil sampling was combined with measurements on soil physical parameters. Soil bulk density was measured in the 5-10 cm layer below the soil surface, in three undisturbed ring samples containing 100 cm^3 soil per plot. Rings were weighed, oven-dried (70 °C) and weighed back. Penetration resistance was measured with an electronic penetrometer (Eijkelkamp, Giesbeek, The Netherlands) with a cone diameter of 1 cm^2 and a 60° apex angle. Cone resistance was recorded per cm of soil depth and expressed as an average value of 10 penetrations in the soil layers of 0-10 cm, 10-20 cm, 20-30 cm and 30-40 cm. Soil structure was determined in 1 block (20 cm x 20 cm x 10 cm) per plot. Soil of this block was assigned by visual observation to crumbs, sub-angular blocky elements and angular blocky elements (FAO, 2006). These were weighed and expressed as a percentage of total fresh soil weight. Three soil cores (0-10 cm, ø of 8.5 cm) per plot were taken to determine the root biomass. The soil in the samples was thoroughly washed out with water after which the roots were oven-dried at 70 °C and the dry matter of the roots was measured.

Grass was harvested four to five times a year. Plots were cut at a stubble height of 6 cm, using a ‘Haldrup’ small-plot harvester (J. Haldrup a/s, Løgstør, Denmark). Grass was weighed and sampled for dry matter (DM). In a second vegetation sample the clover content in the dry matter was determined.

5.2.3 Soil biological parameters

5.2.3.1 Earthworms

Earthworms were sampled in 2 blocks (20 cm x 20 cm x 20 cm) per plot. The blocks were transferred to the laboratory where the earthworms were hand-sorted, counted, weighed (fresh weight without emptying gut) and fixed in alcohol prior to identification. Numbers and biomass were expressed per m^2. Adults were identified to species. A distinction was made between (1) epigeic species (pigmented, living superficially in the litter layer, little burrowing activity), (2) endogeic species (living in the subsoil down to about 20 cm soil depth) and (3) anecic species (relatively large worms, living in vertical burrows from which they collect dead organic matter from the surface at night) (Bouché, 1977). In 2007, before the blocks were hand sorted, on 1 block per plot the earthworm burrows with a diameter >2 mm were counted on horizontal surfaces (20 cm x 20 cm) exposed at 10 cm and 20 cm depth.

5.2.3.2 Nematodes

From the bulk soil sample a sub-sample of 100 ml soil was taken, from which the free-living nematodes were extracted, using an Oostenbrink elutriator (Oostenbrink, 1960). Total numbers were counted and expressed per 100 g fresh soil. Nematodes were fixed in 4% hot formaldehyde, and at least 150 randomly selected nematodes from each sample were identified to genus and, whenever possible, to species. Nematode genera and species were assigned to trophic groups, following Yeates et al. (1993) and allocated to colonizer-persister groups (cp-groups), following Bongers (1990) and Bongers et al. (1995). The Nematode Channel Ratio (NCR) was calculated to express the relative contributions of bacterivorous (B) nematodes to total bacterivorous and fungivorous (F) nematode abundance (NCR=B/(B+F)) (Yeates, 2003). The Maturity Index was calculated as the weighted mean of the individual cp-values, in accordance with Bongers (1990) and Korthals et al. (1996). The Maturity Index is an ecological measure which indicates the condition of an ecosystem based on nematode taxa composition.

5.2.3.3 Microbial parameters

From the bulk soil sample a sub-sample of 200 g field-moist soil was adjusted to 50% WHC (Water Holding Capacity) and pre-incubated at 12 °C for four weeks, to avoid the effects of temperature and moisture fluctuations in the field and to stabilize soil conditions (Bloem et al., 2006). After pre-incubation, fungal and bacterial biomass, bacterial growth rate and Community-Level Physiological Profiles (CLPP) were measured. Soil smears were prepared and measured as described by Bloem and Vos (2004). Fungal hyphae were measured using the grid intersection method. Bacterial numbers and cell volumes were measured by confocal laser scanning microscopy and automatic image analysis (Bloem et al., 1995). Biomass was calculated from biovolume. Bacterial growth rate was determined as the incorporation of [^{3}H] thymidine and [^{14}C]leucine into bacterial DNA and proteins respectively (Bloem and Bolhuis, 2006; Michel and Bloem, 1993). For a more detailed description, see De Vries et al. (2006).

The potential N mineralization rate was determined as the increase in mineral N (ammonium plus nitrate) between week 1 and week 6, during incubation. Soil was homogenized, sieved (5 mm mesh size) and brought to 50-60% of the water holding capacity. Subsamples of 200 g soil were incubated in the dark at 20ºC in 1.5 l air-tight jars supplied with a gas septum, between week 1 and week 6. The first week was not used to avoid effects of soil homogenization. Sub-samples of 80 g soil were extracted with 200 ml of 1 M KCl. After 1 h shaking the extracts were filtered over a paper filter. Mineral N contents (ammonium and nitrate) were determined by Skalar Segmented Flow Analysis (Breda, the Netherlands). In the same jars where the potential N mineralization was measured, the CO_2 evolution was measured weekly by gas chromatography in 2007. The gas chromatograph was a Carlo Erba 6000 with a column switching system, equipped with a 4-m Porapak q and a 2 mmol sieve 5A column. The detector (HWD) temperature was 180ºC, the column temperature was 50ºC, and the injection volume was 1 ml (Bloem et al., 1994). Potential C mineralization was expressed as mg C respired kg^{-1} soil $week^{-1}$. The results of the first week were not used to avoid effects of soil homogenization. In 2003 and 2007 potentially mineralizable C was measured by incubation for seven days at 20°C and 50% WHC. During this period CO_2 was absorbed in alkali (1*N* KOH) followed by titration with 0.1 *N* HCl (Pell et al., 2006). In 2007, in addition to potential N mineralization by aerobic incubation of soil, potentially mineralizable N was measured by anaerobic incubation of a soil sample under water (in slurry) for 1 week at 40°C (Keeny and Nelson, 1982; Canali and Benedetti, 2006). These warm and anoxic conditions are optimal for a quick mineralization of organic matter by anaerobic bacteria. The lack of oxygen prevents conversion of released NH_4 to NO_3 (nitrification) and uncontrolled N losses by denitrification can not occur. The amount of mineral N (NH_4-N) released is a measure of the quality (N content and decomposability) of the organic matter, and thus for biological soil fertility.

5.2.4 Statistical analyses

The data were analyzed with GENSTAT (8th Edition, VSN International, Hemel Hempstead, UK). The treatments were N application rate (0, 40, 80 and 120 kg N ha^{-1}) and manure type (no manure, farm yard manure and normal slurry manure). Treatment effects were analyzed by an unbalanced one-way ANOVA because the lay-out of the trial did not permit the comparison of the N application rate and manure type in a two-way ANOVA. The measurements over the consecutive years (2003 and 2007) were in the same plots, so in principle not independent. By using the repeated measure procedure in GENSTAT we corrected for this dependency. When relationships between parameters were expected, Pearson correlations were determined. Data of nematode taxa were square root transformed and subjected to redundancy analysis (RDA) (CANOCO 4.5, Biometris, Wageningen, The Netherlands). A Monte Carlo permutation test (499 random permutations) was performed to test for statistically significant (P=0.05) relationships between community structure and treatment effects, following multivariate analysis of variance design (Van Dobben et al., 1999).

5.3 Results

5.3.1 Soil chemical, soil physical and crop parameters

Soil organic matter (SOM) was significantly influenced by the amount of manure (expressed as N application rate) and manure type (Table 5.2a, 5.2b). The treatments 120 kg N ha^{-1} and FYM had the highest SOM. SOM in 2007 was significantly lower than 2003, in

Table 5.2a Soil chemical and physical parameters and plant parameters in grass clover fertilized with four nitrogen application rates (0, 40, 80 120 kg N-total ha^{-1}) over two years (2003 and 2007).

Chemical/ Physical	Units	Treatments					Year	Treat.*year
		0	40	80	120	P-value	P-value	P-value
SOM*	g kg dry soil^{-1}	50.0 ab	50.0 ab	48.8 b	53.6 a	0.043	0.004	NS
Organic C**	g C kg dry soil^{-1}	25.5	26.0	25.6	28.8	NS	--	--
Total N*	g N kg dry soil^{-1}	2.41	2.23	2.24	2.38	NS	NS	NS
C/N		10.5 a	12.0 b	11.8 b	12.3 b	<0.001	--	--
pH-KCl*		4.51 b	4.59 b	4.73 a	4.84 a	<0.001	<0.001	0.006
Bulk density**	g cm^{-3}	1.44 a	1.37 b	1.33 b	1.36 b	<0.001	--	--
Penetration resistance								
0-10 cm	mPa	1.37	1.35	1.33	1.34	NS	--	--
10-20 cm	mPa	1.85	1.92	1.90	1.91	NS	--	--
20-30 cm	mPa	2.17	2.22	2.17	2.08	NS	--	--
30-40 cm	mPa	2.12	2.33	2.34	2.17	NS	--	--
Soil structure 0-10 cm**								
Crumb	%	53 a	46 b	51 ab	56 a	0.038	--	--
Sub-angular	%	6	6	6	5	NS	--	--
Angular	%	41	48	43	39	NS	--	--
Grass yield*								
Clover	%	39	37	33	36	NS	<0.001	NS
Total biomass	g DM m^{-2}	1047	1039	1034	1116	NS	<0.001	NS
Roots**								
Biomass	g DM m^{-2}	646	824	740	670	NS	--	--

* Means of 2003 and 2007,

** Measured in 2007 only.

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

that the average SOM in the experiment decreased from 52.2 g per kg dry soil in 2003 to 49.1 g per kg dry soil in 2007. Organic C tended to rise with N application rate (P=0.060). FYM not only had the highest SOM but also the highest soil organic C content. Total N content of the soil was not affected by N application rate, but was significantly higher in the treatment without manure application compared to the treatment with NSM. Total N was positively cor-

Table 5.2b Soil chemical and physical parameters and plant parameters in grass clover without fertilization (0 kg N-total ha^{-1}) or with fertilization of normal slurry manure (NSM) (40, 80 120 kg N-total ha^{-1}) or farm yard manure (FYM) (40, 80 120 kg N-total ha^{-1}) over two years (2003 and 2007).

Chemical/	Units	Treatments				Year	Treat.*year
Physical		0	NSM	FYM	P-value	P-value	P-value
SOM*	g kg dry soil^{-1}	50.0 ab	48.8 b	52.7 a	0.028	0.005	NS
Organic C**	g C kg dry soil^{-1}	25.5 b	25.4 b	28.2 a	0.025	--	--
Total N*	g N kg dry soil^{-1}	2.41 a	2.20 b	2.36 ab	0.032	NS	0.039
C/N		10.5 b	12.3 a	11.7 a	<0.001	--	--
pH-KCl*		4.51 b	4.68 a	4.75 a	0.020	<0.001	0.004
Bulk density**	g cm^{-3}	1.44 a	1.33 b	1.37 b	<0.001	--	--
Penetration resistance							
0-10 cm	mPa	1.37	1.34	1.34	NS	--	--
10-20 cm	mPa	1.85	1.93	1.90	NS	--	--
20-30 cm	mPa	2.17	2.19	2.13	NS	--	--
30-40 cm	mPa	2.12	2.41	2.16	NS	--	--
Soil structure 0-10 cm**							
Crumb	%	53	51	51	NS	--	--
Sub-angular	%	6	6	5	NS	--	--
Angular	%	41	43	44	NS	--	--
Grass yield*							
Clover	%	39	33	37	NS	<0.001	0.015
Total biomass	g DM m^{-2}	1047	1090	1036	NS	<0.001	NS
Roots**							
Biomass	g DM m^{-2}	646	826	681	NS	--	--

* Means of 2003 and 2007,

** Measured in 2007 only.

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

related with clover content in the sward over the two years (r=+0.37, P=0.006), and did not differ between years. The C/N ratio was lowest for the treatments without manure and highest for NSM. pH increased significantly with a higher N application rate and organic manure fertilization had a significantly higher pH than no manure.

Soil bulk density was significantly lower with application of organic manure and positively correlated with clover content in the sward (r=+0.51, P=0.007). The penetration resistance did not differ between the treatments, but was negatively correlated with number of earthworm burrows at 10 cm in the 10-20 cm (r=-0.43, P=0.024) and 30-40 cm soil layer (r=-0.52, P=0.005). The percentage of crumbs was significantly higher at N application rates of 0 and 120 kg N ha^{-1} and was positively correlated with bacterial biomass (r=+0.46, P=0.015), fungal biomass (r=+0.41, P=0.032) and fungal activity (r=+0.54, P=0.004).

Clover content, grass-clover DM yield and root biomass did not differ between the treatments. From 2003 to 2007 the clover content and the grass-clover DM yield decreased from 51% to 20%, and 1100 to 1021 g DM m^{-2}, respectively. Over the two years grass-clover yield was positively correlated with clover percentage (r=+0.46, P<0.001) and with bacterial activity (measured as thymidine incorporation (r=0.32, P=0.021) or leucine incorporation (r=0.33, P=0.014)), and negatively correlated with fungal biomass (r=-0.34, P=0.011), and fungal/bacterial ratio (r=-0.35, P=0.008). In 2007, grass-clover yield was negatively correlated with penetration resistance at 20-30 cm (r=-0.41, P=0.033). In 2007 we measured a positive relationship between the root biomass in the 0-10 cm layer and the penetration resistance at 30-40 cm (r=+0.43, P=0.022).

5.3.2 Soil biological parameters

5.3.2.1 Earthworms

Earthworm abundance did not differ between the treatments manure type and N application rate (Table 5.3a, 5.3b). Neither in 2003 or 2007, earthworm abundance was correlated with pH or content clover in the sward. Compared to 2003, the number of earthworms was higher and the body biomass was lower in 2007. Over the two years the earthworm body mass was negatively correlated with the number of juvenile earthworms (r=-0.57, P<0.001).

Species of adult earthworms identified in the trial were *Lumbricus castaneus, Lumbricus rubellus*, *Aporrectodea caliginosa*, *Allolophora chlorotica* and *Aporrectodea rosea.*

The number of earthworm burrows at 10 cm and 20 cm soil depth was not significantly different between the treatments.

Table 5.3a Earthworm numbers, biomass, species, functional groups and earthworm burrows in grass clover fertilized with four nitrogen application rates (0, 40, 80 120 kg N-total ha^{-1}) over two years (2003 and 2007).

Earthworms	Units	Treatments					Year	Treat.*year
		0	40	80	120	P-value	P-value	P-value
Total number	n m^{-2}	295	323	306	338	NS	0.003	NS
Body biomass	g worm^{-1}	0.51	0.53	0.48	0.51	NS	0.008	NS
Total biomass	g m^{-2}	143	161	145	159	NS	NS	NS
Number of species	n 20x20x20cm^{-1}	2.2	1.9	2.0	2.5	NS	NS	NS
Epigeic adults	%	35	29	22	39	NS	NS	NS
Endogeic adults	%	65	71	78	61	NS	NS	NS
Earthworm burrows*								
10 cm depth	n m^{-2}	138	97	103	131	NS	--	--
20 cm depth	n m^{-2}	200	141	163	138	NS	--	--

* Earthworm burrows were counted in 2007 only.

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

Table 5.3b Earthworm numbers, biomass, species, functional groups and earthworm burrows in grass clover without fertilization (0 kg N-total ha^{-1}) or with fertilization of normal slurry manure (NSM) (40, 80 120 kg N-total ha^{-1}) or farm yard manure (FYM) (40, 80 120 kg N-total ha^{-1}) over two years (2003 and 2007).

Earthworms	Units	Treatments				Year	Treat.*year
		0	NSM	FYM	P-value	P-value	P-value
Total number	n m^{-2}	295	343	302	NS	0.003	NS
Body biomass	g worm^{-1}	0.51	0.51	0.50	NS	0.009	NS
Total biomass	g m^{-2}	143	169	141	NS	NS	NS
Number of species	n 20x20x20cm^{-1}	2.2	2.3	2.0	NS	NS	NS
Epigeic adults	%	35	32	29	NS	NS	NS
Endogeic adults	%	65	68	71	NS	NS	NS
Earthworm burrows*							
10 cm depth	n m^{-2}	138	102	119	NS	--	--
20 cm depth	n m^{-2}	200	138	156	NS	--	--

* Earthworm burrows were counted in 2007 only.

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

5.3.2.2 Nematodes

None of the treatments had a statistically significant effect on the abundance of nematodes (Table 5.4a, 5.4b). However, the percentage of the trophic group category omnivorous nematodes was significantly higher in FYM than in NSM. The number of herbivorous and carnivorous nematodes was increased in 2007, which resulted in a higher percentage of herbivorous and carnivorous nematodes in 2007, while the percentage bacterivorous and omnivorous nematodes decreased.

The life-strategy group distribution differed between 2003 and 2007, with a decrease

Table 5.4a Nematode abundance, throphic groups, life history groups (Cp=colonizer-persister groups) and community structure indices in in grass clover fertilized with four nitrogen application rates (0, 40, 80 120 kg N-total ha^{-1}) over two years (2003 and 2007).

Nematodes	Units	Treatments					Year	Treat.*year
		0	40	80	120	P-value	P-value	P-value
Total number	n 100g soil^{-1}	5290	4875	5707	5842	NS	NS	NS
Bacterivorous	n 100g soil^{-1}	1964	1930	1940	2188	NS	NS	NS
Herbivorous	n 100g soil^{-1}	2940	2577	3266	3212	NS	0.008	NS
Fungivorous	n 100g soil^{-1}	116	120	181	162	NS	NS	NS
Omnivorous	n 100g soil^{-1}	206	178	238	229	NS	NS	0.038
Carnivorous	n 100g soil^{-1}	66	70	82	51	NS	<0.001	NS
Bacterivorous	%	37	39	34	36	NS	0.014	NS
Herbivorous	%	56	53	58	56	NS	0.004	NS
Fungivorous	%	2	3	3	3	NS	NS	NS
Omnivorous	%	4	4	4	4	NS	0.017	NS
Carnivorous	%	1	1	1	1	NS	<0.001	NS
Cp-1	%	20	26	20	23	NS	<0.001	NS
Cp-2	%	63	57	59	60	NS	NS	NS
Cp-3	%	3	4	5	4	NS	<0.001	NS
Cp-4	%	8	10	11	9	NS	<0.001	0.018
Cp-5	%	6	4	4	4	NS	0.043	NS
Number of genera		25	24	26	26	NS	<0.001	NS
Maturity Index(cp1-5)		2.18	2.09	2.21	2.10	NS	<0.001	NS
Nem. Channel Ratio		0.94	0.93	0.93	0.93	NS	NS	NS

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

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of cp-1 (enrichment opportunists) and an increase of cp-3,cp-4 and cp-5 taxa, respectively. This resulted in a significantly higher Maturity Index (cp1-5) in 2007.

In the 2007 sampling, the number of taxa was lower than in 2003. Differences in the nematode taxa composition were mainly related to the year of sampling (Table 5.5, Fig. 5.1). Moreover, there was an effect of N application rate on the occurrence of nematode taxa (Table 5.5; Fig. 5.1).

Table 5.4b Nematode abundance, throphic groups, life history groups (Cp=colonizer-persister groups) and community structure indices in grass clover without fertilization (0 kg N-total ha^{-1}) or with fertilization of normal slurry manure (NSM) (40, 80 120 kg N-total ha^{-1}) or farm yard manure (FYM) (40, 80 120 kg N-total ha^{-1}) over two years (2003 and 2007).

Nematodes	Units	Treatments				Year	Treat.*year
		0	NSM	FYM	P-Value	P-value	P-value
Total number	n 100g soil^{-1}	5290	5682	5268	NS	NS	NS
Bacterivorous	n 100g soil^{-1}	1964	2211	1829	NS	NS	NS
Herbivorous	n 100g soil^{-1}	2940	3065	2972	NS	0.011	NS
Fungivorous	n 100g soil^{-1}	116	152	157	NS	NS	NS
Omnivorous	n 100g soil^{-1}	206	172	258	NS	NS	NS
Carnivorous	n 100g soil^{-1}	66	82	54	NS	<0.001	NS
Bacterivorous	%	37	38	35	NS	0.012	NS
Herbivorous	%	56	55	56	NS	0.004	NS
Fungivorous	%	2	3	3	NS	NS	NS
Omnivorous	%	4ab	3b	5a	0.032	0.027	NS
Carnivorous	%	1	2	1	NS	<0.001	NS
Cp-1	%	20	24	21	NS	<0.001	NS
Cp-2	%	63	59	59	NS	NS	NS
Cp-3	%	3	4	5	NS	<0.001	NS
Cp-4	%	8	10	11	NS	0.005	0.040
Cp-5	%	6	3	4	NS	0.033	NS
Number of genera		25	25	26	NS	<0.001	NS
Maturity Index(cp1-5)		2.18	2.08	2.18	NS	<0.001	NS
Nem. Channel Ratio		0.94	0.93	0.93	NS	NS	NS

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

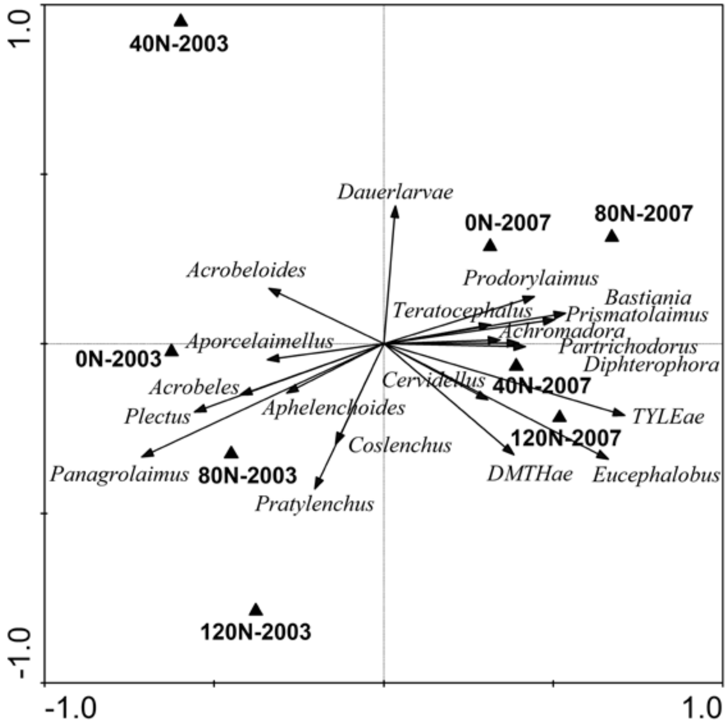

Fig.5.1 Ordination diagram of the nematode taxa based on RDA with the model year (2003, 2007) x nitrogen application rate (0, 40, 80, and 120 kg N-total ha^{-1}). The diagram explains 80.2% of the variance in the fitted abundances of the full model. Only nematode taxa of which >10% of their variance was explained by the axes 1 and 2 were plotted. Abbreviations of taxa: TYLEae, Tylenchidae; DMTHae, Dorylaimoidae + Thornenematinae. The eigenvalues of axis 1 and 2 are 0.144 and 0.029, respectively.

Table 5.5 Percentage explained variance of nematode community composition data and significance of treatment effects.

Treatment	Application rate effects (n=32)		Manure type effects (n=24)	
Year (Y)	14%	***	14%	***
Application rate (AR)	8%	**	--	
Manure type (MT)	-		4%	(NS)
Y x AR	6%	(NS)	--	
Y x MT	--		3%	(NS)

Fitted models: YxAR and YxMT. Significance was determined by the permutation test implemented in CANOCO (Ter Braak 1988), using 499 random permutations. Significance levels: *** p≤0.001; ** 0.001<p≤0.01, * 0.01<p≤0.05; NS P>0.0.05; --, not applicable.

5.3.2.3 Microbial parameters

Bacterial and fungal biomass were not significantly different between the treatments (Table 5.6a, 5.6b). However, fungal/bacterial ratio was affected by N application rate: the highest N application rates (80 and 120 kg N ha) had the lowest fungal/bacterial ratios. Bacterial biomass was lower in 2007 than 2003 while fungal biomass was higher in 2007, which resulted in a higher fungal/bacterial ratio in 2007. Over the two years, the bacterial biomass and fungal/bacterial ratio were correlated with the pH (r=+0.30, P=0.026; r=-0.36, P=0.006).

Bacterial activity (measured as thymidine and leucine incorporation) increased significantly with N application rate and was higher with the fertilization of organic manure (Table 5.6a, 5.6b). Both bacterial and fungal activity were higher in 2007 than in 2003. Over the two years both measures of bacterial activity were also positively correlated with pH (r=+0.45,

Table 5.6a Microbial biomass, activity, potential N mineralization and potentially mineralizable C in grass clover fertilized with four nitrogen application rates (0, 40, 80 120 kg N-total ha^{-1}) over two years (2003 and 2007).

Microbial	Units	Treatment					Year	Treat.*year
		0	40	80	120	P-value	P-value	P-value
Bact. biomass	μg C g dry soil^{-1}	73	64	73	76	NS	NS	NS
Thym. incorp.	pmol g dry soil^{-1} h^{-1}	11 c	17 bc	23 ab	24 a	0.002	<0.001	NS
Leuc. incorp.	pmol g dry soil^{-1} h^{-1}	206 c	247 bc	296 ab	306 a	0.002	<0.001	NS
Fung. biomass	μg C g dry soil^{-1}	42	39	38	33	NS	<0.001	NS
Fung. activity	% of hyphal length	22	26	21	25	NS	<0.001	NS
Fungal/bacterial ratio		0.66 ab	0.76 a	0.54 ab	0.47 b	0.033	<0.001	NS
Pot.N-mineral.	mg N kg dry soil^{-1}wk^{-1}	7.9	7.6	8.3	9.4	NS	NS	NS
Pot.mineral N**	mg N kg dry soil^{-1}	48.7	30.5	48.4	47.2	NS	--	--
Pot.C-mineral.**	mg C kg dry soil^{-1}wk^{-1}	79.2	79.1	87.8	81.0	NS	--	--
Pot.mineral C	mg CO_2 kg dry soil^{-1} wk^{-1}	1170 ab	1092 b	1179 ab	1249 a	0.009	<0.001	0.016
Microbial metabolic quotient (qCO_2)		0.70	0.84	0.74	0.83	NS	--	--

* ES50=Effective soil needed for 50% substrate utilization.

** Measured in 2007 only.

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

P<0.001; r=+0.43, P=0.002).

Potential N mineralization, potentially mineralizable N, potential C-mineralization and microbial metabolic quotient ($q\mathrm{CO}_2$) were not significantly affected by N application rate and manure type (Table 5.6a, 5.6b). In 2007, the potentially mineralizable N was correlated to SOM (r=+041, P=0.031). Potentially mineralizable C was significantly higher at 120 kg N ha^{-1} and lower in 40 kg N ha^{-1} than in the other treatments. Potentially mineralizable C was lower in 2007 than in 2003. Over the two years, the potentially mineralizable C was positively correlated with soil total N (r=+0.27, P=0.048).

Table 5.6b Microbial biomass, activity, potential N mineralization and potentially mineralizable C in in grass clover without fertilization (0 kg N-total ha^{-1}) or with fertilization of normal slurry manure (NSM) (40, 80 120 kg N-total ha^{-1}) or farm yard manure (FYM) (40, 80 120 kg N-total ha^{-1}) over two years (2003 and 2007).

Microbial	Units	Treatment				Year	Treat.*year
		0	NSM	FYM	P-value	P-value	P-value
Bact. Biomass	µg C g dry soil^{-1}	73	75	67	NS	NS	NS
Thym. incorp.	pmol g dry soil^{-1} h^{-1}	11 b	21 a	21 a	0.018	<0.001	0.040
Leuc. incorp.	pmol g dry soil^{-1} h^{-1}	206 b	287 a	277 a	0.026	<0.001	NS
Fung. Biomass	µg C g dry soil^{-1}	42	37	36	NS	<0.001	NS
Fung. Activity	% of hyphal length	22	24	24	NS	<0.001	NS
Fungal/bacterial ratio		0.66	0.55	0.63	NS	<0.001	NS
Pot.N-mineral.	mg N kg dry soil^{-1}wk^{-1}	7.9	8.4	8.5	NS	NS	NS
Pot.mineral. N**	mg N kg dry soil^{-1}	48.7	37.1	47.0	NS	--	--
Pot.C-mineral.**	mg C kg dry soil^{-1}wk^{-1}	79.2	72.2	62.9	NS	--	--
Pot.mineral. C	mg CO_2 kg dry soil^{-1} wk^{-1}	1170	1172	1174	NS	0.001	0.026
Microbial metabolic quotient ($q\mathrm{CO}_2$)		0.70	0.76	0.84	NS	--	--

* ES50=Effective soil needed for 50% substrate utilization.

** Measured in 2007 only.

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

5.4 Discussion

5.4.1 Soil chemical and physical parameters

In our experiment the SOM decreased with an average of 0.79 g kg dry soil^{-1} per year, from 2003 to 2007. At a bulk density of 1.36 g cm^3 this would mean a net decrease of 1420 kg SOM ha^{-1} year^{-1}. In theory an effective annual organic matter input of 3675 kg ha^{-1} is assumed in grasslands, counteracted by an annual 2% decomposition of SOM (± 1400 kg SOM ha^{-1} year^{-1}) (http://www.nmi-agro.nl). So a net increase in SOM would have been expected. Possible explanations for this difference could be 1) the accuracy of SOM measurements (the mean accuracy for sampling and analysis errors was estimated by Hanegraaf et al. (2009) at ± 10%), 2) a lower effective organic matter input by a reduced organic manure application and/or purely cutting management, 3) a higher decomposition caused by higher initial SOM and lime application (Whitehead, 1995), and 4) a lower accumulation of soil organic C with grass-clover than grass (Schils and Snijders, 2004; Schils et al., 2005). Although the 3.14 g kg dry soil^{-1} decrease in SOM is smaller than the accuracy of SOM measurements of individual samples (± 10 % for sampling and determination), the results suggest a tendency towards a decrease in SOM. This is in contradiction with the results of Baars (2002), Schils and Snijders (2004) and De Boer et al. (2007) who showed an increase in SOM in grass(-clover) with a cutting management and no organic manure application. An important difference is the higher initial SOM in our experiment of 55 g kg dry soil^{-1} versus respectively 30, 31 and 33 g SOM kg dry soil^{-1}, and the lower pH of 4.5 versus 5.4, 7.1 and 6.1, respectively. It is assumed that SOM stabilizes at a certain equilibrium content (Hoogerkamp, 1973). Whitehead (1995) stated that changes in management often prevent reaching an equilibrium of organic matter content. The low initial pH of the experimental field made it necessary to apply lime which possibly increased the mineralization and reduced the equilibrium level of SOM. The role of soil biota in the mineralization of SOM will be discussed in the next paragraph. Besides liming, the decrease in SOM could also be caused by the mixture of grass and clover cultivars. Schils and Snijders (2004) measured a lower accumulation of soil organic C with grass-clover than grass only. Van Eekeren et al. (2009b) did not find this difference in soil organic C after two growing seasons, but described a higher potentially mineralizable C with grass-clover, suggesting a greater amount of readily decomposable labile carbon. In this experiment, the SOM was reduced least with the highest N application rate, and FYM had the highest SOM and organic C. Baars (2002) also found the highest SOM with farm yard manure, whereas Van Eekeren et. al (2009a) could not measure differences in organic C between slurry and farm yard manure in grass-only.

Since pasture products (grass or grass-clover) are slightly alkaline, continued harvest and removal of these products will lower the soil pH over time (Upjohn et al., 2005). In the case of grass-clover, much of the herbage N is absorbed from N_2 fixation rather than as negatively charged NO_3^-. Therefore, the soils are even acidified more due to an excess absorption of cations in the grass-clover which is balanced by an excretion of H^+ into the rhizosphere (Yan et al., 1996; Haynes and Williams, 1999). Liming of our experimental plots increased the pH over the years. However, pH increased more in the plots with a higher manure application in general and farm yard manure in particular. This ranking in pH for manure types was also found by Baars (2002) in a fertilization experiment on grass-clover.

Overall we conclude that there was a general trend towards a decrease in SOM which was less for the highest N application rate and the treatment with FYM. Cultivation of grass-clover and a general increase of pH by liming, but also by applying organic manure, probably increased mineralization and thus decreased SOM. Therefore, SOM under grass-clover requires attention, both with and without organic manure application.

5.4.2 Soil biological parameters

We hypothesized that the reduced fertilization treatments would have only little effect on the soil biological parameters measured, since the lower N input from manure would be compensated for by the increased N fixation of clover. Furthermore, the organic matter input of manure was relatively small (Table 5.1) compared to the total annual input of 9000 kg organic matter ha^{-1} (from dead leaves, stubble and roots) of harvested grass-clover with no fertilizer N application (Whitehead, 1995).

For earthworms no significant treatment effects on abundances and species were found. Also Haraldsen et al. (1994) found little effect on earthworm abundance of different types of slurry on a grass-clover ley compared to an unfertilized control. They argued that the positive effect of the control treatment may be related to the higher clover content. Similar results were measured by Schmidt et al. (2003) in a permanent understorey of white clover in winter wheat. Because of the food preference of epigeic earthworms for fresh organic residues (Lavelle, 1998), we expected a shift towards epigeic species with FYM, like under pure grass (Van Eekeren et al., 2009a). However, such a shift could not be found in the present experiment. We have to mention that the maximum N application rate in the present experiment was almost half of the rate in the experiment conducted by Van Eekeren et al. (2009a). The higher number of earthworms in 2007 compared to 2003, was the result of a higher percentage of juveniles. This could have been an effect of the lime application (Edwards and Bohlen, 1996), although no significant correlations were found between pH (range in 2003 was 4.3-4.6 and in 2007 4.6 –5.3, respectively) and earthworm abundance. Therefore, the difference between the years might have been caused by different weather conditions or a combination of effects. For example, the summer in 2003 was very dry which resulted in a low reproduction of earthworms (Edwards and Bohlen, 1996). Moreover, at low pH earthworms may not only be less numerous, but can go into diapause more rapidly upon the onset of dry conditions (Doeksen and Van Wingerden, 1964).

Application of organic manure, and in particular the rate, affected the composition of the nematode fauna. At the functional group level the only effect of the N application rate and manure type was a higher percentage of omnivorous nematodes with FYM compared to NSM. This was not found in an experiment with different fertilizer types under pure grass (Van Eekeren et al., 2009a). The shift in 2007 to a higher abundance and percentage of herbivorous nematodes, and a lower percentage of bacterivorous nematodes could be the result of the lower content percentage in 2007 (average 20% in the DM in 2007 versus 51% in 2003). This is in agreement with observations of Van Eekeren et al. (2009b) who found a reduced percentage of herbivorous nematodes and an increased percentage of dauerlarvae, i.e. the non-feeding immature resting stage of the bacterivorous nematode species belonging to the Rhabditidae, with pure clover. Such shift in the trophic group composition of nematodes with a lower clover content (increase in herbivorous and decrease in bacterivorous nematodes) could be explained by a higher (grass) root biomass) and a lower N availability of N from litter or root exudates in the rhizosphere of a grass-clover with a lower clover content.

Taking into account the two sampling years, the bacterial and fungal biomass were not affected by N application rate and manure type. In 2003 De Vries et al. (2006) found a reduced fungal biomass with increasing N application rates. Compared to the sampling in 2003, fungal biomass was significantly increased in 2007. Again this could be caused by the reduced clover content in the experiment in 2007, the consequently lower N fixation, and the lower N availability from litter or root exudates. De Vries et al. (2006) and Van Eekeren et al. (2009b) found significantly higher fungal biomass in grass with low N fertilization compared to grass-clover mixtures. Despite the non-significant differences between bacterial and fungal biomass, the fungal/bacterial ratio decreased significantly with an increased N application rate, as also found by De Vries et al. (2006). Bacterial activity increased with increased N application rate of organic manure. Also Van Eekeren et al. (2009a) measured increased bacterial activity with the application of organic fertilizers on pure grass. Although the organic matter input did not increase over the years, the bacterial activity was higher in 2007 than in 2003. This could be a year effect but the question arises if bacterial activity was also influenced by other factors. From 2003 to 2007 the pH increased significantly. In 2003 (De Vries et al., 2006), but also over the years 2003 and 2007, bacterial activity was correlated with pH, whereby pH in our experiment was confounded with the N application rate of organic manure. This corroborates results of Haynes (1984) who found a flush of microbial activity following lime applications and Tobor-Kaplon (2006) who found a positive effect of a higher soil pH on bacterial activity. Consequently, the increase in bacterial activity could have increased the mineralization and contributed to a decrease of SOM. Chan and Heenan (1996) showed that liming in the short term resulted in a decrease in soil organic C. Lime application for 42 years on a permanent pasture compared to an unfertilized control (Van Der Wal et al., 2009) resulted in a 2.4 points higher pH-KCl, a tenfold higher bacterial activity, and 188 g SOM kg dry soil $^{-1}$ with, and 215 g SOM kg dry soil $^{-1}$ without lime application (unpublished results).

In conclusion, the treatments had little effect on the abundance of earthworms, nematodes and microbes. There was a year effect on the trophic group composition of nematodes and fungal biomass which was probably related to a change in clover content. The bacterial activity increased with N application rate but was also related to a higher pH. This increase in bacterial activity possibly increased the mineralization and decreased the SOM.

5.4.3 Relevance of changes in soil quality for ecosystem services

Soil biota are key for a range of ecosystem services, including soil structure maintenance, water retention, supply of nutrients and grass production (Swift et al., 2004; Mulder, 2006; Brussaard et al., 2007a; Kibblewhite et al., 2008). Soil structure maintenance is an ecosystem service provided by SOM, roots and soil biota such as earthworms, bacteria and fungi. Under pure grass, with a higher N application rate, Van Eekeren et al. (2009a) found some evidence that solid organic manure contributed more to soil structure maintenance than inorganic fertilizer. Under clover-only, Van Eekeren et al. (2009b) found a less developed soil structure than in pure grass or the mixture of grass-clover. In our experiment, with a tendency of reduced SOM-levels over the years but no significant treatment effects on root, earthworm and microbial parameters, only little effect was measured in 2007 on the soil physical parameters underlying the service of soil structure maintenance. Compared to no manure, the bulk density was lower in FYM and NSM, and was positively correlated with clover content. The latter is in line with Van Eekeren (2009b). Our results suggest that after seven years, with a tendency of reduced SOM levels, there was only little effect of N application rate and manure type on the ecosystem service of soil structure maintenance in grass-clover.

