Soil fungi and nitrogen cycling

Causes and consequences of changing fungal biomass in grasslands

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Soil fungi and nitrogen cycling

Causes and consequences of changing fungal biomass in grasslands

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Abstract

As a result of agricultural intensification, nitrogen losses to the environment have increased and the demand emerged for sustainable agricultural production. It has been suggested that a soil community that is dominated by fungal pathways of decomposition is a feature of grasslands with low potential for nitrogen losses. This thesis focuses on the interaction between fungi and the nitrogen cycle in grassland soils, and attempts to unravel causes and consequences of changing fungal biomass.

I found that fungal biomass increases with reduced fertilisation and with sward age in 48 grasslands of organic dairy farms on sandy soils in the Netherlands. I studied the relationship between fungal biomass and fertilisation rate, manure type and application method in more detail in a field experiment. Here, the positive effect of decreasing fertilisation rates on fungal biomass was expressed already within three growing seasons, whereas manure type or application method did not affect fungal biomass. Grass production was not affected by fertilisation rate under the conditions studied. Fungal biomass correlated negatively to nitrogen leaching, but this is not necessarily a causal relationship.

Because of the differential effects that arbuscular mycorrhizal fungi (AMF) and saprotrophic (decomposer) fungi can have on nutrient cycling, and because they respond differently to changes in management, I tried to quantify their contribution to total fungal biomass. I found that increased fungal biomass in less fertilised soil was not caused by an increase in AMF alone.

Using intact soil columns from the field experiment, I found that fungi contribute considerably to the reduction of nitrogen losses. I quantified nitrogen losses from soil columns with high and low fungal biomass. The soil with higher fungal biomass had lower leaching and denitrification rates because of lower mineralisation and nitrification rates. After addition of ^{15}N-labelled nitrogen, the high fungal biomass soil immobilised more nitrogen into microbial biomass. Plant N uptake did not differ between the soils, which rules out the mechanism of enhanced plant nitrogen uptake through AMF.

Finally, in two field experiments, I showed that seeding of legumes reduces fungal biomass in soil and can therefore not be used to stimulate fungal-based systems with more closed nutrient cycles.

I conclude that lower fertilisation rates result in a soil with higher fungal biomass that retains nitrogen better while maintaining—at least on the short term—production. Therefore, increased fungal biomass is not only a consequence of reduced fertilisation, but also a cause of reduced nitrogen losses to the environment.

Contents

Chapter 1

General Introduction

1 General introduction

1.1 Relevance of the thesis

The intensification of agricultural production has increased crop yield but also poses severe environmental problems (Hansen et al., 2000; Mäder et al., 2002). Because of the increasing use of fertiliser nitrogen, nitrogen losses from agricultural soils have been strongly increasing, too. Nitrogen losses cause eutrophication of aquatic and terrestrial ecosystems, and contribute to atmospheric pollution and the greenhouse effect (Tilman, 1999). In particular, the leaching of nitrate is a problem for groundwater quality and natural ecosystems (Hansen et al., 2001), which has led to regulations such as the EU Nitrate Directive to reduce nitrate leaching (Hansen et al., 2000; Velthof and Oenema, 2001).

Parallel to, and as a consequence of, these regulations there has been an increasing demand for sustainable agriculture in the last decades. To reach a sustainable agricultural production the use of chemical fertilisers, which contain highly mobile inorganic nitrogen and are produced at the expense of large amounts of energy, should be reduced. Integrated farming systems partly, and organic farming systems completely rely on the application of organic fertiliser. To release nutrients from organic matter, decomposition is necessary. The decomposition process is catalysed by enzymes produced by bacteria and fungi (Swift et al., 1979), and decomposition rates are controlled by the quality of the substrate, environmental conditions and the characteristics of the decomposing organisms and their predators (Bloem et al., 1997; Nicolardot et al., 2001). Agricultural systems in which mineralisation provides nutrients for crop growth at the right time and in the right amounts, with a low potential for nutrient losses to the environment, can be considered as relatively sustainable. It is becoming evident that agricultural systems with a soil community that bears the closest resemblance to related natural ecosystems come closest to this ideal (Altieri, 1991; Wardle et al., 1995; Yeates et al., 1997).

A key feature of natural grasslands is a soil community that is dominated by fungal pathways of decomposition (Bardgett et al., 1996). Therefore, it has been suggested that an increase in the ratio between fungal biomass and bacterial biomass, the fungal/bacterial or F/B ratio, is indicative for conversion from an intensive system to a low-input grassland (Bardgett and McAlister, 1999). However, the factors that influence the F/B ratio in agro-ecosystems remain largely unexplored, and the consequences of varying F/B ratios for nutrient cycling have not been investigated so far. Moreover, the rate of response of the F/B ratio to changes in management has been shown to vary greatly. For the F/B ratio to be an indicator for the sustainability of an agricultural system, clarity on these aspects is required.

1.2 Occurrence of fungal-dominated food webs

Fungi and bacteria play a key role in the soil, as they are the main decomposers of organic matter and thereby form the basis of the soil food web. Fungi and bacteria can have very distinct functions in the soil and they each support their own chain of soil fauna (De Ruiter et al., 1993; Wardle and Lavelle, 1997; Wardle et al., 2004). This was, among others, demonstrated by Yeates et al. (1997), who found that a higher fungal biomass coincided with a higher biomass of fungal-feeding nematodes. Therefore, the biomass of fungi relative to bacteria can be used as an indicator for the importance of the two pathways (Fig 1.1), and ecosystems can be characterized to have

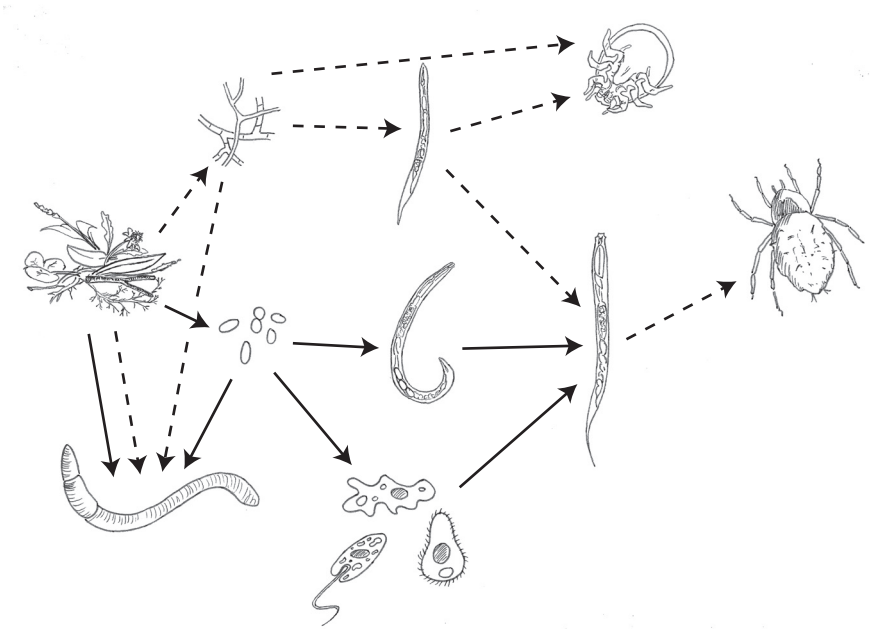


Fig. 1.1. Simplified soil food web, with the fungal decomposition pathway depicted by dashed arrows, and the bacterial decomposition pathway depicted by solid arrows. Fungi decompose organic matter and are subsequently eaten by earthworms, mites, or fungivorous nematodes. Fungivorous nematodes are, in turn, eaten by mitres or predatory nematodes. Bacteria decompose organic matter and are eaten by protozoa or bacterivorous nematodes, which are, in turn, eaten by predatory nematodes. Finally, nematodes are eaten by mites.

either a bacterial-dominated decomposition pathway or a fungal-dominated decomposition pathway.

In general, fungal-dominated decomposition pathways occur in undisturbed, late-successional ecosystems (Coleman et al., 1983; Klein et al., 1995; Ohtonen et al., 1999; Williamson et al., 2005). The occurrence of fungi in these systems is attributed to succession-related changes such as increased substrate humification, nitrogen immobilisation and increased heterogeneity of substrate (Bardgett and Shine, 1999; Holland and Coleman, 1987; Paustian and Schnurer, 1987).

In agricultural soils, fungal biomass is usually lower than in natural systems, but in these systems still fungal biomass is commonly found to be greater than bacterial biomass (Anderson and Domsch, 1975; Sakamoto and Oba, 1994; Schnürer et al., 1986; Zelles et al., 1995). In the Netherlands however, the soil microbial biomass in agricultural systems is usually strongly dominated by bacteria (Bloem et al., 1994; Hassink, 1993; Velvis, 1997). Recent analyses of Dutch grasslands that are managed at relatively low intensity show a much higher contribution of fungi to the microbial biomass than when more intensively managed (Bloem et al., 2004). In addition, when arable fields are abandoned, fungal biomass increases with time since abandonment, although not reaching levels in their natural reference sites (Van der Wal et al., 2006).