Water regulation is an ecosystem service largely provided by SOM, directly through water retention (Gupta and Larson, 1979; Ohu et al., 1987), and by earthworms through their burrowing activities, which stimulate water infiltration and deeper root growth (Hoogerkamp et al.,1983; Clements et al., 1991; Logsdon and Linden, 1992; Edwards and Shipitalo, 1998). Therefore, the higher SOM content in FYM in our experiment is expected to lead to a higher water retention. The number of earthworm burrows was not significantly different between the treatments. Van Eekeren et al. (2009a) measured more earthworm burrows under grass in plots with organic fertilizers compared to the unfertilized control, and Van Eekeren et al. (2009b) found more earthworm burrows under clover. This suggests that clover in this experiment compensated for the effects of a reduced manure application on earthworm burrows. Moreover, the soil penetration resistance did not differ in the treatments, indicating no change in earthworm activity. All together the results suggest that the higher SOM in FYM treatments can increase the water retention but that no differences in water infiltration are expected.

Concerning the ecosystem service of nutrient supply, the highest N application led to the highest SOM, the highest pH, the highest bacterial activity and the highest potentially mineralizable C. However, these treatment effects did not result in significant differences in the grass-clover yield over the years 2003 and 2007. Grass-clover yield was not only positively correlated with bacterial activity, but also with the clover content in the sward, which is an important determinant for N fixation. Baars (2002) found higher grass-clover yields in treatments that received slurry and farm yard manure than in a no-manure treatment. He ascribed this partly to a shortage in potassium in the non-manure treatment. Since in our experiment the potassium and phosphate fertilization for all treatments were the same, only the N application could have made a difference in grass-clover yield. Considering the effect of N application on grass-clover, Schils and Snijders (2004) found that the increased N yield due to N application (up to 190 kg N ha^{-1}) was completely offset by the reduced N fixation. Both Schils (1997) and Baars (2002) argue that N application before the first harvest results in higher DM yield. In our experiment such differences could not be measured. However, we found a decrease in DM yield from 2003 to 2007, which was probably related to the lower clover content in 2007 compared to 2003. The very dry summer of 2003 reduced the clover content at the end of the summer from which it did not recover to the dominance in 2003. It is known that it is difficult to maintain an optimal clover content in accordance to the nitrogen supply capacity of the soil. Management measures to maintain an optimal clover content like choice of a persistent clover cultivar, and an optimal potassium and phosphate supply (Baars, 2002; Van Eekeren et al., 2005b), were taken in this experiment. However, the pH at the start of the experiment was too low for clover. To optimize the pH to the official Dutch fertilization recommendations of grass-clover (pH-KCl 5.2-5.5; http.//www.bemestingsadvies.nl), lime was applied. Possibly, the increase in pH with a subsequently increased bacterial activity, led to an increased N mineralization and N availability, which had a negative effect on the clover content. In experiments of Scheu (2003) and Kreutzer et al. (2004) an increased availability of N by mineralization, through the presence of earthworms, counteracted the dominance of *T. repens* by increasing the biomass of *L. perenne*. The higher N mineralization must have increased the grass production but did not completely compensate for the loss of N availability by fixation of the clover. Based on a theoretical N fixation of 39 g N 1000 g^{-1} white clover according to Baars (2002), the N fixation in 2007 was 7.8 g N m^{-2} compared to 21.3 g N m^{-2} in 2003.

Therefore, we conclude that under grass-clover the ecosystem service of nutrient supply of N is dominated by the N-fixing *Rhizobium* bacteria, which depends on the clover content in the sward. Besides other management options to maintain the clover cover (e.g. persistent clover cultivar, and an optimal potassium and phosphate supply), the initial pH when seeding grass-clover should be within the limits of the recommendations. In the following years the pH should be maintained on a yearly basis to compensate for acidifying effects of clover accounting for the grazing and cutting management, and organic manure application.

5.5 Conclusions

We conclude that with the export of animal manure from organic dairy farms to organic arable farms the soil biological quality under grass-clover pastures will be sustained by the clover if it persists in the grass-clover sward. Most effects of manure type and N application rate on soil biological quality were compensated for by the N fixation of the clover. However, even with the application of organic manure, there was a tendency towards a decrease in SOM, whereas normally there is a net accumulation. One of the causes was probably liming, which increased pH, bacterial activity and mineralization. It is clear that the whole system depends on the clover persistence. Over the years a reduction in clover content resulted in a reduced DM yield, increased abundance and percentage of herbivorous nematodes, decrease of bacterivorous nematodes and increase in fungal biomass. It is likely that, amongst others, an increased N mineralization due to liming had an overall negative effect on the clover content. The higher mineralization must have increased the production but not completely compensated for the loss of the N fixation of the clover. A persistent white clover cultivar in combination with a soil pH within the limits of the recommendations before sowing, should guarantee the persistence of the clover, next to an optimal fertilization of potassium and phosphate.

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Chapter 6

Effects of individual grass species and grass species mixtures on soil quality as related to root biomass and grass yield

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Abstract

For the purpose of feeding value, drought resistance and nitrogen utilization, other grasses (*e.g. Festuca arundinacea* and *Dactylis glomerata*) than the currently widely used perennial rye grass (*Lolium perenne*) are introduced in dairy farming, either as a monoculture or in a mixture. To study the effect of these grasses on yield and soil chemical and biological quality, the three species were sown in a field experiment in monoculture and in two mixtures. Within two growing seasons, the grass species tested under high soil fertility conditions did not show significant effects on most of the tested soil biological parameters. Only for the mixture of *L. perenne* and *D. glomerata* a higher soil NO_3^- and mineral N content were most probably related to a higher bacterial activity, possibly induced by dying roots of *L. perenne*. This was the likely cause of the high aboveground dry matter yield of this mixture. The N-efficiencies of the monocultures of *L. perenne, F. arundinacea* and *D. glomerata* were not different when only considering the aboveground biomass. In *L. perenne* and *F. arundinacea* the total N in root biomass was higher while under *D. glomerata* the NO_3^- in the soil was higher. The lower fraction of mineral N to total N for *L. perenne, F. arundinacea* and the mixture of the two suggests that their organic matter build-up/mineralization ratio was higher than for *D. glomerata*. Furthermore, the mixture of *L. perenne* and *F. arundinacea* showed significantly lower soil mineral N levels than the monocultures of each. We suggest that grassland systems with either *L. perenne* or *F. arundinacea* or both are more sustainable than *D. glomerata*, in terms of reduced nitrogen losses, increased build-up of soil organic matter and probably soil structure. Furthermore, *D. glomerata* should only be used in species mixtures with companion grass species that have vigorous root development and that are able to withstand competition from *D. glomerata.*

Keywords

Grass species, grass mixtures, grass roots, soil nitrogen, soil organic matter, soil quality, microbiology, nematodes

6.1 Introduction

Organic farming and reduced use of external inputs, such as fertilizers and pesticides, implies a greater reliance on self-regulating ecosystem processes (Brussaard et al., 2007a). Soil biota (soil organisms and plant roots) play a key role in these processes. Grassland management directly and indirectly influences the soil biota and their functions (Bardgett, 2005). To develop and optimize sustainable grassland systems, insight is needed into how grassland management influences the soil quality in general and soil biota in particular (Van Eekeren et al., 2007).

For the purpose of specific feeding value (e.g. structural components) and drought resistance, other grasses (*e.g. Festuca arundinacea* and *Dactylis glomerata L.*) than the currently used perennial rye grass (*Lolium perenne L.*) are being introduced on dairy farms. Introduction can be either as a monoculture or in a mixture with L. perenne. Some studies provide evidence that mixtures can improve grassland productivity (Remison and Snaydon, 1980b; Wilson and Newman, 1987) because the species differ in limiting growth factors, growing periods, and root characteristics (Whittington and O'Brien, 1968; Wilson, 1988). Especially complementary root characteristics in a mixture might develop a 'rootscape' that can exploit the soil volume more efficiently for water and nutrients than monocultures of each of the species (Crush et al., 2005).

Plants affect soil chemical and biological quality through various mechanisms: 1) the quantity and quality of resources allocated to the soil, 2) the extent to which plant species deplete nutrients and water from soils, and 3) the modification and formation of habitats for soil biota (Clement and Williams 1964; 1967; Klein et al., 1988; Stone and Buttery 1988; Carter et al. 1994; Wardle, 2002). Since the major source of organic matter in soil is from plant primary production, entering soil as litter-fall, root die-off and rhizodeposition, there is a tight coupling between plant and microbial productivity (Paterson and Sim, 2000). Different functional types of plants give rise to differences in microbial biomass in soils, as well as in microbial activity and community structure, as has been demonstrated in microcosms (Wardle & Nicholson, 1996; Graystone et al., 1998; Innes et al., 2004) as well as in the field (Groffman et al., 1996; Wardle et al., 1999). Similarly, nematodes differ in abundance and community structure in soils on which different functional types of plants are grown (Griffiths et al., 1992; Viketoft et al., 2005).

In grasslands, productivity and soil quality are related to root characteristics. Wardle et al. (1997) suggest that the above-ground effect of plant litter on soil biota is of less importance than that of plant parts belowground. However, effects of plant species on soil biota are generally studied in the upper soil layer, while grass species are likely to differ in root properties at different depths. As a consequence, the effects of grass species on soil biota may differ accordingly between soil layers.

We conducted a field experiment with three grass species used in production grasslands: *Lolium perenne* (Perennial rye grass), *Festuca arundinacea* (Tall fescue), *Dactylis glomerata* (Cocksfoot) and *L. perenne* mixed with either *F. arundinacea* or *D. glomerata*. We measured root biomass, grass yield, and soil chemical and biological parameters at three soil depths; 0-10 cm, 10-20 cm and 20-30 cm. The objectives of this study were 1) to assess the effect of grass species and mixtures on soil chemical and biological quality, 2) to quantify the effect of root biomass on soil quality, and 3) to measure the effect of grass species and mixtures on grass yield. We hypothesised that grasses with the highest root biomass would have the highest soil content of organic C and total N, and that this will be reflected in highest microbial biomass and abundance of nematodes. Furthermore, we hypothesised that mixtures of grasses would have a higher grass yield than that of the highest yielding monoculture.

6.2 Materials and methods

6.2.1 Study site and experimental design

This study was carried out from April 2007 to September 2008 on a grassland on sandy soil (Typic Haploquod according to USDA classification) in the South of the Netherlands (51º39´N, 5º11´E). Five treatments were established in a complete randomized block design with three blocks. The individual plot size was 24 m^2 (3 m x 8 m). The grass treatments were:

LP : *Lolium perenne* (cv. Bargala);
FA : *Festuca arundinacea* (cv. Barolex);
DG : *Dactylis glomerata* (cv. Ambassador);
LP/FA : Mixture of *L. perenne* and *F. arundinacea*;
LP/DG : Mixture of *L. perenne* and *D. glomerata*.

Prior to the experiment, the field was in use as continuous maize land for a minimum of 10 years. Before sowing the grass treatments, dolomitic lime was applied at the rate of 2500 kg ha^{-1}. In 2008 all plots received 250 kg N ha^{-1} from inorganic fertilizer (calcium ammonium nitrate 27%) and 232 kg N-total ha^{-1} from organic fertilizer (dairy manure slurry).

6.2.2 Soil parameters

On 8 September 2008, two growing seasons after the start of the experiment, soil samples for determination of soil chemical and biological parameters were taken. Bulk samples of 70 cores (ø 2.5 cm) at three soil depths (0-10 cm, 10-20 cm, 20-30 cm) were collected per experimental plot, sieved through 1 cm mesh, homogenized and stored at field moisture content at 4°C before analyses. Sub-samples were taken for chemical, microbial and nematode analyses.

6.2.2.1 Soil chemical parameters

Prior to further chemical analysis, samples were oven-dried at 40 °C. Soil dry matter content was determined after oven-drying of approximately 30 g of the bulk sample (in duplicate) at 105 ºC. Soil acidity of the oven-dried samples was measured in 1 M KCl (pH-KCl). Organic C was measured by incineration of dry material at 1150 °C, after which the produced CO_2 was determined by an infrared detector (LECO Corporation, St. Joseph, Mich., USA). For determination of total N, gasses after incineration were reduced to N_2 and detected with a thermal-conductivity detector (LECO Corporation, St. Joseph, Mich., USA).

6.2.2.2 Soil microbiological parameters

From the bulk soil sample, a sub-sample of 200 g field moist soil was adjusted to 50% WHC (Water Holding Capacity) and pre-incubated at 12 °C for four weeks, to avoid the effects of temperature and moisture fluctuations in the field and to stabilize soil conditions (Bloem et al., 2006). After pre-incubation bacterial biomass was determined for all samples. Fungal biomass and bacterial growth rate was only determined for the treatments LP, DG and LP/DG while bacterial biomass was determined for all treatments. Microbial soil smears were prepared and measured as described by Bloem and Vos (2004). Fungal hyphae were measured using the grid-intersection method. Bacterial numbers and cell volumes were measured by confocal laser scanning microscopy and automatic image analysis (Bloem et al., 1995). Bacterial biomass was calculated from bacterial cell volume. Bacterial growth rate was determined as the incorporation of [^{3}H]thymidine and [^{14}C]leucine into bacterial DNA and proteins respectively (Bloem and Bolhuis, 2006; Michel and Bloem, 1993). For a more detailed description, see De Vries et al. (2006).

Potentially mineralizable N was measured for the treatments LP, DG and LP/DG by anaerobic incubation of a soil sample under water (in slurry) for 1 week at 40°C (Keeny and Nelson 1982; Canali and Benedetti 2006). These warm and anoxic conditions are optimal for a quick mineralization of organic matter by anaerobic bacteria. The lack of oxygen prevents conversion of released NH_4^+ to NO_3^- (nitrification), so that N losses by denitrification can not occur. The amount of mineral N (NH_4^+-N) released is a measure of the quality (N-content and decomposability) of the organic matter, and thus for biological soil fertility (Sparling and Schipper 2002).

6.2.2.3 Soil nematode parameters

Free-living nematodes were extracted from a sub-sample of 100 ml field-moist soil, using the Oostenbrink elutriator (Oostenbrink, 1960). Total numbers were counted and expressed per 100 g fresh soil. Nematodes were fixed in hot formaldehyde 4%, and at least 150 randomly selected nematodes from each sample were identified to genus and, whenever possible, to species. Nematode genera and species were assigned to trophic groups, following Yeates et al. (1993), and to colonizer-persister groups (cp-groups), according to Bongers (1990) and Bongers et al. (1995). The Nematode Channel Ratio (NCR) was calculated to express the relative contributions of bacterivorous (B) and fungivorous (F) nematodes to the total microbivorous nematode abundance (NCR = B/(B+F)) (Yeates et al., 2003). The Maturity Index was calculated as the weighted mean of the individual cp-values, in accordance with Bongers (1990) and Korthals et al. (1996). The Maturity Index is an ecological measure which indicates the condition of an ecosystem based on nematode species composition.

6.2.3 Crop parameters

The plots were harvested four times in 2007 and five times in 2008. Grass dry matter yield in the harvests was determined by cutting a strip of 0.84 m x 5 m with a two-wheel tractor. After weighing the fresh biomass, a sub-sample was dried for 48 hours at 70 ºC and analyzed for dry matter (DM) and total N. Total N in the grass samples was only determined in the treatments with monocultures. In order to determine the percentage of the different grass species in the two mixtures, a second sub-sample was taken in the 2nd and 4th harvest at 2008 in which grasses were hand-sorted and dried for 24 hours at 105 ºC.

In September 2008, three soil cores (8.5 cm diameter) at three depths (0-10, 10-20, 20-30 cm) were collected per plot to determine the root biomass. The soil in the samples was carefully washed out with water, after which the roots were oven-dried at 70 °C for 48 hours and the dry matter of the roots was weighed. After drying, the individual samples of roots were analyzed for ash content and total N. Root biomass was expressed as grams of ash-free dry matter (AFDM).

6.2.4 Statistical analyses

The effects of the five grass treatments (LP, FA, DG, LP/FA, LP/DG) and three soil depths (0-10 cm, 10-20 cm, 20-30 cm) were tested using two-way ANOVAs in randomized blocks. Treatment effects were apportioned on the basis of Least Significant Difference (LSD) post-hoc analyses. When relations between parameters were expected, Pearson correlations were determined. Since depth was a dominant factor, the correlations between soil parameters were corrected for depth by substracting the mean of each soil layer. Grass yield was correlated with soil parameters in the 0-10 cm and 0-30 cm soil layer. ANOVAs and correlation analyses were carried out using the GENSTAT statistical software (8th Edition, VSN International, Hemel Hempstead, UK). To assess the effects of the grass and depth treatment on nematode community composition, non-metric multidimensional scalings were used, followed by Analyses of Similarity (ANOSIM) using Primer 5.0 (©2000 PRIMER-E Ltd.).

6.3 Results

6.3.1 Soil chemical parameters

Although DG and LP/DG had the lowest soil organic C and total N, differences were not significant (Table 6.1). Both parameters decreased significantly with soil depth. The amount of NO_3^- decreased with depth and showed a significant decrease in the order LP/DG>DG>LP=FA=LP/FA (Table 6.1; Fig. 6.1). The same held for mineral N, where in addition LP/FA was significantly lowest. The mineral N fraction, expressed as percentage of soil total N, was highest for DG and LP/DG.

There was a tendency for a lower pH in the DG and LP/DG treatments (Table 6.1). When corrected for soil layer, soil pH was negatively correlated with NO_3^- (r=-0.41, P=0.006).

6.3.2 Soil biological parameters

6.3.2.1 Microbial parameters

Soil bacterial biomass did not differ between grass treatments but decreased significantly with depth (Table 6.2). This was similar for fungal biomass, where in addition depth showed a significant interaction effect with grass treatment. This interaction was caused by a slight increase in fungal biomass in DG with depth and a decrease in depth in LP/DG. When corrected for soil layer, bacterial biomass was correlated with soil total N (r=+0.33, P=0.028) and pH (r=-0.35, P=0.017)

Bacterial activity was significantly highest in LP/DG (Table 6.2; Fig. 6.2) and decreased significantly with depth. However, there was a significant interaction effect between the grass treatment and depth (Fig. 6.2). When corrected for soil layer, bacterial activity (thymidine and leucine incorporation, respectively) was significantly correlated with total number of nematodes (r=+0.44, P=0.022; r=+0.51, P=0.007) and fungal biomass (r=+0.39, P=0.047; r=+0.43, P=0.026).

Table 6.1 Soil chemical parameters in grassland with *Lolium perenne* (LP), *Festuca arundinacea* (FA), *Dactylis Glomerata* (DG), a mixture of *L. perenne* and *F. arundinacea* (LP/FA), and a mixture of *L. perenne* and *D. glomerata* (LP/DG) at three soil depths (0-10 cm, 10-20 cm, 20-30 cm).

Chemical	Unit	Mean of Grass treatment over three soil depths						Mean of soil Depth over five grass treatments				G*D
		LP	FA	DG	LP/FA	LP/ DG	P-value	0-10	10-20	20-30	P-value	P-value
Organic C	g C kg dry soil^{-1}	16.0	16.4	14.7	17.3	15.3	0.280	17.8a	15.1b	14.9b	*0.007*	0.755
Total N	g N kg dry soil^{-1}	0.96	0.99	0.83	1.02	0.95	0.218	1.08a	0.92b	0.85c	*0.006*	0.989
NH_4^+	mg NH_4^+ N kg dry soil^{-1}	12.6	12.8	11.6	10.0	13.1	0.079	11.7	12.0	12.3	0.786	0.811
NO_3^-	mg NO_3^- N kg dry soil^{-1}	5.6C	5.3C	8.9B	5.6C	12.7A	*<0.001*	11.7a	5.0b	6.2b	*<0.001*	0.088
Mineral N	mg N kg dry soil^{-1}	18.2C	18.0C	20.5B	15.6D	25.8A	*<0.001*	23.5a	16.9c	18.6b	*<0.001*	0.491
Fraction (%) mineral N of total N		1.9B	1.8B	2.5A	1.6B	2.8A	*<0.001*	2.3	1.9	2.2	0.085	0.278
C/N		16.7	16.9	18.0	17.1	16.4	0.467	16.8	16.6	17.7	0.268	0.380
pH-KCl		5.10	5.02	4.98	5.11	4.97	0.072	5.60a	4.72b	4.78b	*<0.001*	0.705

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

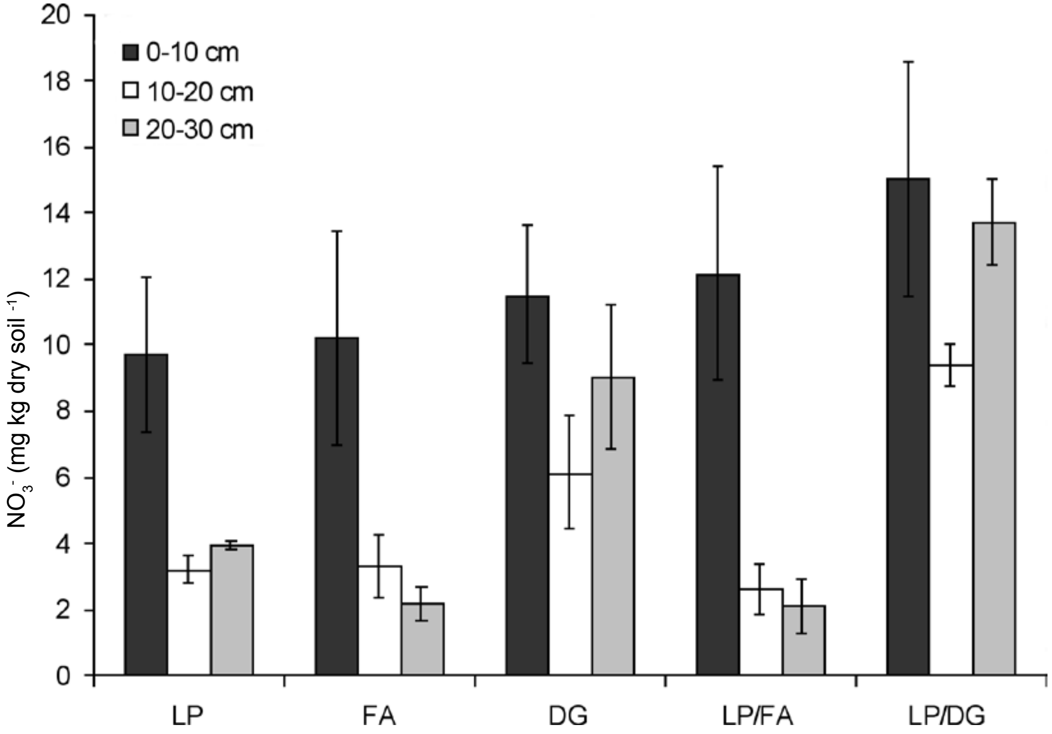

Figure 6.1 NO_3^- in grassland with *Lolium perenne* (LP), *Festuca arundinacea* (FA), *Dactylis Glomerata* (DG), a mixture of *L. perenne* and *F. arundinacea* (LP/FA), and a mixture of *L. perenne* and *D. glomerata* (LP/DG) at three soil depths (0-10 cm, 10-20 cm, 20-30 cm).

Potentially mineralizable N was significantly highest in LP and decreased significantly with depth (Table 6.2). When corrected for soil layer, potentially mineralizable N was correlated with total N (r=+0.43, P=0.027), but not to root biomass.

6.3.2.2 Nematodes

The grass treatments did not affect the total number of nematodes or the abundance of any of the trophic groups (Table 6.3). There were some significant differences between grass treatments in the proportions of trophic groups, but the differences in absolute terms were small. The abundance of all trophic groups except carnivorous nematodes, decreased with depth. For the herbivorous nematodes there was an interaction between the grass treatment

Table 6.2 Microbial parameters in grassland with *Lolium perenne* (LP), *Festuca arundinacea* (FA), *Dactylis Glomerata* (DG), a mixture of *L. perenne* and *F. arundinacea* (LP/FA), and a mixture of *L. perenne* and *D. glomerata* (LP/DG) at three soil depths (0-10 cm, 10-20 cm, 20-30 cm).

Microbial	Unit	Mean of Grass treatment over three soil depths						Mean of soil Depth over five grass treatments				G*D
		LP	FA	DG	LP/FA	LP/DG	P-value	0-10	10-20	20-30	P-value	P-value
Bacterial biomass	µg C g dry soil^{-1}	12.8	15.3	12.7	14.0	12.2	0.894	17.6a	11.4b	11.2b	0.035	0.566
Thymydine incorporation	pmol g dry soil^{-1} h^{-1}	20B	--	21B	--	26A	0.043	45a	15b	7c	<0.001	0.007
Leucine incorporation	pmol g dry soil^{-1} h^{-1}	467B	--	495Bb	--	574A	0.002	917a	392b	227c	<0.001	0.003
Fungal biomass	µg C g dry soil^{-1}	11.3	--	8.9	--	11.0	0.463	14.1a	9.0b	8.1b	0.025	0.041
Active fungal hyphae	percentage length	9.8	--	14.7	--	5.1	0.273	4.4	16.6	8.7	0.128	0.338
Pot.mineralizable N	mg N kg dry soil^{-1}wk^{-1}	20.7A	--	14.9B	--	15.0B	0.005	27.5a	13.0b	10.1c	<0.001	0.862

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

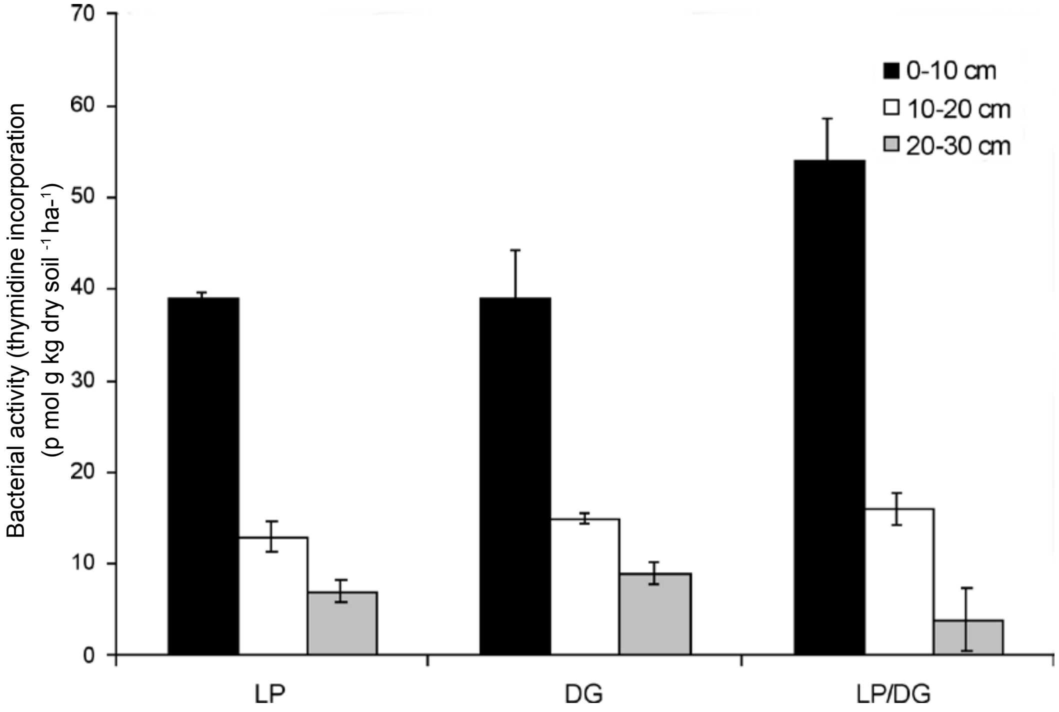

Figure 6.2 Bacterial activity in grassland with *Lolium perenne* (LP), *Dactylis Glomerata* (DG) and a mixture of *L. perenne* and *D. glomerata* (LP/DG) at three soil depths (0-10 cm, 10-20 cm, 20-30 cm).

and depth (Fig. 6.3). The DG-treatment had less herbivorous nematodes at 0-10 cm depth than the other treatments, and more herbivorous nematodes at 20-30 cm depth than the LP/FA-treatment. The number of herbivorous nematodes in the mixture of LP/DG was similar to that in LP in the 0-10 and 10-20 cm soil layer, while it resembled DG in the 20-30 cm soil layer. When corrected for soil layer, the number of herbivorous nematodes was correlated with root biomass (r=+0.39, P=0.009).

The cp-2 group dominated with 62-65% the grass treatments (Table 6.3). The effect of the grass treatments on the cp-groups resulted in significant differences in the cp-1, cp-3 and cp-5 group. The differences in cp-group composition resulted in significant differences in the Maturity Index between the grass treatments. DG had the highest MI, and FA and LP/DG the lowest. The percentage of the cp-2 group decreased with depth while the percentage of the cp-3, cp-4 and cp-5 group increased. This was reflected in a higher MI with increasing soil depth.

Table 6.3 Nematode parameters in grassland with *Lolium perenne* (LP), *Festuca arundinacea* (FA), *Dactylis Glomerata* (DG), a mixture of *L. perenne* and *F. arundinacea* (LP/FA), and a mixture of *L. perenne* and *D. glomerata* (LP/DG) at three soil depths (0-10 cm, 10-20 cm, 20-30 cm).

Nematode	Unit	Mean of Grass treatment over three soil depths						Mean of soil Depth over five grass treatments				G*D
		LP	FA	DG	LP/FA	LP/DG	P-value	0-10	10-20	20-30	P-value	P-value
Total number	n 100g soil^{-1}	3089	2831	2667	2828	3095	0.208	4677a	2373b	1656c	<0.001	0.199
Bacterivorous	n 100g soil^{-1}	1510	1527	1265	1463	1565	0.432	2810a	909b	679b	<0.001	0.938
Dauerlarvae	n 100g soil^{-1}	121	130	217	120	141	0.090	222a	143b	73b	<0.001	0.425
Herbivorous	n 100g soil^{-1}	1162	973	933	1011	1180	0.191	1371a	1123b	661c	<0.001	0.043
Fungivorous	n 100g soil^{-1}	104	61	71	79	47	0.062	86	68	64	0.310	0.811
Omnivorous	n 100g soil^{-1}	102	63	60	64	80	0.299	128a	57b	37b	<0.001	0.460
Carnivorous	n 100g soil^{-1}	89	77	120	90	83	0.299	60b	74b	142a	<0.001	0.738
Bacterivorous	%	46	50	43	48	46	0.227	60a	38b	42b	<0.001	0.550
Dauerlarvae	%	4b	5b	8a	5b	4b	0.027	5	6	5	0.186	0.247
Herbivorous	%	39	37	38	36	43	0.356	29c	47a	39b	<0.001	0.076
Fungivorous	%	4A	2AB	3AB	4A	2B	0.032	2b	3ab	4a	0.008	0.381
Omnivorous	%	3	2	2	2	2	0.145	3	2	2	0.495	0.307
Carnivorous	%	4	4	6	5	4	0.080	1c	3b	9a	<0.001	0.301
Cp-1	%	13B	21A	13B	14B	18AB	0.039	14	16	17	0.290	0.490
Cp-2	%	64	62	63	65	65	0.788	77a	64b	51c	<0.001	0.504
Cp-3	%	4AB	3AB	3AB	5A	2B	0.003	2b	4a	5a	<0.001	0.001
Cp-4	%	14	12	15	11	13	0.132	6c	13b	19a	<0.001	0.201
Cp-5	%	5AB	3C	6A	4BC	3BC	0.026	1c	4b	7a	<0.001	0.671
Number of taxa		27.8	29.4	28.9	30.8	30.6	0.352	28.7	29.6	30.2	0.712	0.487
MI (cp1-5)		2.34AB	2.14C	2.39A	2.26BC	2.19C	0.005	2.04c	2.26b	2.49a	<0.001	0.384
NCR		0.92C	0.96AB	0.94BC	0.92C	0.97A	0.010	0.97a	0.93b	0.92	<0.001	0.351

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

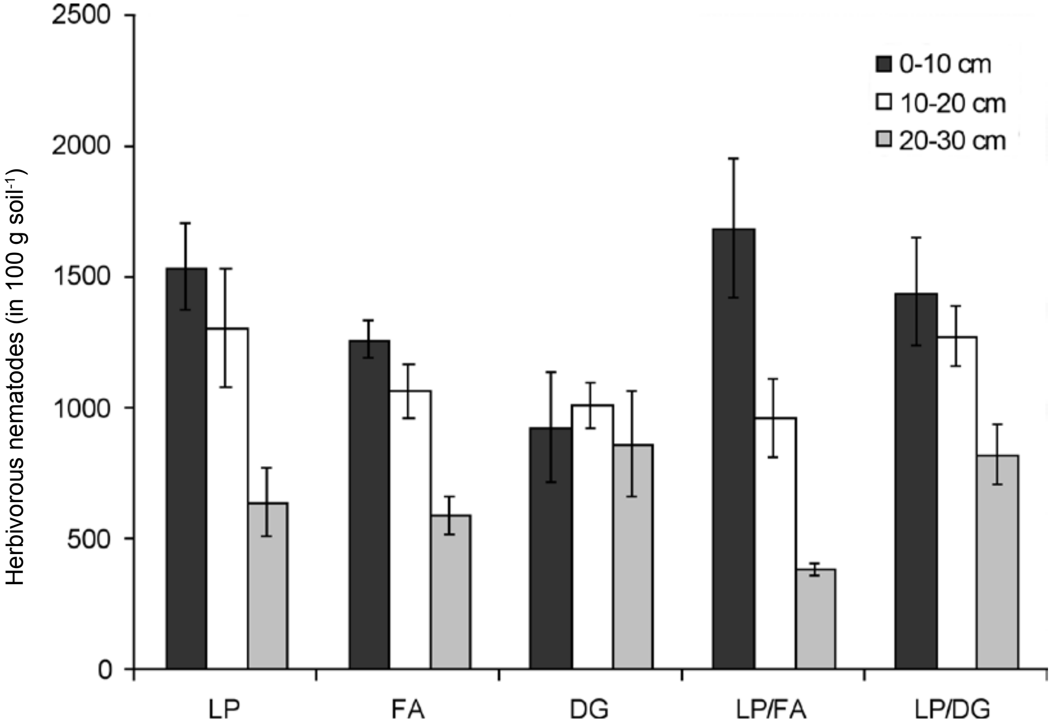

Figure 6.3 Herbivorous nematodes in grassland with *Lolium perenne* (LP), *Festuca arundinacea* (FA), *Dactylis Glomerata* (DG), a mixture of *L. perenne* and *F. arundinacea* (LP/FA), and a mixture of *L. perenne* and *D. glomerata* (LP/DG) at three soil depths (0-10 cm, 10-20 cm, 20-30 cm).

The number of nematode taxa did not differ for the grass treatments and depths. The multidimensional scaling of the nematode species composition (Fig. 6.4) shows that the depth of sampling had a significant effect on species composition ($r=0.98$, $P<0.001$) whereas grass treatments did not. These community changes were mainly due to the effects of depth on bacterivorous nematode species.

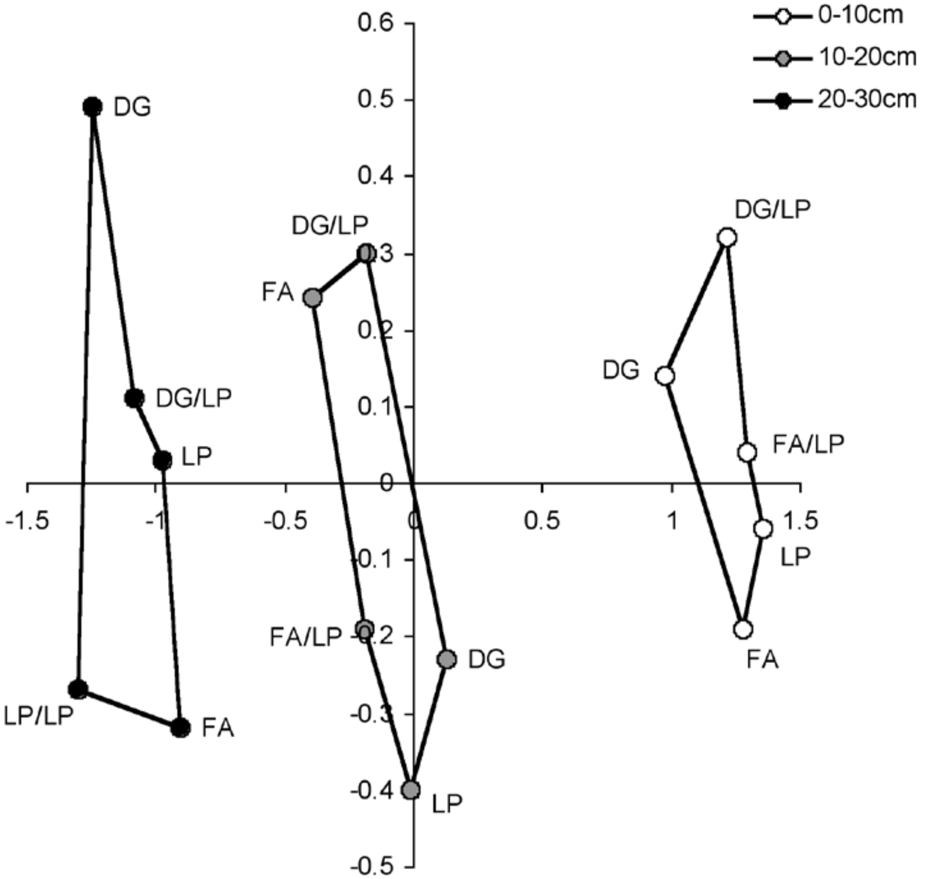

Figure 6.4 The non-metric multidimensional scaling (nMDS) based on Bray-Curtis similarity between nematode species compositions in the grass and depth treatments (stress=0.04). Lines connect treatments of which nematode communities did not differ. Differences between the communities of each of the three soil depths were significant (ANOSIM: r=0.98, P<0.001). Compositions did not differ between grass treatments.