Increased fungal/bacterial (F/B) biomass ratios in extensively managed grasslands or abandoned agricultural fields are consistent with other reports (Bailey et al., 2002; Bardgett and McAlister, 1999; Donnison et al., 2000b; Grayston et al., 2001; Holtkamp et al., 2008; 2006; Zeller et al., 2001). Removal of sheep grazing from heavily grazed upland grasslands in the United Kingdom has been shown to increase fungal biomass (Bardgett et al., 1993a; Bardgett et al., 1996) and cessation of liming stimulates fungal growth (Bardgett et al., 1996), whereas application of manure or inorganic fertiliser reduces fungal biomass (Bardgett et al., 1999; Bittman et al., 2005; Bloem et al., 2004). These shifts in the abundance of fungi and bacteria related to grassland management have so far been attributed to quantity (Mawdsley and Bardgett, 1997) and quality (Grayston et al., 2001) of root exudates, changes in quality and quantity of input of litter or animal faeces (Bardgett et al., 1996), and plant productivity and composition (Donnison et al., 2000b). In addition, application of manure or organic material with a high C/N ratio might stimulate fungi over bacteria, although this has never been demonstrated in the field (Rousk and Bååth, 2007; Vinten et al., 2002).

Although the negative effects of fertiliser or manure application on fungal biomass are mostly ascribed to these indirect effects through changes in organic matter quantitiy and quality, direct toxic or inhibiting—or sometimes stimulating—effects of inorganic nitrogen on fungi have also been reported (Donnison et al., 2000a; Fog, 1988). The mechanisms for the direct negative effects have not been studied, but Fog (1988) suggests that inorganic nitrogen can depress ligninolysis or the production of non-specific enzymes by fungi. Also, as a result of nitrogen addition, increased formation of toxic or recalcitrant compounds may occur. These negative effects are greatest with recalcitrant organic matter.

One of the most important factors influencing the abundance of fungi is pH. It is generally accepted that the biomass of fungi relative to bacteria is higher in more acidic soils because of their greater tolerance of acidity (Alexander, 1977; Bååth and Anderson, 2003; Blagodatskaya and Anderson, 1998). In a study of upland grasslands, Bardgett et al. (1993a) showed that in acidic, ungrazed fields, fungal biomass was more abundant than in less acidic, heavily grazed fields, whereas earthworms and Collembola showed the opposite.

Furthermore, in arable soils, bacteria usually dominate under conventional tillage, while the contribution of fungi is increased increases under no-tillage. In a study by De Ruiter et al. (1994), the bacterial decomposition pathway was more dominant under conventional tillage, whereas the fungal decomposition pathway dominated under no tillage. Tillage increases the direct contact between bacteria and substrate, encouraging bacterial growth, while mycelial networks are destroyed (Beare et al., 1997; Frey et al., 1999).

One of the challenges in understanding the effects of fertilization and other agricultural

measures on fungi is the potentially differential effects on saprotrophic and arbuscular mycorrhizal fungi. AMF form a distinct group of soil fungi, and can make up a substantial amount of the total fungal biomass in agricultural soils (Gosling et al., 2006). Although it has been demonstrated that AMF are able to decompose organic matter, their contribution to decomposition is much smaller than that of saprotrophic fungi (Hodge et al., 2001). AMF play an important role in plant nutrition, as most agricultural crops form associations with AMF. AM fungal root colonisation has been shown to be higher in organically managed agricultural systems compared to conventional systems (Mäder et al., 2000a; Mäder et al., 2002) and decreases with fertilisation (Bradley et al., 2006; Johnson et al., 2003; Sparling and Tinker, 1978). It has been suggested that AMF are more susceptible to disturbance and fertilisation than saprotrophic fungi (Bradley et al., 2006; Kabir et al., 1997b).

It has been proposed that legumes stimulate fungal-based food webs with relatively closed nutrient cycles, because legumes depend on P acquisition through arbuscular mycorrhizal fungi (AMF) and on N acquisition through N-fixing bacteria in root nodules (Almeida et al., 2000; Cleveland et al., 1999; Sprent, 2001). Furthermore, legumes are known to have higher root colonization of arbuscular mycorrhizal fungi (AMF) compared to grass (Ryan and Ash, 1999; Tisdall and Oades, 1979). Interestingly, positive correlations have also been found between white and red clover and *saprotrophic* fungal biomass. So, seeding of legumes might induce a below-ground food web that resembles that of related species-rich grasslands and, therefore, has been proposed to promote fungal-based food webs (Smith et al., 2003; Smith et al., 2008).

Because of the differential effects that saprotrophic and AM fungi have on nutrient cycling, however, they may well respond differently to changes in management. Therefore, a reduced fungal biomass as a result of increased fertilisation might be caused by any combination of increase and decrease of the two groups that results in a net reduction of total fungal biomass, while a reduction in saprotrophic fungal biomass would have other consequences for nutrient cycling than a reduction in AM fungal biomass. Hence, to evaluate the causes and consequences of changes in fungal biomass on nutrient cycling, the relative contribution of the two groups to total fungal biomass has to be quantified.

1.3 Impacts of fungi on nitrogen cycling

Decomposer fungi are considered to be more efficient in substrate utilization than bacteria. They have been proposed to have higher carbon assimilation efficiencies, and thus to mineralise less carbon per unit substrate (Alexander, 1977; Anderson and Domsch, 1975; Holland and Coleman, 1987). Because carbon and nitrogen mineralisation are coupled stoichiometrically, fungi should mineralise less nitrogen than bacteria (Hessen et al., 2004). However, Six et al. (2006) concluded, in a comprehensive literature review, that there is significant overlap in the range of reported growth yield efficiencies of bacteria and fungi, based on laboratory culture studies. Thiet et al. (2006) compared growth yield efficiencies of microbial communities with high and low F/B ratios and concluded that fungi did not have higher growth yield efficiencies than bacteria, but that was with glucose as a substrate.

Conflicting hypotheses abound on the impact of fungi on mineralisation. Fungi have higher carbon-to-nitrogen (C/N) ratios than bacteria (Bloem et al., 1997; Van Veen and Paul, 1979).

As a consequence, they need less nitrogen and—depending on the substrate quality—will or will not excrete the surplus. On the other hand, fungi grow on more recalcitrant substrates (with higher C/N ratios) that contribute less to nitrogen mineralisation, and are able to use them more efficiently than bacteria (Hunt et al., 1987; Newman, 1985). This has been attributed to greater rates of production of extracellular cellulolytic enzymes, lower nitrogen requirements (Alexander, 1977), and an ability to colonize non-labile substrates more rapidly than bacteria (Gray and Baxby, 1968; Tribe, 1960). In addition, fungal hyphae form a more stable nitrogen pool in the soil than bacteria, because they are more persistent than bacterial cells (Martin and Haider, 1979). Moreover, grazing on fungi by fungivorous fauna results in lower nitrogen mineralisation per unit of consumed biomass than grazing on bacteria (Chen and Ferris, 2000). Although fungivorous nematodes and bacterivorous nematodes have similar C/N ratios, fungal hyphae contain less nitrogen than bacterial cells and thus less nitrogen is excreted by fungivorous nematodes. Furthermore, fungal-feeding fauna generally have smaller biomass and lower turnover rates than their bacterial-feeding counterparts (Sohlenius, 1980; Sohlenius et al., 1988). Because of all these different properties of fungi, bacteria and their predators, their effect on net mineralisation is hard to predict, although most properties imply a lower net mineralisation in fungal-based food webs.

Very few experiments have been done to compare nitrogen mineralisation between fungal- and bacterial-dominated systems. After contamination with bacteria of an experiment to measure the effects of fungal-feeding nematodes on mineralisation, Chen and Ferris (1999) found that net mineralisation rates increased. In contrast, in a pot experiment, Ingham et al. (1985) found higher net mineralisation rates in treatments with both bacteria and fungi compared to treatments with only bacteria. In spite of the lack of experimental evidence, simulations of the soil food web model first described by Hunt et al. (1987) show that fungi contribute less to net nitrogen mineralisation than bacteria (De Ruiter et al., 1993; Hunt et al., 1987). Moreover, model simulations show that an important part of nitrogen mineralisation in arable farming systems may be due to grazing, and that grazing by fungivorous nematodes contributes far less to net mineralisation than grazing by bacterivorous nematodes (De Ruiter et al., 1994). In contrast, simulations of the same model show that in pine forest ecosystems fungi contribute more to net nitrogen mineralisation than bacteria, but, again, grazing by bacterivorous fauna accounts for the largest part of net mineralisation (Berg et al., 2001; Schröter et al., 2003). Osler and Sommerkorn (2007) summarised that in agricultural fields, microbes are most important for nitrogen mineralisation, whereas in forest ecosystems where nitrogen may be more limiting, microbial contributions to net nitrogen mineralisation decline and the contribution of soil fauna increases.

Immobilisation and mineralisation of nitrogen occur simultaneously in soil. Net nitrogen mineralisation occurs when microbes are carbon-limited, while net immobilisation occurs when they are nitrogen-limited (Bardgett, 2005). A critical factor determining the balance between mineralisation and immobilisation is the C/N ratio of the substrate. Roughly, if substrate C/N ratios are higher than 30, microbes become N limited and start immobilising exogenous sources of inorganic nitrogen (Hodge et al., 2000). Heterotrophic microorganisms preferentially take up ammonium over nitrate (Recous et al., 1990). Especially fungi are thought to have the potential to immobilise readily available nitrogen in their biomass, because they can access spatially separated recalcitrant substrates and inorganic nitrogen simultaneously with their extensive hyphal networks (Frey et al., 2003; Holland and Coleman, 1987). Because of their filamentous growth form,

they would also be able to access, and thus immobilise, the non-mobile ammonium better than bacteria (Myrold and Posavatz, 2007). Fungi have been shown to immobilise nitrogen in forest soils (Jones and Richards, 1978). Although in general, fungal-dominated systems are assumed to have higher immobilisation of nitrogen than bacterial-dominated systems (Schimel and Bennett, 2004), experimental comparisons between fungal- and bacterial-dominated communities have scarcely been made. It has been shown though that microbes in extensively managed grasslands, with a (presumed) larger proportion of fungi relative to bacteria, immobilise more nitrogen than their more intensively managed, bacterial-rich, counterparts (Bardgett et al., 1993b; Bardgett et al., 2003).

Another way in which fungi can affect nitrogen cycling in the soil is by increasing aggregate formation by entangling soil particles with their hyphae and by excreting extracellular polysaccharides that glue soil particles together (Beare et al., 1997; Helfrich et al., 2008; Tisdall and Oades, 1982). This phenomenon is most prominent under reduced tillage (Beare et al., 1997). Aggregates can physically protect plant and microbial derived soil organic matter and thereby reduce its mineralisation (Bossuyt et al., 2002; Pulleman and Marinissen, 2004; Simpson et al., 2004), but a recent study showed that fungal-associated aggregates do not contribute to long-term stabilisation of soil organic matter (Helfrich et al., 2008).

By affecting mineralisation rates, fungi would also indirectly change nitrification and denitrification rates in soil. If mineralisation rates in fungal-based systems are lower than in bacterial-based systems, less ammonium will be present and subject to nitrification. In turn, less nitrate will be present and subject to denitrification. Although denitrification has long been considered a prokaryotic process, it has become clear that fungi are also capable of denitrification (Shoun et al., 1992). In semi-arid soils, denitrification has shown to be controlled by fungi, through heterotrophic nitrifier denitrification fueled by mineralisation of proteins (Crenshaw et al., 2008; McLain and Martens, 2005; McLain and Martens, 2006). However, evidence is also present that fungi dominate denitrification in temperate, agricultural grasslands (Laughlin and Stevens, 2002). Because many fungi lack the enzyme nitrous oxide reductase, N_2O is the main product of fungal denitrification. However, Cavigelli and Robertson (2000) found that in a conventionally tilled agricultural field a greater proportion of total denitrification remained as N_2O compared to a never-tilled successional field, whereas the successional field would be more fungal-rich than the agricultural field. Moreover, nitrate availability has been shown to be a stronger control on denitrification than microbial community composition (Cavigelli and Robertson, 2000; Wallenstein et al., 2006).

Arbuscular mycorrhizal fungi (AMF), which can make up a substantial amount of the total fungal biomass in agricultural soils (Gosling et al., 2006), can directly affect nitrogen cycling. They have been demonstrated to be able to decompose organic matter, although their contribution to decomposition is much smaller than that of saprotrophic fungi (Hodge et al., 2001). AMF are mostly associated with an increased plant uptake of phosphorus, but they also enhance the uptake of inorganic and organic nitrogen (Hawkins et al., 2000; Hodge et al., 2001; Mäder et al., 2000b). Apart from these direct effects, AMF can also have indirect effects on nutrient cycling. Van der Heijden et al. (1998) showed that mycorrhizal fungal diversity determined plant biodiversity and productivity, and Marschner and Baumann (2003) showed that the presence of AMF in maize changed the structure of soil bacterial communities.

Finally, it has been suggested that fungal-based systems would be more resistant to stresses such as drying and rewetting. It has been shown, for instance, that fungal-rich, unimproved

grassland soils have lower leaching of inorganic and organic nitrogen after drying and rewetting than their bacterial-rich, improved counterparts (Gordon et al., 2008). On the other hand, although fungal-based systems are considered to be more resistant to such stresses, they are also more susceptible to changes in space and time than bacterial-based systems. Fungi use larger areas of resource and have lower dispersal rates, thus they have a lower ability to recolonise disturbed soil than bacteria (Hedlund et al., 2004).

In figure 1.2 the main mechanisms through which fungi can reduce nitrogen losses are summarised. First, fungal-based food webs would have lower mineralisation and nitrification

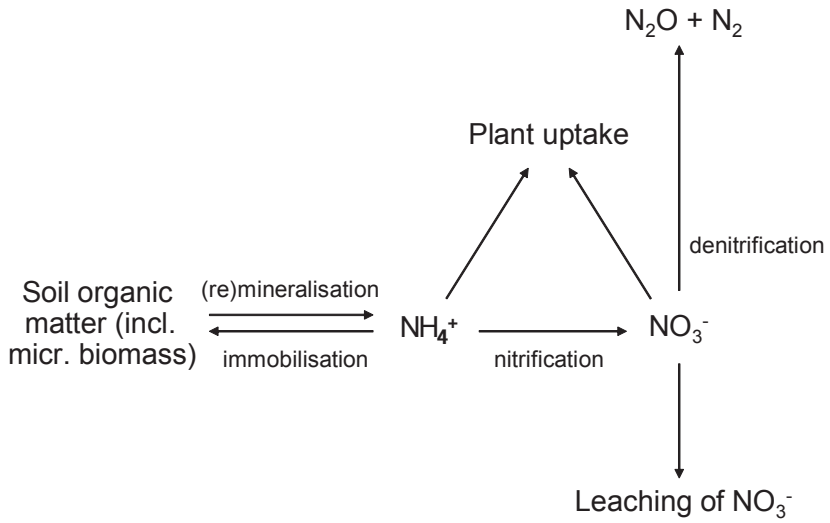


Fig. 1.2. Nitrogen pools and loss pathways that can be affected by fungi. See text for explanation.

rates than bacterial-based food webs because of higher carbon assimilation efficiencies, slower degradation of fungal hyphae, lower nitrogen excretion by fungal-feeding fauna, or increased aggregate formation. In addition, fungi would immobilise readily available ammonium, and AMF could enhance plant nitrogen uptake. Via these mechanisms, nitrate availability in soil would be reduced, resulting in lower nitrate leaching and lower denitrification rates and thus lower gaseous losses.

1.4 Objectives and hypotheses

As described in the two previous sections, a high soil fungal biomass, relative to bacterial biomass, has repeatedly been suggested to be indicative for systems with low nitrogen losses. Not only are fungi reduced by fertiliser application, they have also been proposed to affect nitrogen cycling

themselves. Therefore, the main question of this thesis is: Is an increased fungal biomass in soil not only a consequence of reduced fertilisation, but can it also reduce nitrogen losses to the environment? By answering this question, I want to evaluate whether a higher F/B ratio can be used as an indicator for agricultural systems with low nitrogen losses. The first step to achieve this goal is to find out which management practices or soil characteristics determine fungal biomass. Second, I will need to quantify the contribution of AMF to total fungal biomass. Third, the effect of different F/B ratios or fungal biomass on nitrogen cycling has to be established, with particular interest for mineralisation and leaching potential. Fourth, if an increased fungal biomass is not only a consequence but also a cause of lower nitrogen losses, agricultural management that stimulates soil fungi has to be explored. Given these research needs, I have formulated four hypotheses that will be tested in this thesis:

1. Fungal biomass increases
   a. as a consequence of reduced fertilisation
   b. when farm yard manure is applied superficially compared to slit injection of slurry
   c. with age in grasslands with low cultivation frequency
2. Increased fungal biomass is predominantly the result of increased densities of AMF, which are (in itself) indicative for a low nutrient availability
3. The higher the fungal biomass in soil, the lower the nitrogen losses to the environment and the higher the crop nitrogen uptake efficiency
4. Fungal biomass in soil can be increased by seeding of legumes

1.5 Experimental approach

Hypothesis one will be tackled in field observations and field experiments. Field observations are a useful tool to relate fungal and bacterial biomass to management practices, nutrient status, organic matter content, clay content, pH and vegetation characteristics. I will sample a wide range of agricultural grasslands with different management, and use multivariate techniques to determine major management practices and soil characteristics that determine fungal and bacterial biomass. To distinguish between management factors such as manure type, nitrogen application level or application techniques, I will sample the experimental field trial at the organic farm "Aver Heino". This is a factorial experiment with treatments manure type (no manure, farm yard manure and slurry) and nitrogen level (0, 40, 80, 120 kg N ha^{-1} y^{-1}).