6.3.3 Crop parameters

The total grass DM-yield decreased in the order LP/DG > DG > FA > LP/FA > LP (Table 6.4). The grass N-yield was not significantly different between the three monocultures. In the mixture with LP/FA, *F. arundinacea* constituted 41% of the DM in the 2nd harvest and

Table 6.4 Crop parameters in grassland with *Lolium perenne* (LP), *Festuca arundinacea* (FA), *Dactylis Glomerata* (DG), a mixture of *L. perenne* and *F. arundinacea* (LP/FA), and a mixture of *L. perenne* and *D. glomerata* (LP/DG) at three soil depths (0-10 cm, 10-20 cm, 20-30 cm).

Crop parameter	Unit	Mean of Grass treatment over three soil depths						Mean of soil Depth over five grass treatments				G*D
		LP	FA	DG	LP/FA	LP/DG	P-value	0-10	10-20	20-30	P-value	P-value
Root biomass												
AFDM	g m^{-2}	513A	524A	383B	530A	376B	*0.010*	1034a	217b	135c	<0.001	0.008
Total N[1]	g N m^{-2}	21.5A	18.1AB	13.7B	21.9A	14.7B	*0.023*	--	--	--	--	--
C/N[1]		28.8	30.4	30.3	28.1	27.6	0.790	--	--	--	--	--
Grass yield												
Total DM	g DM m^{-2}	1383D	1534BC	1569AB	1430CD	1641A	*0.003*	--	--	--	--	--
Total N	g N m^{-2}	39.5	42.2	42.5	--	--	0.290	--	--	--	--	--
Root/shoot		0.89A	0.72AB	0.51B	0.85A	0.50B	*0.015*					

[1] Only measured in the 0-10 cm soil layer.

Values followed by the same letter within a row are not statistically different at the 5% error level for the main treatment effect.

37% in the 4th harvest. In the mixture with LP/DG, *D. glomerata* dominated with 88% of the DM in the 2nd harvest and 99% in the 4th harvest. In the 0-10 cm soil layer, both DM- and N-yield were correlated with mineral N (r=+0.56, P=0.030; r=+0.70, P=0.038, respectively), NO_3^- (r=+0.52, P=0.045; r=+0.86, P=0.003, respectively) and percentage of cp-1 nematodes (r=+0.64, P=0.011; r=+0.88, P=0.002, respectively). Comparable relations were found with soil parameters in the 0-30 cm soil layer with the exception that bacterial activity (thymidine incorporation) was also correlated with DM-yield (r=+0.67, P=0.049).

Root biomass was significantly higher in LP, FA and LP/FA, than in the treatments DG and LP/DG (Table 6.4). A lower root biomass of DG and LP/DG, combined with a lower N-content of the root biomass (data not shown) resulted in a significantly lower root total N in the 0-10 cm soil layer for these two treatments. For root biomass there was a significant

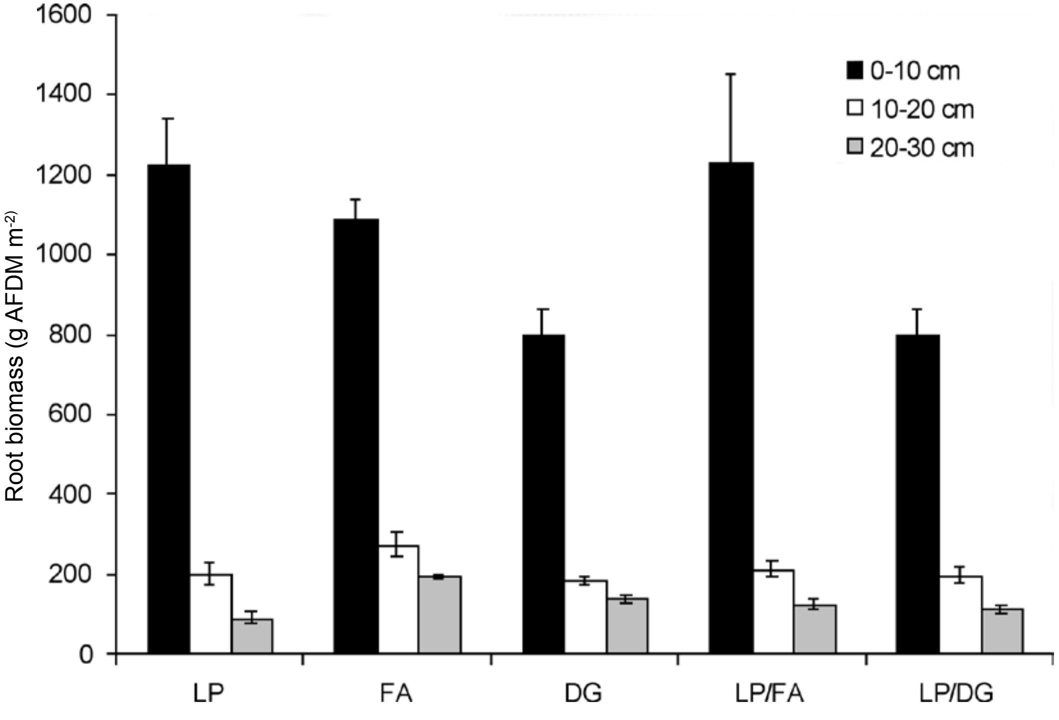

Figure 6.5 Root biomass in grassland with *Lolium perenne* (LP), *Festuca arundinacea* (FA), *Dactylis Glomerata* (DG), a mixture of *L. perenne* and *F. arundinacea* (LP/FA), and a mixture of *L. perenne* and *D. glomerata* (LP/DG) at three soil depths (0-10 cm, 10-20 cm, 20-30 cm).

interaction effect between grass treatments and depth in that the effect of depth was strongest in the LP, FA and LP/FA treatments and weaker in the DG and LP/DG treatments (Fig. 6.5), because LP, FA and LP/FA had a higher root biomass in the 0-10 cm than DG and LP/DG. FA had the highest root biomass in the 10-20 cm and 20-30 cm soil layer of all grass treatments. When corrected for soil layer, root biomass was correlated with soil pH (r=+0.4, P=0.007) and the number of herbivorous nematodes (r=+0.39, P=0.009), but not to soil mineral N
The root (AFDM in 0-30 cm) to shoot ratio was significantly highest for LP and LP/FA while the ratio was lowest for DG and LP/DG.

6.4 Discussion

6.4.1 Soil chemical parameters

The soil organic C and total N decreased with soil depth. Since the experimental field had been under continuous maize cultivation with conventional ploughing for at least 10 years, at the start of the experiment the organic matter was well mixed throughout the plough layer (about 25 cm). After two growing seasons under grass, the on average higher soil organic C and total N in the 0-10 cm soil layer, suggests an accumulation of organic C and total N with grass (Haynes, 2000). The grass treatments with *D. glomerata* (DG and LP/DG) had a lower soil organic C and total N. Although differences were not significant, this coincided with a significantly lower potentially mineralizable N in these treatments compared to LP, whereby potentially mineralizable N was correlated with total N in our present (r=+0.43, P=0.027) and other studies (chapter 7). Furthermore, in other research, with a longer experimental period and another sampling depth, differences in total soil organic C and total N between grass species have been found. Clement and Williams (1964; 1967) reported lower soil organic C and total N in the 0-7.5 cm soil layer of a three years old sward of *D. glomerata*/white clover compared to *L. perenne*/white clover. Later, Carter et al., 1994 showed differences between grass species in total N in the 0-15 cm soil layer after a 4 year period. Clement and Williams (1967) and Carter et al. (1994) explained differences in soil organic C and total N, from differences in organic matter build up depending on the biomass production and the amounts of unharvested material (roots, leaves etc.). According to Whitehead (1995) roots are the most important source of organic matter and N from harvested grassland. In our experiment, root biomass of the treatments with *D. glomerata* was 25% lower than from the other grass treatments. Next to difference in organic matter production and build-up, part of the difference in soil organic C and total N may have been caused by a difference in C- and

N-mineralization. Our results showed that the treatments with *D. glomerata* (DG and LP/DG) had increased concentrations of NO_3^- and higher fractions mineral N to total N, which may indicate higher N-mineralization. Especially the higher fraction mineral N to total N for the treatments with *D. glomerata* (DG and LP/DG) suggest a lower organic matter build- up/mineralization ratio for the treatments with *D. glomerata* compared to the treatments with *L. perenne* and *F. arundinacea* (LP, FA, LP/FA). The higher concentrations of NO_3^- under *D. glomerata* may also be partly a result of a lower N-uptake. The higher NO_3^- content of the soil under *D. glomerata* (DG and LP/DG) was most pronounced in the 10-20 cm and 20-30 cm soil layers (Fig. 6.1). Despite the higher NO_3^- level, the total DM- and N-yield of DG was not significantly higher than that of FA. Apparently, *D. glomerata* did not utilize the higher NO_3^- availability for higher net production but possibly even leached NO_3^- to deeper soil layers. Because root biomass was lowest in the treatments with *D. glomerata*, the lower uptake of NO_3^- may have been caused by its less intensive root system in the 0-10 cm soil layer. Thorup-Kristensen (2001) related higher subsoil nitrate residues among others to a lower rooting intensity. In our present study, root biomass was more related to the grass treatments and not correlated with the nitrogen availability in the soil like in Ennik et al. (1980) and Van Eekeren et al. (2009a).

The pH was highest in the 0-10 cm soil layer, possibly due to the application of lime at the start of the experiment. However, the grass treatments with *D. glomerata* had a tendency for a lower pH. This is in support of Bardgett et al. (1999b) who reported that soil pH can indeed vary between grass species. In our experiment a lower soil pH could be caused by a higher mineralization of organic matter, a higher removal of the aboveground grass yield and/or leaching of NO_3^- (Upjohn et al., 2005). From our results, DM-yield could explain the tendency for a difference in pH. However, the negative relationship between pH and NO_3^-, could be also be a confirmation of a higher N-mineralization and leaching of NO_3^- in the treatments with *D. glomerata.*

We hypothesised that grasses with the highest root biomass would have the highest soil content of organic C and total N. In our results, *L. perenne* and *F. arundinacea* had the highest root biomass but no significantly higher levels of organic C and total N. The potentially mineralizable N was lower in the *D. glomerata* treatments, which could be an early indicator for differences between the grasses in build-up and mineralization of organic C and N. The difference between the grass treatments in the fraction mineral N to total N suggests that the non-significant differences in organic C and total N were determined by differences in the organic matter build up/mineralization ratio.

6.4.2 Soil biological parameters

Bacterial and fungal biomass in the 0-10 cm layer were relatively low for grassland on sandy soils, which is probably a result of the cropping history of the experimental field (Bloem et al., 2006; Van Eekeren et al., 2008, 2009a, 2009b). Fungal biomass was also low, but relatively high compared to bacterial biomass. Overall microbial biomass was not affected by the grass treatments. This could depend on the species tested. Groffman et al. (1996) and Griffiths et al. (1992), also did not find differences in microbial biomass between *D. glomerata*, *F. arundinacea* and *L. perenne*, but they did find differences with other grass species. Moreover, Bardgett et al. (1999b) and Innes et al. (2004) showed that *L. perenne* gave rise to a lower soil microbial biomass than *Holcus lanatus* L.. Groffman et al. (1996) found that, next to grass species, microbial biomass varied with soil type and texture and argued that the variation in microbial biomass C is a function of variation in labile C input to the soil. Wardle and Ghani (1995b) found strong correlations between microbial biomass C and soil organic C, and total N in soils with a more than two-fold range in soil organic C and total N. This supports our results that bacterial biomass is associated more with soil total N than with root biomass. In the longer term the difference between grasses in the organic matter build up/mineralization ratio will probably result in bigger differences in soil organic C and total N, and hence, in differences in microbial biomass (Haynes, 2000).

In our experiment, bacterial activity between *L. perenne* and *D. glomerata* did not differ. Again this could be species-dependent as Bardgett et al. (1999b) and Innes et al. (2004) did find lower microbial activity under *L. perenne* than under *H. lanatus*. Although there was no difference between *L. perenne* and *D. glomerata* in bacterial activity, the LP/DG treatment showed a higher bacterial activity than both monocultures. This was unexpected since the LP/DG treatment consisted in the 4th harvest for 99% of *D. glomerata*. Next to bacterial activity, the treatment with LP/DG had a higher NO_3^- and mineral N level than DG. The shift from 12% *L. perenne* in the 2nd harvest to 1% *L. perenne* in the 4th harvest may have caused the differences between the mixture (LP/DG) and the monoculture (DG). Dying roots of *L. perenne* in the LP/DG-treatment may have caused a higher bacterial activity, followed by higher N-mineralization and higher NO_3^- and mineral N levels.

The higher bacterial activity or growth rate in the LP/DG-treatment, did not result in a larger bacterial biomass. Possibly, an increase in bacterial biomass was prevented by microbivores like bacterivorous nematodes and protozoa (Bloem et al., 1994). In our results, this was supported by the positive relation between bacterial biomass and the number of bacterivorous nematodes, although the number of bacterivorous nematodes was not affected by the grass treatments. Also for most other thropic groups of nematodes no effect's of the grass treatments were found. This supports Griffiths et al. (1992) who also did not find a difference between *L. perenne* and *F. arundinacea*, but did find differences in bacterivorous nematodes between these two species and *Poa pratensis*. Viketoft et al. (2005) showed that the nematode community under *D. glomerata* can differ from those under other grass species. However, Verschoor et al. (2001) measured minor differences in nematodes between grass species but major changes with time of non-fertilisation. They suggested that a lower nutritional quality of the food source, due to decreased nutrient concentrations and increased levels of defensive compounds in the plant, was the major driver of changes in herbivorous nematode communities. Similarly, Groffman et al. (1996) and Innes et al. (2004) showed that microbial properties of grass species can differ greatly between soils with different fertility.

We hypothesised that microbial biomass and abundance of nematodes would be highest in the plant treatment with the highest root biomass. However, only herbivorous nematodes were related to root biomass. Apparently our grass species tested under high soil fertility conditions did not have significant effects on most of the soil biological parameters within two growing seasons. Soil biological parameters did only partly explain the lower potentially mineralizable N, the higher NO_3^- , the higher mineral N and the higher fraction of mineral N to total N in the treatments with *D. glomerata* (DG and LP/DG). These differences were probably mainly caused by a lower organic matter build up/mineralization ratio in the treatments with *D. glomerata* compared to the other grass treatments.

6.4.3 Crop parameters

The higher grass DM-yields of the monoculture treatments FA and DG compared to LP is in agreement with production levels presented under cutting in the Dutch recommended variety list (Anonymous, 2007). The higher root biomass of LP and FP compared to DG and the distribution of roots over the soil layers was in line with results of Garwood and Sinclair (1979). Although *D. glomerata* is commonly regarded as a drought-resistant species, they did not find evidence that it was deeper-rooted or more effective in its exploitation of soil water than *L. perenne.* DG even had the lowest root/shoot ratio. Thus, DG appears to allocate relatively more resources (water, C and N) to aboveground production. Since the N-supply (from deposition, soil and fertilization) and the aboveground N-output was comparable, the N-efficiency of these grasses was not different when only taking into account the aboveground biomass. In FA and LP more N was mobilized in root biomass while under DG this was available as NO_3^- and possibly subject to leaching. The lower fraction of mineral N to total N for LP and FA suggests that their organic matter build up/mineralization ratio was higher. Carter et al. (1994) also measured a greater degree of soil structural stability with *F. arundinacea* than *D. glomerata*. Thus, as a monoculture, the grasses *L. perenne* and especially *F. arundinacea* seem more suitable for sustainable grassland systems than *D. glomerata*.

In the mixture of LP/FA, with an approximate composition of 60% *L. perenne* and

40% *F. arundinacea*, both grasses were more or less equally represented. In contrast, the mixture with LP/DG was dominated up to 99% by *D. glomerata*. This confirms the strong competitive abilities of *D. glomerata* in mixtures with *L. perenne* (Mott, 1982; Remison and Snaydon, 1980a). A higher frequency of harvesting in our experiment could have reduced the competition of *D. glomerata* (Remison and Snaydon, 1980a).

The grass yield of the LP/FA mixture was comparable with that of the proportion of each species multiplied with the grass yield of the species in monoculture. In the LP/DG-mixture the grass yield was higher than that of the highest yielding monoculture (overyielding, Trenbath, 1974). Some researchers suggest that overyielding in mixtures is caused by complementary root characteristics allowing for more efficient use of nutrients and water (Whittington and O'Brien, 1968; Berendse 1981; Wilson, 1988). In our experiment, the root biomass and distribution of the grass mixtures (LP/FA and LP/DG) reflected the proportion of species in the mixture. Since the LP/DG consisted of 99% *D. glomerata* in the 4th harvest, this meant a comparable root distribution to that of DG. Therefore, the tendency of overyielding of the LP/DG treatment in our experiment was most probably not the result of the root characteristics but caused by the dying of *L. perenne* roots. This was confirmed by a higher bacterial activity, a higher mineralization of N, higher NO_3^- levels and finally a higher grass yield. We do not conclude that overyielding of a mixture of *L. perenne* and *D. glomerata* can not be the result of complementary root characteristics. In potential, the roots of *L. perenne* were supplementary to the roots of *D. glomerata* in the 0-10 cm soil layer. The higher NO_3^- levels under *D. glomerata* combined with a high root biomass of *L. perenne* in the 0-10 cm soil layer could have resulted in a higher N-uptake and finally overyielding in the mixture. Wasilewska (1995) measured in a mixture with 5 grass species including *D. glomerata* and *L. perenne,* a higher root biomass and a higher grass yield than in a monoculture of *D. glomerata*.

Next to the possible overyielding of mixtures, the LP/FA treatment had the lowest soil mineral N content, and thus, the lowest risk of N leaching. Since the root biomass of the LP/FA was not significantly different from the monocultures and the root distribution over the soil layers was not conclusively complementary, this could not be linked to root characteristics measured in our experiment. Possibly other root characteristics played a role, for example the effective deeper rooting for water extraction of *F. arundinacea* in comparison to *L. perenne* and *D. glomerata*, and the low weight per unit length of root of *L. perenne* compared to *F. arundinacea* (Garwood and Sinclair, 1979). Hence, with a comparable root biomass, the length of root per unit volume of soil for *L. perenne* was double that of *F. arundinacea* (Garwood and Sinclair, 1979).

We hypothesised that mixtures of grasses with complementary root characteristics would overyield in comparison with the monocultures. For a mixture of *L. perenne* and *D. glomerata* this overyielding potential on the basis of root characteristics occurred indeed, but in our experiment this could be explained by extra mineralization of dying roots of *L. perenne*. The significantly lower soil mineral N in the mixture of *L. perenne* and *F. arundinacea* than in the monocultures could not be related to complementary root characteristics measured. Other root characteristics like effective depth of rooting and/or length of root per unit volume may have played a role.

6.5 Conclusions

The grass species LP, FA and DG and the mixtures LP/FA and LP/DG, grown under high soil fertility conditions did not have significant effects on most of the tested soil biological parameters within two growing seasons. Only for the mixture of *L. perenne* and *D. glomerata* a higher soil NO_3^- and mineral N content were most probably related to higher bacterial activity, possibly induced by dying roots of *L. perenne*. This was the likely reason for the overyielding in aboveground dry matter of the mixture of *L. perenne* and *D. glomerata*. The N-efficiencies of the monocultures of *L. perenne*, *F. arundinacea* and *D. glomerata* were not different when only considering the aboveground biomass. In *L. perenne* and *F. arundinacea* the N in the root biomass was higher while under *D. glomerata* the NO_3^- in the soil was higher. The lower fraction of mineral N to total N for *L. perenne, F. arundinacea* and the mixture of the two suggests that their organic matter build-up/mineralization ratio was higher. Furthermore, the mixture of *L. perenne* and *F. arundinacea* showed significantly lower soil mineral N levels than the monocultures of each. We suggest that grassland systems with either *L. perenne* or *F.arundinacea* or both are more sustainable than *D. glomerata*, in terms of reduced nitrogen losses, increased build-up of soil organic matter and probably soil structure. Furthermore, *D. glomerata* should only be used in species mixtures with companion grass species that have vigorous root development and that are able to withstand competition from *D. glomerata*.

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Chapter 7

Ecosystem services in grassland associated with biotic and abiotic soil parameters

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Abstract

Biotic soil parameters have so far seldom played a role in practical soil assessment and management of grasslands. However, the ongoing reduction of external inputs in agriculture would imply an increasing reliance on ecosystem self-regulating processes. Since soil biota play an important role in these processes and in the provision of ecosystem services, biological soil parameters should be an integral part of soil assessment. The general objective of the current study is to investigate to what extent biotic soil parameters provide additional value in soil quality assessment of grassland on sandy soils. We measured abiotic and biotic soil parameters together with process parameters underlying ecosystem services in 20 permanent production grasslands. Cross-validated stepwise regression was used to identify abiotic and biotic soil parameters that explained the soil ecosystem services soil structure maintenance, water regulation, supply of nutrients, and grassland production, respectively.

Process parameters underlying the ecosystem service soil structure maintenance such as bulk density and the percentage of sub-angular blocky elements were mainly influenced by SOM and its qualities. The correlations between penetration resistance at 0-10 cm and the percentage of soil crumbs with earthworms suggested a relationship to earthworm activity. Parameters underlying the service of water regulation showed no clear relationship to biotic soil parameters. Water infiltration rate in the field was explained by the penetration resistance at 10-20 cm. Process parameters underlying the service of nutrients' supply such as the potentially mineralizable C and N were mainly determined by soil total N. The potential C and N mineralization were more related to biotic soil parameters, whereby each parameter was the other's antithesis. The grassland production without N fertilization viz the nitrogen supply capacity of the soil measured as N yield, was mainly explained by soil organic matter (SOM) and soil moisture, and to a lesser extent by soil total N. One gram of SOM per kg of dry soil corresponded to 3.21 kg N yield ha^{-1}, on top of a constant of 15.4 kg N ha^{-1}. The currently applied calculations in the Dutch grassland fertilization recommendation, underestimated in 85% of the production grasslands, the measured nitrogen supply capacity of the soil by on average 42 kg N ha^{-1} (31%). This legitimizes additional research to improve the currently applied recommendations for sandy soils. The response of N yield to N fertilization ranged from 35-102%. This wide range emphasizes the importance of a better recommendation base to target N fertilizer. The response of N yield to N fertilization was predicted by the total number of enchytraeids, the underlying mechanism of which needs further investigation on different soil types. This knowledge can be important for the optimal use of fertilizer and its consequences for environmental quality.

Keywords

Ecosystem services, grassland, soil quality, soil biota, soil structure, water regulation, nutrient supply, grass production, apparent nitrogen recovery

7.1 Introduction

Soil quality is globally acknowledged as the major factor determining yield and quality of crops. Although many definitions exist, agronomic soil quality can be defined as the sustained ability of a soil to (i) provide enough water and nutrients to crops, (ii) maximize the use efficiency of external inputs, (iii) minimize negative influences on the environment and (iv) sustain soil biodiversity. In The Netherlands, soil quality of production grasslands has not been a matter of concern in the last few decades. Nutrients and irrigation could be applied in abundance and could thus compensate for a lack, if any, of agricultural soil quality. Legislative restrictions have, however, reduced the use of organic and inorganic fertilizers (Vellinga, 2006), and irrigation. This has led to a renewed interest in the potential for optimizing yield and nutrient use efficiency by improving soil quality.

Soil quality can be assessed by parameters based on chemical, physical and biological properties. Soil quality of permanent grassland is generally assessed on the basis of a number of abiotic parameters (e.g. soil organic matter, total N, pH, K-HCl and P-Al). Biological soil properties have so far seldom played a role in practical soil assessment. However, the reduced use of external inputs implies a greater reliance on self-regulating processes (Brussaard et al., 2007). Soil biota play an important role in these processes and in the associated provision of various ecosystem services, such as supply of nutrients to plants, maintenance of soil structure, water regulation and grass production (Brussaard et al., 1997; Swift et al., 2004; Mulder, 2006; Kibblewhite et al., 2008). Therefore, biotic soil parameters could play a role in future soil assessment. Relationships between soil biota, soil ecosystem services including grass yield and/or soil quality, have been established in different microcosms and field experiments. Bacteria and fungi govern nutrient supply via nutrient mineralization and immobilization (De Ruiter et al., 1993). In regard to the service of soil structure maintenance, there is evidence that the polysaccharides produced by bacteria bind aggregates together, and that fungal hyphae entangle soil particles and smaller aggregates into larger aggregates (Tisdall and Oades, 1979; Six et al., 2002; Mäder et al., 2002).

A relationship between microbial biomass nitrogen and nitrogen uptake by grass was detected by Hassink (1995a). Protozoans, nematodes, Collembolans and mites affect nutrient cycling through grazing on micro-organisms and excretion of nutrients (Ingham et al., 1985; Griffiths, 1989; Bardgett and Chan, 1999; Vreeken-Buijs et al., 1997), and thus increase the N content and growth of grass (Ingham, 1985; Griffiths, 1989). Earthworms and enchytraeids increase nutrient cycling processes through fragmentation and mixing (Mackay et al, 1982; Clements at al., 1991; Brown, 1995; Cole et al., 2000; Mulder et al., 2006; Postma-Blaauw et al., 2006). Furthermore, they affect soil structure through the production of faecal pellets, promotion of humification and creation of pores (Hoogerkamp et al., 1983; Clements et al., 1991), and support water regulation through burrows, stable crumb formation and root growth stimulation (Logsdon and Linden, 1992; Bouché and Al-Addan, 1997; Haria et al., 1998). Introduction of earthworms has led to an increase in grass production (Stockdill, 1982; Hoogerkamp et al., 1983; Baker, 1998). Hence, it is well established that the soil biota play vital roles in the functioning of the ecosystem and associated ecosystem services, including grassland production.

To date, the observed relationships are virtually not translated into biotic soil indicators useful for soil quality assessment, although references to biotic soil parameters (Rutgers et al., 2009) and the effect of grassland management on soil biota (van Eekeren et al., 2008, 2009a, b) have recently been assessed. The general objective of this study is to investigate to what extent biotic soil parameters have indicative and explanatory value in soil quality assessment of grassland on sandy soils. At one sampling point, abiotic and biotic soil parameters were measured together with process parameters underlying ecosystem services in 20 production grasslands with comparable management histories. In the growing season, grass yield at 0 kg N ha^{-1} and response of grass yield to N fertilizer was measured in experimental plots fertilized with 0, 150 and 300 kg N ha^{-1} yr^{-1}. Measured soil parameters were used to explain process parameters underlying the following ecosystem services:

Soil structure maintenance

We hypothesized that soil structure maintenance is positively influenced by SOM, and the biomass of roots, bacteria, fungi and earthworms.

Water regulation

We hypothesized that water regulation is positively correlated with the biomass of earthworms, thanks to the positive effect of their burrowing activities on soil structure, penetration resistance and hence water infiltration and root development.

Nutrient supply

We hypothesized that nutrient supply of the soil is positively influenced by the quality of SOM (organic C, total N and C/N ratio) and correlated with the abundance and biomass of micro-organisms, microbivorous grazers (protozoans, nematodes, micro-arthropods), and their predators (nematodes and mites).

Grass production

We hypothesized that the dry matter yield of grassland and the response to N fertilization are explained by one or more of the process parameters for nutrient supply.

7.2 Materials and methods

7.2.1 Experimental sites

The experiment was conducted in 2006, on 20 permanent grasslands on sandy soil distributed over ten conventional dairy farms. The grasslands were selected using the following criteria: sandy soil, minimum age of the sward of three years, and a botanical composition with a minimum of 65% grass cover (mainly *Lolium perenne* L.) and maximum 2% legumes (Table 7.1). On 15 grasslands, the historical management was mixed grazing and cutting, while on five it was purely cutting. On average, the 20 grasslands received, the year before the experiment was conducted, 222 ± 70 kg N ha^{-1} in inorganic fertilizer and 260 ± 67 kg N-total ha^{-1} in organic fertilizer, mainly cattle manure slurry. On all grasslands, the manure slurry was slit injected.

On each grassland, an experimental field (15 m x 9 m) was laid out in February 2006. The first 10 m of the experimental field was split into three plots of 10 m x 3 m and the last 5 m in one plot of 5 m x 9 m. Over the three plots (10 m x 3 m), a fertilization treatment was randomized. Plots were fertilized with 0, 150 and 300 kg N ha^{-1} yr^{-1} with calcium ammonium nitrate (CAN, 27% N), respectively. Of the annual N fertilization, 33% was given before the first growing period, 27% before the second, 23% before the third and 17% before the fourth and last growing period. The remaining 5 m x 9 m plot was not fertilized with N and was used to determine soil quality properties. All plots, except the 5 m x 9 m plot, received ample fertilization of P, K and S.

Weather data were recorded at the weather stations in Heino and IJsselstein. Average rainfall during the growing season 2006 (1 May until 31 October) was 570 mm (Heino) and 521 mm (IJsselstein). The average temperature during this period was 14.0 ºC (Heino) and 14.5 ºC (IJsselstein). The summer season of 2006 was characterized by a very dry month of July.

7.2.2 Soil sampling

Soil samples were taken between 28 April and 2 May 2006. Per unfertilized 5 m x 9 m plot, a field-moist bulk sample of 70 cores (0-10 cm, ø 2.3 cm) was collected randomly, sieved through 1 cm mesh, homogenized and stored at 4°C until analysis. The bulk sample was split into sub-samples for abiotic and biotic (nematode and microbiological) analysis. An overview of all measurements is given in Table 7.2. Details of sampling methods are given in the following sections.

Table 7.1 Coordinates, soil type, age and botanical composition of the 20 grasslands sampled.

No.	Coordinates		Soil type (USDA)	Age (years)	Botanical composition (%)			
	‘North	‘East			*L. perenne*	Other grasses	Legumes	Herbs
1	52º44	6º27	Plaggeptic Haploquod	8	64	28	0	8
2	52º44	6º27	Plaggeptic Haploquod	3	73	27	0	0
3	52º54	6º27	Typic Haploquod	6	68	11	0	21
4	52º54	6º27	Typic Haploquod	6	75	9	0	17
5	52º42	6º21	Plaggeptic Haploquod	7	85	11	0	4
6	52º42	6º21	Typic Haploquod	7	82	12	0	6
7	52º49	6º31	Typic Haploquod	4	76	17	2	6
8	52º50	6º29	Typic Humaquept	6	69	24	0	7
9	52º40	6º22	Typic Humaquept	8	63	35	0	3
10	52º41	6º22	Plaggeptic Haploquod	5	88	9	0	4
11	51º51	5º88	Typic Haploquod	4	50	32	1	18
12	51º51	5º89	Typic Haploquod	4	66	25	0	10
13	51º54	5º84	Typic Haploquod	8	80	9	0	11
14	51º54	5º84	Typic Haploquod	12	17	72	0	11
15	51º53	5º90	Typic Haploquod	4	94	2	0	4
16	51º53	5º90	Typic Haploquod	4	95	3	1	2
17	51º54	5º84	Typic Haploquod	5	92	6	0	2
18	51º50	5º84	Typic Haploquod	5	95	5	0	1
19	51º58	5º83	Plaggeptic Haploquod	4	95	1	0	4
20	51º58	5º83	Typic Haploquod	4	97	2	0	2

7.2.3 Abiotic soil parameters

Soil moisture contents were measured in the 5-10 cm layer below the soil surface, in three undisturbed ring samples containing 100 cm^{-3} soil per unfertilized 5 m x 9 m plot. Samples were weighed, oven dried (70 °C), and re-weighed to determine moisture content.

Soil dry matter content was determined after oven-drying of approximately 30 g of the bulk sample (in duplicate) at 105 ºC. Prior to chemical analysis, samples were oven-dried at 40 °C. Soil acidity of the oven-dried samples was measured in 1 M KCl (pH-KCl). Soil Organic Matter (SOM) was determined by loss-on-ignition (Ball, 1964). Total Carbon (C) was measured by incineration of dry material at 1150 °C, after which the CO$_2$ produced was determined by an infra-red detector (LECO Corporation, St. Joseph, Mich., USA). Hot Wa-

Table 7.2 Overview of soil parameters measured.

Set	Type of parameter	Parameter
Abiotic (17)	Physical (6)	Clay(<2µm), silt (>2<50), loam (<50), fine sand (>50<210), coarse sand (>210<2000), Soil moisture
	Organic matter and its characteristics (7)	SOM, HWC, DOC, C-total, Total N, C/N ratio, C-percentage of SOM
	Inorganic chemical (4)	pH-KCl, P-total, P-Al, K-HCl
Biotic 39)	Roots (1)	Root biomass
	Earthworms (7)	Total number, total biomass, number of taxa, number and percentage of epigeic and endogeic adults
	Enchytraeids (9)	Total number, total biomass, number of taxa, number and percentage of *Fridericia*-group, *Marionina*-group and *Enchytraeus*-group
	Micro-arthropods (5)	Total number of micro-arthropods, number and percentage of mites, number and percentage of Collembolan
	Nematodes (11)	Total number of nematodes, number of taxa, Maturity Index, number and percentage of fungivorous nematodes, of herbivorous, bacterivorous, and predacious nematodes
	Microbial (6)	Bacterial biomass, bacterial activity, fungal biomass, fungal activity, physiological activity and diversity of bacterial community
Process (17)	Soil structure maintenance (8)	Bulk density, penetration resistance at 0-10 cm, 10-20 cm, 20-30 cm and 30-40 cm, percentage of crumbs, sub-angular blocky elements, and angular blocky elements
	Water regulation (5)	Water infiltration rate, number of earthworm burrows at 10 cm and 20 cm depth, number of roots at 10 cm and 20 cm depth
	Supply of nutrients (4)	Potential C mineralization, potentially mineralizable C, potential N mineralization, potentially mineralizable N

(..) = Number of parameters

ter Extractable Carbon (HWC) was analyzed according to the method of Ghani et al.(2003). Field-moist samples were extracted with 30 ml distilled water for 30 minutes, centrifuged for 20 minutes and filtered. Then a further 30 ml distilled water was added to the sediments, shaken for 10 seconds and left for 16 hours in a hot-water bath at 80 °C. Dissolved Organic Carbon (DOC) was determined by extraction in $CaCl_{2.}$ For determination of total Nitrogen (N), evolved gasses after incineration were reduced to N_2 and detected with a thermal-conductivity detector (LECO Corporation, St. Joseph, Mich., USA). Several phosphorous fractions (aluminum-bound, water-extractable and total P) were determined according to standard methods (Bronswijk et al., 2003). Total potassium (K) in solution was determined using flame photometry after extraction of soil with HCl (0.1 M) and oxalic acid (0.5 M) in a 1:10 M:V ratio and filtration (Bronswijk et al., 2003).

Soil particle analysis was done by a Beckman Coulter LS-230 laser with software version 3.01 and firmware version 2.02. Particle analysis was performed after removal of $CaCO_3$ with 1 M HCl (at 80-95 °C), and addition of de-ionized water, and of 30% H_2O_2 to remove organic matter (at 80-95 °C).

7.2.4 Biotic soil parameters

7.2.4.1 Roots

Three soil cores (0-10 cm, ø of 8.0 cm) per unfertilized 5 m x 9 m plot were taken randomly to determine the root biomass. The soil in the samples was thoroughly washed out with water over a sieve with a mesh size of 2mm. All roots were collected, oven-dried at 70 °C and the dry matter of the roots was measured.

7.2.4.2 Earthworms

Earthworms were sampled in 2 blocks (20 cm x 20 cm x 20 cm) per unfertilized 5 m x 9 m plot. The blocks were transferred to the laboratory where the whole block was broken down and the earthworms were hand-sorted, counted, weighed and fixed in alcohol prior to identification. Numbers and biomass were expressed per m^2. Adults were identified according to species. A distinction was made between (1) epigeic species (pigmented, living superficially in the litter layer, little burrowing activity), (2) endogeic species (living in burrows at approximately 10-15 cm depth) and (3) anecic species (relatively large worms, living in vertical burrows from which they collect dead organic matter from the surface at night) (Bouché, 1977). The sampling method with blocks of 20 cm x 20 cm x 20 cm is known to underestimate especially the number of *L. terrestris*.

7.2.4.3 Enchytraeids

Per unfertilized 5 m x 9 m plot, three enchytraeid samples were taken using a separable core sampler of 15 cm length with a diameter of 5.8 cm, holding 6 PVC rings of 2.5 cm high. The enchytraeids were extracted from the soil in the rings with a modified wet extraction method (Didden and Römbke, 2001; Römbke et al., 2006). The organisms were counted, measured and identified using a light microscope. Adults were identified according to species and juveniles to genus. Based on length, the fresh weight was calculated according to Abrahamsen (1973). The observed species were subdivided into three functional groups (1) *Fridericia*, (2) *Marionina* and (3) *Enchytraeus* (Didden and Römbke, 2001).

7.2.4.4 Micro-arthropods

Per unfertilized 5 m x 9 m plot, three samples for micro-arthropods were collected with a core sampler of 15 cm length with a diameter of 5.8 cm, holding 3 PVC rings of 2.5 cm high. Micro-arthropods were extracted from the soil by placing the soil sample rings in a Tullgren funnel (Siepel and Van de Bund, 1988; Römbke et al., 2006). The temperature in the upper part of the funnel was set at 30 ºC and kept at 5 ºC in the lower part. The organisms moved downwards to escape the heat, dropped through a funnel and collected in a bottle containing 70% ethanol. The total extraction time was one week. Collembola and mites were counted separately but not identified.