To test hypothesis two, I will assess the contribution of AMF to total fungal biomass in samples from different soil types and with different fertilisation rates, with focus on soils I will use for further experiments. Hypothesis three will be tackled in several greenhouse experiments, using soils with different fungal biomass. Ideally, I would modify the fungal biomass in one soil to obtain different fungal biomass treatments. However, modifying microbial biomass in soil without affecting other soil properties is impossible. For instance, selective inhibitors have been used to eliminate fungi or bacteria in soil, but problems with specificity have been reported, as well as unpredicted effects on the longer term. The addition of straw has been shown to increase fungal biomass in soil, but this also changes organic matter quantity and quality of the soil used. Therefore, I will search for two soils that differ widely in fungal biomass, whilst other soil properties differ as little as possible. I will use intact soil columns to prevent disruption of

mycelial networks and to maintain the original vegetation. In a factorial set up, I will determine the effect of fungal biomass on nitrogen pools and fluxes.

Finally, I will use field experiments to evaluate whether seeding of legumes can stimulate fungal biomass in the field.

1.6 Thesis outline

The four hypotheses stated in section 1.4 are central to this thesis. In the next chapters, I will address these hypotheses subsequently.

Chapters 2 and 3 of this thesis address the first hypothesis stated in section 1.4. Chapter 2 focuses on the management and soil characteristics that determine fungal biomass and the F/B ratio. It provides the results of field observations in 48 Dutch grassland sites of different management and age. Chapter 3 describes the results of a field experiment with applications of either no manure at all, or of farm yard manure or slurry at different rates. Combined, these chapters give an overview of what causes changes in fungal biomass in agricultural grasslands. Also, in Chapter 3, to anticipate on hypothesis three, the relationship between fungal biomass and the nitrogen balance and nitrogen leaching is touched on.

In Chapter 4 I describe three incubation experiments in which I quantify the contribution of AMF to total fungal biomass, in order to test hypothesis 2. In these experiments, I monitor the dynamics of different groups of fungi in soil during a four-week incubation.

Chapter 5 addresses the hypothesis that nitrogen losses decrease with higher fungal biomass in soil (hypothesis 3). I describe the results of a series of greenhouse experiments in which I quantify gaseous and aqueous nitrogen losses from two soils; one with a high fungal biomass and one with a low fungal biomass. I use ^{15}N-labeled fertiliser to disentangle the mechanisms responsible for differences in nitrogen losses caused by differences in fungal biomass.

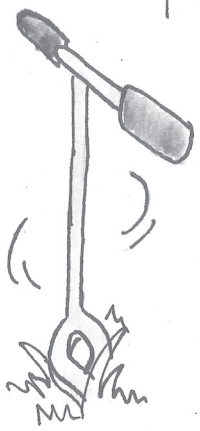
In Chapter 6 the results of two field experiments are described in which I test the fourth hypothesis whether seeding of legumes can promote fungal biomass.

In Chapter 7 I synthesise the results of the previous chapters, again with the hypotheses as a guideline. In this chapter, I answer the question whether an increased fungal biomass in soil is not only a consequence of reduced fertilisation, but can also reduce nitrogen losses to the environment. I evaluate the use of the F/B ratio or fungal biomass as an indicator for nitrogen losses from soil. Also, I explore the possibilities for stimulating fungal biomass to reduce nitrogen losses from agricultural grasslands. I interpret the significance of my results in the context of a changing world with increasing demands for production of food and biofuel.

Chapter 2

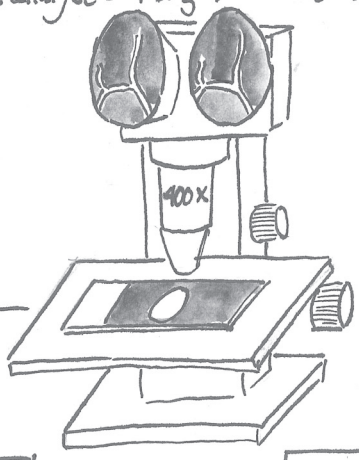
In 48 Dutch grasslands of differing age...

...I took soil samples...



...recorded plant species...

...and analysed fungal biomass.



Fungal biomass in pastures increases with AGE and REDUCED N INPUT

2 Fungal biomass in pastures increases with age and reduced N input

Franciska T. de Vries, Jaap Bloem, Nick van Eekeren, Lijbert Brussaard and Ellis Hoffland

Abstract

Previous studies have shown that soil fungal biomass increases towards more natural, mature systems. Shifts to a fungal-based soil food web have previously been observed with abandonment of agricultural fields and extensification of agriculture. In a previous field experimental we found increased fungal biomass with reduced N fertilisation. Here, we explore relationships between fungi, bacteria, N input and grassland age on real dairy farms in the Netherlands. We hypothesised that also in pastures that are still in production there is a negative relationship between fungal biomass and fertilisation, and that fungal biomass increases with grassland age in pastures that are still in production. We expected the fungal/bacterial biomass ratio to show the same responses, as this ratio has often been used as an indicator for management changes. We sampled 48 pastures from 8 organic dairy farms. Sites differed in age and fertilisation rate. We determined fungal and bacterial biomass, as well as ergosterol (a fungal biomarker). Fungal and bacterial biomass, and ergosterol, showed a negative relationship with N application rate, and correlated positively with organic matter percentage. In old pastures, fungal biomass and ergosterol were higher than in younger pastures. Because bacterial biomass responded in the same way as fungal biomass, the F/B ratio remained constant, and can therefore – in our dataset – not be used as an indicator for changing management. We conclude that the changes in fungal and bacterial biomass were driven by changes in organic matter quality and quantity. The negative relationship we found between N application rate and fungal biomass adds to earlier work and confirms the presence of this relationship in pastures with relatively small differences in management intensities. Earlier studies on shifts in fungal biomass focused on ex-agricultural fields or restoration projects. Here we show that fungal biomass is also higher in older agricultural pastures.

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2.1 Introduction

Soil fungi and bacteria play an important role in nutrient cycling, as they decompose organic matter. In this process, nutrients are released but can also be immobilised, depending on environmental conditions, the characteristics of the decomposing organisms and their predators, and the quality of the substrate (Bloem et al., 1997). Fungi and bacteria both support their own "chain" of soil fauna (De Ruiter et al., 1993; Wardle and Lavelle, 1997), and therefore, their biomass can be considered an indicator for the relative importance of these two pathways of the soil food web that are formed by fungivores or bacterivores and their predators.

Fungal biomass increases towards more natural, mature systems (Klein et al., 1995; Ohtonen et al., 1999; Williamson et al., 2005). Several articles report a shift towards the fungal pathway with extensification of agriculture (Bardgett and McAlister, 1999; Donnison et al., 2000b; Yeates et al., 1997) or abandonment of agricultural fields (Van der Wal et al., 2006; Zeller et al., 2001). These shifts are attributed to succession-related changes such as increased substrate lignification, N immobilisation and increased heterogeneity of substrate (Bardgett and Shine, 1999; Holland and Coleman, 1987; Paustian and Schnurer, 1987) as well as to reduced additional stresses such as N fertilisation (Bittman et al., 2005; Lovell et al., 1995) and tillage (Beare et al., 1997; Frey et al., 1999).

Although total microbial biomass has been reported to increase with ageing of systems, this increase was caused by an increase in fungal biomass only, as bacterial biomass remained constant. As a consequence, the fungal/bacterial biomass ratio increased (Klein et al., 1995; Ohtonen et al., 1999; Van der Wal et al., 2006). Also, bacterial biomass generally does not increase with extensification of agricultural management (Bardgett and McAlister, 1999; Bittman et al., 2005; Frey et al., 1999). Bacterial biomass can increase with the application of organic manure compared to inorganic fertiliser or no fertiliser (Bittman et al., 2005; Sakamoto and Oba, 1994).

For these reasons, a higher fungal/bacterial ratio can be seen as an indicator for systems, in which organic matter decomposition and N mineralisation dominate the provision of plant nutrients for crop growth. Nitrogen cycling in such systems is supposed to be more efficient because mineralisation can act as a slow release fertiliser with low nitrogen losses (Bloem et al., 1997; Bloem et al., 1994; Ingham et al., 1985), and also because of a more efficient crop nutrient uptake through mycorrhizal fungi (Mäder et al., 2002; Smith and Read, 1997).

Indeed, there are indications that an increased fungal/bacterial (F/B) ratio and/or fungal biomass is typical for grassland systems with low nutrient losses to the environment. In a field experiment in the Netherlands, we found an increase in fungal biomass with a lower fertilisation rate and a lower clover percentage of the sward. A higher fungal biomass coincided with a lower nitrate leaching (De Vries et al., 2006). As a next step, in the present study, we explore the relationship between fungal biomass and fertilisation rate across a range of agricultural grasslands on real farms in the Netherlands.

Although a great deal of research has been done on fungal biomass development in natural chronosequences, not much is known about the effect of ageing of agricultural grasslands on fungal biomass. In the Netherlands, agricultural grasslands are usually ploughed and reseeded every 5 to 10 years. This practice temporarily improves the physical and chemical quality of the soil and reduces the number of weed plants in the sward (Velthof et al., 2002). Ploughing also stimulates the mineralisation of organic nitrogen, which may lead to increased leaching

losses. As swards age, nitrogen is accumulated and organic matter content increases (Cuttle and Scholefield, 1995; Shepherd et al., 2001).

Therefore, we hypothesise that the fungal biomass decreases if fertilisation rates are higher, and increases with grassland age. We expect the bacterial biomass to remain constant in both cases, so the F/B ratio will decrease and increase, respectively, and therefore can be used to indicate these shifts in fungal and bacterial abundance. Furthermore, we expect the fungal biomass to be positively correlated to organic matter percentage. In old grasslands, organic matter percentage will be higher and plant species composition will be different from young grasslands. To test these hypotheses we sampled 48 grassland fields from 8 organic farms in the Netherlands.

Fig. 2.1. Locations of the 8 farms in the Netherlands. See table 2.1 for farm and field names and characteristics

2.2 Materials and methods

2.2.1 Sampling sites

We sampled 48 grasslands from 8 organic dairy farms on sandy soils across the Netherlands. Farms were located in different regions in the Netherlands (Fig. 2.1). At each farm, a number of pastures was sampled (Table 2.1). The pastures differed in age, which was defined as the period in years since the last seeding. All fields were grass-clover pastures and received slurry fertilisation, ranging from 62 to 244 kg N ha^{-1}. Furthermore, farms and fields differed in the intensity of fertilisation, grazing, mowing, and irrigation.

2.2.2 Soil and vegetation characteristics

Soil samples were collected in October 2003. For each field a bulk sample of 30 cores (0-10 cm depth, 3.5 cm diam.) was collected, sieved (5mm mesh size), homogenised and stored at field moisture content at 4 °C before analysis. A sub sample of 200 g wet soil was pre-incubated at 50% WHC (water holding capacity) at 12°C for four weeks to stabilise soil conditions and to avoid effects of temperature and moisture fluctuations in the field (Bloem et al., 2006). After this pre-incubation fungal biomass and bacterial biomass and growth rate were measured.

Soil moisture content was determined after oven-drying approximately 30 g of the bulk sample (in duplicate) at 105 °C. Prior to further chemical analysis, bulk samples were oven-dried at 40 °C. pH 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). Organic matter percentage was determined by loss-on-ignition (Ball, 1964).

Table 2.1. Farm and field characteristics.

Farm	Soil type (USDA 1975)	Field	Age (y)	Mowing (# cuts y^{-1})	Grazing (# cuts y^{-1})	Irrigation in 2003	Slurry application (kg ha^{-1} y^{-1})
AM	Sandy, siliceous, mesic, Typic Humaquept	AM3	2	2	4	yes	107
		AM4	3	2	3	yes	107
		AM17	7	1	4	yes	125
BT	Sandy, siliceous, mesic, Typic Haploquod	BT4+5	4	2	1	no	92
		BT7	3	0	5	no	92
		BT8	8	1	4	no	92
		BT10	3	1	4	no	92
		BT13	8	3	1	no	92
		BT16	2	0	4	no	92
		BT18	5	1	3	no	92
		BT19	33	0	5	no	92
		BT24	1	3	1	no	92
BW	Sandy, siliceous, mesic Plaggept	BW4	6	1	6	yes	166
		BW7	2	2	5	no	184
		BW16	3	5	0	no	184
DO	Sandy, siliceous, mesic, Typic Humaquept	DO1	11	2	2	no	62
		DO2	4	2	2	no	71
		DO3/1	33	1	4	no	169
		DO6/1	7	2	3	no	83
		DO8	3	1	3	no	83
		DO9	3	1	5	no	83
		DO10	5	1	4	no	169
		DO11	40	1	5	no	83
		DO12	5	4	0	no	169
EO	Sandy, siliceous, mesic, Typic Humaquept	EO1	7	2	5	no	95
		EO7	6	1	5	no	95
		EO9	6	2	5	no	113

Table 2.1 continued

Farm	Soil type (USDA 1975)	Field	Age (y)	Mowing (# cuts y^{-1})	Grazing (# cuts y^{-1})	Irrigation in 2003	Slurry application (kg ha^{-1} y^{-1})
JE	Sandy, siliceous, mesic, Typic Haploquod	JE1	11	2	5	no	171
		JE2	3	2	5	no	187
		JE4	12	0	7	no	93
		JE5	12	0	7	no	93
		JE9	2	2	5	no	140
		JE10	6	2	5	no	140
		JE12	10	2	5	no	140
		JE14	2	3	0	no	202
		JE22	2	3	0	no	202
ML	Sandy, siliceous, mesic, Typic Humaquept	ML2A	1	3	1	no	114
		ML6A	3	1	4	yes	114
		ML6B	2	2	3	yes	65
		ML7A	7	1	5	yes	114
		ML7B	25	0	5	yes	65
		ML8A	4	0	6	yes	114
		ML8B	5	0	6	yes	114
		ML10	1	1	4	yes	114
		ML11	43	0	3	yes	65
MS	Sandy, siliceous, mesic, Typic Humaquept	MS4	5	1	5	no	141
		MS23	3	4	0	no	187
		MS25	8	2	0	no	244

Furthermore, some standard Dutch fertiliser recommendation measurements were done (Evers et al., 2000). Water-extractable phosphorus (Pw) was determined as described by Sissingh (1971) and ammonium lactate-acetic acid-extractable *P* (P-Al) was determined as described by Schouwenburg and Walinga (1967). Extracts were analysed by spectrophotometry. Potassium content (K-HCl) was analysed by shaking approximately 10 g of soil with 100 ml of extraction solution (0.2 M HCl and 0.4 M oxalic acid). The suspension was shaken for 1 hour, filtered and measured by Eppendorf Elex 6361 Flame-AES.

Vegetation measurements were done to monitor differences in grassland species. The abundance of *Lolium perenne* L., *Trifolium repens* L. and herbaceous species was estimated visually as cover percentage. The total number of species was recorded for each field.

2.2.3 Fungal and bacterial biomass and activity

Microscopic slides were prepared as described by Bloem and Vos (2004). Slides for counting fungi were stained with Differential Fluorescent Stain (DFS) solution (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). Hyphal length was measured using an epifluorescence microscope at 400x magnification. Total hyphal length was calculated using

the grid intersection method (Bloem et al., 1995). Biovolume was calculated using the equation $V = (\pi/4)W^2(L-W/3)$, where W = width (µm) and L = length (µm). Fungal biomass was calculated assuming a mean hyphal diameter (width) of 2.5 µm and a specific carbon content of $1.3x10^{-13}$ g C μm^{-3} (Bakken and Olsen, 1983; Veen and Paul, 1979).

Ergosterol, a sterol only found in fungal cell membranes, was used as a specific biomarker for fungal biomass. Ergosterol was extracted from soil and quantified by HPLC as described by Bååth (2001).

Microscopic slides for determination of bacterial numbers were prepared in the same way as slides for fungal counting, except that bacterial slides were stained with the fluorescent protein dye 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF). Bacterial numbers, cell volumes and number of dividing cells 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 3.1×10^{-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, respectively (Bloem and Bolhuis, 2006; Michel and Bloem, 1993). For a detailed description, see De Vries et al. (2006).

Total microbial activity was determined by measuring soil respiration. Fourty g dried, ground (2 mm) and rewetted (60% WHC) soil was incubated for 7 days at 20°C. CO_2 was trapped in 0.1 M NaOH solution, which was analysed for total C by a TOC analyzer.

Potential N mineralisation was determined by incubating 200 g homogenised and sieved (< 2 mm) soil in 1.5 l airtight jars at 20°C in the dark for 6 weeks. 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 mineralisation 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).

2.2.4 Statistical analysis

F/B ratios were transformed for statistical analysis using the arcsine square root to meet the requirements of normality and homogeneity. Regression analyses with the factor age were biased, because a group of older fields heavily determined the relationships of soil and microbial parameters with age. Therefore, we focussed on the difference between young and old fields. Fields that had been seeded less than 10 years ago were considered young, and fields that had been seeded over 10 years ago were considered old. This cut-off was chosen because in grasslands older than 10 years, organic matter accumulation usually approaches an equilibrium and nitrogen accumulation declines (Cuttle and Scholefield, 1995; Hassink, 1994; Velthof and Oenema, 2001). To test the differences between the groups, a T-test was performed. To obtain relationships between fungal biomass and N application rate, regression analyses were done. These tests were done using the statistical package SPSS (SPSS Inc., Chicago, Illinois).