7.2.4.5 Nematodes

For determination of number and species of nematodes, a sub-sample of 450 g of field moist soil was taken from the bulk sample. Approximately 100 g of this was put in a suspension from which the free-living nematodes were extracted, using the Oostenbrink elutriator (Oostenbrink, 1960). Total numbers were counted and expressed per 100 g fresh soil. Nematodes were fixed in hot formaldehyde (4%), and at least 150 randomly selected nematodes from each sample were identified according to genus and, whenever possible, to species. The Maturity Index was calculated as the weighted mean of the individual cp-values, in accordance with Bongers (1990) and Bongers et al. (1995) as an index of soil quality.

7.2.4.6 Microbial parameters

Microbiological analyses were performed on another 200 g of field moist soil, adjusted to 50% of the water-holding capacity, and pre-incubated at 12 °C for four weeks. Incubation was performed to stabilize soil conditions (Bloem et al., 2006). After pre-incubation, fungal and bacterial biomass, bacterial growth rate and Community-Level Physiological Profiles (CLPP) were determined. Microbial soil smears were prepared and measured as described by Bloem and Vos (2004). Fungal hyphae were measured using the grid-intersection method. Bacterial numbers and cell volumes were measured by confocal laser scanning microscopy and automatic image analysis (Bloem et al., 1995). Bacterial biomass was calculated from the bacterial cell volume. Bacterial growth rate was determined as the incorporation of [^{3}H]thymidine into bacterial DNA and proteins respectively (Bloem and Bolhuis, 2006; Michel and Bloem, 1993). For a more detailed description, see De Vries et al. (2006).

The CLPPs of the bacterial communities in the soil extracts were determined with ECO-plates from BIOLOG Inc. (Hayward, USA). These plates contain a triplicate set of 31 different carbon substrates, a control, a freeze-dried mineral medium and a tetrazolium redox dye. For each bacterial extract, a dilution series was made using 10 mM BisTris buffer at pH 7. Each dilution series (3^{-1} until 3^{-12}) was used to inoculate four ECO-plates with a volume of 100 µl per well. The color formation in the plate was measured every 8 hours for 7 days with a plate reader spectrophotometer at 590 nm. The CLPPs were calculated from the color formation in the wells, and corrected for inoculum density using a regression approach applied to the average well color development (AWCD) as described by Rutgers et al. (2006). To survey the bacterial community activity in the ECO-plate, the AWCD was calculated after 7 days of incubation. The CLPP-slope parameter was calculated from the color development in the ECO-plates. This parameter indicates the rate at which the capacity of the soil to degrade a set of carbon and energy substrates disappears upon dilution. A low slope parameter is indicative of a slow disappearance rate and can be considered as a measure of high physiological diversity (Rutgers et al., 2006). In addition, the amount of extracted soil necessary for conversion of 50% of all substrates in ECO plates was determined, which is a measure of physiological activity in the bacterial community. A low amount of extracted soil necessary is indicative of a high degree of activity.

7.2.5 Soil process parameters

7.2.5.1 Soil structure maintenance

Soil bulk density was measured in the 5-10 cm layer below the soil surface, in three undisturbed ring samples containing 100 cm^{-3} soil per unfertilized 5 m x 9 m plot. Rings were weighed, oven-dried (70 °C), and re-weighed. Penetration resistance was measured using an electronic penetrometer (Eijkelkamp, Giesbeek, The Netherlands) with a cone diameter of 1 cm^2 and a 60° apex angle. Cone resistance was recorded per cm of soil depth and expressed as an average value of 20 penetrations per unfertilized 5 m x 9 m plot in the soil layers of 0-10 cm, 10-20 cm, 20-30 cm and 30-40 cm. Soil structure was determined in 2 blocks (20 cm x 20 cm x 10 cm) per unfertilized 5 m x 9 m plot. Soil in this block was assigned by visual observation of crumbs, sub-angular blocky elements and angular blocky elements (FAO, 2006). These were weighed and expressed as a percentage of total fresh soil weight. On horizontal surfaces (20 cm x 20 cm) at 10 cm and 20 cm depth, the total number of roots was counted and expressed per

7.2.5.2 Water regulation

Per unfertilized 5 m x 9 m plot, water infiltration was measured at 3 randomly chosen spots. A PVC pipe of 15 cm high (ø of 15 cm) was driven into the soil to a depth of 10 cm, after which 500 ml of water was poured into the ring. The number of minutes it took for the 500 ml water to infiltrate was recorded and calculated to give an infiltration rate per minute. Earthworm burrows with a diameter >2 mm were counted on horizontal surfaces (20 cm x 20 cm) exposed at 10 cm and 20 cm depth. On the same horizontal surfaces, the number of roots were counted.

7.2.5.3 Supply of nutrients

The potential C mineralization was measured in soil which was homogenized, sieved (5 mm mesh size) and brought to 50-60% of the water-holding capacity. Sub-samples of 200 g soil were incubated in the dark at 20 ºC in 1.5 l air-tight jars supplied with a gas septum. CO_2 evolution was measured weekly by gas chromatography over a 6-week period of incubation. The gas chromatograph was a Carlo Erba 6000 with a column switching system, equipped with a 4 m Porapak q and a 2 mmol sieve 5A column. The detector (HWD) temperature was 180ºC, the column temperature was 50 ºC, and the injection volume was 1 ml (Bloem et al., 1994). Potential C mineralization was expressed as mg C respired kg^{-1} soil $week^{-1}$, averaged over the last five weeks. The first week's results were not used in order to avoid soil homogenization effects.

Potentially mineralizable C was measured by incubation of dried (35 ºC for 48 hours) and re-wet soil samples for seven days at 20 °C and 50% WHC. During this period, CO_2 was absorbed in alkali (1*N* KOH) followed by titration with 0.1 *N* HCl (Pell et al., 2006).

The potential N mineralization rate (aerobic) was determined in the same jars used for potential C mineralization measurements, and during the same 6 week incubation period. Potential N mineralization was determined as the increase in mineral N (ammonium plus nitrate) in the five last weeks. The first week's results were not used in order to avoid soil homogenization effects. NH_4 and NO_3 contents were determined by Segmented Flow Analysis, after extraction of 80 g of field moist soil with 200 ml of 1 M KCl, shaken for 1 hour and filtered over a paper filter.

The potentially mineralizable N (anaerobic) was determined by anaerobic incubation of a soil sample under water for 1 week at 40 °C (Keeny and Nelson, 1982; Canali and Benedetti, 2006) These warm and anoxic conditions are optimal for a quick mineralization of organic matter by anaerobic bacteria. The lack of oxygen prevents conversion of released NH_4^+ to NO_3^- (nitrification) and uncontrolled N losses by denitrification cannot occur.

7.2.6 Grass production

Grass was harvested four times (10-15 May, 26 June, 11-15 August and 2-3 October). Plots were cut at a stubble height of 6 cm, using a 'Haldrup' small-plot harvester (J. Haldrup a/s, Løgstør, Denmark). Grass was weighed and sampled for dry matter (DM) and total N analysis. DM was determined after drying at 70 °C. Dry material was analyzed for total N (Kjeldahl) and residual moisture content (105 °C).

Dry Matter (DM) yield as a function of N level was modeled based on an exponential curve:

$$Y = \alpha_0 i + (\alpha_1 i * (1 - e^{-\rho * Ngift}) + \varepsilon_{ij}$$

with the terms:

Y DM yield;

$\alpha_0 i$ DM yield intercept, or DM yield of field *i* with 0 kg N ha^{-1};

$\alpha_1 i$ DM yield response to N fertilizer when N level is infinite; so with infinite N level, maximal yield per field is $\alpha_0 + \alpha_1 i$;

ρ velocity parameter for yield increase;

ε_{ij} random variance between plots within a field , $\varepsilon_{ij} \sim N(0, \sigma_V^2)$.

The model has been adapted in Genstat 8 with Residual Maximum Likelihood (REML). The non-linear parameters have been estimated according to an iterative procedure, based on 1st order Taylor-approach.

N yield as function of N level was modeled by a linear trend:

$$Y = \beta_{0i} + \beta_{1i} * Ngift + \varepsilon_{ij}$$

with the terms:

Y N yield;

β_{0i} N yield intercept, or N yield of field i with 0 kg N ha^{-1};

$\beta_1 i$ N yield response to N fertilizer; or the slope of the linear correlation between N yield and N application;

ε_{ij} random field effect, $\varepsilon_{ij} \sim N(0, \sigma_V^2)$.

Assumptions were that the variance on each field was the same and that the correlation between N yield and N application was linear.

In the statistical analyses of the experiment, we used the intercepts of these two equations representing the DM yield and N yield at 0 kg N ha^{-1}. Furthermore, we used the maximum DM yield and slope of the N yield representing the response to N fertilization or the 'apparent' N recovery of fertilizer (ANR).

The nitrogen supply capacity of the soil, defined as the non-fertilizer N supply including atmospheric deposition (Hassink, 1995a), was calculated from soil total N, according to the Dutch grassland fertilization recommendation. For a grassland with an age of 4-6 years, the formula used was 78+28.36 * (g total N/kg soil)$^{1.0046}$ (http://www.bemestingsadvies.nl).

7.2.7 Statistical analyses

Data analysis was performed with Matlab (version 7.6.0 R2008a, Mathworks). If necessary, parameters were log-transformed for heteroscedacity to improve the normality of their distributions. Pearson correlations were calculated for all pairs of parameters. Cross-validated stepwise regression was applied to find subsets of parameters that most accurately explained the response parameters. Response parameters were either soil process parameters underlying ecosystem services (section 2.6) or grass production parameters (section 2.2). Soil process parameters were explained by a set of soil parameters (abiotic or biotic) or a combination of the two. Grass production parameters were explained by a set of abiotic or biotic soil or process parameters or all possible combinations of these. Sets of potential regression models were generated using a minimum of one and a maximum of three variables. For each maximum number of parameters, the best regression model was selected by the highest cross-validated R^2. Final selection of reliable subsets of explanatory parameters was based on random permutation tests. Random permutation tests were performed on the complete stepwise regression procedure described. The permuted response parameter and the true response parameter were restricted to have a Pearson correlation below 0.5. For each maximum number of parameters, we tested in 999 permutations whether the permuted cross-validated R^2 was equal to or above 90% of the true cross-validated R^2. In other words, we assessed whether there was a significant 10% gap between the true cross-validated R^2 and the distribution of all permuted cross-validated R^2s. Random permutation tests resulting in a P-value below 0.05 were considered valid.

7.3 Results

This section starts with the analysis of soil processes underlying the ecosystem services soil structure maintenance, water regulation and nutrient supply. They are related to abiotic and/or biotic soil parameters. This is followed by the explanation of grass production by abiotic or biotic soil or process parameters or a combination of these. In general, data examination followed the pattern of correlation analysis followed by a stepwise regression procedure for the different process parameters underlying an ecosystem service. Many significant correlations were established (Table 7.3, Table 7.7, Annex 7.1).

7.3.1 Soil structure maintenance

The process parameters measured to assess the service of soil structure maintenance were bulk density, penetration resistance and visual soil structure (Table 7.2). The results are examined in this sequence.

Bulk density had the highest correlation with the abiotic soil parameters Hot Water extractable Carbon and SOM (Table 7.3, Annex 7.1). In the stepwise regression procedure, a significant model could only be established using HWC (Table 7.4).

Penetration resistance is a result of soil density, grinding action and humidity of the soil. In the 0-10 cm soil layer it was negatively correlated with biotic soil parameters such as number of earthworm taxa, total number of earthworms and number of endogeic adult earthworms (Table 7.3, Annex 7.1). However, these correlations did not lead to a significant model after the random permutation tests on the complete stepwise regression procedure with one or more parameters. Penetration resistance at 10-20 cm was positively correlated with fungal activity, and negatively with the number and percentage of *Enchytraeus* enchytraeids (Table 7.3, Annex 7.1). In contrast to the penetration resistance at 0-10 cm, the resistance at 10-20 cm could be explained by several regression models with biotic soil parameters in which fungal activity was the main explanatory parameter (Table 7.4). Penetration resistance at 20-30 cm

Table 7.3 Highest 3 correlations of soil process parameters underlying the different ecosystem services and abiotic and biotic soil parameters. Complete overview of correlations in Annex 7.1.

Service and parameters	Highest correlating abiotic and biotic parameters (all significant at the <0.05 probability level)
Soil structure maintenance	
Bulk density	HWC (-0.64), SOM (-0.56), Number of bacterivorous nematodes (-0.55)
Penetration resistance 0-10 cm	Number of earthworm taxa (-0.63), Number of earthworms (-0.62), Number of endogeic earthworms (-0.55)
Penetration resistance 10-20 cm	Fungal activity (+0.78), Number and % of *Enchytraeus* enchytraeids (-0.65, -0.61)
Penetration resistance 20-30 cm	Number of herbivorous nematodes (-0.62), Number of *Enchytraeus* enchytraeids (-0.6), total N (-0.57
Penetration resistance 30-40 cm	Number of epigeic earthworms (+0.61), Number of enchytraeids (-0.56), % Collembola (-0.56)
% Crumbs	Number of earthworms (+0.51)
% Sub-angular blocky elements	Organic C (-0.54), SOM (-0.54), Number of earthworm taxa (0.47)
% Angular blocky elements	Number of earthworms (-0.46), Number of earthworm taxa (-0.46)
Water regulation	
Water infiltration rate	Fungal activity (+0.6), Number and % of *Enchytraeus* enchytraeids (-0.54, -0.50)
Number of earthworm burrows at 10 cm	Bacterial activity (-0.51), Number of mites (+0.52), Bacterial biomass (+0.6)
Number of earthworm burrows at 20 cm	Number of nematode taxa (+0.69), Number and % of *Marionina* enchytraeids (-0.66, -0.64)
Number of roots at 10 cm	Number of bacterivorous nematodes (-0.56), % *Enchytraeus* enchytraeids (-0.55). Number of *Marionina* enchytraeids (+0.47)
Number of roots at 20 cm	Number of micro-arthropods (-0.61), Physiological activity (+0.47), Number of Collembola (-0.46)
Supply of nutrients	
Potentially mineralizable C	Number of nematode taxa (+0.73), Total N (+0.71), % *Marionina* enchytraeids (-0.63)
Potentially mineralizable N	Total N (+0.83), Number of nematode taxa (+0.76), % *Marionina* enchytraeids (-0.75)
Potential C mineralization	% Mites (+0.55), Silt (+0.52), Loam (+0.52)
Potential N mineralization	% Collembola (+0.54), Number of enchytraeids taxa (+0.53), Coarse sand (-0.53)

Table 7.4 Explanation of soil process parameters underlying the ecosystem service soil structure maintenance. The cross-validated R^2 and P-values of all significant ($P<0.05$) models were calculated using only one parameter of either the set of abiotic (A) or biotic (B) soil parameters, or a combination of the two. The same procedure was followed for a maximum of two or three parameters.

Response parameter	cvR^2	P-value	Set	Explanatory parameter(s)
Bulk density	0.27	0.039	A	-HWC
Penetration resistance				
0-10 cm	--	NS	--	
10-20 cm	0.51	0.003	B	+Fungal activity
	0.69	0.005	B	+Fungal activity, -Number of *Enchytraeus* enchytraeids
	0.73	0.016	B	+Fungal activity, -Number of *Enchytraeus* enchytraeids,+Number of enchytraeid taxa
20-30 cm	--	NS	--	
30-40 cm	--	NS	--	
Soil structure elements				
Crumbs %	--	NS	--	
Sub-angular %	0.44	0.034	A	-SOM, -C/N ratio
Angular %	--	NS	--	

and 30-40 cm were amongst others negatively correlated to the total number of enchytraeids (Table 7.3, Annex 7.1), but none of the models was significant after the random permutation tests.

For the percentage of soil crumbs and angular blocky elements, no regression models could be fitted. Nevertheless, the percentage of crumbs showed a positive correlation with the total number of earthworms and a negative correlation with penetration resistance at 0-10 cm (Table 7.3, Annex 7.1). Since soil crumbs and angular blocky elements are the other's antithesis, this was vice versa for angular blocky elements. The percentage of sub-angular blocky elements was negatively correlated with SOM and parameters characterizing organic matter quality. The percentage of sub-angular blocky elements could be significantly explained by a model containing SOM and soil C/N ratio as parameters (Table 7.4).

7.3.2 Water regulation

Water regulation was assessed using the process parameters water infiltration rate per minute, the number of earthworm burrows and the number of grass roots at 10 and 20 cm depth.

From the abiotic and biotic soil parameters, water infiltration rate was, like penetration resistance at 10-20 cm, correlated with fungal activity, and the number and percentage of *Enchytraeus* enchytraeids (Table 7.3, Annex 7.1). However, the water infiltration rate had a stronger negative correlation with penetration resistance at 10-20 cm and 20-30 cm (Annex 7.1). In the stepwise regression procedure, the water infiltration rate could only be explained by penetration resistance at 10-20 cm when one parameter out of 16 soil process parameters was allowed (Table 7.5).

The number of earthworm burrows at 10 and 20 cm depth was not significantly correlated with water infiltration. Earthworm burrows at 10 cm depth were correlated with abiotic and biotic soil parameters (Table 7.3, Annex 7.1). However, the number of earthworm burrows at 10 cm depth could not be explained with a significant model with one or more abiotic or biotic soil parameters. The number of earthworm burrows at 20 cm depth was correlated with several abiotic and biotic soil parameters. In the stepwise regression procedure, the number burrows was

Table 7.5 Explanation of soil process parameters underlying the ecosystem service water regulation. The cross-validated R^2 and P-values of all significant ($P<0.05$) models were calculated using only one parameter of either the set of abiotic (A) or biotic (B) soil parameters, or a combination of the two. The same procedure was followed for a maximum of two or three parameters. For infiltration rate also a model with 16 soil process (P) parameters was tested.

Response parameter	cvR^2	P-value	Set	Explanatory parameter(s)
Water infiltration rate	0.36	0.016	P	-Penetration resistance at 10-20 cm
Earthworm burrows				
10 cm depth	--	NS	--	
20 cm depth	0.58	0.011	A,B	+DOC, -Bacterial activity
	0.70	0.025	A,B	+DOC, -Bacterial activity, +P-total
Number of roots				
10 cm depth	0.66	0.049	B	+Total biomass of enchytraeids, -Number of bacterivorous nematodes, -Number of *Enchytraeus* enchytraeids
20 cm depth	--	NS	--	

explained by DOC, bacterial activity and soil total P when a maximum of three parameters was allowed out of the combined set of 17 abiotic and 39 biotic parameters (Table 7.5).

The number of roots at 10 cm depth was negatively correlated with number of bacterivorous nematodes and percentage of *Enchytraeus* enchytraeids, and positively with soil bulk density (Table 7.3, Annex 7.1). In the stepwise regression procedure, the number of roots was explained by total biomass of enchytraeids, number of bacterivorous nematodes and number of *Enchytraeus* enchytraeids (Table 7.5). The number of roots at 20 cm was positively correlated with the number of micro-arthropods, but did not result in an explanatory model (Table 7.3, Annex 7.1)

7.3.3 Supply of nutrients

The nutrient supply capacity of the soil depends on the composition of the organic material and the decomposition and mineralization processes. In these processes bacteria, fungi, microbivorous fauna and their predators play an important role. The ecosystem service supply of nutrients was assessed with the process parameters potentially mineralizable C and N, and potential C and N mineralization.

Potentially mineralizable C and N were positively correlated (r=+0.66, P=0.002) (Annex 7.1). Moreover, both parameters were positively correlated with soil total N, and biotic soil parameters that are in turn related to total N. Amongst others, soil total N was positively correlated with total number of nematodes and number of nematode taxa, and negatively with percentage of *Marionina* enchytraeids (Table 7.3, Annex 7.1). In regression models, the potentially mineralizable C and N was explained by total N or number of nematode taxa, in case only one abiotic or biotic soil parameter was allowed (Table 7.6). In a model with two abiotic parameters, total N and loam fraction explained a higher percentage of the variance of the potentially mineralizable N. When all abiotic and biotic soil parameters were allowed in the regression model, the bacterial activity explained extra variation above total N, whereby bacterial activity was again correlated with loam fraction.

Potential C mineralization and potential N mineralization were not significantly correlated with each other, but were correlated with a variable number of contrasting biotic soil parameters (Table 7.3, Annex 7.1). The potential N mineralization was positively correlated with the fungal biomass, the number of predaceous nematodes, the Maturity Index, the number of *Fridericia* enchytraeids and the percentage of Collembola, and negatively with the number of mites. The potential C mineralization was negatively correlated with the bacterial biomass, the Maturity Index and the percentage of endogeic earthworms, and positively correlated with the percentage of mites. However, despite several correlations, potential C mineralization and potential N mineralization could not be significantly explained by a regression model with one or more parameters from the abiotic and/or biotic soil parameter sets (Table 7.6).

Table 7.6 Explanation of soil process parameters underlying the ecosystem service supply of nutrients. The cross-validated R^2 and P-values of all significant ($P<0.05$) models were calculated using only one parameter of either the set of abiotic (A) or biotic (B) soil parameters.

Response parameter	cvR^2	P-value	Set	Explanatory parameter(s)
Field respiration	--	NS	--	
Pot. C mineralization	--	NS	--	
Pot. N mineralization	--	NS	--	
Pot. mineralizable C	0.36	0.016	A	+Total N
	0.41	0.019	B	+Number of nematode taxa
Pot. Mineralizable N	0.61	0.001	A	+Total N
	0.51	0.011	B	+Number of nematode taxa
	0.72	0.002	A	+Total N, -Loam fraction
	0.65	0.016	B	-Percentage *Marionina* enchytraeids,+Percentage Collembola within micro-arthropods
	0.71	0.005	A,B	+Total N, -Bacterial activity
	0.79	0.013	A,B	+Total N, +Number of nematode taxa, -Number of adult endogeic earthworms

7.3.4 Grass production

7.3.4.1 DM and N yield intercept

Dry Matter (DM) yield intercept and the Nitrogen (N) yield intercept were used as two standards for grass production parameters at 0 kg N ha^{-1} fertilization. The DM yield intercepts over the different experimental fields ranged from 3363 to 10120 kg DM ha^{-1} (Fig. 7.1). N yield intercepts ranged from 78 kg to 263 kg N ha^{-1} (Fig. 7.2).

DM yield intercept was correlated among others with soil moisture, SOM and total biomass of enchytraeids (Table 7.7). Soil moisture was selected out of 17 abiotic soil parameters in a significant model (P=0.002) with a cross-validated R^2 of 0.67 (Table 7.8). One percent of soil moisture in the 5-10 cm soil layer in spring explained 427 kg DM yield ha^{-1}.

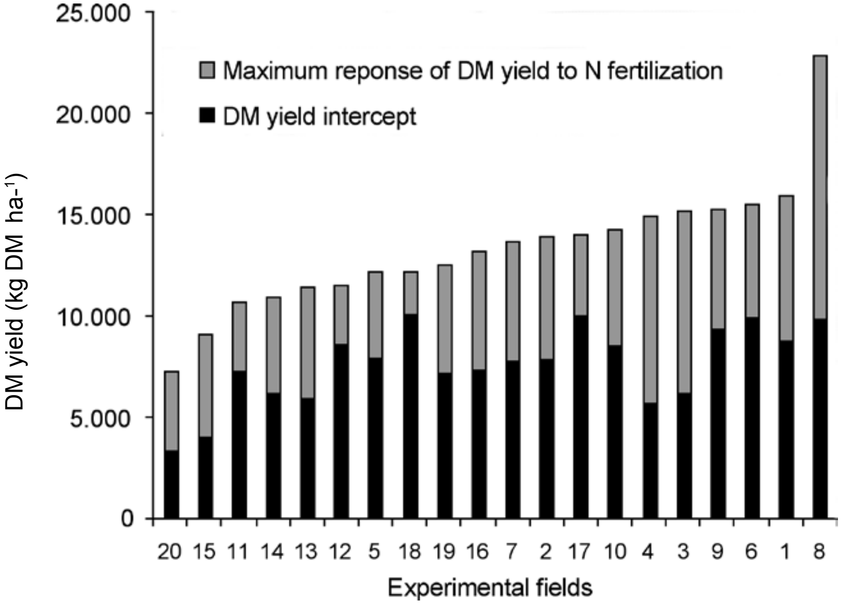

Fig. 7.1 DM yield intercept and maximum response of DM yield to N fertilization.

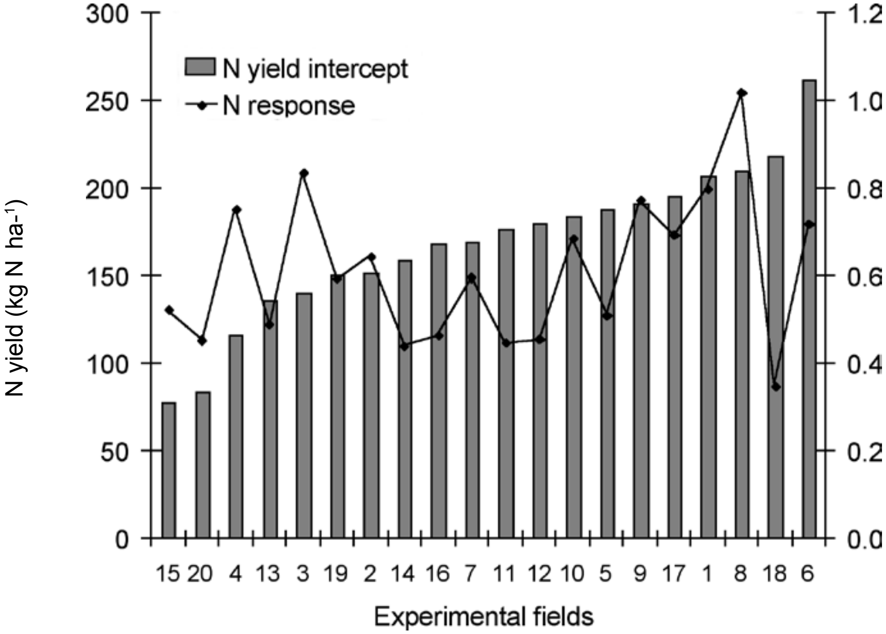

Fig. 7.2 N yield intercept (kg N ha^{-1}) and response of N yield to N fertilization (N yield per kg N fertilized ha^{-1}).

Table 7.7 Significant correlations of intercept and response of DM yield and N yield on N fertilization with soil quality parameters.

Set	Parameters	Intercept		Response on N fertilization	
		DM yield	N yield	DM yield	N yield
	DM yield intercept	+1**	+0.93**		
	N yield intercept	+0.93**	+1**		
	Response DM yield N fert.			+1**	+0.86**
	Response N yield N fert.			+0.86**	+1**
Abiotic soil parameters	Clay	+0.69**	+0.59**		
	Loam	+0.6**	+0.52*		
	Silt	+0.54*	+0.47*		
	SOM	+0.77**	+0.82**		
	C-percentage		-0.53*		
	C-tot	+0.66**	+0.67**		
	C/N ratio			-0.5*	-0.62**
	HWC	+0.58**	+0.67**		
	DOC	+0.61**	+0.49*		
	N-tot	+0.72**	+0.77**		
	Soil moisture	+0.84**	+0.77**		
Biotic soil parameters	Number of epigeic adult earthworms		-0.52*		
	Number of endogeic adult earthworms				+0.45*
	% of epigeic adult earthworms				-0.53*
	% of endogeic adult earthworms			+0.48*	+0.67**
	Number of earthworm taxa				+0.41*
	Total number of enchytraeids	+0.72**	+0.61**		+0.69**
	Total biomass of enchytraeids	+0.78**	+0.7**		+0.59**
	Number of *Enchytraeus* enchytraeids			+0.45*	+0.68**
	Number of *Fridericia* enchytraeids				+0.46*
	% of mites				-0.47*
	Total number of nematodes	+0.5*	+0.61**		
	Number of bacterivorous nematodes		+0.52*		
	Number of fungivorous nematodes	+0.45*	+0.47*		
	% of bacterivorous nematodes			-0.62**	-0.59**
	% of herbivorous nematodes			+0.5*	+0.48*
	Maturity Index of nematodes			+0.53*	+0.56*
	Number of nematode taxa		+0.51*		+0.47*
	Fungal biomass				+0.47*
	Bacterial activity	+0.51*			
	Physiological activity	+0.57**	+0.49*		+0.48*
	Root biomass		-0.46*		
Process soil parameters	Bulk density		-0.46*		
	Sub-angular blocky elements				+0.45**
	Penetration resistance 0-10 cm				-0.56**
	Penetration resistance 20-30cm		-0.47*		
	Penetration resistance 30-40 cm	+0.53*	-0.58**		
	Earthworm burrows at 20 cm			+0.58**	+0.46*
	Pot. Mineralizable N	+0.5*	+0.64**		+0.49*
	Pot. Mineralizable C	+0.45*	+0.57**		

*Significant at the 0.05 probability level, **Significant at the 0.01 probability level.

Table 7.8 Explanation of grass production parameters from soil quality parameters. The cross-validated R^2 and P-values of all significant ($P<0.05$) models were calculated using only one explanatory parameter of either the set of abiotic (A) or biotic (B) soil parameters or the set of soil process (P) parameters, or a combination of the three. The same procedure was followed for a maximum of two or three parameters.

Response parameter	cvR^2	P-value	Set	Explanatory parameter(s)
Intercept				
DM yield	0.67	0.002	A	+Soil moisture
	0.50	0.006	B	+Total biomass of enchytraeids
	0.79	0.001	A	+Soil moisture, -Fine sand fraction
	0.59	0.027	B	+Total biomass of enchytraeids, + Number of bacterivorous nematodes
	0.83	0.001	A	+Soil moisture, -Fine sand fraction, +SOM
	0.85	0.005	A,B	+Soil moisture, -Fine sand fraction, +Physiological activity
	0.83	0.004	A,P	+Soil moisture, -Fine sand fraction, +Field respiration
N yield	0.59	0.001	A	+SOM
	0.35	0.025	B	+Total biomass of enchytraeids
	0.68	0.002	A	+SOM, +Soil moisture
	0.56	0.022	B	+Total biomass of enchytraeids, +Number of bacterivorous nematodes
	0.74	0.002	A,B	+SOM, +Total biomass of enchytraeids
	0.70	0.003	A,P	+SOM, +Percentage sub-angular blocky elements
	0.76	0.002	A	+SOM, +Soil moisture, -C-total
	0.79	0.014	A,B	+SOM, +Total biomass of enchytraeids, -Number of taxa of enchytraeids
N response				
DM yield	--	NS	--	
N yield	0.36	0.047	B	+Total number of enchytraeids
	0.58	0.046	B	+Total number of enchytraeids, +Maturity Index of nematodes
	0.7	0.020	B	+Total number of enchytraeids, +Maturity Index of nematodes, -Number of bacterivorous nematodes

When soil moisture was removed from the abiotic data set, SOM was the next-best parameter (cvR^2=0.51, P=0.007). Total biomass of enchytraeids was selected as the best explanatory biotic soil parameter. However, biomass of enchytraeids was also positively correlated with soil moisture and SOM (Annex 7.1). When two abiotic parameters were allowed, fine sand fraction was added to the model with soil moisture. When two biotic parameters were allowed, the number of bacterivorous nematodes was added to the model with total biomass of enchytraeids.

DM and N yield intercepts were correlated (Table 7.7). Correlations with soil parameters were comparable for both intercepts, although ranking in the correlations could differ. Both intercepts were correlated with soil moisture and SOM, but soil moisture explained more of the variance in the DM yield intercept than SOM, whereas SOM explained more of the variance in the N yield intercept (Table 7.8). When one abiotic parameter was allowed, SOM was selected as the best explanatory parameter for the N yield intercept (cvR^2=0.59, P=0.001). One gram of SOM per kg dry soil meant 3.21 kg N yield ha^{-1}, on top of a constant of 15.4 kg N ha^{-1}. When SOM was removed from the abiotic soil parameter set, the N yield intercept could be significantly explained from soil moisture (cvR^2=0.52, P=0.003) or total N (cvR^2=0.48, P=0.007). With one biotic parameter in the model, total biomass of enchytraeids was selected. Next to correlations with soil moisture and SOM, total biomass of enchytraeids was positively correlated with soil total N (Annex 7.1). Models with two or three parameters, produced by best sub-set selection, were dominated by SOM as one of the explanatory parameters. When two biotic soil parameters were allowed, N yield intercept was explained by the total biomass of enchytraeids and the number of bacterivorous nematodes, just as we found with the DM yield intercept.

In order to compare the theoretical nitrogen supply capacity of the soil calculated from the total N according to the Dutch grassland fertilization recommendation (section 7.2.6) with the N yield intercept from this experiment, both were plotted against SOM (Fig. 7.3). It can be seen that the nitrogen supply calculated from soil total N underestimated the measured N yield intercept from 32 g SOM kg dry $soil^{-1}$ onwards. In this range of grassland soils, the difference between calculated and measured values ranged from -36 to 103 kg N ha^{-1}. On average, it meant a 24% higher nitrogen supply capacity than calculated according to the Dutch grassland fertilization recommendation. These differences are significantly explained by the model that includes soil moisture in addition to C-percentage in SOM (cvR^2=0.62, P=0.006). When three parameters were allowed, the differences are explained by a model of soil moisture, C-percentage in SOM and total number of mites (cvR^2=0.76, P=0.033).

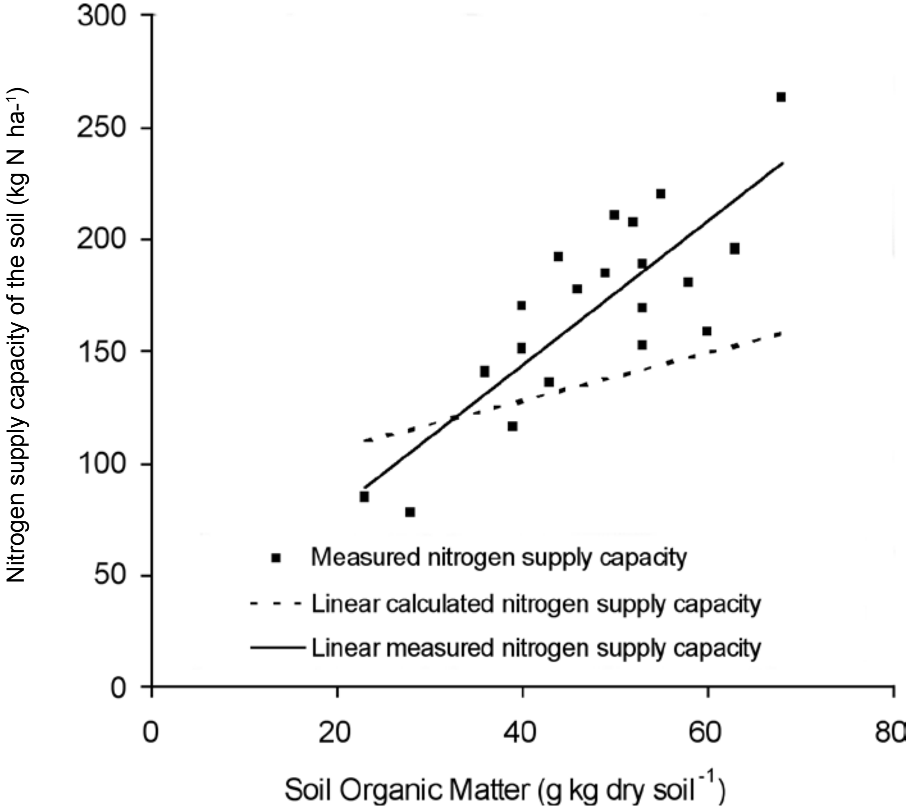

Fig. 7.3 Measured nitrogen supply capacity (N yield intercept) (kg N ha^{-1}) plotted against SOM (g kg dry soil^{-1}) and its linear regression ($R^2=0.67$, cross-validated $R^2=0.59$, $P<0.001$), compared with the nitrogen supply capacity of the soil calculated from total N according to the Dutch grassland fertilization recommendation (section 7.2.6, http://www.bemestingsadvies.nl).

7.3.4.2 Response of DM- and N yield to N fertilization

The maximum DM response to N fertilizer ranged from 2109 to 12992 kg DM ha^{-1} on top of the production without fertilizer (Fig. 7.1).The response of N yield to N fertilizer ranged from 0.35 to 1.02 kg N yield per kg N ha^{-1} applied (Fig. 7.2). Yield intercepts and yield responses to N fertilization were not correlated (Table 7.7).