To analyse the response of microbial parameters to management, vegetation and soil characteristics, a principal component analysis (PCA) and a redundancy analysis (RDA) were done with Canoco 4.5 (Ter Braak and Smilauer, 2002). Microbial characteristics were used as species data, while soil, vegetation and management factors were used as environmental variables. PCA is an indirect ordination method in which the variation in species composition is

determined from the species data only. RDA is the canonical form of PCA and selects the linear combination of environmental variables that gives the smallest residual sum of squares. A large reduction in explained variance from the axes of a RDA compared to a PCA indicates that the environmental variables measured do not represent the relation between soil, vegetation and management characteristics and microbial characteristics very well (Ter Braak, 1995). Scaling was focused on inter-species correlations and species scores were divided by standard deviation. Species scores were centered and standardized. Monte Carlo permutation tests (499 permutations under reduced model) were performed to test the significance of the eigenvalues of the first canonical axes and all canonical axes together of the RDA.

2.3 Results

2.3.1 N application effects on microbial properties

Fungal and bacterial biomass showed a weak but significant decrease with increasing N application rate ($P = 0.014$ and $P = 0.016$, respectively, Fig. 2.2A,B). In addition, ergosterol was negatively affected by N application ($P = 0.015$, Fig. 2.2C). The ratio of the two biomasses, however, did not show any relationship with N application rate (Fig. 2.2D). Bacterial growth rate, measured as thymidine incorporation, remained constant (Fig 2.2E), whereas leucine incorporation showed a significant positive relationship with N application rate ($P = 0.001$, Fig. 2.2F).

2.3.2 Differences between young and old pastures

Fungal biomass in old fields was significantly higher than in young fields ($P = 0.007$, Fig. 2.3A), whereas bacterial biomass was not significantly different between young and old pastures ($P = 0.16$, Fig. 2.3B). Total microbial biomass, however, was just not significantly different between young and old sites ($P = 0.07$, Fig. 2.3C). Similar to fungal biomass, ergosterol content was also higher in old fields than in young fields ($P < 0.001$, Fig. 2.3D). Despite the fungal biomass being higher in old fields, and bacterial biomass showing no difference between young and old fields, the ratio of fungal and bacterial biomass was not significantly higher in old pastures ($P = 0.14$, Fig. 2.3E). Finally, although microbial biomass was not different in young and old fields, respiration was significantly higher in old fields than in young fields ($P = 0.017$, Fig. 2.3F).

Organic matter percentage and soil N were significantly higher in the old fields ($P = 0.001$ and $P < 0.001$, Fig. 2.4A,B). However, potential nitrogen mineralisation was not affected by field age ($P = 0.18$, Fig. 2.4C).

The vegetation also showed distinct differences between young and old pastures. Clover cover was reduced strongly in old sites, while herbs were more abundant in old pastures than in young pastures. A higher number of species was present in old pastures (Table 2.2).

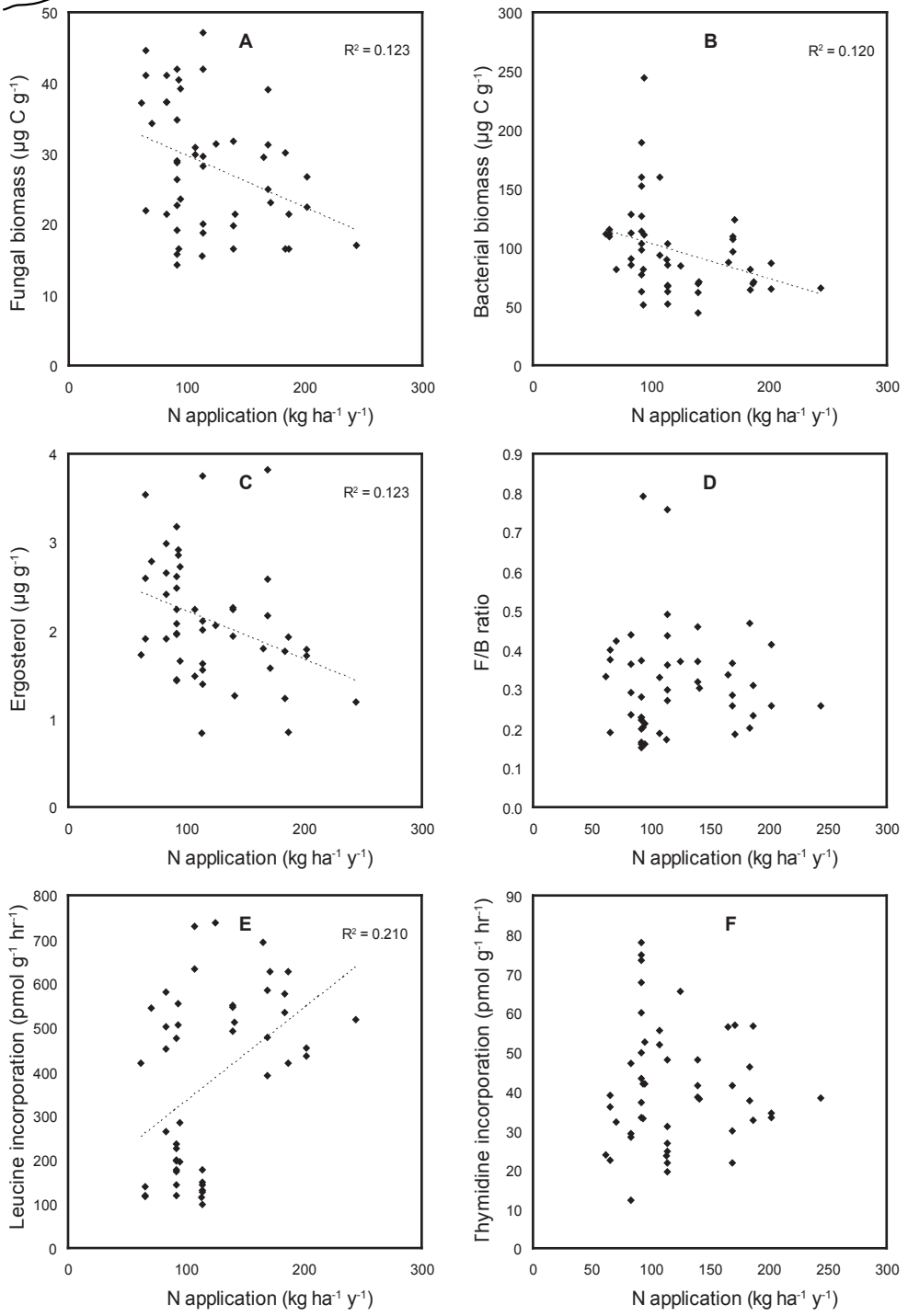

Fig 2.2. Relationships between microbial properties and N application.