In contrast to the intercepts, the N fertilizer responses were not significantly correlated with the different abiotic soil parameters, except for a negative correlation with the C/N ratio in the soil (Table 7.7). Both yield response parameters were correlated with numerous

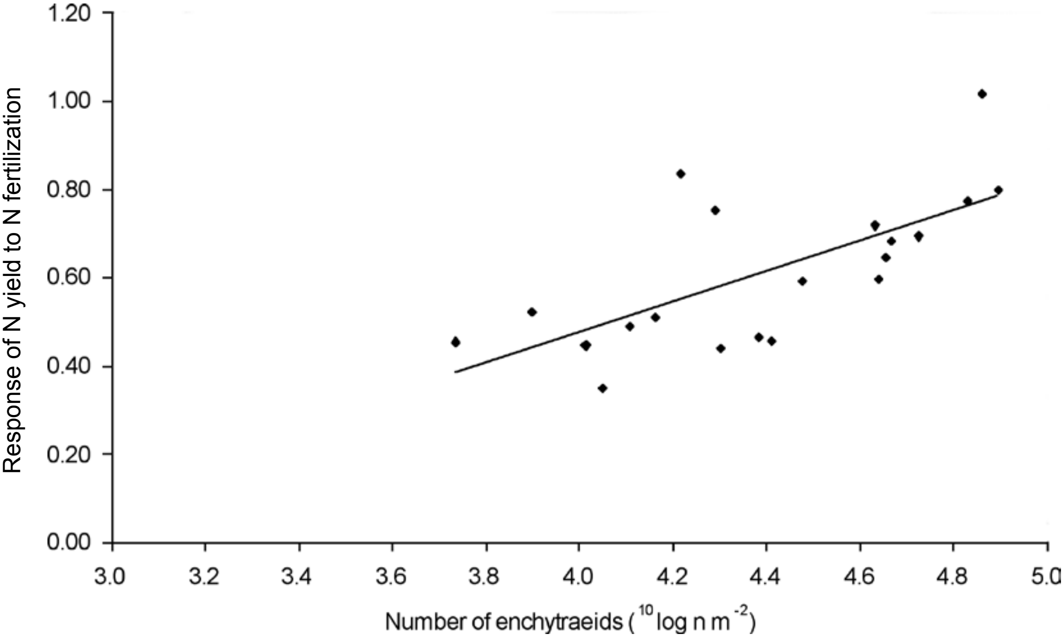

Fig. 7.4 Linear regression between total number of enchytraeids (^{10}log n m^{-2}) and response of N yield to N fertilization (R^2=0.47, cross validated R^2=0.36, P=0.047).

biotic soil parameters such as number of *Enchytraeus* enchytraeids, percentage of endogeic adult earthworms, and percentage of bacterivorous nematodes. Despite these correlations, the response of DM yield to N fertilizer could not significantly be explained by a regression model of abiotic and/or biotic soil parameters. However, the response of N yield to N fertilization was significantly explained by the total number of enchytraeids (cvR^2=0.36, P=0.047) (Table 7.8) (Fig. 7.4). On top of the number of enchytraeids, the Maturity Index and the number of bacterivorous nematodes explained extra variance in the data set. However, this variance was mainly caused by two grasslands out of the 20. When these two grasslands were left out of the analysis, the cvR^2 increased to 0.53 (P=0.006) with total number of enchytraeids as explanatory parameter. Both yield response parameters had a tendency towards a significant model with three soil process parameters: +Potentially mineralizable N, +Bulk density and -Penetration resistance at 0-10 cm (cvR^2=0.44, P=0.074 for DM-yield; cvR^2=0.51, P=0.058 for N yield).

7.4 Discussion

7.4.1 Soil structure maintenance

In our experiment, the variation in bulk density was best explained by Hot Water-extractable Carbon (HWC), which is regarded as one of the key labile components of SOM responsible for soil micro-aggregation (Haynes, 2005). Both bulk density and HWC were strongly correlated with SOM. Soil organic particles weigh less than mineral soil particles, which makes soil bulk density highly dependent on SOM (Locher and Bakker, 1990). Clements et al. (1991) showed that earthworms decreased the soil bulk density. However, the influence of earthworms on bulk density was not confirmed in this study. Penetration resistance at 0-10 cm could not be explained from the measured abiotic or biotic soil parameters. However, the negative correlations between penetration resistance and biotic soil parameters suggest that earthworms in general and endogeic earthworms in particular, decrease the penetration resistance at 0-10 cm. In experiments by Hoogerkamp et al. (1983) and Clements et al. (1991), earthworms decreased the soil penetration resistance in the 0-10 cm soil layer.

Penetration resistance at 10-20 cm could be explained from the biomass of active fungal hyphae in the 0-10 cm soil layer. This correlation was not expected. Fungal activity was positively correlated with other soil structure parameters that are indicative of a poor soil structure (penetration resistance at 0-10 cm and 20-30 cm, and angular blocky elements) (Annex 7.1). A poor soil structure may lead to an accumulation of organic material which could have stimulated fungal growth. The positive correlation of fungal activity with surface feeding epigeic earthworms could be an indication of the accumulation of organic material.

Crumbs and angular blocky elements could not be explained from abiotic and/or biotic soil parameters, but they were, like penetration resistance at 0-10 cm, correlated with total number of earthworms. Van Eekeren et al. (2009a) also found a positive correlation between earthworm biomass and the percentage of crumbs in the 0-10 cm soil layer. The percentage of sub-angular blocky elements was explained by SOM and C/N ratio in a regression model, and negatively correlated with C-total and HWC. This suggests that sub-angular blocky elements are indicative of the quality of SOM.

We had hypothesized that the process parameters underlying the ecosystem service soil structure maintenance would be influenced by abiotic soil parameters like SOM and biotic parameters related to the presence and activity of roots, bacteria, fungi and earthworms. We conclude that bulk density and the percentage of sub-angular blocky elements are mainly influenced by SOM quantity and quality. Penetration resistance at 10-20 cm is explained by fungal activity, but the cause and effect are not completely clear. The correlations between penetration resistance at 0-10 cm and percentage of soil crumbs with earthworms suggest a relationship with earthworm activity.

7.4.2 Water regulation

Effects of biological activity on the service of water regulation have been measured in the field via the number of earthworm burrows, number of roots and via water infiltration. For number of roots and number of earthworms there were some relationships with biotic soil parameters but cause and effect were still difficult to separate. We could not even measure a relationship between earthworm burrows and earthworm biomass, whereas Van Eekeren et al. (2009) established such a relationship in a field experiment.

Water infiltration rate is influenced by soil type, soil texture, soil structure, earthworm burrow numbers, earthworm species, stable organic matter and initial soil water content (Lowery et al., 1996; Sarrantonio et al., 1996; Bouché and Al-Addan, 1997; Edwards and Shipitalo, 1998). In our experiment, water infiltration was not correlated with initial soil water content. The variance in water infiltration was best explained through a regression model with penetration resistance at 10-20 cm as an explanatory parameter. The average penetration resistance in our experiment increased from 1.40 MPa in the 0-10 cm layer to 2.13 MPa in the 10-20 cm layer (data not shown), suggesting that the higher penetration resistance in the 10-20 cm was a barrier to water infiltration.

Our hypothesis of a positive correlation between water infiltration and earthworm activity had to be rejected. A first explanation could be that no anecic species were found in the 0-20 cm soil layer, which may be due to the absence, but more likely the non-capture of *Lumbricus terrestris*. Burrows of these earthworms play an important role in water infiltration (Bouché and Al-Addan, 1997). A second explanation could be that only in experiments with extreme treatments (e.g. with or without earthworms), can an increased water infiltration rate be linked to earthworm presence (Hoogerkamp et al.,1983; Clements et al., 1991) or earthworm burrow numbers (Joschko, 1989). A third explanation could be that the negative effect of soil compaction by trafficking of heavy machinery and/or animal trampling precluded a possible positive effect of earthworms on water infiltration in some of the grasslands.

7.4.3 Supply of nutrients

The correlation between potentially mineralizable N and C has previously been described by Haney et al. (2008). Monaco et al. (2008) measured a correlation between potentially mineralizable N and soil respiration measured in air-dried soil (which we call potentially mineralizable C) in the 0-15 cm and 15-30 cm soil layers. In the current experiment, both parameters were strongly correlated with total N and number of nematode taxa. The soil parameters, total N and nematode taxa were related. The total number of nematodes increased with a higher soil total N, which probably increased the number of nematode taxa. Few references are available where potentially mineralizable N and C have been related to biotic soil parameters. Some authors have suggested that the potentially mineralizable N measured by anaerobic incubation may serve as a substitute for microbial biomass determinations, since it apparently involves killing and mineralizing the obligate aerobes and correlates with microbial biomass C as determined by fumigation extraction (Drinkwater et al., 1996; Schipper and Sparling, 2000). However, in our experiment, potentially mineralizable N was not correlated with bacterial biomass determined by microscopy (Annex 7.1). Franzluebbers (1999), Haney et al. (2001) and Hermann and Witter (2002) have suggested that the rapid C mineralization after the drying and re-wetting of samples is indicative for the labile fraction of organic matter, which in turn is closely associated with a soil's agronomic history. Drinkwater et al. (1996) stated that differently managed soils can have similar levels of total N, but very different N mineralization potential, indicating differences in SOM quality. Monaco et al. (2008) confirmed this for potentially mineralizable N when different organic materials were applied, and Van Eekeren et al. (2009b) showed this for potentially mineralizable C when clover was cultivated. In this experiment, the recent management and fertilization history of the different grasslands were rather similar, which could have resulted in a comparable SOM quality, explaining the strong correlation between potentially mineralizable N and total N.

Contrary to potentially mineralizable C and N, potential C and N mineralization were not significantly correlated with total N in the current study. The difference may be explained by the different methods used. Both the potentially mineralizable C and N are biochemical assays, whereby aggregates are destroyed and the total flushes of CO_2 and NH_4^+, respectively, are measured. Potentially mineralizable C was determined after drying and re-wetting of soil which increases the flush of CO_2 in the first week after the disturbance. Potentially mineralizable N was determined by incubation in a slurry at 40 °C under anaerobic conditions. This promotes net N mineralization because less N is immobilized in anaerobic conditions than in aerobic conditions (Patrick and Reddy, 1972; Yadvinder-Singh et al., 2005). In contrast, potential C and N mineralization rates are biological assays measured in moist samples from week 1 to week 6, omitting the first week (flush after disturbance). Different authors (Jenkinson and Powlson, 1976; Franzluebbers et al., 1996; Pell et al., 2006; Canali and Benedetti, 2006) point out that the potential C and N mineralization are representative of the process of mineralization in an undisturbed field-moist soil (basal respiration and basal N mineralization). In our experiment, both potential N and C mineralization were mainly correlated with biotic soil parameters (Annex 7.1), including microbial biomass, nematode MI and percentage of mites within the micro-arthropods. For these three biotic soil parameters, both potential

C and N are the other's antithesis, whereby a high potential N mineralization represents a matured soil food web with predacious nematodes and a high nematode Maturity Index. This in contrast to a high potential C mineralization which has next to a lower nematode Maturity Index, a higher percentage of mites. A higher percentage of mites within the micro-arthropods suggests litter accumulation (Mulder and Elser, 2009; Smeding et al., 2005).

We had hypothesized that nutrient supply of the soil was positively influenced by the quality of SOM and correlated with the abundance and biomass of micro-organisms, microbivorous grazers and their predators. We conclude that potentially mineralizable C and N are mainly determined by soil total N. The potential C and N mineralization are suggested to be more related to biotic soil parameters, whereby the two parameters are the other's antithesis.

7.4.4 Grass production

7.4.4.1 DM and N yield intercepts

One of the major objectives of this study was to identify soil parameters that explain grassland production. The DM and N yield intercept of the most productive grassland was three times higher than the least productive grassland. The best abiotic parameter to explain the DM yield intercept was soil moisture, while SOM was the best explanatory parameter for N yield intercept. Hassink (1996) also found a correlation between the N yield at 0 kg N ha^{-1} of mineral soils and SOM in the 0-5 cm soil layer (r=+0.51). Hassink (1995a) concentrated his work on the relationship between the N yield at 0 kg N ha^{-1} and the N-organic in the soil and found correlations of r=+0.38 in the 0-5 cm layer and r=+0.70 in the 0-20 cm layer of sandy and clayey soils. Parfitt et al. (2005) related DM production of grasslands fertilized with 0 to 90 kg N ha^{-1} to the total N in 0-20 cm (r=+0.87) (soils formed in silty mudstones and quartzo-feldspathic loess).

In the Dutch grassland fertilization recommendation (http://www.bemestingsadvies.nl) the nitrogen supply capacity of the soil, defined as the non-fertilizer nitrogen supply including atmospheric deposition, is estimated from soil total N. This relationship is based on the work of Hassink (1995a, 1996). In the range of soils sampled in this experiment, the nitrogen supply capacity on the basis of his work was underestimated in 17 of the 20 grasslands. In this study, part of the difference between the N yield intercept and the nitrogen supply capacity calculated from total N could be explained by soil moisture and C-percentage of the SOM. Apart from these differences, the underestimation of the measured nitrogen supply capacity by the standard calculation method, could be the higher soil C/N ratios in the soils sampled by Hassink (1995a). The latter would be in line with a correction made to the standard calculation method by a commercial laboratory in The Netherlands on the basis of the C/N ratio in the soil (Reijnenveld, personal communication). Differences in C/N ratios in the sandy soils sampled by Hassink (1995a) and this experiment could be explained by the experimental sites and their fertilization history. In the research of Hassink (1995a), some experimental sites were located in the North-East of The Netherlands, which had a relatively high SOM (>100 g kg dry soil^{-1}) for sandy soils and probably contained a stable humus with its origin in peat. Moreover, in the experiments of Hassink (1995a), the nitrogen supply capacity was measured on experimental plots without fertilization for several years, while the 0 kg N ha^{-1} plots in the current experiment were without fertilizer for one year only. The fertilization history in our experiment is more representative of day-to-day practice. Therefore, the explanation of the nitrogen supply capacity of grassland on sandy soils with a SOM < 80 g kg dry soil^{-1} could be more realistic with the results of our experiment. The average underestimation of 42 kg N ha^{-1} (31%) for the 17 grasslands with an underestimated nitrogen supply capacity legitimizes new research to modify the currently used recommendations.

DM and N yield intercept could also be significantly explained by total enchytraeid biomass, although the cross-validated R^2 was lower than that of soil moisture and SOM. In the current experiment, the enchytraeid biomass was closely related to soil moisture, SOM or total N in the soil. Mikola et al. (2001) and Van der Wal et al. (2009) measured a positive correlation between enchytraeid abundance and the harvested shoot mass. Since soil organic matter and microbes are the main food sources of enchytraeids (Didden et al., 1994), the observed correlation could be a bottom-up response to food abundance. In our experiment, the number of bacterivorous nematodes explained extra variation of the DM and N yield intercept, in addition to enchytraeid biomass. These parameters, which were measured in spring before fertilization, are probably indicative of readily available nutrients in the growing season.

Of the process parameters underlying the ecosystem service nutrient supply, potentially mineralizable N was correlated most strongly with both DM and N yield intercept. However, no significant regression model could be established. Parfitt et al. (2005) related potential N mineralization (0-8 weeks) with pasture DM yield ($r=0.87$; $P<0.003$) and N yield ($r=0.95$; $P<0.001$). The potentially mineralizable N is frequently used as an indicator of the potential N supplying capacity of a soil (Drinkwater et al., 1996; Curtin and McCallum, 2004; Russell et al., 2006).

We had hypothesized that the dry matter yield of grassland would be explained by one or more of the process parameters for nutrient supply. In our experiment, however, an abiotic soil parameter (SOM) best explained the nitrogen supply capacity, and not a soil process parameter such as potentially mineralizable N. Therefore, we conclude that for the explanation of the nitrogen supply capacity of the soil, the focus should be on abiotic parameters such as moisture content, SOM and total N.

7.4.4.2 Response of DM and N yield to N fertilization

The response of N yield to N fertilization or the 'apparent' N recovery (ANR) of fertilizer by grassland is usually between 50 and 80% (Whitehead, 1995). In the current experiment, the response of N yield to N fertilization ranged from 35-102%. For the grassland with the lowest N response, this would mean that 149 kg N ha^{-1} of the average 222 kg N ha^{-1} inorganic fertilizer applied on these 20 grasslands in 2005, was not harvested as N in the grass compared with the grassland with the highest response. This shows the importance of explaining this response in order to better target the N fertilizer, not only from an economic point of view but also to prevent losses to the environment.

The response of DM and N yield to N fertilization could not be explained by abiotic soil parameters, although both responses were negatively correlated with soil C/N ratio (in the range of 11.2 to 19.1). This appears obvious: with decreasing C/N ratio, less fertilizer N will be immobilized, i.e. more will be available for plant uptake and dry matter production.

The response of N yield to N fertilization could be explained by the total number of enchytraeids. It is remarkable that a biotic parameter explained the response to inorganic N fertilizer. A possible explanation could be that enchytraeids indicate a nutrient-rich environment. However, different from the N yield intercept, the response of N yield to N fertilization was not correlated with other abiotic soil parameters representing a nutrient-rich environment, such as total N. Furthermore, just as by Hassink (1995b), the response of N yield to N fertilization was not related to the N yield intercept (Fig. 7.3, Table 7.6). A second explanation could be the effect of the historical grass production on the enchytraeid abundance measured in the spring of 2006. In fact, the enchytraeid abundance could be an indication of the potential production capacity with N fertilization. As already mentioned, Mikola et al. (2001) and Van der Wal et al. (2009) measured a positive correlation between enchytraeid abundance and the harvested shoot mass. Yeates (1968) also postulated that enchytraeids appear to act as an indicator of favorable conditions. A third possible explanation is the role of enchytraeids in decomposition processes. Research of Didden (1993) and Mulder (2006) on clayey as well as sandy soils suggest that enchytraeids are a key functional group of the decomposition community. Enchytraeids have been shown to strongly regulate ecosystem processes of organic matter decomposition and nutrient (C and N) mineralization (Cole et al., 2002; Mulder and Elser, 2009). Therefore, the total number of enchytraeids may be indicative of a balanced decomposition of organic matter and a high N response to N fertilization. We recommend that the mechanisms behind this relationship be further investigated.

The response of N yield to N fertilizer was correlated with potentially mineralizable N and there was a tendency towards an explanatory model with three soil process parameters, including potentially mineralizable N. We had hypothesized that the response to N fertilization would be explained by one or more of the process parameters for nutrient supply. However, we have to conclude that a biotic soil parameter explained more of the variance.

7.4.5 Biotic soil parameters in soil quality assessment?

The main objective of this study was to investigate to what extent biotic soil parameters have added value in soil quality assessment of grassland on sandy soils. For the explanation of soil process parameters underlying soil ecosystem services, various biotic soil parameters indeed played a role. However, the majority of the process parameters and the N yield intercept were best explained by "traditional" abiotic parameters such as soil moisture content, SOM and total N. Only for the explanation of the N response to N fertilization, a biotic parameter (number of enchytraeids) had added value over and above abiotic parameters. An explanation for this could be that the fertilization on these 20 grasslands was so high in the past that ecosystem self-regulating processes were overruled. Ritz et al. (2009) stated that the majority of soil processes are driven by soil biota. This would suggest that abiotic parameters are intermediate to soil biota and the processes that result from their activity. Again this could be explained by a higher variation in biological soil parameters compared to abiotic parameters, and/or by soil processes being governed by different soil biota so that one-to-one relationships are less likely to become established. Hence, for soil quality assessment of grassland on sandy soils, the focus should be on abiotic parameters such as soil moisture content, SOM and total N. On other soil types these findings could be different. For the explanation of the N response to N fertilization, the mechanism behind the correlation between the number of enchytraeids and the N use efficiency has to be further investigated.

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ANNEX 7.1 Significant pearson correlations (P<0.05) (+ = positive correlation, - = negative correlation)

	DM-yield intercept	N yield intercept	DM-yield resonse	N yield response	Clay	Silt	Loam	Fine sand	Coarse sand	SOM	HWC	DOC	C-total	N-total	Soil moisture	C/N ratio	C-percentage of SOM	P-total	P-Al	K-HCl	pH-KCl	Root biomass	Total number of earthworms	Total biomass of earthworms	Number of earthworm taxa
DM-yield intercept		+0.93			+0.69	+0.54	+0.60			+0.77	+0.58	+0.61	+0.66	+0.72	+0.84										
N yield intercept	+0.93				+0.59	+0.47	+0.52			+0.82	+0.67	+0.49	+0.67	+0.77	+0.77		-0.51					-0.46			
DM-yield response				+0.86												-0.50				-0.41					
N yield response			+0.86													-0.62		+0.55							
Clay	+0.69	+0.59				+0.69	+0.79	-0.60	+0.60	+0.46		+0.59	+0.47	+0.67	+0.48	-0.45		+0.69		+0.47					
Silt	+0.54	+0.47			+0.69		+0.99			+0.54			+0.51		+0.47					+0.52					
Loam	+0.60	+0.52			+0.79	+0.99		-0.46	+0.46	+0.55		+0.45	+0.53		+0.50					+0.54					
Fine sand					-0.60		-0.46		-1									-0.60							
Coarse sand					+0.60		+0.46	-1										+0.60							
SOM	+0.77	+0.82			+0.46	+0.54	+0.55				+0.85		+0.92	+0.78	+0.63										
HWC	+0.58	+0.67								+0.85			+0.83	+0.66	+0.46								-0.49		
DOC	+0.61	+0.49			+0.59		+0.45						+0.49	+0.59	+0.66			+0.47							
C-total	+0.66	+0.67			+0.47	+0.51	+0.53			+0.92	+0.83	+0.49		+0.79	+0.59							-0.47			
N-total	+0.72	+0.77			+0.67					+0.78	+0.66	+0.59	+0.79		+0.60	-0.62		+0.56							
Soil moisture	+0.84	+0.77			+0.48	+0.47	+0.50			+0.63	+0.46	+0.66	+0.59	+0.60	1				-0.51			-0.48			
C/N ratio			-0.5	-0.62	-0.45									-0.62				-0.67							
C-percentage of SOM		-0.51																							
P-total				+0.55	+0.69			-0.60	+0.60			+0.47		+0.56		-0.67									
P-Al															-0.51										
K-HCl					+0.47	+0.52	+0.54																		-0.54
pH-KCl																								-0.50	
Root biomass		-0.46											-0.47		-0.48										
Total number of earthworms											-0.49													+0.68	+0.72
Total biomass of earthworms																					-0.50		+0.68		+0.70
Number of earthworm taxa																				-0.54			+0.72	+0.70	
% of epigeic adult earthworms				-0.53												+0.62		-0.45							
% of endogeic adult earthworms			+0.48	+0.67												-0.60		+0.46							
Number of epigeic adult earthworms		-0.52												-0.54		+0.57						0.60			
Number of endogeic adult earthworms				+0.45																			+0.70	+0.68	+0.81
Total number of potworms	+0.72	+0.61		+0.69	+0.59					+0.51			+0.53	+0.70	+0.60	-0.47		+0.51							
Total biomass of potworms	+0.78	+0.70		+0.59	+0.49					+0.46				+0.61	+0.74	-0.47									
Number of potworm taxa																									
% of fridericia potworms								+0.46	-0.46																
% of marionina potworms		-0.41			-0.51								-0.45	_0.73		+0.60		-0.66							
% of enchytraeus potworms																		+0.57							
Number of fridericia potworms				+0.46										+0.46											
Number of marionina potworms														-0.46											
Number of enchytraeus potworms			+0.45	+0.63	+0.51									+0.67		-0.55		+0.68							
Total number of mytes and springtails																									
Number of mytes																									
Number of springtails											+0.45			+0.50		-0.55									
% of mytes				-0.47												+0.45	+0.52								
% of springtails										+0.46					+0.48		-0.51		-0.49						
Total number of nematodes	+0.50	+0.61								+0.60	+0.59	+0.58	+0.56	+0.68	+0.51										
Number of nematode taxa		+0.51		+0.47										+0.63		-0.70			-0.52						
Maturity Index of nematodes			+0.53	+0.56															-0.46						
% of fungivorous nematodes																									
% of herbivorous nematodes			+0.5	+0.48												-0.81		+0.50							
% of bacterivorous nematodes			-0.62	-0.59								-0.51				+0.79		-0.54							
% of predacious nematodes																									
Number of fungivorous nematodes	+0.45	+0.47										+0.49		+0.46		-0.45									
Number of herbivorous nematodes												+0.52		+0.67		-0.77		+0.57							
Number of bacterivorous nematodes		+0.52								+0.64	+0.65		+0.61	+0.45						+0.52			-0.44		
Number of predacious nematodes																									
Bacterial biomass																			-0.77						
Bacterial activity	+0.51				+0.53	+0.76	+0.75								+0.56					+0.52					
Fungal biomass				+0.47											+0.45				-0.73						
Fungal activity																									
Physiological activity	+0.57	+0.49		+0.48	+0.52									+0.46		-0.65		+0.51							
Physiological diversity																									
Bulk density		-0.46								-0.56	-0.64		-0.53												
Penetration resistance 0-10 cm				-0.56																+0.49			-0.62		-0.63
Penetration resistance 10-20 cm																		-0.57							
Penetration resistance 20-30 cm		-0.47												-0.57		+0.52		-0.57							
Penetration resistance 30-40 cm	-0.53	-0.58								-0.52				-0.48								0.50			
% Crumbs																							+0.51		
% Sub-angular blocky elements				+0.45						-0.54	-0.46		-0.57			-0.47									+0.47
% Angular blocky elements																							-0.46		-0.46
Water infiltration rate																		+0.56							
Earthworm burrows at 10 cm																	+0.45								
Earthworms burrows at 20 cm																-0.50		+0.55							
Number of roots at 10 cm													-0.45							-0.40					+0.46
Number of roots at 20 cm																									
Potential C mineralization						+0.52	+0.52					+0.47													
Potentially mineralizable C	+0.45	+0.57								+0.51	+0.45		+0.52	+0.70		-0.45						-0.47			
Potential N mineralization								+0.53	-0.53																
Potentially mineralizable N	+0.50	+0.64		+0.49						+0.62	+0.63	+0.50	+0.61	+0.83		-0.58		+0.53							

ANNEX 7.1 Significant pearson correlations (P<0.05) (+ = positive correlation, - = negative correlation) (continued)

	% of epigeic adult earthworms	% of endogeic adult earthworms	Number of epigeic earthworms	Number of endogeic earthworms	Total number of potworms	Total biomass of potworms	Number of potworm taxa	% of Fridericia potworms	% of Marionina potworms	% of Enchytraeus potworms	Number of Fridericia potworms	Number of Marionina potworms	Number of Enchytraeus potworms	Total number of mites and springtails	Number of mites	Number of springtails	% of mytes	% of springtails	Total number of nematodes	Number of nematode taxa	Maturity Index of nematodes	% of fungivorous nematodes	% of herbivorous nematodes	% of bacterivorous nematodes	% of predacious nematodes
DM-yield intercept					+0.72	+0.78													+0.50						
N yield intercept			-0.52		+0.61	+0.70													+0.61	+0.51					
DM-yield response		+0.48											+0.45								+0.53		+0.50	-0.62	
N yield response	-0.53	+0.67		+0.45	+0.69	+0.59					+0.46		+0.63				-0.47			+0.47	+0.56		+0.48	-0.59	
Clay					+0.59	+0.49			-0.51				+0.51												
Silt																									
Loam																									
Fine sand								-0.46																	
Coarse sand								-0.46																	
SOM					+0.51	+0.46												+0.46	+0.60						
HWC																+0.45			+0.59						
DOC																			+0.58					-0.51	
C-total					+0.53				-0.45										+0.56						
N-total			-0.54		+0.70	+0.61			-0.73		+0.46	-0.46	+0.67			+0.50			+0.68	+0.63					
Soil moisture					+0.60	+0.74												+0.48	+0.51						
C/N ratio	+0.62	-0.60	+0.57		-0.47	-0.47			+0.60				-0.55			-0.55	+0.45			-0.70			-0.81	+0.79	
C-percentage of SOM																	+0.52	-0.51							
P-total	-0.45	+0.46			+0.51				-0.66	+0.57			+0.68										+0.50	-0.54	
P-Al																		-0.49		-0.52	-0.46				
K-HCl																									
pH-KCl																									
Root biomass			+0.60																						
Total number of earthworms				+0.70																					
Total biomass of earthworms				+0.68											-0.45										
Number of earthworm taxa				+0.81																					
% of epigeic adult earthworms		-0.76	+0.72	-0.47									-0.49							-0.49			-0.52	+0.47	
% of endogeic adult earthworms	-0.76		-0.51	+0.67	+0.60	+0.55							+0.54				-0.49								
Number of epigeic adult earthworms	+0.72	-0.51														-0.58	+0.47	-0.53		-0.68					
Number of endogeic adult earthworms	-0.47	+0.67				+0.45																			
Total number of potworms		+0.60				+0.91			-0.45		+0.55		+0.73												
Total biomass of potworms		+0.55		+0.45	+0.91								+0.45												
Number of potworm taxa								+0.53			+0.55	+0.52			-0.51		-0.48	+0.53							
% of fridericia potworms							+0.53				+0.89					+0.51	-0.45	+0.50					+0.51		
% of marionina potworms					-0.45					-0.68	-0.46	+0.87	-0.73						-0.65	-0.63					
% of enchytraeus potworms									-0.68			-0.57	+0.84										+0.47		
Number of fridericia potworms					+0.55		+0.55	+0.89	-0.46				+0.57			+0.49		+0.52					+0.55		
Number of marionina potworms							+0.52		+0.87	-0.57				-0.48					-0.56	-0.53					
Number of enchytraeus potworms	-0.49	+0.54			+0.73	+0.45			-0.73	+0.84	+0.57												+0.51		
Total number of mytes and springtails												-0.48			+0.57	+0.81				+0.51					
Number of mytes							-0.51							+0.57			+0.66	-0.63							
Number of springtails			-0.58					+0.51			+0.49			+0.81			-0.72	+0.73		+0.64	+0.65		+0.52	-0.52	+0.56
% of mytes		-0.49	+0.47				-0.48	-0.45							+0.66	-0.72		-0.93			-0.66		-0.47	+0.55	-0.62
% of springtails			-0.53				+0.53	+0.50			+0.52				-0.63	+0.73	-0.93		+0.50	+0.47	+0.63			-0.51	+0.56
Total number of nematodes									-0.65			-0.56						+0.50		+0.65					
Number of nematode taxa	-0.49		-0.68						-0.63			-0.53		+0.51		+0.64		+0.47	+0.65		+0.48			-0.48	
Maturity Index of nematodes																+0.65	-0.66	+0.63		+0.48			+0.45	-0.59	+0.82
% of fungivorous nematodes																									
% of herbivorous nematodes	-0.52							+0.51		+0.47	+0.55		+0.51			+0.52	-0.47				+0.45			-0.89	
% of bacterivorous nematodes	+0.47															-0.52	+0.55	-0.51		-0.48	-0.59		-0.89		-0.53
% of predacious nematodes																+0.56	-0.62	+0.56			+0.82			-0.53	
Number of fungivorous nematodes									-0.44										+0.66	+0.51		+0.91			
Number of herbivorous nematodes								+0.49	-0.64	+0.49	+0.57	-0.47	+0.56			+0.60	-0.50	+0.61	+0.73	+0.67	+0.47		0.81(0)	-0.78	
Number of bacterivorous nematodes									-0.50			-0.51							+0.85						
Number of predacious nematodes																+0.63	-0.61	+0.68	+0.70	+0.65	+0.74			-0.54	+0.84
Bacterial biomass		+0.50													-0.49		-0.54	+0.60							
Bacterial activity																									
Fungal biomass			-0.56																	+0.46					
Fungal activity	+0.46																								
Physiological activity	-0.59	+0.57			+0.62	+0.65																	+0.52	-0.47	
Physiological diversity																									
Bulk density																									
Penetration resistance 0-10 cm		-0.48		-0.55		-0.46																			
Penetration resistance 10-20 cm	+0.45									-0.61			-0.65										-0.49		
Penetration resistance 20-30 cm	+0.51				-0.45				+0.55	-0.50			-0.60	-0.47		-0.55			-0.48	-0.51			-0.48		
Penetration resistance 30-40 cm	+0.48	-0.48	+0.61		-0.53	-0.56										-0.55	+0.50	-0.56		-0.47					
% Crumbs																									
% Sub-angular blocky elements																									
% Angular blocky elements																									
Water infiltration rate										+0.54			+0.50												
Earthworm burrows at 10 cm															+0.52										
Earthworms burrows at 20 cm									-0.62	+0.47		-0.66							+0.54	+0.69				-0.45	
Number of roots at 10 cm										-0.55		+0.47													
Number of roots at 20 cm														-0.61	-0.45	-0.46									
Potential C mineralization		-0.46															+0.55				-0.45				
Potentially mineralizable C			-0.55						-0.63			-0.55		+0.50		+0.50			+0.53	+0.73					
Potential N mineralization							+0.53	+0.49			+0.52				-0.47		-0.47	+0.54			+0.46				+0.48
Potentially mineralizable N			-0.61		+0.54	+0.46			-0.75			-0.54	+0.58	+0.47		+0.67	-0.45	+0.58	+0.73	+0.76					

ANNEX 7.1 Significant pearson correlations ($P<0.05$) (+ = positive correlation, - = negative correlation) (continued)

	Number of fungivorous nematodes	Number of herbivorous nematodes	Number of bacterivorous nematodes	Number of predacious nematodes	Bacterial biomass	Bacterial activity	Fungal biomass	Fungal activity	Physiological activity	Physiological diversity	Bulk density	Penetration resistance 0-10 cm	Penetration resistance 10-20 cm	Penetration resistance 20-30 cm	Penetration resistance 30-40 cm	% Crumbs	% Sub-angular blocky elements	% Angular blocky elements	Waterinfiltartion rate	Earthworm burrows at 10 cm	Earthworm burrows at 20 cm	Number of roots at 10 cm	Number of roots at 20 cm	Potential C mineralization	Potentially mineralizable C	Potential N mineralization	Potentially mineralizable N
DM-yield intercept	+0.45					+0.51			+0.57						-0.53										+0.45		+0.50
N yield intercept	+0.47		+0.52						+0.49		-0.46			-0.47	-0.58										+0.57		+0.64
DM-yield response																											
N yield response							+0.47		+0.48			-0.56					+0.45										+0.49
Clay						+0.53			+0.52																		
Silt						+0.76																		+0.52			
Loam						+0.75																		+0.52			
Fine sand																										+0.53	
Coarse sand																										-0.53	
SOM			+0.64								-0.56				-0.52		-0.54								+0.51		+0.62
HWC			+0.65								-0.64						-0.46								+0.45		+0.63
DOC	+0.49	+0.52																						+0.47			+0.50
C-total			+0.61								-0.53						-0.57					-0.45			+0.52		+0.61
N-total	+0.46	+0.67	+0.45						+0.46					-0.57	-0.48										+0.70		+0.83
Soil moisture						+0.56	+0.45																				
C/N ratio	-0.45	-0.77							-0.65					+0.52			-0.47				-0.50				-0.45		-0.58
C-percentage of SOM																				+0.45							
P-total		+0.57							+0.51				-0.57	-0.57					+0.56		+0.55						+0.53
P-Al					-0.77		-0.73																				
K-HCl			+0.52			+0.52						+0.49										-0.40					
pH-KCl																											
Root biomass															+0.50										-0.47		
Total number of earthworms			-0.44									-0.62				+0.51		-0.46									
Total biomass of earthworms																											
Number of earthworm taxa												-0.63					+0.47	-0.46				+0.46					
% of epigeic adult earthworms								+0.46	-0.59				+0.45	+0.51	+0.48												
% of endogeic adult earthworms					+0.50				+0.57			-0.48			-0.48									-0.46			
Number of epigeic adult earthworms							-0.56								+0.61										-0.55		-0.61
Number of endogeic adult earthworms										-0.51		-0.55															
Total number of potworms									+0.62					-0.45	-0.53												+0.54
Total biomass of potworms									+0.65			-0.46			-0.56												+0.46
Number of potworm taxa																										+0.53	
% of fridericia potworms		+0.49								+0.47																+0.49	
% of marionina potworms		-0.64	-0.50											+0.55							-0.62				-0.63		-0.75
% of enchytraeus potworms		+0.49											-0.61	-0.50					+0.54		+0.47	-0.55					
Number of fridericia potworms		+0.57								+0.46																+0.52	
Number of marionina potworms		-0.47	-0.51																		-0.66	+0.47			-0.55		-0.54
Number of enchytraeus potworms		+0.56											-0.65	-0.60					+0.50								+0.58
Total number of mytes and springtails														-0.47									-0.61		+0.50		+0.47
Number of mytes					-0.49															+0.52			-0.45			-0.47	
Number of springtails		+0.60		+0.63										-0.55	-0.55								-0.46		+0.50		+0.67
% of mytes		-0.50		-0.61	-0.54										-0.50									+0.55		-0.47	-0.45
% of springtails		+0.61		+0.68	+0.60										-0.56											+0.54	+0.58
Total number of nematodes	+0.66	+0.73	+0.85	+0.70										-0.48							+0.54				+0.53		+0.73
Number of nematode taxa	+0.51	+0.67		+0.65			+0.46							-0.51	-0.47						+0.69				+0.73		+0.76
Maturity Index of nematodes		+0.47		+0.74						+0.45														-0.45		+0.46	
% of fungivorous nematodes	+0.91																										
% of herbivorous nematodes		+0.81							+0.52				-0.49	-0.48													
% of bacterivorous nematodes		-0.78		-0.54					-0.47												-0.45						
% of predacious nematodes				+0.84						+0.53																+0.48	
Number of fungivorous nematodes		+0.50	+0.46																		+0.53						+0.50
Number of herbivorous nematodes	+0.50			+0.71									-0.49	-0.62							+0.56						+0.65
Number of bacterivorous nematodes	+0.46										-0.55											-0.56			+0.46		+0.51
Number of predacious nematodes		+0.71								+0.52				-0.48												+0.50	+0.53
Bacterial biomass							+0.54													-0.49				+0.45			
Bacterial activity																				-0.48				+0.45			
Fungal biomass					+0.54																				+0.48	+0.50	
Fungal activity												+0.45	+0.78	+0.56		-0.46			-0.60								
Physiological activity																							-0.47				
Physiological diversity																											
Bulk density			-0.55											+0.50	+0.53							+0.50			-0.50		
Penetration resistance 0-10 cm								+0.45								-0.60		+0.61									
Penetration resistance 10-20 cm		-0.49						+0.78						+0.83					-0.68								
Penetration resistance 20-30 cm		-0.62		-0.48				+0.56			+0.50		+0.83		+0.55				-0.50								-0.56
Penetration resistance 30-40 cm											+0.53			+0.55													-0.55
% Crumbs								-0.46				-0.60						-0.94		+0.54							
% Sub-angular blocky elements																		-0.64				+0.50					
% Angular blocky elements												+0.61				-0.94	-0.64			-0.55							
Water infiltration rate								-0.60					-0.68	-0.50													+0.47
Earthworm burrows at 10 cm					-0.49	-0.48										+0.54		-0.55			+0.55						
Earthworms burrows at 20 cm	+0.53	+0.56																		+0.55					+0.46		+0.64
Number of roots at 10 cm			-0.56								+0.50						+0.50										
Number of roots at 20 cm									-0.47																		
Potential C mineralization					-0.45	+0.45																					
Potentially mineralizable C			+0.46				+0.48				-0.50										+0.46						+0.66
Potential N mineralization				+0.50			+0.50																				
Potentially mineralizable N	+0.50	+0.65	+0.51	+0.53										-0.56	-0.55				+0.47		+0.64				+0.66		

Chapter 8

General discussion

8 General discussion

This chapter aims to integrate the results from the various experiments presented in this thesis. The effects of various grassland management measures on soil biota are discussed first (8.1). These effects are related to the delivery of ecosystem services, including grassland production (8.2). The implications of these results for grassland management on sandy soils are discussed in section (8.3).