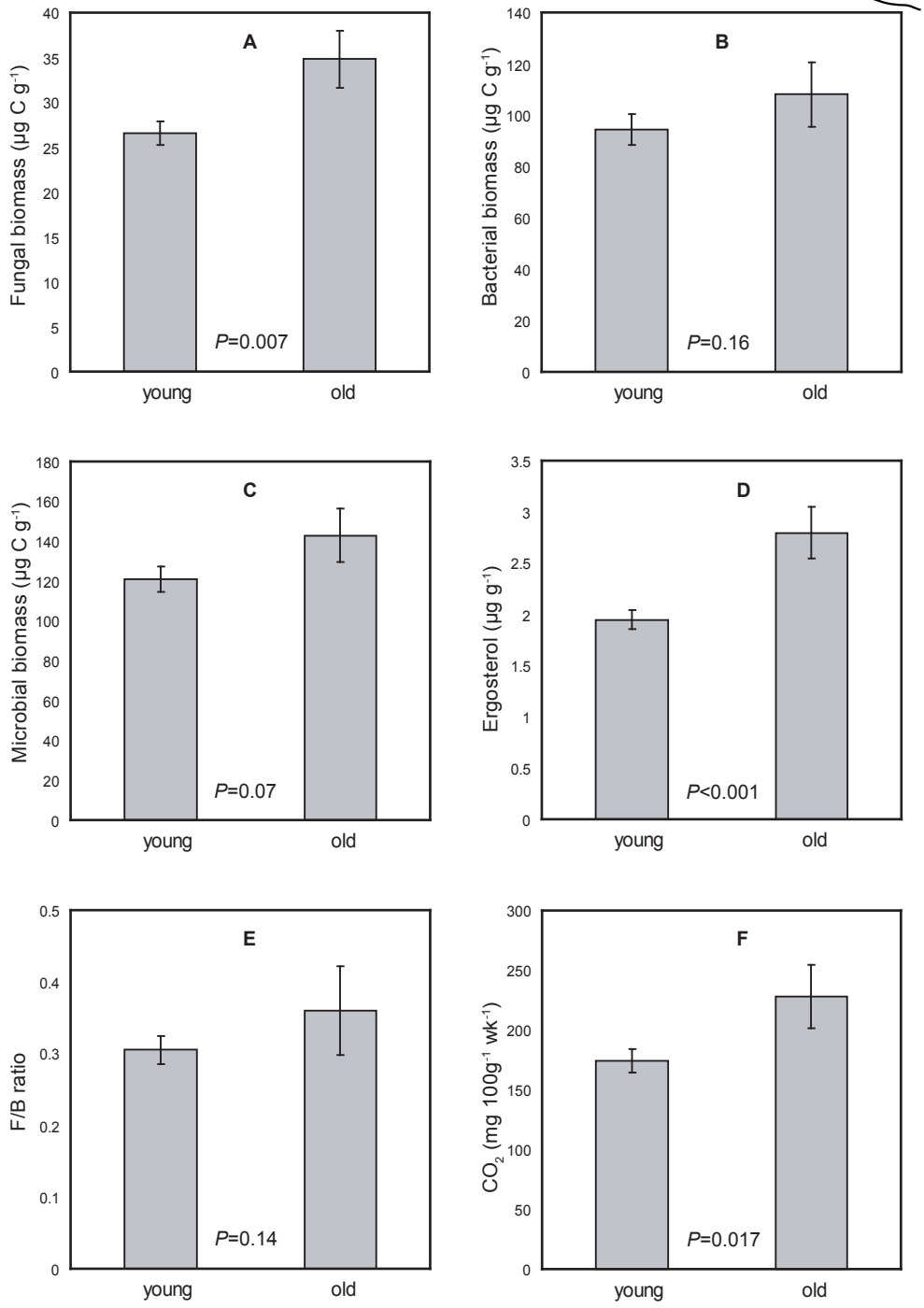

Fig. 2.3. Differences of microbial properties between young and old fields. Bars indicate ± 1 SE.

2.3.3. Relations of microbial properties with management and soil characteristics

The first two axes of PCA explained 24.4% and 18.5% of the variance of "species" data, respectively. RDA restricts the axes to linear combinations of the environmental variables. Therefore, the first two RDA axes explain less than the PCA axes, namely 14.4% and 9.9% of the variance. The first two species-environment correlations are 0.84 and 0.71. Although the explained variance was low, the 1st canonical axis had a P-value of 0.014 and the P-value of all canonical axes together was 0.002.

The RDA model with manual selection of environmental variables included organic matter, N application, clover percentage and K-HCl. The *P* value of this model was 0.002. The first

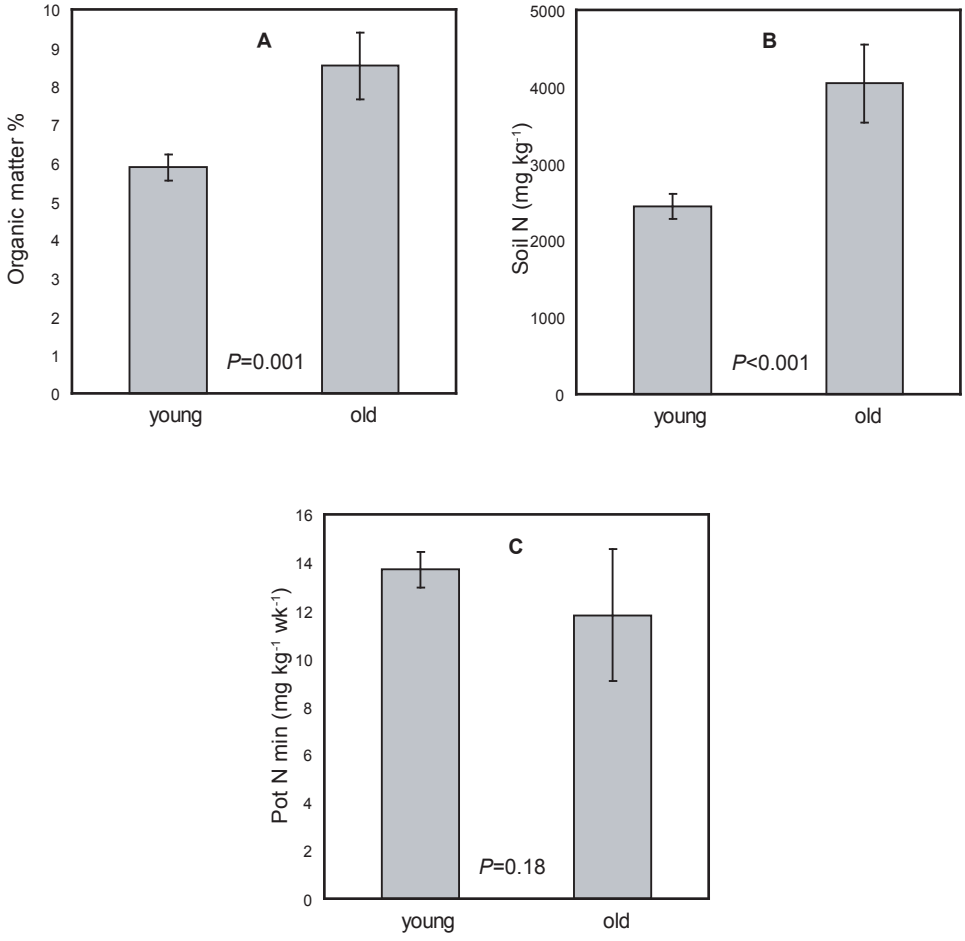

Fig. 2.4. Differences of organic matter characteristics between young and old fields. Bars indicate ± 1 SE.

axis explained 10.5% of species data and 48% of species-environment relation; the second axis explained 6.3% of species data and 26.4% of species-environment relation. Because the first axis (X-axis) explains the largest part of the variance, environmental variables that are situated along this axis are the most important explaining variables.

The triplot of this manually selected RDA model (Fig. 2.5) shows that fungal biomass, ergosterol, and respiration were correlated because these three arrows point in the same direction.

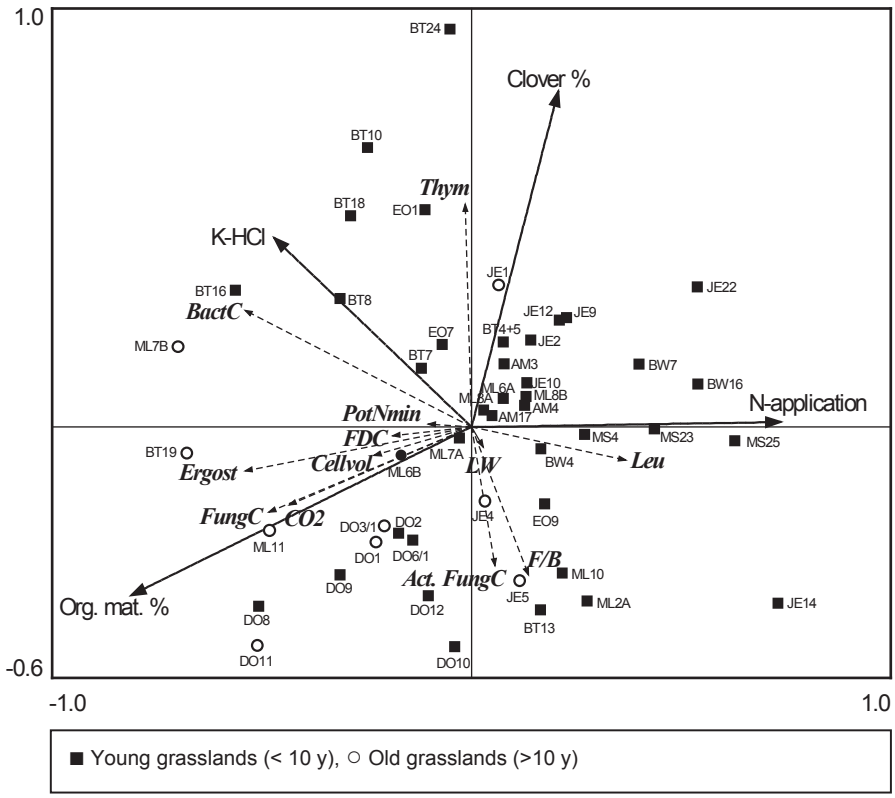

Fig 2.5. RDA triplot depicting the relations between microbial properties and management and soil characteristics. Only manually selected soil and management characteristics that significantly explained variance in microbial properties are shown (dashed arrows). Microbial parameters are represented by solid arrows, codes are: Act. FungC, microscopically determined active fungal biomass carbon (µg C g^{-1}); BactC, bacterial biomass carbon (µg C g^{-1}); FDC, frequency of dividing bacterial cells (% of total cells); Cellvol, bacterial cell volume (μm^3) ; CO_2, respiration (µg $100g^{-1}$ wk^{-1}); Ergost, ergosterol (µg g^{-1}); F/B, fungal/bacterial biomass ratio; FungC, fungal biomass (µg C g^{-1}); Leu, leucine incorporation (pmol g^{-1} h^{-1}); Thym, thymidine incorporation (pmol g^{-1} h^{-1}). The rest of the codes are self-explaining. Markers represent young and old fields, field names are listed in table 1.