8.1 Effects of management on soil biota

The first objective of this thesis was to gain insight into the effects of grassland management measures on soil biota in sandy soils. Whereas chapters 2 to 6 present the effects of individual management measures, this section aims to provide an integrated discussion of management effects on earthworms, nematodes and microbes, in terms of abundance and/or biomass, functional groups and species composition.

8.1.1 Earthworms

Abundance and biomass

Earthworm abundance and biomass were most clearly affected by cropping system. In the long-term experiment with different cropping systems (chapter 2) earthworm abundance in permanent arable land was only 12% of the abundance in permanent grassland. Reduced numbers of earthworms were also found in the arable phase of the ley-arable crop rotation (chapter 2). In addition to the direct effects of soil tillage (mechanical damage, increased predation by birds, loss of insulating vegetation layer), decreased resource quantity and quality in the arable phase are major drivers of decreases in earthworm abundance (Wardle, 1995; Edwards and Bohlen, 1996). The importance of resource quantity and quality for earthworm abundance in comparison to immediate effects of soil tillage was shown by Schmidt et al. (2003). Compared to conventionally grown wheat, they found that direct drilling of wheat into a permanent clover resulted in much higher earthworm numbers than direct drilled wheat in a grain stubble. Therefore, I conclude that, in the arable phase of ley-arable crop rotations, earthworm abundance is affected more by the decrease in resource quantity and quality than by mechanical damage due to soil tillage. This conclusion has important implications for the development of sustainable maize cropping systems. Moreover, it suggests that the common practice on dairy farms of re-sowing grass immediately after killing and plowing the old grass sward, may be less harmful to earthworms than is currently assumed; this merits further research.

In the cropping system experiment (chapter 2), the abundance of earthworms in temporary grass-clover ley recovered within 2 years to the level of permanent grassland, whereas earthworm biomass recovered within 3 years. The high proportion of white clover in the grass-clover ley may have accelerated earthworm recovery (Fraser et al., 1994; Haraldsen et al., 1994; Yeates et al., 1998; Postma-Blaauw et al., 2010). A similar positive effect of clover on earthworm biomass was measured in the experiment on clover introduction (chapter 3). Swards of clover-only had higher earthworm numbers and biomass than swards of grass-only with or without inorganic fertilizer. In the same experiment a negative correlation between root C/N ratio and earthworm abundance was found, which emphasizes the importance of resource quality (nitrogen content) for earthworms. Similar results were found by Laossi et al. (2008), although they concluded that it remained difficult to disentangle the effects of resource quantity and quality. Next to a clover effect, we measured a higher number of earthworm burrows (a measure of earthworm activity (Pommeresche and Løes, 2009)) with the application of organic fertilizers compared to inorganic fertilizer (chapter 4). Previous studies have reported a positive effect of N-input from both inorganic and organic fertilizers on earthworm abundance and biomass (Cotton and Curry, 1980; Edwards and Lofty, 1982), as long as negative effects on soil pH were limited (Edwards and Lofty, 1982; Ma et al., 1990). Since some inorganic fertilizers (Ma et al., 1990) and clover N-fixation (Yan et al., 1996) can lead to a decrease in soil pH, and organic fertilizers may increase soil pH (chapters 4 and 5), N-effects on earthworms can be confounded with pH-effects. In the experiment comparing organic and inorganic fertilizers on grass-only, the lower soil pH could explain the lower density of earthworm burrows under inorganic fertilizer (chapter 4). Based on the results of these various experiments, I conclude that nitrogen input positively affects the abundance and biomass of earthworms in grassland soil, by increasing resource quantity, and particularly resource quality (a lower C/N ratio). The origin of the N-input seems less important as long as negative effects on soil pH are limited.

Ecological groups and species composition

Cropping systems significantly influenced earthworm species composition. In the long-term cropping experiment (chapter 2), arable soil contained primarily endogeic adults, whereas permanent and temporary grassland soil included both endogeic and epigeic adults (chapter 2; Edwards and Bohlen, 1996). Anecic adults were dominant only in permanent grassland (chapter 2). Already in the first year of the arable phase of the ley-arable crop rotation, epigeic earthworm abundance had decreased, probably due to soil tillage and lower resource quantity and quality available from the arable crop. In the grassland phase of the ley-arable crop rotation, epigeic earthworms (mainly *Lumbricus rubellus*) recovered quickly, whereas anecic species (mainly *Aporrectodea longa*) failed to recover to abundance levels in permanent grassland (chapter 2). I conclude that a ley-arable crop rotation, which can be sustainable in terms of nutrient use efficiency (Nevens and Reheul, 2001, 2003), supports the abundance of epigeic species but minimizes the abundance of anecic earthworms.

The different responses of these two ecological groups to ley-arable crop rotation could be explained by their different capacity to regenerate in the ley-phase. Epigeic species generally have a shorter generation time and higher fecundity compared to anecic species (Bouché, 1977; Edwards and Bohlen, 1996). As such, epigeic earthworms can be designated as r-strategists, characterized by early reproduction, short generation time, short lifespan, small adult body size and high fecundity. Similarly, anecic earthworms can be designated as K-strategists, characterized by late reproduction, long generation time, long lifespan,large adult body size and low fecundity. Anecic species therefore need a more stable environment to thrive, which explains the absence of anecic species under arable land (chapter 2). Various authors have found higher numbers of anecic earthworms in minimal tillage systems where crop residues are left on the soil surface, compared to conventional tillage systems (Friebe and Henke, 1992; Pitkänen and Nuutinen, 1998). Schmidt et al. (2003) found even higher abundances of anecic species in no tillage systems with a permanent clover crop. The combination of no tillage and a stable food supply on the soil surface provides an ideal environment for anecic species.

The identification of key species is considered a prerequisite to understanding ecosystem functioning (Heemsbergen, 2009). Both epigeic and anecic species, in particular *L. rubellus* and *A. longa*, play a key role in the functioning of the soil-plant-system (*L. rubellus*, Heemsbergen et al., 2004; *A. longa*, section 8.2.2). However, the difference in recovery of these two ecological groups after a major ecological stress shows that the priority of sustainable grassland management should be with anecic species rather than epigeic species. In The Netherlands, the two most common anecic species are *A. longa* and *Lumbricus terrestris*. On only 30% of the dairy farms on sandy soils in The Netherlands, adults of these two species are present. Where anecic adults are present, they can comprise up to 28% of total adult earthworm abundance. However, it has been suggested that the relatively low anecic earthworm abundance on dairy farms on sandy soils is due to the sampling method used, which is known to underestimate especially the number of *L. terrestris* (unpublished results Biological Indicator System for Soil Quality). Therefore, I suggest that more insight is needed into the present and potential populations of these anecic earthworms, and the influence of present grassland management practices other than crop rotation, particularly presently used re-sowing techniques and the frequency of grassland renovation.

Surface applications of solid manures to grass-only tended to increase the relative abundance of epigeic earthworms (chapter 4). Since epigeic species feed on the soil surface and live in the upper soil layer, surface applications of solid manures could benefit surface feeding (epigeic and anecic) earthworms (Edwards and Lofty, 1982; Whalen et al., 1998). Competition for food at the soil surface could also be the reason that slit injection of slurry increased the abundance of anecic earthworms compared to epigeic earthworms (De Goede et al., 2003). In our comparison of 20 production grasslands (chapter 7), the total and relative abundance of epigeic earthworms was positively correlated with soil C/N ratio, and the relative abundance of endogeic earthworms was negatively correlated with soil C/N ratio. These results suggest that litter availability was higher on the surface of soils with a high C/N ratio, compared to soils with a low C/N ratio. Endogeic species ingest more soil, and feed mainly on humified organic matter with an estimated C/N ratio of about 12 (Edwards and Bohlen, 1996; Van Vliet et al., 2007), which could be the reason that they prefer soils with low C/N ratios. I suggest that epigeic earthworms are more abundant in soils with a high C/N ratio and that their abundance may increase when solid manures are applied to the soil surface. Furthermore, the relative abundance of endogeic earthworms is likely to be higher in soils with a low soil C/N ratio.

8.1.2 Nematodes

Abundance of trophic groups

Herbivorous nematodes were the most dominant trophic group in grassland soil, and their abundance was positively correlated with root biomass (chapters 3, 4, 6). However, in our comparison of 20 grasslands the total and relative abundances of herbivorous nematodes were negatively correlated with soil C/N ratio (chapter 7). This suggests that, in addition to being related to root biomass, herbivorous nematode abundance is also related to root quality (N content). Todd (1996) reported a significant correlation between the abundance of herbivorous nematodes and root N content, and Verschoor et al. (2001) measured a reduced density of herbivorous nematodes after long-term cessation of fertilization. I suggest that herbivorous nematode is related to both root quantity and quality.

Herbivorous nematode abundance responded quickly to management changes. In the ley phase of the ley-arable crop rotation (chapter 2), it took only 6 months for herbivorous nematode abundance to increase to levels found in permanent grassland. Likewise, in the arable phase of this crop rotation it took only 6 months for herbivorous nematode abundance to decrease to levels found in permanent arable land. This is in agreement with Böstrom and Sohlenius (1986), who showed that herbivorous nematode abundance can quickly follow root system development. The fertilizer experiment in chapter 4 showed an increased herbivorous nematode abundance in response to an increase in root biomass, the latter being due to reduced N-availability. Therefore I suggest that herbivorous nematode abundance is affected by management choices that affect root quantity and quality, such as cropping system, N-input, types of fertilizer, clover introduction and grass species composition.

The abundance of bacterivorous nematodes, the second largest trophic group in grassland soil, was also found to be related to their food resource, but less clearly than for herbivorous nematodes. Rotavation of a 3-year old grass-ley and the subsequent large input of organic matter resulted in an increase of bacterivorous nematode abundance (chapter 2). This increase was mainly due to the family Rhabditidae, which is known to rapidly increase in response to a resource pulse (chapter 2; De Goede et al., 1993; Ettema and Bongers, 1993; Yeates, 2003). Interestingly, bacterivorous nematode abundance was not correlated with bacterial biomass in any of the experiments in this thesis, and only one experiment showed a correlation with bacterial activity (chapter 4). According to Wardle and Yeates (1993) and Wardle and Lavelle (1997), bacterivorous nematodes indeed show extremely rapid increases with the addition of a food resource, but they soon enter into predator-prey cycles in which the sudden abundance of bacterivorous nematodes is regulated by predatory nematodes. This tri-trophic effect might explain the differences that were observed between permanent grassland (>36 years old) and temporary grassland (≤ 3 years old) (chapter 2): permanent grassland had a higher bacterial biomass, a comparable bacterivorous nematode abundance, and a higher abundance of predatory nematodes (chapter 2). In conclusion, I suggest that bacterivorous nematodes increase after a resource pulse, but in stable environments such as permanent grassland they are subsequently regulated top-down, by predators.

Relatively to their food source (fungal and bacterial biomass), fungivorous nematode abundance was lower than bacterivore abundance in all experiments (chapters 2, 3, 4, 5, 6, 7). Wardle and Yeates (1993) attributed this to differences in resource quality. Bacteria have relatively little protection against nematode grazing, while fungi possess a range of anti-grazing defenses (morphological and chemical) (Wardle and Yeates, 1993). In the experiments of chapters 3 to 6, fungivorous nematodes were not affected by any of the following management measures: N-input, clover introduction, fertilizer type and grass species composition. However, their food resource, fungal biomass, was found to be negatively affected by N-input from N-fixation and inorganic fertilizer (chapter 3). The only management measure that affected fungivorous nematodes was cropping system. Fungivorous nematode abundance was higher in grassland than arable land, while their food resource (fungal biomass) did not significantly differ between these land uses (chapter 2). Similar results were found by Postma-Blaauw (2010). A possible explanation is that fungivorous nematodes favor saprophytic fungi over mycorrhizal fungi, like was found for fungivorous collembola by Klironomos et al. (1999).

In the cropping system experiment we measured a higher mycorrhizal colonization in arable land compared to grassland in 2003 (chapter 2; unpublished results) which could explain why fungivorous nematode abundance was lower in arable soil. This merits further research.

` The low number of fungivorous nematodes relative to bacterivorous nematodes resulted in a high Nematode Channel Ratio (Bacterivorous nematodes/Bacterivorous + Fungivorous nematodes). These results confirm the general observation that agricultural soils in the Netherlands are dominated by the bacterial-based energy channel of decomposition (Bloem et al., 1994; Velvis, 1997). Only when comparing 'extreme opposites' such as grassland versus arable land (chapter 2), grass-only versus clover-only (chapter 3) and no fertilization versus fertilization (chapter 4), bacterivorous nematode dominance was found to be reduced in the first-mentioned treatment of each pair.

Management measures such as N-input, fertilizer type, clover introduction and grass species had little effect on the total and relative abundances of omnivorous and carnivorous nematodes (chapters 3 to 6). However, the higher abundance of omnivorous and carnivorous nematodes in permanent grassland (>36 years old) compared to temporary grassland (≤ 3 years old), suggests an effect of grassland age (chapter 2). This was also found by Postma-Blaauw et al. (2010). Likewise, permanent grassland and permanent arable land had higher relative abundances of omnivorous and carnivorous nematodes than temporary grassland and temporary arable land (chapter 2). Yeates et al. (1998) and Postma-Blaauw et al. (2010) also found higher carnivore and omnivore abundances in monoculture systems (grassland and arable land). Therefore I suggest that it takes a stable food supply over several years to develop predator-prey cycles and to maintain steady populations of omnivorous and carnivorous nematodes. To what extent an increased abundance of carnivorous nematodes in deeper soil layers (chapter 6) plays a role in surviving variation of food supply within a year is not known. Little is known on the effect of re-sowing grass directly after killing and plowing the old grass sward (common practice on Dutch dairy farms) on omnivorous and carnivorous nematodes. This calls for further research.

Nematode diversity

Management effects on the relative abundance of omnivorous and carnivorous were reflected in the maturity of the system (Bongers, 1990). As was observed for omnivorous and carnivorous nematodes, the nematode Maturity Index (cp1-5) was mainly affected by cropping system, and was highest in permanent grassland (>36 years old) (chapter 2). The effect of grassland age was also shown by Wasilewska (1994), who found that nematode taxa known to be r-strategists or colonizers dominated in younger meadows, while K-strategists or persisters dominated in older meadows. However, as was observed for the relative abundance of omnivorous and carnivorous nematodes, the Maturity Index was higher in permanent arable soil than temporary grassland soil. Therefore I suggest that, like for omnivorous and carnivorous nematodes, a stable food supply over several years is important for the maturity of the nematode community.

In our comparison of 20 production grasslands (chapter 7) total nematode abundance and number of taxa were found to decrease with lower soil total N. Verschoor et al. (2001) also measured a decrease in nematode density and species richness after cessation of fertilization. On the other hand, the number of nematode taxa also decreased with N-inputs through N-fixation or fertilizer applications (chapter 3). I suggest that nematode thrive in stable, nutrient rich soil conditions, but that the number of taxa can be reduced after a resource pulse that selectively stimulates colonizers.

Cropping system, clover introduction, and N-application rate on grass-clover determined the taxonomic composition of the nematode community (chapters 2, 3, 5). Taxonomic composition in temporary grassland soil in the first year after rotavation was intermediate between arable land and permanent grassland, but from the second year onwards it closely resembled taxonomic composition in permanent grassland soils. This suggests that nematode taxonomic composition in grassland soils is negatively affected by resource pulses, but recovers relatively quickly.

8.1.3 Microbes

Biomass

Bacterial biomass was affected by cropping system (chapter 2). Permanent grassland had a 50% higher bacterial biomass than permanent arable land. Microbial biomass is often found to be correlated with soil organic C and/or soil total N (Fromm et al., 1993; Wardle and Ghani, 1995b; Haynes and Tregurtha, 1999). However, in the cropping experiment (chapter 2) SOM did not significantly explain the variation in bacterial biomass. A possible explanation is the different ratio of microbial biomass to soil carbon in grassland soil versus arable soil (Wardle, 2002). Moreover, Wardle and Ghani (1995b) suggested that the correlation between microbial biomass and organic C can only be found in soils with a wide range of organic C. Another factor which could play a role is soil available P. In our comparison of 20 production grasslands (chapter 7) bacterial biomass was negatively correlated with soil P-Al (ranging between 28-72 mg 100 g^{-1} soil). Mulder et al. (2003) found a negative relation between bacterial biomass and livestock units in sandy soils of dairy grasslands. Soil available P in dairy grasslands can be an expression of historical livestock intensity, but the mechanism behind the negative correlation of P-Al with bacterial biomass remains unclear. Therefore, I suggest that further research is needed to disentangle the relations between bacterial biomass,organic C and available P.

In the experiment on clover introduction (chapter 3), where organic C and available P did not significantly differ between treatments, bacterial biomass was negatively affected by the introduction of clover-only, and positively related to root biomass. The lower bacterial biomass in clover-only could be due to a specific clover effect, or to the lower root biomass in this treatment. De Vries et al. (2006) measured a lower bacterial biomass in grass-clover than in grass, and Wardle and Nicholson (1996) measured a different bacterial biomass under *L. perenne* than under several dicotyledonous species. The relation between bacterial biomass and root biomass could not be established in the experiment with three grass species with different root biomass (chapter 6). In the same experiment, bacterial biomass was correlated with soil total N. Therefore, I conclude that in grassland soils with little variation in organic C and available P, the introduction of dicotyledonous species like clover can alter the bacterial biomass; specifically that high clover percentages in grass-clover may decrease the bacterial biomass in the soil, while increasing the bacterial biomass in the rhizosphere (Mawdsley and Bardgett, 1997).

In our comparison of 20 production grasslands (chapter 7), fungal biomass was also negatively correlated to soil P-Al. Although soil phosphorous content is known to control the level of colonization by mycorrhizal fungi (Ryan et al., 2000), the positive correlation between fungal and bacterial biomass in the comparison of 20 production grasslands suggests that the same mechanisms which play a role for bacterial biomass also apply to fungal biomass. This warrants further research.

In terms of management effects, fungal biomass was not found to be affected by cropping system (chapter 2), type of fertilizer (chapters 4, 5) or grass species (chapter 6), possibly because available P interacted with management effects. The only significant management effect on soil fungal biomass was a negative effect of N input from N-fixation or inorganic fertilization (chapter 3), where available P did not differ between treatments. Comparable results were found by De Vries et al. (2006), who measured a lower fungal biomass in grass-clover with N-fixation, compared to grass-only with a low level of N-fertilization but comparable soil available P. On organic dairy farms De Vries et al. (2007) found that soil fungal biomass increased with grassland age, which was related to the lower clover content of older grasslands, and the consequently lower N-fixation in these soils. A similar pattern was observed in our experiment with different fertilizer types on grass-clover (chapter 5), in which over the years the proportion of clover decreased and fungal biomass increased. Bardgett et al. (1999) attributed the negative effects of nitrogen on fungi to changes in vegetation and organic matter characteristics, while De Vries (2009) suggested a direct, inhibitory effect of nitrogen on fungal growth. The experiment in chapter 3 showed a negative N-effect on fungal biomass within two growing seasons, without significant changes in organic matter characteristics or vegetation composition other than clover introduction. Therefore, I conclude that, in grasslands, N-input through N-fixation or fertilizer application is a management factor that directly reduces soil fungal biomass, provided that soil available P does not change. Thus, reducing N-input will lead to an increase in soil fungal biomass. However, the effect on saprophytic fungi may differ from the effect on mycorrhizal fungi: Tisdall and Oades (1979) and Ryan et al. (2000) found higher mycorrhizal colonization on clover roots than grass roots. Thus, clover may have a positive effect on mycorrhizal fungi, and a negative effect (N-input) on saprophytic fungi (chapter 3). This merits further study.

The generally low fungal to bacterial biomass ratio agrees with the high Nematode Channel Ratio measured in these grassland soils (8.1.2), and confirms the observation that Dutch agricultural soils are generally dominated by the bacterial-based energy channel of decomposition (Bloem et al., 1994; Velvis, 1997). It has been suggested that an increase in the fungal to bacterial biomass ratio is indicative of conversion from intensive to low-input grassland management (Bardgett and McAllister, 1999). Like De Vries et al. (2006, 2007), we found several correlations between fungal/bacterial ratio and grassland management (chapters 3, 5), but these changes were mainly caused by N-input effects on fungal biomass. Therefore I endorse the conclusion of De Vries (2009) that soil fungal biomass more accurately reflects grassland management effects than the fungal to bacterial biomass ratio.

Activity

While no significant management effects on fungal activity (measured as the ratio of active hyphae to total hyphal length) were detected, bacterial activity (measured as thymidine and leucine incorporation, which reflects growth rate) was significantly lower in grassland soil than in arable soil (chapter 2). Bacterial activity increased after organic fertilizer application on grass-only (chapter 4) and grass-clover (chapter 5). The positive effect of organic fertilizers (labile organic matter) on bacterial activity was confounded by the positive effect of organic fertilizers on soil pH (chapters 4 and 5). Soil pH was positively correlated with bacterial activity (chapter 5; De Vries et al., 2006; Tobor-Kaplon, 2006). In two experiments, liming and high soil pH resulted in an overall increase in bacterial activity (chapters 2, 5). In our comparison of 20 production grasslands (chapter 7), bacterial activity correlated strongly with clay, silt and loam content. Rutgers et al. (2008) and Sonneveld et al. (2009) found a higher bacterial activity in clayey soils compared to sandy soils. Therefore, I conclude that the clay, silt and loam content of sandy soils determines baseline bacterial activity, whereas management measures that increase soil pH (liming, organic fertilizer) and input of labile organic material (roots, organic fertilizers) increase bacterial activity in grass-only and grass-clover systems.

The physiological activity of the bacterial community (measured as the amount of soil extract needed to convert 50% of all substrates in ECO plates; lower amounts indicate higher physiological activity) was higher in grassland soil than arable soil (chapter 2). In our comparison of 20 production grasslands (chapter 7) high physiological activity was related to low soil C/N ratio, high soil total N, high relative abundance of endogeic earthworms, low relative abundance of epigeic earthworms, and high enchrytreaid biomass and abundance. This suggests a positive relation between physiological activity and N-availability, such as the trends observed in chapter 3: higher physiological activity in the clover-only treatment, and in grass-only after inorganic fertilizer application. However, these trends were not observed in the experiment comparing the effect of different organic and inorganic fertilizers (chapter 4), nor in a comparison of semi-natural grassland with dairy grasslands by Rutgers et al. (2008. Therefore the relation between N-availability and the physiological activity of soil bacteria remains unclear, and warrants further research.

Physiological diversity of bacterial communities

The bacterial community in permanent grassland soil (>36 years old) had a higher physiological diversity (measured as the slope parameter of CLPPs, where a low slope indicates a high diversity) than in temporary grassland (≤3 years old) (chapter 2). Rutgers et al. (2008) measured in semi-natural grassland on sandy soils a higher physiological diversity than in dairy grassland. Both older grasslands and semi-natural grasslands have in common that they have been 'disturbance-free' for longer than young grasslands, and have had more time to accumulate SOM (Sonneveld and Bouma, 2003) and to shift towards a more diverse vegetation (De Vries et al., 2007). In our comparison of 20 production grasslands (chapter 7), physiological diversity of the bacterial community was not related to SOM, but there were positive correlations with total and relative abundance of predatory nematodes and the nematode Maturity Index (chapter 7), which suggests that the physiological diversity also depends on a stable food supply over years. However, whereas predatory nematodes and the Maturity Index did not differ between permanent arable land and temporary grassland , physiological diversity was higher in temporary grassland than in permanent arable land (chapter 2). As primary decomposers, bacteria are obviously directly influenced by the resource quality in grasslands. Moreover, the soil bacterial community in permanent grassland distinguished itself from the bacterial community in temporary grassland in its ability to decompose a distinct set of substrates in the ECO-plates (chapter 2). Most likely the different vegetation composition of these grasslands has played a role. The vegetation of the permanent grassland was indeed more diverse than in the temporary grassland (Nevens and Reheul, 2003). Therefore, I suggest that the physiological diversity of bacterial communities in grassland soils depends on a stable food supply over years, but also on the specific resource quality under grassland, which in turn depends on vegetation composition.

8.2 Effect of management on ecosystem services

The second objective of this thesis was to explore the effect of grassland management measures on the ecosystem services associated with soil biota: soil structure maintenance, water regulation, and supply of nutrients. Dairy farmers would like to see these ecosystems services result in a stable grassland production grass of good feeding quality and an efficient use of water and nutrients. Therefore this section will also explore grassland management effects on “the ultimate ecosystem service”, grass production.

Although it is difficult to establish direct relationships between soil biota and ecosystem services based on field data, the field experiments in this thesis provide some room to explore these relationships. Species may be functionally redundant due to the presence of other species in the same functional group (Andrén and Balandreau, 1999; Laakso and Setälä, 1999; Bardgett, 2005), or their functional contribution may be positively or negatively affected by interactions with other species (Heemsbergen et al., 2004). In pot experiments with low species diversity (e.g. Griffiths, 1989; Laakso and Setälä, 1999; Postma-Blaauw et al., 2005, 2006) and field experiments where complete functional groups were either absent or under stress (e.g. Clements et al. 1991; Hoogerkamp et al., 1983; Haria et al., 1998), direct relations between soil biota and ecosystem services were found. It appears that relations between soil biota and ecosystem services in field experiments are more easily demonstrated for soil biota that have low abundance, that have a specific function that is not easily taken over by other soil biota, and that are sensitive to management (Postma-Blaauw et al., 2010). But other than that, it remains extremely difficult to disentangle cause and effect, due to the interactive, cyclical and complex nature of the plant-soil system (Syers and Springett, 1983). Furthermore, there is often a discrepancy between the scale (from microfilm to the landscape scale) at which soil biota and processes (ecosystem services) are measured, and the scale at which soil biota influence these processes (Lavelle et al., 2006). The process parameters measured in this thesis were selected primarily based on their relevance for farmers, and are not per se related to the functional spatial scales of soil organisms.

In this section I discuss the relations between abiotic and biotic soil parameters and various process parameters representing ecosystem services. The effects of management measures on these processes are also discussed.

8.2.1 Soil structure maintenance

The process parameters measured to assess management effects on soil structure maintenance were bulk density, penetration resistance and visual soil structure.

Bulk density

In our comparison of 20 production grasslands (chapter 7), variation in soil bulk density (5-10 cm soil layer) was significantly explained by Hot Water-extractable Carbon (HWC). Both bulk density and HWC were also strongly correlated with SOM. Because soil organic particles weigh less than mineral soil particles, soil bulk density is highly dependent on SOM (Locher and Bakker, 1990). The effect of SOM on bulk density was reflected by the lower soil bulk density in permanent grassland compared to temporary grassland (chapter 2), the tendency for a lower soil bulk density in grasslands fertilized with solid organic manures rather than inorganic fertilizer (chapter 4), and the lower soil bulk density in grass-clover after the application of organic manures compared to a no-manure control (chapter 5).

In the experiment with a ley-arable crop rotation (chapter 2), the temporary arable land had a lower soil bulk density than the temporary grassland with comparable SOM, probably due to annual tillage of the arable soil. In chapter 4, we found a negative correlation between the relative abundance of adult epigeic earthworms and bulk density. Earthworms in general are known to decrease soil bulk density by creating large macropores, and epigeic earthworms in particular are known for their activity in the 0-10 cm soil layer (Francis and Fraser, 1998). However, in our experiment (chapter 4), the correlation between bulk density and epigeic earthworms was lost when bulk density was corrected for SOM. In contrast, in a long-term field experiment, soil bulk density was lower in the treatment with earthworms (even when corrected for SOM) than in the treatment without earthworms (Clements et al., 1991). It is possible that these effects can only be measured in long term field experiments with rather ‘extreme’ treatments.

I conclude that bulk density is mainly affected by management measures that influence SOM, and by tillage. Therefore, maintaining permanent grassland, applying organic manures in general and solid organic manure in particular, increase SOM and reduce soil bulk density. Grass-clover appears to accumulate less soil organic C than grass (Schils and Snijders, 2004; Schils et al., 2005), and therefore grass-clover could lead to a higher soil bulk density than grass. The higher root biomass and the lower fraction of mineral N to total N in soils with the grass species *L. perenne* and *F. arundinacea* compared to *D. glomerata* suggests that the first two grass species accumulate more SOM in the soil and could decrease soil bulk density in the long term.

Penetration resistance

In this thesis, management measures that significantly reduced soil penetration resistance were the introduction of clover-only (effects in the 20-30 cm soil layer) (chapter 3) and the application of solid manures (effects in the 0-10 cm soil layer) (chapter 4).

In the experiment comparing grass-only, grass-clover and clover-only (chapter 3), penetration resistance at 20-30 cm was negatively correlated with earthworm biomass. In the same experiment, clover-only had a higher number of earthworm burrows, which can be regarded as a measure of earthworm activity (Pommeresche and Løes, 2009). In the experiment with different fertilizer types on grass-clover (chapter 5), the penetration resistance at 10-20 cm and 30-40 cm was also negatively related to the number of earthworm burrows.

The significantly lower penetration resistance of the 0-10 cm layer with the application of solid manures (chapter 4) was partly explained by an increase in SOM, but could also have been caused by the larger epigeic earthworm populations in these soils (although the population differences were not significant). Epigeic earthworms live in the top soil layer and their activity may have loosened the soil. In our comparison of 20 production grasslands (chapter 7), significant negative correlations between penetration resistance and different earthworms parameters were found. However, penetration resistance in the 0-10 cm, 20-30 cm and 30-40 cm soil layers was not significantly explained by the measured soil abiotic or biotic parameters in a stepwise regression procedure (chapter 7). In experiments where earthworms were either introduced or killed, the presence of earthworms decreased soil penetration resistance in the 0-10 cm soil layer (Hoogerkamp et al., 1983) or even deeper soil layers (Clements et al., 1991). I suggest that penetration resistance is affected by earthworm activity, but treatments with and without earthworms are necessary to demonstrate significant effects. Furthermore, our experiments have made clear that application of solid manures reduces penetration resistance of the top soil layer, and that the introduction of clover-only reduces the penetration resistance of deeper soil layers.

In the previous paragraph I suggested that penetration resistance is affected by soil biota, especially earthworms. But since penetration resistance is also directly and indirectly related to the habitat of these soil biota, it is difficult to disentangle cause and effect. For example, in our comparison of 20 production grasslands (chapter 7), penetration resistance of the 10-20 cm soil layer was positively correlated to active fungal hyphae in the 0-10 cm soil layer. It remains unclear whether fungal activity caused higher penetration resistance, or whether higher fungal activity was caused by a higher penetration resistance. Fungal activity was not affected by any of the management measures investigated in this thesis. Moreover, in our comparison of 20 production grasslands (chapter 7), fungal activity was positively correlated to soil structure parameters that are indicative of poor soil structure. This suggests that a higher penetration resistance in deeper soil layers creates conditions that affect soil biota in the upper soil layer, as well as grass production (chapters 5, 7). Therefore, I would like to make a case for more research on processes in deeper soil layers, to better understand their effects on soil biota in the upper soil layer and on the functioning of the soil-plant system.

Visual soil structure

In our comparison of 20 production grasslands (chapter 7), the percentage of subangular blocky elements (intermediate to crumbs and angular blocky elements) was explained by a regression model including SOM and soil C/N ratio as variables. In the same comparison, soil crumbs and angular blocky elements could not be significantly explained by any of the measured abiotic and biotic soil parameters in a stepwise regression procedure (chapter 7). However, there was a positive correlation between the percentage of soil crumbs and the number of earthworms (chapter 7). A similar correlation was found in the fertilizer application experiment (chapter 4). The percentage of soil crumbs was also higher in grassland soil than in arable soil (chapter 2), and higher in the grass-only treatment compared to the clover-only treatment (chapter 3). An explanation for the positive effect of grass on the percentage of soil crumbs is most likely its extensive fine-root system (Robinson and Jacques, 1958). Organic material released by grass roots is known to stabilize micro-aggregates directly or indirectly by providing nutrients to rhizosphere micro-organisms, which in turn may excrete soil-stabilizing substances. Furthermore, roots and mycorrhizal fungal hyphae physically entangle micro-aggregates, turning them into macro-aggregates (Miller and Jastrow, 1990). Therefore, I suggest that the proportion of crumbs is positively affected by SOM, earthworms, and grass roots with their associated rhizoflora in general and mycorrhizal fungi in particular.

Management measures to improve soil structure could therefore aim for higher SOM, earthworm populations, and root biomass and density. Measures to increase SOM have already been discussed in the text on bulk density (8.2.1). Earthworm populations can be stimulated with N-inputs that increase both resource quantity and quality (8.1.1). Root density is higher in temporary grassland than permanent grassland (chapter 2), and root biomass increases with lower N-availability (chapter 4; Ennik et al., 1980) and with specific grass species such as *L. perenne* and *F. arundinacea* (chapter 6). However, since the effect of N-availability on grass root biomass could be opposite to its effect on earthworm abundance, management measures aimed at a higher ratio of crumbs to angular blocky elements may lead to conflicting results. Lastly, I suggest that the high proportion of crumbs of grass-clover, intermediate to grass-only with and without fertilization (chapter 4) has important consequences for soil structure management, and merits further research.

8.2.2 Water regulation

Water regulation is an ecosystem service that is closely linked to the ecosystem service of soil structure maintenance. It can be subdivided in water infiltration and water retention in the root zone. Process parameters measured were penetration resistance, earthworm burrows, soil moisture and root growth.

Penetration resistance

In our comparison of 20 grasslands (chapter 7), the variation in water infiltration rate was significantly explained by penetration resistance at 10-20 cm depth. Therefore, I conclude that water infiltration is determined to a large extent by soil penetration resistance.

Management to enhance water infiltration should therefore include measures to reduce soil penetration resistance, for instance by promoting earthworm activity (8.2.1.). Based on the results of this thesis, the application of solid manures may reduce the penetration resistance of the top soil layer (chapter 4), and the introduction of clover-only can reduce the penetration resistance of deeper soil layers (chapter 3).

Earthworm burrows

Whereas water infiltration rate was significantly explained by penetration resistance, it was not significantly related to any of the earthworm parameters measured (chapter 7). Although penetration resistance is likely affected by earthworms (8.2.1), and penetration resistance, in turn, is an important determinant of water infiltration rate, it remains difficult to directly relate earthworm abundance and/or earthworm burrows to water infiltration rate, unless earthworm abundance is experimentally manipulated (Hoogerkamp et al., 1983; Joschko et al., 1989; Clements et al., 1991).

The relation between water infiltration rate and penetration resistance is not always straightforward either: we measured a twofold higher water infiltration rate in permanent grassland compared to temporary grassland and arable treatments (chapter 2; unpublished results), while penetration resistance did not differ (chapter 2). Within the treatments it was not possible to correlate earthworm parameters with water infiltration rate. An explanation could be that annual soil tillage in the arable treatments positively affected water infiltration while negatively affecting earthworm populations. When only comparing between grass treatments, permanent grassland had a higher earthworm biomass, a higher number of earthworm burrows and a greater dominance of anecic species, compared to temporary grassland. Anecic earthworms are known for their vertical, large-diameter burrows that open to the soil surface, and a twofold higher infiltration rate per unit of biomass compared to epigeic and endogeic earthworms (Bouché and Al-Addan, 1997). Therefore, I suggest that water infiltration is not only a function of penetration resistance but also influenced by earthworm burrows and the presence of anecic earthworms. In this thesis, management measures that increased the number of earthworm burrows were the preservation of permanent grassland (chapter 2), the introduction of clover-only (chapter 3) and the application of organic manure on grass-only (chapter 4). Anecic earthworms are positively influenced by a stable food supply, and may increase with farm yard manure application (Edwards and Lofty, 1982; Whalen et al., 1998).

The positive effect of anecic earthworms on water infiltration is important, considering that in the last 100 years rainfall in the Netherlands has increased by 18%, and in the last 50 years the number of days with more than 50 mm of rainfall has increased from 5.4 to 9.0 (anonymous, RNMI; Boxel and Cammeraat, 1999). However, potential disadvantages of too many earthworm burrows are increased bypass flow (Van Stiphout et al., 1987) and greater nutrient leaching (Edwards et al., 1992; Dominiguez et al., 2004). This may apply particularly to anecic burrows, since various experiments with predominantly endogeic species have shown that earthworm burrows act as preferential pathways for water only in saturated soil conditions (Pitkänen and Nuutinen, 1998; Francis and Fraser, 1998).

Soil moisture

In our comparison of 20 grasslands (chapter 7), soil moisture in the 0-10 cm soil layer was strongly correlated with SOM and its characteristics. Therefore I suggest that water retention can be improved through management measures that increase SOM: preservation of permanent grassland (chapter 2), application of organic manure in general (chapter 4) and farm yard manure in particular (chapter 5). The selection of grass species may also play a role (chapter 6). Grass-clover accumulates less organic C than grass (Schils and Snijders, 2004; Schils et al., 2005), and therefore could result in lower soil water retention.

Root growth

In our comparison of 20 grasslands (chapter 7) and the fertilization experiment on grass-clover (chapter 5), root biomass in the 0-10 cm soil layer was positively correlated with the penetration resistance in the 30-40 cm soil layer. In other words, soil compaction in the 30-40 cm soil layer resulted in more roots in the top soil layer. The same phenomenon was observed by Bouwman and Arts (2000), who compared root growth in slightly versus heavily compacted soils. Although total root biomass was the same in both treatments, root biomass in the heavily compacted soil was entirely confined to the upper soil layer, whereas 20% of the root biomass in the slightly compacted soil was found below 20 cm depth. This means that differences in penetration resistance of deeper soil layers should be taken into account when interpreting treatment differences in root biomass in the top 0-10 cm soil layer. Moreover, these results suggest that a high penetration resistance of deeper soil layers could result in an uneven distribution of roots over the soil profile. In this thesis, the only management measure that led to a lower penetration resistance of deeper soil layers was the introduction of clover-only (chapter 3). This lower penetration resistance was correlated with a higher earthworm biomass. Therefore, I suggest that earthworm activity in deeper soil layers could decrease penetration resistance of these layers, which in turn could result in more even distribution of roots over the soil profile.

In the long-term cropping experiment (chapter 2), temporary grassland had almost twice the number of roots at 10 and 20 cm depth than permanent grassland, while penetration resistance did not differ between these treatments. It is generally accepted that younger grass swards have more and deeper roots than older grass swards, but there is no explanation in the literature. More earthworm activity (Logdson and Linden, 1992) or less root herbivory in the temporary grassland do not explain the differences in our experiment, because the abundance of earthworms and herbivorous nematodes were similar or lower in temporary grassland compared to permanent grassland. Botanical composition may have played a role: permanent grassland contained less *L. perenne* than temporary grassland, and also included *D. glomerata* (chapter 6). Moreover, root numbers in the temporary grassland may have been increased by the lower SOM, lower total N, and subsequently lower mineral N-availability. In the experiment with the application of different fertilizer types to grass-only, higher root biomass was also linked to lower N-availability (chapter 4). In conclusion, I suggest that grass root biomass and density can be increased through management choices such as temporary grassland (chapter 2), lower N-availability (chapter 4) and grass species such as *L. perenne* and *F. arundinacea* (chapter 6). In this context clover-only would be a lesser choice, since it has a lower root biomass than grass (chapter 4).

8.2.3 Supply of nutrients

To quantify the ecosystem service ‘supply of nutrients’ I focused on mineralization processes and the supply of N. The process parameters measured in the different experiments were potentially mineralizable C and N (anaerobic incubation), and potential C and N mineralization (aerobic incubation).

Potentially mineralizable C and N

In our comparison of 20 grasslands (chapter 7), potentially mineralizable C and N were significantly explained by soil total N. Also in other experiments of this thesis, potentially mineralizable C and/or N were related to total N and/or SOM (chapters 2, 4, 5 and 6). Both potentially mineralizable C and N were also correlated with numerous biological soil parameters (chapters 3, 4, 7). However the strongest correlation was with total N (chapter 7).

Potentially mineralizable C and N were affected by the same management measures as soil total N and SOM. However there were some apparent differences: 1) whereas soil total N and SOM did not significantly differ between temporary arable land and temporary grassland (chapter 2), potentially mineralizable C was significantly higher in temporary grassland; 2) potentially mineralizable C was higher in grass-clover and clover-only than in grass-only (chapter 3), while organic C and total N did not differ between these treatments; 3) potentially mineralizable N significantly differed between fertilizer treatments (chapter 4)and grass treatments (chapter 6), while there were no differences in organic C and total N. These results suggest that the relation between potentially mineralizable C and N on the one hand and total N and/or SOM on the other hand, are not always straightforward. It appears that potentially mineralizable C and N are particularly sensitive to changes in organic matter and to labile fractions of SOM (Drinkwater et al., 1996; Monaco et al., 2008). In addition to management measures that increase total N and SOM, potentially mineralizable C and N also increases in soils with grass (chapters 2), clover (chapters 2, 3), and organic fertilizers (chapter 4).

Potential C and N mineralization

In contrast to potentially mineralizable C and N, potential C mineralization did not correlate with total N or SOM (chapters 4, 5, 7), nor did potential N mineralization (chapters 2, 3, 4, 5, 7). Potentially mineralizable C and N are assessed in biochemical assays, in which soil aggregates are crushed/destroyed and the total flushes of CO_2 or NH_4^+ are measured, whereas potential C and N mineralization are measured in biological assays, in moist soil samples during 6 weeks, disregarding the initial flush due to soil disturbance in the first week. Various authors (Jenkinson and Powlson, 1976; Franzluebbers et al., 1996; Pell et al., 2006; Canali and Benedetti, 2006) have suggested that potential C and N mineralization reflect the mineralization process in undisturbed field-moist soil (basal respiration and basal N mineralization).

In our comparison of 20 grasslands (chapter 7), potential C and N mineralization were primarily related to biotic soil parameters, but these correlations were not strong enough to result in a significant regression model. Potential N-mineralization was positively correlated with microbial biomass, number of predatory nematodes, nematode Maturity Index, total and relative abundance of springtails, and enchytraeid diversity (chapter 7). Parfitt et al. (2005) also found significant correlations between potential N mineralization and microbial biomass and nematode diversity. In a soil microcosm experiment, Postma-Blaauw et al. (2005) observed that N mineralization increased with the diversity of nematode life strategy groups. In conclusion I suggest that high potential N mineralization is an indication of a well-developed soil food web. Potential N mineralization was positively affected by grassland in general, permanent grassland in particular (chapter 2), and clover introduction (chapter 3). Compared to organic fertilizers, potential N mineralization was negatively affected by inorganic fertilizer (chapter 4).

Patterns of potential C mineralization were completely opposite to N mineralization, in so far that potential C mineralization correlated negatively with microbial biomass, nematode Maturity Index, and relative abundance of endogeic earthworms. It correlated positively with the relative abundance of mites (chapter 7). When different fertilizers were applied on grassland potential C mineralization was positively correlated with the number of epigeic earthworms (chapter 4). The positive correlation between potential C mineralization and relative abundance of mites suggests an effect of litter accumulation (Mulder and Elser, 2009); taken together with the positive correlation to epigeic earthworms this suggests a soil food web that primarily operates near the soil surface (Smeding et al., 2005). Furthermore, in the experiment with different fertilizer types on grassland (chapter 4), potential C mineralization and metabolic quotient (ratio between potential C mineralization and microbial biomass) were 30% higher in treatments with farm yard manure and composted yard manure compared to a treatment with normal manure slurry (although this difference was not significant). A high metabolic quotient has been suggested to indicate low C substrate use efficiency (Wardle and Ghani, 1995a). When nutrients are limited (e.g. in substrates with high C/N ratio or high C/mineral N ratio) bacteria respire the carbon but are not able to grow. In the same fertilizer experiment (chapter 4) the bacterial communities in the farm yard manure and composted yard manure treatments showed a higher capability to degrade carbohydrate substrates in CLPP assays, compared to bacterial communities in the other treatments. In conclusion, I suggest that high potential C mineralization is an indication of limited N supply, low substrate use efficiency of soil bacteria, and a soil food web that primarily operates in the upper soil layer. The only management measure that increased potential C mineralization was the application of farm yard manure and composted yard manure on grassland (chapter 4). This effect was not observed when farm yard manure was applied on grass-clover (chapter 5). Apparently, clover N-fixation compensated for the higher C/N ratio of farm yard manure.

8.2.4 Grass production

For dairy farmers, grass production (quantity and quality) is the ultimate ecosystem service, made possible by soil ecosystem services such as soil structure maintenance, water regulation and nutrient supply in particular. In this thesis, parameters to assess grass production were Dry Matter (DM) and Nitrogen (N) yield at 0 kg N ha^{-1} and the response of DM and N yield to N fertilizer.

DM and N yield at 0 kg N ha^{-1}

In our comparison of 20 grasslands (chapter 7), DM and N yield at 0 kg N ha^{-1} were best explained by abiotic parameters: soil moisture, SOM and to a lesser extent soil total N. Hassink (1995a, 1996) found a similar relation between grass N yield at 0 kg N ha^{-1} and SOM and organic N.

Although different authors (Jenkinson and Powlson, 1976; Franzluebbers et al., 1996; Pell et al., 2006; Canali and Benedetti, 2006) have suggested that potential C and N mineralization reflect the mineralization process in undisturbed field-moist soil, in our comparison of 20 grasslands (chapter 7) these parameters were not correlated with DM and N yield at 0 kg N ha^{-1}. Parfitt et al. (2005) found significant correlations between potential N mineralization and pasture DM and N yield, but they measured N mineralization including the first week's flush which is due to disturbance of the soil sample. In that case the method seems less representative for undisturbed field-moist soil, and the outcome more closely resembles potentially mineralizable N. In our comparison of 20 grasslands (chapter 7), potentially mineralizable C and N were correlated with N yield at 0 kg N ha^{-1}, but to a lesser degree than SOM, soil moisture and total N.

As for the biotic parameters, only enchytraeid biomass explained DM and N yield at 0 kg N ha^{-1} (chapter 7). This relation, which was not as strong as was observed for the abiotic parameters discussed in the previous paragraph, most likely reflects a bottom-up response of enchytraeids to food abundance i.e. grass yield (Didden et al., 1994). Although the majority of soil processes are driven by soil biota (Ritz et al., 2009), the relation between soil biota and processes are hardly ever linear. SOM, for example, is the stable result of most (if not all) soil biological activity, while at the same time it provides food and habitat for soil biota, and feeds the soil food web via its effects on plant growth. This is illustrated by the N yield at 0 kg N ha^{-1} of the permanent grassland in the long-term cropping experiment (chapter 2): measured N yield at 0 kg N ha^{-1} over 2002-2004 was 224 kg N ha^{-1} (Bommelé, 2007), while the N yield calculated on the basis of SOM (regression model Fig. 7.3) was 210 kg N ha^{-1}. This result suggests that, also in permanent grassland on sandy soils, with a stable soil environment and a well-developed soil food web, N yield can be estimated fairly accurately on the basis of SOM. Therefore I conclude that SOM, soil moisture and to a lesser extent soil total N explain DM and N yield at 0 kg N ha^{-1} most accurately in the range of soils sampled. Management measures to increase SOM have already been discussed in section 8.1.1.

Response of DM and N yield to N fertilization

In contrast to DM and N yield at 0 kg N ha^{-1}, the N-yield response to fertilization in our comparison of 20 production grasslands was significantly explained by only one biotic soil parameter, i.e. the number of enchytraeids (chapter 7). The only abiotic soil parameter that significantly correlated with the N-yield response to fertilization was soil C/N ratio (chapter 7). As discussed earlier in this chapter, direct relations between processes and soil biotic parameters are not easy to demonstrate in grasslands with complete soil food webs and diverse soil communities. The significant relation between enchytraeid abundance and N-yield response to fertilization could therefore indicate a major interaction in the functioning of the soil-plant system. Although it is difficult to disentangle cause and effect in this interactive, cyclical and complex system (Syers and Springett, 1983), a possible explanation could be a relation between enchytraeid abundance and grass production in the year preceding sampling (Mikola et al., 2001; Van der Wal et al., 2009), i.e. a bottom-up response of enchytraeids to food abundance (Didden et al., 1994). As fertilization rates in the year preceding our experiment were comparable between the 20 grasslands, it is this yield potential that we want to explain and predict. Therefore, I suggest that enchytreaid abundance can be an indication of the N yield response to N fertilization.

However, the question remains why N yield response to N fertilization differs from one grassland to another, and what we can learn from its relationship to enchytreaid abundance. Didden (1993), Laakso and Setälä (1999) and Mulder (2006) suggest that enchytreaids are a key functional group of the decomposer community. Enchytreaids have been shown to strongly regulate ecosystem processes such as organic matter decomposition and nutrient (C and N) mineralization (Cole et al., 2002; Mulder and Elser, 2009). In our experiment, the enchytraeid community in grassland soils with a high N yield response to N fertilization was generally dominated by Enchytraeus. Furthermore, these soils also showed a high relative abundance of endogeic earthworms, a high number of earthworm burrows at 20 cm, a relatively low abundance of bacterivorous nematodes and a high nematode Maturity Index (chapter 7, table 7.6). Thus, the high abundance of enchytreaids was mainly caused by an increase of Enchytraeus, which like endogeic earthworms mainly feed on humified organic matter with a C/N ratio of about 12 (Edwards and Bohlen, 1996; Schouten et al., 2002; Van Vliet et al., 2007). The increase of Enchytraeus coincided with an increase in the total and relative abundance of endogeic earthworms. The activity of these earthworms, which live down to about 40 cm depth (Edwards and Bohlen, 1996), most likely caused the high number of earthworm burrows at 20 cm depth (section 8.2.2). At the same time, increased enchytreaid activity probably contributed to the lower penetration resistance in the 0-10 cm soil layer. Here the question arises whether the abundances of Enchytraeus and endogeic earthworms were higher because of the lower soil C/N ratio and higher humified organic matter content, or because they contributed to a decomposition process resulting in more humified organic matter. The lower relative abundance of bacterivorous nematodes and the higher nematode Maturity Index suggest a well-developed soil food web with top-down regulation of bacterivorous nematodes. The higher potentially mineralizable N shows that there was more soil total N, probably in a labile form (section 8.2.3).

In contrast, grassland soils with a lower N-yield response to fertilization were characterized by lower enchytraeid abundance, higher soil C/N ratio, higher relative abundances of epigeic earthworms and mites, and lower physiological activity of the bacterial community The distinct difference between soil food webs with high enchytraeid abundance and soil food webs with high mite abundance was also recognized in an empirical study on soil food webs in sandy soils of 49 Dutch dairy farms (Van Eekeren et al., 2005d). Like with potential C mineralization (section 8.2.3), the high relative abundance of mites suggests an effect of litter accumulation (Mulder and Elser, 2009), which taken together with the higher relative abundance of epigeic earthworm suggests a soil food web that primarily operates near the soil surface (Smeding et al., 2005). The lower bacterial physiological activity could be attributed to lower N-availability (section 8.1.3).

Not surprisingly N-availability plays a role in both situations The question remains whether a higher N yield response to N fertilizer is primarily due to a higher N-availability or to a better decomposition process. To answer this question further research is needed.

In terms of management choices, the experiment with different types of fertilizer applications on grassland may provide some starting points. Figure 8.1 outlines schematically the two distinctly different plant-soil system with low N yield response to fertilizers versus a

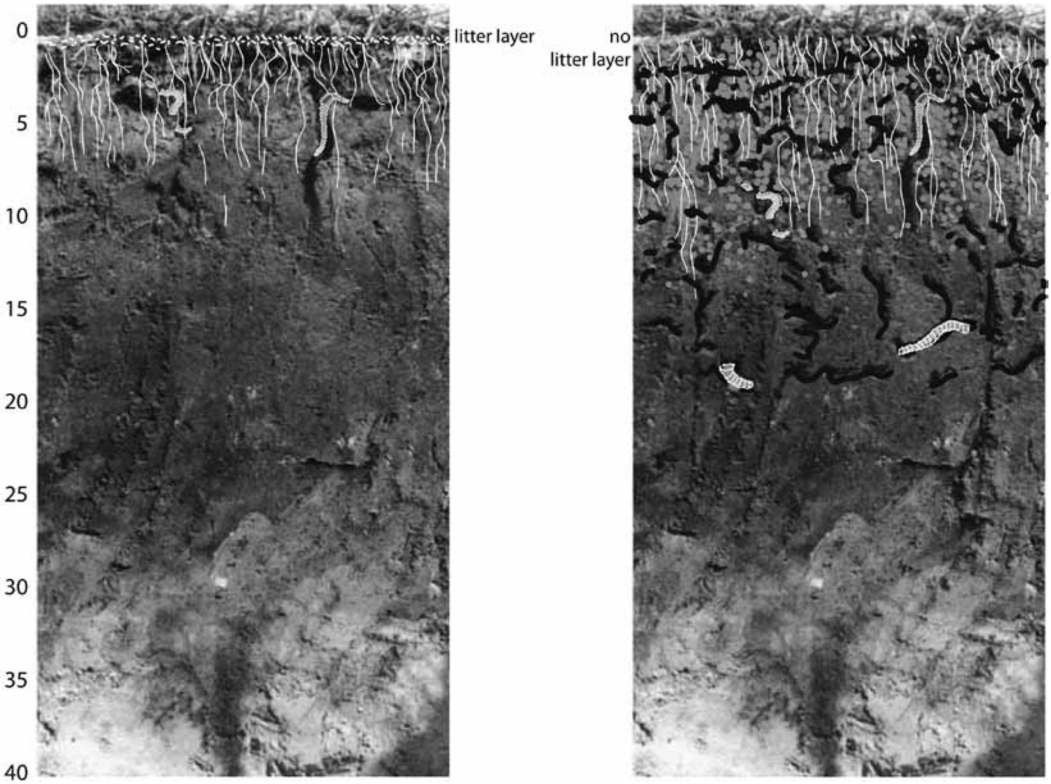

Fig. 8.1 Schematic outline of a plant-soil system with low N yield response to fertilizers (left) versus a system with high N yield response (right). The soil food web in grasslands with low N yield response primarily operates near the soil surface where litter has accumulated, while in soils with a high N yield response the food web operates also in deeper soil layers.

soil with high N yield response. The soil food web in grasslands with low N yield response primarily operates near the soil surface where litter has accumulated, while in soils with a high N yield response the food web operates also in deeper soil layers. Soil profiles in grasslands treated with pesticides for 20 years versus soil profiles of untreated grassland show a similar disparity, with litter accumulation in the treated plots, and earthworm activity in deeper soil layers in the untreated plots (Clements et al., 1991). Similarly, compared to fertilization with normal manure slurry, applications of farm yard manure and composted yard manure tended to increase the relative abundance of epigeic earthworms, the potential C mineralization and the metabolic quotient (chapter 4). These results suggest that in N-limited soils, organic fertilizers with high C/N ratios lead not only to an inefficient use of applied organic C, but also to a slower decomposition process that primarily takes place on or just below the soil surface, resulting in an suboptimal functioning of the soil-plant cycle. Therefore I suggest a rational use of organic fertilizer, with more attention for whether the C/N ratio of the organic fertilizer “fits” the soil-plant system it is applied to.

8.3 Implications for grassland management

Grassland management for soil structure maintenance

Based on the parameters bulk density, penetration resistance and visual soil structure, the ecosystem service of soil structure maintenance is determined by SOM, earthworms and roots, and the micro-organisms sustained by SOM and roots (bacteria, fungi and mycorrhizal fungi). Management measures to enhance this ecosystem service may conflict, since they sometimes have opposite effects on SOM, earthworms and roots (section 8.2.1). Based on the results of this thesis, the best management practice for enhancing the ecosystem service of soil structure maintenance would probably be the cultivation of a semi-permanent grass-clover mixture or a semi-permanent grass-only with moderate N fertilization (chapter 2, 3), with grasses with a dense and deep root system in the grass mixture (chapter 6). For grass-only, fertilization with slurry and farm yard manure would be the best choice in this context (chapter 4).

Grassland management for water regulation

Which management practices would contribute to better water infiltration and water retention in the root zone? Water infiltration is to a large extent a function of penetration resistance, which is likely to be affected by earthworm activity (section 8.2.1). Earthworms, in turn, are positively affected by resource quantity and quality, which depend on N-input. Water retention in the root zone is improved by SOM and root proliferation throughout the soil profile. The latter depends on N-availability (chapter 4; Ennik et al., 1980) and grass species characteristics (chapter 6). Like for soil structure maintenance, management practices to stimulate one factor could unintentionally inhibit another: the N-input needed to promote earthworm abundance would also reduce root distribution over the soil profile. However, the latter also depends on penetration resistance and therefore indirectly on earthworm abundance and activity (section 8.2.1). For earthworms it is mainly resource quality (low C/N ratio) (chapter 3) rather than resource quantity that determines their abundance (section 8.1.1). Thus, for the ecosystem service of water regulation the challenge will be to find an optimal N-input that supports both earthworm activity and root proliferation throughout the soil profile: this clearly merits further research. The best management choice would probably be the cultivation of a semi-permanent grass-clover mixture or a semi-permanent grass-only with moderate N fertilization (chapter 2, 3), with grasses with a dense and deep root system in the grass mixture (chapter 6).

Another example of conflicting effects on water regulation is the effect of anecic earthworms. Anecic earthworms have been found to increase water infiltration (Bouché and Al-Addan, 1997), but their burrows could also lead to increased bypass flow and therefore reduced water retention within the root zone (Van Stiphout et al., 1987). On the other hand their activity could reduce penetration resistance of deeper soil layers and their burrows could function as preferential paths serving roots in deeper soil layers (Logsdon and Linden, 1992). Springett (1985) showed that the need for the services of anecic species also depends on local conditions. She introduced anecic earthworms to pastures with shallow root systems due to wet winter conditions. Here, anecic earthworm introductions resulted in increased surface infiltration rates, higher total soil porosity at 10-20 cm, and greater root biomass at 15-20 cm. In terms of management, anecic earthworms require a stable environment (chapter 2) and prefer soil surface food resources such as farm yard manure (Edwards and Lofty, 1982; Whalen et al., 1998).

Grassland management for supply of nutrients

Potentially mineralizable C and N were positively affected by management measures that increased total N, SOM, and grass(roots) (chapter 2, 6), by clover introduction (chapter 2, 3) and by organic fertilizers (chapter 4). Potential N mineralization was higher in grassland in general and permanent grassland in particular (chapter 2), and increased after clover introduction (chapter 3). Potential N mineralization was negatively affected by the use of inorganic fertilizers, compared to organic fertilizers (chapter 4). The application of farm yard manure and composted yard manure on grassland appeared to increase potential C mineralization, suggesting microbial N limitation (chapter 4, section 8.2.3). For the ecosystem service of nutrient supply, the best combination of management practices would probably be a permanent grass-clover, with grass species with a dense and deep root system, and moderate fertilization with slurry and/or farm yard manure. The latter is a matter for discussion, because in grass-clover the service of N supply is primarily provided by N fixing *Rhizobium* bacteria, and the addition of organic manure may have little added effect on nutrient supply in this system (chapter 5).

Grassland management for production

Based on the previous paragraphs, the best grassland management scheme for enhancing the ecosystem services of soil structure maintenance, water regulation, and nutrient supply in sandy soils appears to be a semi-permanent grass-clover or a semi-permanent grass-only with moderate N-fertilization, with grass species with a dense and deep root system, and fertilization with organic fertilizers. As for management for grass production, Bommelé (2007) has shown that a grass-white clover mixture had significantly higher DM-yields over 4 N-application rates (0, 100, 300, 400 kg N ha^{-1}) than permanent grassland (>36 years old), provided that the grass-clover was sown after an arable phase (3 years' or 36 years' arable land). Taken this into account, the various management choices can be specified according to four scenarios of grass N yield at 0 kg N ha^{-1} and response of grass N yield to N fertilizer (Fig. 8.2).

I. Low N yield at 0 kg N ha^{-1}, Low N-yield response to N fertilizer

In grassland soils where both N yield at 0 kg N ha^{-1} and the response to fertilizer N are low, it is difficult to obtain good yields with the present legislative restrictions on the use of organic and inorganic fertilizers. Therefore the best management choice for these grasslands would be the introduction of grass-clover, to make use of N-fixation. To improve the soil's nitrogen supply capacity SOM should be increased. The first step to increase SOM will be to aim for a semi-permanent grass-clover system where the frequency of re-sowing is reduced to a minimum. The optimal starting point of this grass-clover would be after an arable crop, starting at a suitable soil pH, and using a persistent clover variety (Van Eekeren et al., 2005c; Bommelé, 2007). Grass species choice for the grass-clover mixture should focus on root characteristics (density and depth) and SOM accumulation (chapter 6). Yearly maintenance of pH (liming) and fertilization with phosphate, potassium and sulfur should be based on soil conditions and crop nutrient withdrawal (Frame et al., 1998; Baars, 2002; Van Eekeren et al., 2005b). If necessary, clover should be oversown yearly to keep clover in the mixture. A second step to increase SOM would be through an increase in organic fertilizer addition, particularly using organic fertilizers with high C/N ratio. However, fertilization with organic manure may reduce clover percentage (Baars, 2002). The effect of straw application in autumn on grass-clover production and SOM accumulation should be investigated. Grazing management should be

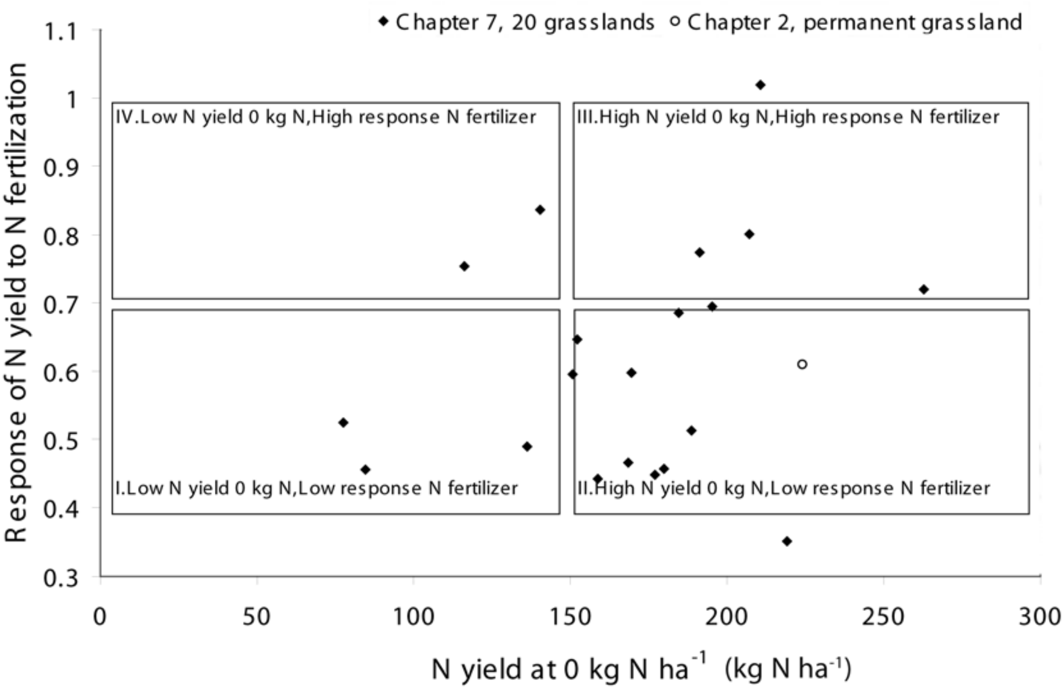

Fig. 8.2 Four scenarios of grass N yield at 0 kg N ha^{-1} and response of grass N yield to N fertilizer. The plotted points are the production parameters of the 20 grasslands from chapter 7 and the permanent grassland from chapter 2 (data from Bommelé, 2007).

directed towards maintaining clover percentage in the first place and root development in the second place. Clover will increase earthworm abundance and activity. SOM, earthworms, roots and micro-organisms (bacteria, fungi and mycorrhizal fungi) will contribute to the ecosystem services of soil structure maintenance and water regulation (chapter 3). Fertilization with farm yard manure could increase anecic earthworm species (provided they are already present), and therefore improve water infiltration rates in soils where infiltration is limited (Edwards and Lofty, 1982; Springett, 1985; Whalen et al., 1998). The described management in this section would also be the preferred management to move from scenario I to III.

II. High N yield at 0 kg N ha^{-1}, Low response N yield on N fertilizer

For grassland soils with a high N yield at 0 kg N ha^{-1} and low response of N yield on N fertilizer grass-clover is optional but not advised when it requires plowing of the grass sward. Grass species choice should focus on root characteristics (density and depth) (chapter 6). The preferred organic fertilizer for grass-only is normal manure slurry. Applications of farm yard manure with high C/mineral N ratio should be limited, to prevent that the soil food web becomes "soil-surface-based" and applied C is inefficiently used (8.2.3). Injection of slurry could be used to stimulate endogeic and anecic earthworms (De Goede et al., 2003). Grazing management should be directed towards root development. For grass-only, optimal N application rates and strategic timing of N application for enhancing root development should be further investigated (section 8.2.1; chapter 4; Ennik et al., 1980).

III. High N yield at 0 kg N ha^{-1}, High response N yield on N fertilizer

Ideal situation, there is no need to adjust present grassland management.

IV. Low N yield at 0 kg N ha^{-1}, High response N yield on N fertilizer

For the management of grassland soils with high N-yield response to fertilization but low N yield at 0 kg N ha^{-1}, the objective should be to increase SOM while maintaining the same high N-yield response to N fertilizer. In these soils, the most effective management choice to increase SOM would be to allow the grassland to age, and possibly to apply more organic fertilizer.

8.4 Overall conclusion and suggestions for further research

In this thesis I have shown that managing the basic soil qualities SOM, soil moisture, soil total N and soil C/N ratio is pertinent to achieving sustainable grassland production on sandy soil. SOM plays an important role in all ecosystem services, including grass production at 0 kg N ha^{-1}. I have also shown a significant correlation between enchytraeid abundance and N yield response to N fertilization. Taking into account that the grasslands in this study represented a broad range of N yield responses to N fertilization, this novel result could be very valuable information in identifying the most efficient use of fertilizer. However, further research into the mechanisms underlying this relationship is warranted. My hypothesis is that a high enchytraeid abundance reflects a soil food web that operates across the entire soil profile, whereas a low enchytraeid abundance reflects a less developed soil food web that operates mainly near the soil surface. A plant-soil cycle that involves shallow as well as deeper soil layers, in which deep (grass) roots and endogeic and anecic earthworms play prominent roles, uses available nutrients and water much more efficiently, leading to a more stable grass production over the season and over the years, which substantially reduces economic risks for farmers. Therefore, future research should focus on developing plant-soil systems that take advantage of a greater part of the soil profile.

Furthermore, I have shown that on sandy soils a semi-permanent grass-clover or a semi-permanent grass-only with moderate N-fertilization, with grass species with a dense and deep root system, and fertilization with organic fertilizers, is the most favorable combination of management measures for sustaining the ecosystem services of soil structure maintenance, water regulation and nutrient supply. The specific management choices can be specified according to four scenarios of N yield at 0 kg N ha^{-1} and response of grass N yield to N fertilizer described in section 8.3. My finding that the presently used fertilization recommendation model underestimated the soil nitrogen supply capacity of the majority of soils in my study, taken together with the fact that these soils represented a broad range of N response to N fertilizer, legitimates reconsideration of the present fertilizer recommendations.

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Summary

Introduction

Until recently the soil quality of production grasslands was generally not a matter of great concern. Nutrients and irrigation could always be applied in abundance, which compensated for a possible deterioration in soil quality. However, recent legislative restrictions on the use of fertilizers and irrigation, and a quest for sustainable farming systems have drawn renewed attention to the functioning of the soil and the ecosystem services it provides.

Legislative restrictions on the use of fertilization are the most severe for production grasslands on sandy soils, because these are most vulnerable to nitrate leaching, At the same time, these soils have the lowest inherent quality for agricultural production, which necessitates external inputs such as fertilizers and irrigation. Therefore, managing the quality of sandy soils and the ecosystem services they provide is of utmost importance for sustainable grassland production.

In addition to soil chemical and physical quality, soil biological quality is one of the pillars of soil quality management. The average fresh biomass of soil biota in the sandy soils of Dutch dairy farms has been found to amount to 2700 kg ha^{-1}, which is more than the biomass of livestock aboveground. Soil biota play an important role in the provision of ecosystem services such as soil structure maintenance, water regulation, nutrient supply, and grassland production, the ultimate ecosystem service for dairy farmers. Grassland management may influence soil biota directly, or indirectly. The interactions between grassland management measures and their effects on plants, soil biota and ecosystem services are cyclical and complex. For developing and optimizing sustainable grassland systems more insight is needed into the effect of grassland management measures on soil biota and the ecosystem services they provide. This knowledge would allow to make better use of these ecosystem services, and could help to limit inadvertent negative effects of management measures on soil biota and ecosystem functioning.

Therefore, the two main objectives of this thesis were 1) to gain insight into the effect of grassland management measures on the abundance, activity and species richness of soil biota in sandy soils; and 2) to explore the effect of grassland management measures on the ecosystem services provided by soil biota, such as soil structure maintenance, water regulation, supply of nutrients, and ultimately, grass production. Following a general introduction (chapter 1), chapters 2 to 6 report and discuss effects of various grassland management measures on earthworms, nematodes and microbes, and the ecosystem services associated with these biota. In chapter 7 the interrelationships between soil biological quality and ecosystem services are analyzed based on data collected in 20 grasslands. The thesis is concluded by a general discussion of the results and the implications for grassland management (chapter 8).

Management measure: cropping system and grassland age

Earthworms, nematodes, bacteria and fungi were sampled during three years in a 36-year-old cropping experiment (chapter 2). Permanent grassland was compared with permanent arable land and a ley-arable crop rotation. In this rotation, a period of three years of grass ley production (temporary grassland) alternated with three years of arable crop production (temporary arable land).

Cropping system and age significantly affected earthworm populations. Already in the first year of temporary arable cropping, earthworm numbers had dropped to levels comparable to permanent arable land. In contrast, it took three years for earthworm abundance and biomass to recover in the grass ley period, to levels comparable to permanent grassland. Based on these results and results of other experiments, I conclude that, in the arable phase of ley-arable crop rotations, earthworm abundance is affected more by the decrease in resource quantity and quality than by mechanical damage due to soil tillage. This conclusion has important implications for the development of sustainable maize cropping systems, when ecosystem services of earthworms want to be utilized. Moreover, it suggests that the common practice on dairy farms of re-sowing grass immediately after killing and plowing the old grass sward, may be less harmful to earthworms than is currently assumed; this merits further research (section 8.1.1).

When analyzing earthworm response at the functional group level, I found that, in the grass ley phase of the ley-arable crop rotation, it were mainly endogeic and epigeic earthworms that recovered quickly from the arable phase, whereas anecic species largely failed to recover to abundance levels found in permanent grassland. I conclude that a ley-arable crop rotation, although considered sustainable in terms of nutrient use efficiency, supports the abundance of endogeic and epigeic earthworms but limits the abundance of anecic species. Since anecic earthworms can play a role in the ecosystem service of water regulation, more insight is needed into the present and potential populations of anecic earthworms, and the effect of grassland management practices other than crop rotation, particularly presently used re-sowing techniques and the frequency of grassland renovation, on this earthworm functional group.

Within one to two years after starting temporary grassland or temporary arable land, the abundance of herbivorous and microbivorous nematodes resembled abundance levels observed in permanent grassland and permanent arable land, respectively. Nematode species richness showed the same pattern, but the nematode Maturity Index and the relative abundance of omnivorous and carnivorous nematodes in the temporary treatments remained significantly lower than in the respective permanent treatments. Therefore I suggest that it takes a stable food supply over several years to develop predator-prey cycles and maintain steady populations of omnivorous and carnivorous nematodes.

Within two years after starting temporary grassland or temporary arable land, soil bacterial activity (measured as thymidine and leucine incorporation, which reflects growth rate) and physiological activity (measured as the amount of soil extract needed to convert 50% of all substrates in ECO plates) resembled activity levels in permanent grassland and permanent arable land, respectively. However, the physiological *diversity* of the bacterial community (measured as the slope parameter of CLPPs) in temporary grassland never reached the high level observed in permanent grassland, which clearly stood out from all other treatments.

These results suggest that in the ley phase of the ley-arable crop rotation most soil biota quickly recover from the arable phase, reaching overall abundance and activity levels observed in permanent grassland. However, recovery is not always complete at the species and functional group level, and therefore specific ecosystem services of soil biota may be impaired or lost. Take the example of anecic earthworms and the service of water regulation. Specific for the ecosystem service of nutrient supply, in the ley-arable crop rotation, levels of SOM, soil total N, potential N mineralization and potentially mineralizable C were intermediate between permanent arable land and permanent grassland. Therefore, in terms of soil quality, permanent grassland is the preferred land use. For maize production, a ley-arable crop rotation is preferable to permanent arable cropping systems, but the ley phase should not be considered a replacement for permanent grassland. In other words, farmers are advised not to replace all their permanent grassland with ley-arable crop rotations.

Management measure: clover introduction

The effect of clover introduction on soil biota and ecosystem services was studied in an experiment in which white clover-only and a white clover-grass mixture without fertilization were compared to grass-only with and without fertilization (chapter 3).

Compared to grass-only and grass-clover, clover-only had a lower root biomass and lower shoot and root C/N ratios. The quantity and quality (C/N ratio) of aboveground and belowground plant biomass in grass-clover (20-30 % clover in the dry matter) was comparable to grass fertilized with 150 kg N ha^{-1} of inorganic fertilizer.

Earthworm populations were positively affected by clover introduction. Swards of clover-only had higher earthworm numbers and biomass than swards of grass-only with or without inorganic fertilizer. Based on these results and the results of other experiments, I conclude that nitrogen input –whether through N fixation or N fertilization – positively affects the abundance and biomass of earthworms in grassland soil, through increasing resource quantity, and particularly resource quality (lower C/N ratio) (section 8.1.1).

The lower root biomass and higher earthworm biomass in clover-only resulted in a poorer visual soil structure (more angular blocky elements) but a higher potential water infiltration rate (lower penetration resistance and higher number of earthworm burrows). These results suggested that clover promotes the ecosystem service of waterregulatie, but is less conducive to soil structure maintenance.

In our experiment, earthworm biomass and the number of earthworm burrows at 20 cm depth in grass-clover were intermediate between levels measured in clover-only and grass-only fertilized with inorganic fertilizer. Compared to grass-only without fertilizer and grass-only with fertilizer, grass-clover had an intermediate score for visual soil structure. Moreover, the mixture of grass-clover showed a higher potentially mineralizable C than grass-only, which suggests that the positive effect of clover on the supply of nutrients through the soil food web is maintained in the mixture. These results suggest that a mixture of grass and clover has an optimal combined effect on the ecosystem services measured. This merits further research.

Management measure: inorganic and organic fertilizers on grass

A grassland fertilization experiment was conducted to compare the effects of inorganic fertilizer and four different organic fertilizers (including two kinds of adjusted manure slurry) on soil biota and ecosystem services in grassland (chapter 4).

Organic fertilizers increased soil organic C, total N, the bacterial activity and the supply of nutrients through soil food web interactions. Also, the number of earthworm burrows was higher in the organic fertilizer treatments, which suggests that organic fertilizer applications better support the ecosystem service of water regulation than inorganic fertilizer. Furthermore, compared to inorganic fertilizer the application of solid yard manure (one of the adjusted manure slurries tested) resulted in a significantly lower soil penetration resistance, which suggests that this type of organic fertilizer better supports the ecosystem service of soil structure maintenance than inorganic fertilizer.

Within the group of organic fertilizers tested, no significant differences were found in their effects on soil organic C, abundances of soil biota and potential N mineralization rate. However, applications of farm yard manure and composted yard manure, which both have a higher C/N ratio, tended to increase the relative abundance of epigeic earthworms (surface litter feeding earthworms), the potential C mineralization and the metabolic quotient. These results suggest that organic fertilizers with high C/N ratios may lead to an inefficient use of applied organic C and a slower decomposition process that primarily takes place on or just below the soil surface, resulting in an suboptimal functioning of the plant-soil cycle. Therefore, I suggest a rational use of organic fertilizer, with more attention for whether the fertilizer C/N ratio “fits” the soil-plant system it is applied to (section 8.2.4).

Management measure: manure types and N application rates on grass-clover

In order to meet the demand of organic arable farms for organic manure, organic dairy farms could export animal manure to arable farms, thereby reducing the amount of manure available for fertilizing their own grass-clover pastures. To examine whether this off-farm nutrient export has implications for the biological soil quality of grass-clover pastures, we compared the effect on soil biota of four different application rates of two different manure types (manure slurry and farm yard manure) on grass-clover (chapter 5).

Reduced manure application rates did not affect the soil biological quality of grass-clover as long as the clover persisted. Farm yard manure application to grass-clover did not increase the relative abundance of epigeic earthworms, potential C-mineralization or metabolic quotient, unlike farm yard manure effects on grass-only swards (chapter 4). These results suggest that clover N fixation had a dominant influence on soil biological quality, and compensated for reduced N application. However we did observe that, even with normal organic manure application rates, SOM tended to decrease over the years. In addition to the inaccuracy of SOM measurements and the high initial SOM in our experiment, liming may have played a role. Liming probably increased bacterial activity and mineralization rates, and this could decrease SOM in the long-term.

In sum, I conclude that it is possible to sustain on-farm soil biological quality of grass-clover pastures despite off-farm export of animal manure, as long as the clover content of these pastures remains sufficient, and measures are taken to maintain SOM.

Management measure: grass species and species mixtures

To examine the effects of individual grass species and species mixtures on soil quality, a field experiment was conducted comparing *Lolium perenne* (Perennial rye grass), *Festuca arundinacea* (Tall fescue) and *Dactylis glomerata* (Cocksfoot) in monocultures and mixtures (chapter 6).

After two growing seasons, we found little significant differences between the various monocultures and mixed cultures with respect to soil microbiological and nematode parameters. However, root biomass was significantly higher in monocultures of *L. perenne* and *F. arundinacea* compared to *D. glomerata*, which suggests that the ecosystem service of soil structure maintenance is better supported by the first two grass species. In addition, the ratio of soil mineral N to total N was lower in monocultures of *L. perenne* and *F. arundinacea* and in the mixed culture of these two species, compared to the other treatments. This suggests that *L. perenne* and *F. arundinacea* contribute more to organic matter build-up and/or less to mineralization, than *D. glomerata*. Furthermore, soil mineral N levels were significantly reduced in the mixed culture of *L. perenne* and *F. arundinacea*, compared to levels in the monocultures of these grass species. Therefore, I suggest that grassland systems with either *L. perenne* or *F.arundinacea* or both are more sustainable than *D. glomerata*, in terms of reduced nitrogen losses, increased build-up of soil organic matter and probably soil structure. *D. glomerata* should only be used in species mixtures with companion grass species that have vigorous root development and that are able to withstand competition from *D. glomerata*.

Ecosystem services in grassland associated with biotic and abiotic soil parameters

In 20 grasslands on sandy soils, biotic and abiotic soil parameters were measured simultaneously with process parameters that reflect various ecosystem services. Correlation analysis and stepwise regression was applied to identify which abiotic and biotic soil parameters best explain soil ecosystem services (chapter 7).

Parameters underlying the ecosystem service soil structure maintenance, such as bulk density and the percentage of sub-angular blocky elements, were mainly influenced by SOM content and SOM characteristics. Penetration resistance at 0-10 cm was negatively correlated with earthworm abundance and percentage soil crumbs positively, which suggests a positive contribution of earthworms to soil structure maintenance.

Process parameters for water regulation were not clearly related to any of the biotic soil parameters. Water infiltration rate in the field was explained only by an abiotic soil parameter, soil penetration resistance at 10-20 cm.

Process parameters underlying the ecosystem service nutrients supply, such as potentially mineralizable C and N, were most strongly related to soil total N. On the other hand, potential C and N mineralization showed various correlations with biotic soil parameters. Potential N mineralization was positively correlated to microbial biomass, number of predatory nematodes, Maturity Index, total and relative abundance of Collembola, and enchytraeid diversity. Potential C mineralization was negatively correlated to microbial biomass, Maturity Index, and relative abundance of endogeic earthworms. Both parameters appear to represent contrasting processes in the soil (8.2.3).

Grassland production without N fertilization (viz the N supply capacity of the soil), was mainly explained by soil organic matter (SOM) and soil moisture, and to a lesser extent by soil total N. According to our regression model, each gram of SOM per kg dry soil adds 3.21 kg N yield ha^{-1} to the baseline N yield of of 15.4 kg N ha^{-1}. Measured nitrogen supply capacities were generally higher than estimates based on the presently used fertilization recommendation model for Dutch grasslands: for 85% of the grasslands (n=20) in our study, this standard model underestimated the measured nitrogen supply capacity of the soil by 42 kg N ha^{-1} (31%).

In contrast to the N supply capacity of the soil, the N yield response to fertilization was significantly explained by a biotic soil parameter, i.e. the number of enchytraeids. In the grasslands of our study, N yield response to N fertilization ranged from 35-102%. This wide range underscores the need for an indicator of fertilizer use efficiency. I suggest that enchytraeid abundance can be an indication of N yield response to N fertilization, but that further research is needed to understand the underlying mechanisms.

Implications for grassland management

The results of the field experiments discussed in chapters 2 to 7 have various implications for sustainable grassland management. To make better use of soil ecosystem services, and to prevent inadvertent negative effects of management measures on soil biota and their associated ecosystem services, the following recommendations can be made (see also chapter 8):

- For soil structure maintenance the best management practice would probably be the cultivation of a semi-permanent grass-clover mixture or a semi-permanent grass-only with moderate N fertilization, with grasses with a dense and deep root system in the grass mixture. For grass-only, fertilization with slurry and farm yard manure would be the best choice in this context.
- With regard to the ecosystem service of water regulation, the best management practice would probably be the cultivation of a semi-permanent grass-clover mixture or a semi-permanent grass-only with moderate N fertilization, with grasses with a dense and deep root system in the grass mixture. When water infiltration is a problem, and anecic earthworms are present, they can be stimulated with farm yard manure.
- The best management practice to support the ecosystem service of nutrient supply would probably be the cultivation of a permanent grass-clover, with grass species with a dense and deep root system, and using moderate fertilization with slurry and/or farm yard manure.
- The best management practice which combines aforementioned ecosystem services on sandy soils appears to be a semi-permanent grass-clover or a semi-permanent grass-only with moderate N-fertilization, with grass species with a dense and deep root system, and fertilization with organic fertilizers.

The specific management choices can be specified according to four scenarios of N yield at 0 kg N ha^{-1} and response of grass N yield to N fertilizer (Fig. 8.2):

I. Low N yield at 0 kg N ha^{-1}, Low N-yield response to N fertilizer
II. High N yield at 0 kg N ha^{-1}, Low response N yield on N fertilizer
III. High N yield at 0 kg N ha^{-1}, High response N yield on N fertilizer
IV. Low N yield at 0 kg N ha^{-1}, High response N yield on N fertilizer

These scenarios are further elaborated in section 8.3.

Overall conclusion and suggestions for further research

In this thesis I have shown that managing the basic soil qualities SOM, soil moisture, soil total N and soil C/N ratio is pertinent to achieving sustainable grassland production on sandy soil. SOM plays an important role in all ecosystem services, including grass production at 0 kg N ha^{-1}. I have also shown a significant correlation between enchytraeid abundance and N yield response to N fertilization. Taking into account that the grasslands in this study represented a broad range of N yield responses to N fertilization, this novel result could be very valuable information in identifying the most efficient use of fertilizer. However, further research into the mechanisms underlying this relationship is warranted. My hypothesis is that a high enchytraeid abundance reflects a soil food web that operates across the entire soil profile, whereas a low enchytraeid abundance reflects a less developed soil food web that operates mainly near the soil surface. A plant-soil cycle that involves shallow as well as deeper soil layers, in which deep (grass) roots and endogeic and anecic earthworms play prominent roles, uses available nutrients and water much more efficiently, leading to a more stable grass production over the season and over the years, which substantially reduces economic risks for farmers. Therefore, future research should focus on developing plant-soil systems that take advantage of a greater part of the soil profile.

Furthermore, I have shown that on sandy soils a semi-permanent grass-clover or a semi- permanent grass-only with moderate N-fertilization, with grass species with a dense and deep root system, and fertilization with organic fertilizers, is the most favorable combination of management measures for sustaining the ecosystem services of soil structure maintenance, water regulation and nutrient supply. The specific management choices can be specified according to four scenarios of N yield at 0 kg N ha^{-1} and response of grass N yield to N fertilizer described in section 8.3. My finding that the presently used fertilization recommendation model underestimated the soil nitrogen supply capacity of the majority of soils in my study, taken together with the fact that these soils represented a broad range of N response to N fertilizer, legitimates reconsideration of the present fertilizer recommendations.

Samenvatting

Inleiding

Tot voor kort bestond er weinig interesse voor de bodemkwaliteit onder productiegrasland. Een verslechtering hiervan kon immers worden opgevangen door meer te bemesten of beregenen. Echter wettelijke beperkingen stellen hier duidelijke grenzen. Daarnaast wordt gezocht naar verduurzaming van landbouwmethoden. Tezamen heeft dit het functioneren van de bodem en haar ecosysteemdiensten weer onder de aandacht gebracht.

Op *zandgrond* zijn de beperkingen wat betreft bemesting het hoogst. Deze grond is ook het meest gevoelig voor verliezen van mineralen naar het milieu. Daarbij hebben deze gronden de laagste natuurlijke bodemvruchtbaarheid. Hierdoor zijn juist weer externe inputs uit bemesting en beregening noodzakelijk. Scherp management van de bodem en haar ecosysteemdiensten heeft hier –vanwege dit dilemma- de hoogste prioriteit voor een duurzame graslandproductie.

Naast chemische en fysische bodemkwaliteit is bodem*biologische* kwaliteit een van de peilers onder bodemvruchtbaarheid. De gemiddelde levende biomassa van het bodemleven van een melkveebedrijf op zandgrond bedraagt 2700 kg ha^{-1} (laag: 0-10/20 cm). Dit is meer dan de biomassa van het vee boven de grond. Het bodemleven draagt sterk bij aan ecosysteemdiensten; behoud van bodemstructuur, waterregulatie, nutriëntenvoorziening en uiteindelijk de grasproductie als ultieme dienst voor veehouders. Aan de andere kant beïnvloeden veehouders met het management van het grasland weer het bodemleven. De interactie tussen managementmaatregelen en (het effect op) de plant, het bodemleven en de ecosysteemdiensten zijn cyclisch en complex. Om duurzame graslandsystemen te ontwikkelen en optimaliseren, is meer inzicht nodig in het effect van managementmaatregelen op het bodemleven en de ecosysteemdiensten waarin ze voorzien. Deze kennis geeft ons de kans om deze ecosysteemdiensten te optimaliseren én beter te benutten. Daarnaast kan het helpen om onbedoelde neveneffecten van managementmaatregelen op de samenstelling, omvang en functioneren van het bodemleven te voorkomen.

Aan dit proefschrift lagen twee hoofddoelstellingen ten grondslag: 1) inzicht krijgen in het effect van graslandmanagementmaatregelen op de aantallen, activiteit en soortenrijkdom van bodemleven in zandgrond; en 2) het verkennen van het effect van graslandmanagementmaatregelen op de ecosysteemdiensten zoals bewerkstelligd *door* dit bodemleven. Ecosysteemdiensten zoals: bodemstructuur, waterregulatie, nutriëntenvoorziening en uiteindelijk grasproductie.

Na een algemene inleiding (hoofdstuk 1), worden in hoofdstuk 2 tot en met 6, de effecten van verschillende graslandmanagementmaatregelen op regenwormen, nematoden en microben naast de betrokken ecosysteemdiensten besproken. In hoofdstuk 7 worden de verschillende relaties tussen bodemleven en ecosysteemdiensten geanalyseerd op basis van gegevens verzameld op twintig locaties productiegrasland op zandgrond. Dit proefschrift sluit af met een algemene discussie van de resultaten en de consequenties voor graslandmanagement (hoofdstuk 8).

Managementmaatregel: teeltsysteem en leeftijd van grasland

Regenwormen, nematoden, bacteriën en schimmels zijn gedurende drie jaar bemonsterd in een zesendertig jaar oud experiment met drie teeltsystemen (hoofdstuk 2). Een systeem van oud grasland en continu bouwland werd vergeleken met een vruchtwisseling van gras en akkerbouw. In deze vruchtwisseling, werd drie jaar grasland (tijdelijk grasland) afgewisseld met drie jaar bouwland (tijdelijk bouwland).

De behandelingen hadden een significant effect op de regenwormpopulatie. Al in het eerste jaar van de vruchtwisseling van gras naar bouwland namen de aantallen regenwormen af tot het niveau in continu bouwland. Daarentegen duurde het drie jaar voordat regenwormaantallen en biomassa waren hersteld -in de graslandfase van de vruchtwisseling- tot het niveau van oud grasland. Gebaseerd op deze resultaten en op resultaten van andere experimenten, concludeer ik dat in de bouwlandfase van een vruchtwisseling, de regenwormaantallen meer worden beïnvloed door afname van voedselkwantiteit en -kwaliteit dan door mechanische schade van grondbewerking. Dit heeft belangrijke consequenties voor de ontwikkeling van duurzame systemen voor de maïsteelt als de ecosysteemdiensten van regenwormen gebruikt willen worden. Daarnaast suggereert het dat de huidige praktijk van melkveebedrijven -om grasland direct opnieuw in te zaaien nadat de oude grasmat is doodgespoten en geploegd- mogelijk minder schadelijk is voor regenwormen dan wordt gedacht. Dit vraagt om nader onderzoek.

Bij de analyse van regenwormen in ecologische groepen, vond ik dat -in de graslandfase van de vruchtwisseling- vooral de aantallen bodembewoners en strooiselbewoners herstelden van de bouwlandfase, terwijl de pendelaars niet herstelden tot de aantallen in oud grasland. Ik concludeer dat een vruchtwisseling van gras en akkerbouw -die wordt gezien als duurzaam op het gebied van nutriëntenbenutting- de ecologische groepen van bodembewoners en strooiselbewoners in stand houdt, maar de aantallen pendelaars beperkt. Aangezien regenwormen in de groep van pendelaars een rol spelen in de ecosysteemdienst van waterregulatie, is er meer inzicht nodig in de huidige en potentiële populaties van deze pendelaars. Dit naast het effect van ander graslandmanagement op deze ecologische groep van regenwormen. Hierbij moet er met name gekeken worden naar de huidige frequentie en praktijk van het herinzaaien van grasland.

Binnen één tot twee jaar na de start van de tijdelijke grasland- of bouwlandfase waren de aantallen plantetende, bacterie-etende en schimmeletende nematoden vergelijkbaar met aantallen in het respectievelijke oud grasland of continu bouwland. De soortenrijkdom van nematoden liet hetzelfde patroon zien, maar de “Maturity Index” van nematoden en het percentage allesetende en roofnematoden in tijdelijk gras- en bouwland bleven significant lager dan het oude grasland en continue bouwland. Daarom stel ik dat er een stabiel voedselaanbod nodig is om predator-prooicycli te ontwikkelen en stabiele populaties van allesetende en roofnematoden te onderhouden.

De bacteriële activiteit herstelde zich binnen twee jaar na de start van de tijdelijke grasland- en bouwlandfase tot niveaus in respectievelijk oud grasland of continue bouwland. Echter de fysiologische *diversiteit* van de bacteriële gemeenschap (gemeten via de helling van de “Community-Level Physiological Profiles” op ECO-platen) in tijdelijk grasland haalde niet het niveau van oud grasland, wat zich duidelijk onderscheidde van alle andere behandelingen.

Deze resultaten suggereren dat in de graslandfase van de vruchtwisseling, het meeste bodemleven snel hersteld van de bouwlandfase, waarbij dezelfde aantallen en activiteitenniveaus worden gemeten als in oud grasland. Echter, dit herstel is niet altijd compleet op soort- en functioneel groepsniveau, waardoor specifieke ecosysteemdiensten van het bodemleven mogelijk verminderen of verloren gaan. Zie ook het al genoemde voorbeeld van het verlies van de ecologische groep van pendelaars door vruchtwisseling en de gekoppelde ecosysteemdienst van waterregulatie.

Voor de ecosysteemdienst van nutriëntenvoorziening geldt het volgende. De niveaus van organische stof , N-totaal, potentiële N-mineralisatie en potentieel mineraliseerbare C lagen in de behandelingen met vruchtwisseling van gras- en bouwland tussen die van continu bouwland en oud grasland in. Daarom is wat betreft bodemvruchtbaarheid oud grasland het geprefereerde landgebruik. Voor de teelt van snijmaïs heeft een vruchtwisseling de voorkeur boven continu bouwland, maar het tijdelijke grasland moet niet ten koste gaan van al het oude grasland op een melkveebedrijf.

Managementmaatregel: introductie van klaver

Het effect van de introductie van klaver op bodemleven en ecosysteemdiensten is onderzocht in een experiment met puur witte klaver, gras-klaver zonder bemesting, en gras met en zonder bemesting (hoofdstuk 3).

Vergeleken met gras en gras-klaver, had puur witte klaver een lagere wortelbiomassa en lagere C/N-ratio’s in de bovengrondse en ondergrondse plantbiomassa. De kwantiteit en kwaliteit (C/N-ratio) van de gras-klaver plantbiomassa (20-30 % klaver in de droge stof) in de boven- en ondergrond, was vergelijkbaar met de behandeling van gras bemest met 150 kg N ha^{-1} uit kunstmest.

De regenwormpopulatie werd positief beïnvloed door de introductie van klaver. De behandeling met puur witte klaver resulteerde in meer regenwormen in aantallen en biomassa, dan de behandelingen met gras met of zonder bemesting. Op basis van deze resultaten en de resultaten van andere experimenten, concludeer ik dat input van stikstof – door stikstof*binding* óf door N-bemesting – een positief effect heeft op de aantallen en biomassa van regenwormen. Dit door een toename van de kwantiteit en met name de kwaliteit van voedsel (lagere C/N ratio) (paragraaf 8.1.1).

De lagere wortelmassa en hogere biomassa van regenwormen in puur witte klaver resulteerde in een slechtere bodemstructuur (meer scherpblokkige elementen), maar ook in een hogere potentiële waterinfiltratie (lage bodemweerstand en hogere aantallen wormengangen). Deze resultaten suggereren dat klaver de ecosysteemdienst van waterregulatie positief beïnvloedt, maar door de lagere wortelmassa niet bevorderlijk is voor het behoud van de bodemstructuur.

Uit dit experiment bleek dat de behandeling van gras-klaver wat betreft de biomassa van wormen en het aantal wormengangen op 20 cm diepte tussen de behandelingen van puur witte klaver en gras met kunstmest lag. Wat betreft de score van gras-klaver voor bodemstructuur lag deze tussen die van gras met en zonder bemesting. Daarnaast had gras-klaver een hogere potentiële mineraliseerbare C dan gras, wat suggereert dat het positieve effect van puur witte klaver op de nutriëntenvoorziening zich voortzet in het mengsel. Deze resultaten suggereren dat de positieve effecten van een monocultuur van gras of witte klaver op de gemeten ecosysteemdiensten, optimaal worden gecombineerd in gras-klaver. Dit verdient verder onderzoek.

Managementmaatregel: kunstmest en organische mest op gras

In een bemestingsproef op grasland zijn de effecten van kunstmest en vier verschillende organische mestsoorten (inclusief drijfmest van melkkoeien met een eiwitarm rantsoen en drijfmest gecomposteerd met bermmaaisel) op bodemleven en ecosysteemdiensten gemeten (hoofdstuk 4).

Ten opzichte van kunstmest verhoogde de organische mestsoorten het organische stofgehalte, de N-totaal, de activiteit van de bacteriën en de mineralisatie. Ook was met organische mest het aantal wormgangen hoger, wat suggereert dat organische mest beter de ecosysteemdienst van waterregulatie ondersteunt dan kunstmest. Daarnaast hadden de behandelingen met vaste mest en drijfmest gecomposteerd met bermmaaisel een lagere bodemweerstand dan de behandeling met kunstmest. Dit suggereert dat deze organische mestsoorten het behoud van de bodemstructuur beter ondersteunen dan kunstmest.

Tussen de verschillende organische mestsoorten onderling waren er geen verschillen in het effect op organische stof, bodemleven of potentiële N-mineralisatie. Echter in de behandelingen met vaste mest en drijfmest gecomposteerd met bermmaaisel, was er een tendens naar een hoger percentage strooiselbewonende regenwormen (regenwormen die zich voeden met strooisel op de bovengrond), een hogere potentiële C-mineralisatie en een hoger metabolische quotiënt (ratio van potentiële C-mineralisatie of te wel bodemademhaling en microbiële biomassa). Beide mestsoorten hadden een hoge C/N-ratio. Deze resultaten suggereren dat aanwending van organische mest met een hoge C/N-ratio op grasland, leidt tot een inefficiënt gebruik van de organische stof. Daarnaast tot een langzamer afbraakproces dat hoofdzakelijk in de bovenlaag van de bodem plaatsvindt. Dit resulteert uiteindelijk in een suboptimaal functioneren van de plant-bodemcyclus. Daarom, stel ik een bewuster gebruik van organische mest voor, met meer aandacht voor de C/N ratio van de mest, passend bij het bodem-plant systeem waarbij het wordt toegepast (o.a. paragraaf 8.2.4).

Managementmaatregel: mestsoort en N-bemestingniveaus op gras-klaver

Om aan de vraag van biologische akkerbouwers naar mest van biologische oorsprong te voldoen, zouden biologische melkveebedrijven een gedeelte van hun mest kunnen afvoeren naar akkerbouwbedrijven. Logischerwijs resulteert dit in een vermindering van de bemesting op een biologische melkveebedrijf. In een bemestingsexperiment met vier N-niveaus en twee mestsoorten (drijfmest en vaste mest) is onderzocht wat deze afvoer van mest zou betekenen voor de biologische bodemkwaliteit onder gras-klaver.

Een lagere organische bemesting had geen effect op de biologische bodemkwaliteit onder gras-klaver, mits het klaveraandeel op peil bleef. Dit resultaat suggereert dat de stikstoffixatie van klaver een dominant effect had op de bodembiologische kwaliteit en het lagere bemestingsniveau compenseerde. Hoewel de biologische bodemkwaliteit niet afnam, was er zelfs met normale bemestingsniveaus, een afname in de organische stof in de bodem. Naast de onnauwkeurigheid van de organische stofbepaling en het relatief hoge organische stof gehalte bij het begin van het experiment, heeft mogelijk bekalking een rol gespeeld. Door dit experiment te bekalken is hoogstwaarschijnlijk de bacteriële activiteit gestegen en daardoor de mineralisatie, wat uiteindelijk heeft geresulteerd in een afname van de organische stof.

Samenvattend, concludeer ik dat het mogelijk is om de biologische bodemkwaliteit van gras-klaver op zandgrond te behouden, ondanks afvoer van organische mest naar een biologisch akkerbouwbedrijf. Het is echter wel belangrijk dat het klaveraandeel op peil blijft en tevens maatregelen worden genomen om het organische stofgehalte in de bodem te behouden.

Managementmaatregel: grassoorten en mengsels

Het effect van individuele grassoorten en mengsels op bodemkwaliteit is onderzocht in een experiment waarin Engels raaigras (*Lolium perenne*), rietzwenk (*Festuca arundinacea*) en kropaar (*Dactylis glomerata*) in monocultuur en in mengsels met elkaar vergeleken werden (hoofdstuk 6).

Na twee seizoenen, waren er weinig significante verschillen tussen de verschillende grassoorten en de mengsels op het gebied van de gemeten bodembiologische parameters (microbiologisch en nematoden) zichtbaar. Echter, de wortelmassa van Engels raaigras en rietzwenk was significant hoger dan van kropaar. Aangezien wortels belangrijk zijn voor bodemstructuur, suggereert dit dat Engels raaigras en rietzwenk beter de ecosysteemdienst van bodemstructuur onderhouden. Daarnaast was de ratio van N-mineraal en N-totaal in de bodem lager in de behandelingen met Engels raaigras, rietzwenk en het mengsel van deze twee, in vergelijking met de andere behandelingen. Dit suggereert dat Engels raaigras en rietzwenk meer dan kropaar bijdragen aan de opbouw van organische stof en/of minder aan de mineralisatie. Bovendien was de N-mineraal in de bodem van het mengsel van Engels raaigras en kropaar nog lager dan in de monoculturen van beide grassen. Daarom suggereer ik dat graslandsystemen met Engels raaigras of rietzwenk, of een mengsel van beide grassen, duurzamer zijn wat betreft stikstofverliezen, opbouw van organische stof en behoud van bodemstructuur, dan grasland met kropaar. Kropaar zou alleen gebruikt moeten worden in mengsels met grassoorten met een goede wortelgroei en soorten die kunnen concurreren met kropaar.

Ecosysteemdiensten in grasland gerelateerd aan biotische en abiotische bodemparameters

In twintig graslandpercelen op zandgrond, zijn naast biotische - en abiotische bodemparameters, verschillende procesparameters gemeten die betrekking hebben op de verschillende ecosysteemdiensten. Middels correlaties en een “stepwise” regressieprocedure is gekeken welke biotische - en abiotische bodemparameters het beste deze procesparameters van ecosysteemdiensten verklaren (hoofdstuk 7)

Parameters die betrekking hebben op de ecosysteemdienst 'behoud van bodemstructuur', zoals bodemdichtheid en het percentage afgerond blokkige elementen, werden met name beïnvloed door het organische stofgehalte en de organische stofkenmerken. De bodemweerstand in de laag 0-10 cm was negatief gecorreleerd met wormenaantallen en het percentage kruimels in de bodem positief. Dit suggereert dat wormen een positieve bijdrage leveren aan het behoud van bodemstructuur.

Procesparameters voor waterregulatie waren niet duidelijk gecorreleerd aan één van de bodembiotische parameters in deze twintig graslandpercelen. Waterinfiltratie werd alleen verklaard door een abiotische bodemparameter, namelijk de bodemweerstand op 10-20 cm diepte.

Procesparameters die betrekking hebben op de ecosysteemdienst 'nutriëntenvoorziening', zoals potentieel mineraliseerbare C en N (anaëroob), waren sterk gerelateerd aan de N-totaal in de bodem. Daarentegen lieten juist de potentiële C- en N-mineralisatie (aëroob) verschillende correlaties zien met biotische bodemparameters. De potentiële N-mineralisatie correleerde positief met de microbiële biomassa, aantal predator nematoden, "Maturity Index" van de nematoden, aantal en percentage springstaarten en de diversiteit in potwormsoorten. De potentiële C-mineralisatie correleerde negatief met microbiële biomassa, "Maturity Index" van de nematoden, en het percentage endogene regenwormen. Beide parameters lijken representatief voor tegengestelde voedselwebben in de bodem (paragraaf 8.2.3).

Graslandproductie zonder N-bemesting (zijnde het N-leverend vermogen van de grond) werd met name verklaard door het organische stofgehalte in de bodem, het bodemvocht, en in mindere mate door N-totaal. In het regressiemodel betekende elke gram organische stof per kg droge grond, 3,21 kg N-opbrengst per ha. Het gemeten N-leverend vermogen van de verschillende percelen in dit experiment was over het algemeen hoger dan de schatting op basis van berekeningen uit het huidige bemestingsadvies. Op zeventien van de twintig percelen in dit experiment werd het stikstofleverend vermogen met de berekeningen uit het huidige bemestingsadvies onderschat met 42 kg N per ha (31%).

In tegenstelling tot het N-leverend vermogen, werd de respons van N-opbrengst op bemesting enkel verklaard door één biotische bodemparameter, zijnde het aantal potwormen. In de graslandpercelen van dit experiment, varieerde de respons van N-opbrengst op N-bemesting van 35 tot 102%. Deze grote variatie tussen percelen geeft het belang aan van een indicator om de efficiëntie van bemesting op een graslandperceel in te schatten. Het aantal potwormen zou een indicator kunnen zijn van de respons van N-opbrengst op bemesting, maar verder onderzoek is nodig om onderliggende mechanismen te begrijpen.

Consequenties voor graslandmanagement

De resultaten van de veldexperimenten besproken in hoofdstuk 2 tot en met 7 hebben verschillende consequenties voor duurzaam graslandmanagement. Om ecosysteemdiensten beter te benutten, en onbedoelde neveneffecten van managementmaatregelen op bodemleven en gerelateerde ecosysteemdiensten te voorkomen, zijn de volgende aanbevelingen opgesteld (zie ook hoofdstuk 8):

- Voor het behoud van bodemstructuur is waarschijnlijk de teelt van een semi-permanent gras-klavermengsel of semi-permanent puur gras met een gematigde bemesting de beste managementpraktijk. Beide dan wel met grassen met een intensief en diep wortelstelsel. Voor puur gras zou de bemesting bij voorkeur een combinatie moeten zijn van drijfmest en vaste mest.
- Voor de ecosysteemdienst waterregulatie, is de beste managementpraktijk waarschijnlijk de teelt van een semi-permanent gras-klavermengsel of semi-permanent puur gras met een gematigde bemesting. Beide met grassen met een intensief en diep wortelstelsel. Als waterinfiltratie een probleem is en pendelende regenwormen aanwezig zijn, kunnen deze met vaste mest worden gestimuleerd voor een betere waterinfiltratie
- De beste managementpraktijk om de ecosystemendienst ‘nutriëntenvoorziening’ te ondersteunen, is waarschijnlijk de teelt van een semi-permanent gras-klavermengsel, met grassen met een intensief en diep wortelstelsel en een gematigde bemesting met drijfmest en/of vaste mest.
- De beste management praktijk die bovenstaande ecosysteemdiensten op zandgrond combineert, lijkt de teelt van een semi-permanent gras-klavermengsel of semi-permanent puur gras met een gematigde bemesting. Beide met grassen met een intensief en diep wortelstelsel en bemesting met organische mest.

Deze management opties kunnen nader worden toegespitst voor de volgende vier praktijksituaties:

I. Lage N-opbrengst bij 0 kg N ha^{-1}, Lage respons van N-opbrengst op N-bemesting
II. Hoge N-opbrengst bij 0 kg N ha^{-1}, Lage respons van N-opbrengst op N-bemesting
III. Hoge N-opbrengst bij 0 kg N ha^{-1}, Hoge respons van N-opbrengst op N-bemesting
IV. Lage N-opbrengst bij 0 kg N ha^{-1}, Hoge respons van N-opbrengst op N-bemesting

Deze situaties en bijbehorende maatregelen zijn verder beschreven in paragraaf 8.3.

Overkoepelende conclusie en suggesties voor verder onderzoek

In dit proefschrift heb ik laten zien dat management van de basiskwaliteiten van de bodem zijnde: organische stof, bodemvocht, N-totaal en C/N-ratio belangrijk is voor een duurzame grasproductie. Organische stof speelt een belangrijk rol in alle ecosysteemdiensten, inclusief de graslandproductie bij 0 kg N-bemesting. Daarnaast heb ik heb een duidelijke relatie laten zien tussen aantallen potwormen en de response van N-opbrengst op bemesting. Aangezien de variatie van response van grasgroei op N-bemesting tussen percelen heel groot is, lijkt dit resultaat een belangrijke ingang om mest zo effectief mogelijk in te zetten. Verder onderzoek naar de onderliggende mechanismen van deze relaties is nodig. Mijn hypothese is dat een hoog aantal potwormen een bodemvoedselweb vertegenwoordigt, dat van een dieper bodemprofiel gebruik maakt. Terwijl een laag aantal potwormen een minder ontwikkeld bodemvoedselweb representeert, dat enkel in de bovengrond opereert. Een plant-bodemcyclus welke zowel gebruik maakt van de bovengrond als diepere grondlagen -waarin diepe wortels en regenwormen (met name bodembewoners en pendelaars) een belangrijke rol spelen- maakt beter gebruik van de beschikbare nutriënten en water. Uiteindelijk leidt dit tot een *stabielere productie* in het seizoen en over de jaren, wat het risico van veehouders substantieel kan verlagen. Daarom moet toekomstig onderzoek zich richten op het ontwikkelen van plant-bodemsystemen die gebruik maken van een groter deel van het bodemprofiel.

Daarnaast heb ik laten zien dat op zandgrond de teelt van een semi-permanent gras-klavermengsel of semi-permanent puur gras met gematigde N-bemesting te prefereren is voor duurzame ecosysteemdiensten als behoud van bodemstructuur, waterregulatie en nutriëntenvoorziening. Beide maatregelen met grassen met een intensief en diep wortelstelsel en een bemesting van organische mest. De managementopties kunnen worden gespecificeerd volgens vier praktijksituaties van opbrengst bij 0 kg N ha^{-1} beschreven in paragraaf 8.3. Ik doe twee constateringen die gecombineerd een heroverweging van het huidige bemestingsadvies van grasland rechtvaardigen: 1) De constatering dat het huidige bemestingsadvies het N-leverend vermogen van het merendeel van de graslandpercelen in mijn studie onderschat en 2) Het feit dat tussen deze locaties de respons van N-opbrengst op bemesting sterk varieerde.

Curriculum Vitae

Nick van Eekeren was born on May 27, 1966 in Helmond, the Netherlands. From 1978 to 1984 he attended the Carolus Borromeus College in Helmond for his secondary education. During his youth he worked on a mixed pig and dairy farm, which generated his interest in agriculture, and made him decide to study Animal Production at Wageningen University, where he started in 1984. In the period 1988 to 1990 he did both his internship and his thesis work on Tropical Animal Production and Grassland Production at the Fibrous Feed Utilization Project (FFUP) in Sri Lanka.

After obtaining his Master of Science degree in Tropical Animal Production at Wageningen University in 1990, he started to work as a teacher of Animal Husbandry at the secondary agriculture school (MAS) in Helmond. From 1991-1994 he worked for the Dutch Directorate General of International Cooperation (DGIS) at the Range Management Project Malakand Division in Pakistan, to develop and implement a communal grazing system for mountainous ranges. In his second assignment for DGIS (1994-1997), he set up a participatory research and development programme at the regional centre for agriculture (CRPA) of Kaya, Burkina Faso. During his sabbatical leave he worked on the project “The farmer as an experiential scientist”, with the organic agriculture team of the Dutch agricultural extension service (DLV).

His interest in working together with farmers on the development of sustainable agricultural systems brought him into contact with his present employer, the Louis Bolk Institute, in 1997. As a senior researcher at the Louis Bolk Institute he has been working on different aspects (soil, plant and animal production) of dairy farming, and has been coordinating the institute’s research on agro-biodiversity. One of the research themes he is currently working on is biological soil quality on dairy farms, which resulted in this PhD thesis.

List of Publications (related to soil quality)

For publications of Nick van Eekeren on grassland and animal production see http://www.louisbolk.nl.

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PE&RC PhD Education Certificate

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

Review of literature (5.6 ECTS)
- Leven onder de graszode (2003)
- Soil Biota in grassland, its ecosystem services and the impact of management (2007)

Writing of project proposal (7 ECTS)
- Impact of grassland management on soil biota and ecosystem services on sandy soils (2004)

Post-graduate courses (3 ECTS)
- Optimisation of nutrient cycling and soil quality for sustainable grasslands (2005)
- PhD Course soil ecology (2007)

Laboratory training and working visits (2.4 ECTS)
- Crop rotation and soil biota; University of Ghent (2002-2005)

Invited review of (unpublished) journal (1 ECTS)
- Microorganism related submission; Applied Soil Ecology (2009)

Deficiency, refresh, brush-up courses (3.6 ECTS)
- Applied statistics (2006)

Competence strengthening / skills courses (2.8 ECTS)
- Project management (1995)
- Scientific writing (2006)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)
- Workshop meeting soil quality (2002)
- Network meeting role of mycorrhiza in sustainable land management (2004)
- Network meeting application of mycorrhizal fungi (2005)

Discussion groups / local seminars / other scientific meetings (4.8 ECTS)
- Dutch soil quality monitoring network (2001-2009)

International symposia, workshops and conferences (15 ECTS)
- Grassland Farming Balancing Environmental and Economic Demands; EGF Denmark; poster presentation (2000)
- Organic Grassland Farming; EGF Germany; poster presentation (2001)
- Sward Dynamics, N-Flow and Forage Utilisation in Legume-Based Systems; Italy; poster presentation (2001)
- Multi-Function Grasslands, Quality Forages, Animal Products and Landscapes; EGF France; poster presentation (2002)
- Adaptation and Management of Forage Legumes Strategies for Improved Reliability in Mixed Swards; Sweden; poster presentation (2004)
- Land Use Systems in Grassland Dominated Regions; EGF Switzerland; poster presentation (2004)
- Sustainable Grassland Productivity; EGF Spain; poster presentation (2006)
- Permanent and Temporary Grassland: Plant, Environment, Economy; EGF Belgium; oral presentation (2007)
- Multifunctional Grasslands and Rangelands in a Changing World IGC-IRC China; poster presentation (2008)

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