Table 2.2. Comparison of means ± 1 SE of vegetation characteristics for young and old pastures. *P* values are given for the significance of the difference.

Vegetation characteristic	Young fields	Old fields	*P*
Clover cover (%)	29.0 ± 2.9	6.9 ± 3.0	<0.001
Lolium cover (%)	36.6 ± 2.6	32.3 ± 3.8	0.232
Herb cover (%)	7.1 ± 0.9	13.9 ± 2.4	0.002
Number of species per field	14.1 ± 0.5	16.1 ± 0.7	0.034

As the arrow of N application rate points in the opposite direction, these three microbial properties were negatively correlated with N application rate. In the same way, fungal biomass, ergosterol, and respiration were positively correlated with organic matter percentage.

Bacterial biomass was explained best by potassium content in the soil and was, like fungal biomass, positively correlated with organic matter percentage and negatively correlated with N application level. F/B ratio, active fungal biomass, and—to a lesser extent—total fungal biomass, were inversely correlated with clover percentage. Leucine incorporation correlated with N application level while thymidine incorporation showed a positive relationship with clover percentage.

From the triplot it appeared that the old pastures had a high resemblance, because they were situated near each other, in the lower left quadrant of the triplot.

2.4 Discussion

The results support the hypotheses that fungal biomass in pastures decreases with N fertilisation and increases with grassland age. The decrease of fungal biomass with fertiliser application is in accordance with studies in upland grassland systems (Bardgett and McAlister, 1999; Donnison et al., 2000b; Grayston et al., 2001). However, in these studies, the fields sampled were of different grassland types, and the range of management intensities was wide. Here, we used a narrow range of management intensities, and grassland species composition between fields differed marginally. The increase we found of fungal biomass with grassland age is also in accordance with previous studies (Allison et al., 2005; Klein et al., 1995; Van der Wal et al., 2006). However, these studies focus on ex-arable or natural systems, while our results show that also in agricultural grasslands that are still in production, an increase of fungal biomass with grassland age can be seen.

Both fungal biomass and ergosterol decreased with increasing N fertilisation (Fig. 2.2). Of all environmental variables included in the statistical analyses, N fertilisation was the major one explaining variance in microbial properties of the soil. Also organic matter content, clover cover and potassium content explained part of the variance of fungal biomass and other microbial characteristics (Fig 2.5).

The variance of fungal biomass explained by N fertilisation (Fig. 2.2) was low in this study, because the range of N application rates was relatively narrow, and unfertilised fields were absent in the dataset. In an earlier experimental field trial, we only used one soil type and a more

balanced N application gradient, and therefore the relationship between N application and fungal biomass was stronger (De Vries et al., 2006).

There are two possible explanations for a decreasing fungal biomass with nitrogen addition. Some articles claim that the negative effect of nitrogen addition on fungi is indirect, through changes in plant species composition resulting in changes in organic matter quality and quantity, changes in plant-specific exudates and through alterations in nutrient competition between plants and rhizosphere micro-organisms (Bardgett and McAlister, 1999; Bardgett et al., 1999; Lovell et al., 1995). Others claim that the addition of inorganic nitrogen has a direct toxic or inhibiting – or sometimes even stimulating – effect on fungi (Donnison et al., 2000a; Fog, 1988). Here, we found a negative relationship between organic matter content and N application rate (Fig. 2.5), but this could also be a result of farmers applying higher amounts of slurry to pastures with a lower organic matter percentage. Although we did not distinguish arbuscular mycorrhizal fungi, they respond similarly to fertiliser addition as saprotrophic fungi, but the effects are mainly attributed to P (Abbott and Robson, 1991).

Not only fungal biomass but also bacterial biomass decreased as N application increased, while organic matter content decreased (Fig. 2.2B). This observation contradicts studies that show an increase of bacterial biomass with manure application (Bittman et al., 2005; Bohme et al., 2005; Marschner et al., 2003; Peacock et al., 2001) and our own previous field experiment in which bacterial biomass was not affected by N application (De Vries et al., 2006). We suppose that in our present study the decrease of bacterial biomass with increasing N application is a consequence of the lower organic matter percentage in fields that received higher N rates. Decrease of bacterial biomass is frequently attributed to a lower organic matter percentage (Anderson and Domsch, 1989; Hassink et al., 1991; Schnürer et al., 1985). Because both fungal biomass and bacterial biomass decreased with increasing N fertilisation, the F/B ratio was not affected by N application.

We also confirmed our second hypothesis: fungal biomass was higher in older grasslands (Fig. 2.3A). Bacterial biomass also showed an increase in old pastures, but the difference with young pastures was not statistically significant (Fig. 2.3B). As a consequence, the F/B ratio did not differ between young and old fields (Fig. 2.3E). In the RDA triplot the old pastures were separated from the young pastures (Fig. 2.5), although age was not included in the set of significant explaining variables. The significance of organic matter percentage and clover cover in the RDA model shows the importance of organic matter quantity and quality for microbial properties. This underlines that the differences between young and old fields can be attributed to organic matter characteristics.

We found a negative correlation between fungal biomass and clover cover (Fig. 2.5), similar to our previous study (De Vries et al., 2006). Smith et al. (2003), however, found a positive relationship of fungal PLFA with legumes. In our sites, the presence of white clover decreased with grassland age, while in the trial of Smith et al., the presence of legumes decreased with the addition of fertiliser. We found that the effect of white clover on fungal biomass was less important than the N application effect (Fig 2.5.). The clover effect could, in this case, be mediated through age. The negative relationship between white clover and fungal biomass can be attributed to the less dense root system of white clover compared to perennial rye grass (Schortemeyer et al., 1997), to the higher ammonium exudation rates of white clover (Paynel and Cliquet, 2003), or to the lower C/N ratio of the litter (Neergaard et al., 2002).

We cannot explain potassium as a variable explaining variation in fungal biomass (Fig. 2.5).

Soil potassium concentrations can be coupled to clover growth: as a result of the high K demand of clover, levels of soil potassium under clover pasture quickly decline (Baars, 2002). Earlier, we found a higher potassium content in the soil in grass plots than in grass-clover plots (De Vries et al., 2006) (unpublished data). If the potassium content of the soil simply follows the percentage clover in the sward, it is a response variable to management rather than a determining factor for microbial characteristics.

From the higher organic matter content and the larger soil N pool in old pastures, we would expect the potential nitrogen mineralisation to increase. Due to higher mineralisation rates, old grasslands are claimed to have a higher nitrogen leaching than young grasslands (Cuttle and Scholefield, 1995; Shepherd et al., 2001). However, we did not find a difference in potential N mineralisation between young and old pastures (Fig. 2.4C). In old pastures the more recalcitrant soil organic matter will be less mineralisable, while in young pastures the higher amount of clover (Table 2.2) will result in an increase of mineralisable N.

In addition to changes in organic matter quantity and quality, reduced disturbance might also play a role in stimulating fungal biomass. Fungal biomass has repeatedly been shown to be higher in no-tillage systems compared to conventional tillage systems, because tillage destroys the mycelial network (Beare et al., 1997; Frey et al., 1999; Wardle, 1995). Presumably, AMF are even more susceptible to tillage than saprotrophic fungi (Kabir et al., 1997). Similar to tillage, establishing or ploughing and reseeding grasslands destroys fungal hyphae. In older pastures, fungal biomass has had more time to recover from these disturbances, and hence fungal biomass will be higher.

Finally, we conclude that the hypotheses we formulated were partly confirmed. We found a negative relationship of fungal and bacterial biomass with N application rate, and a positive relationship of fungal biomass with grassland age. Because organic matter quantity and quality appeared to be tightly correlated with fungal and bacterial biomass, we consider this as the most important driving factor in both cases. Further experimental studies are needed to confirm this. We have shown that the negative relationship between fungal biomass and N application rate is present in a range of Dutch grasslands with relatively small differences in management intensity. Also, we have shown that not only in ex-agricultural fields or restoration projects fungal biomass is positively related to grassland age, but also in agricultural grasslands that are still in production. Because both fungal and bacterial biomass were affected by N fertilisation as well as by pasture age, the F/B ratio remained constant and can not be used as an indicator for shifts in fungal and bacterial abundances.

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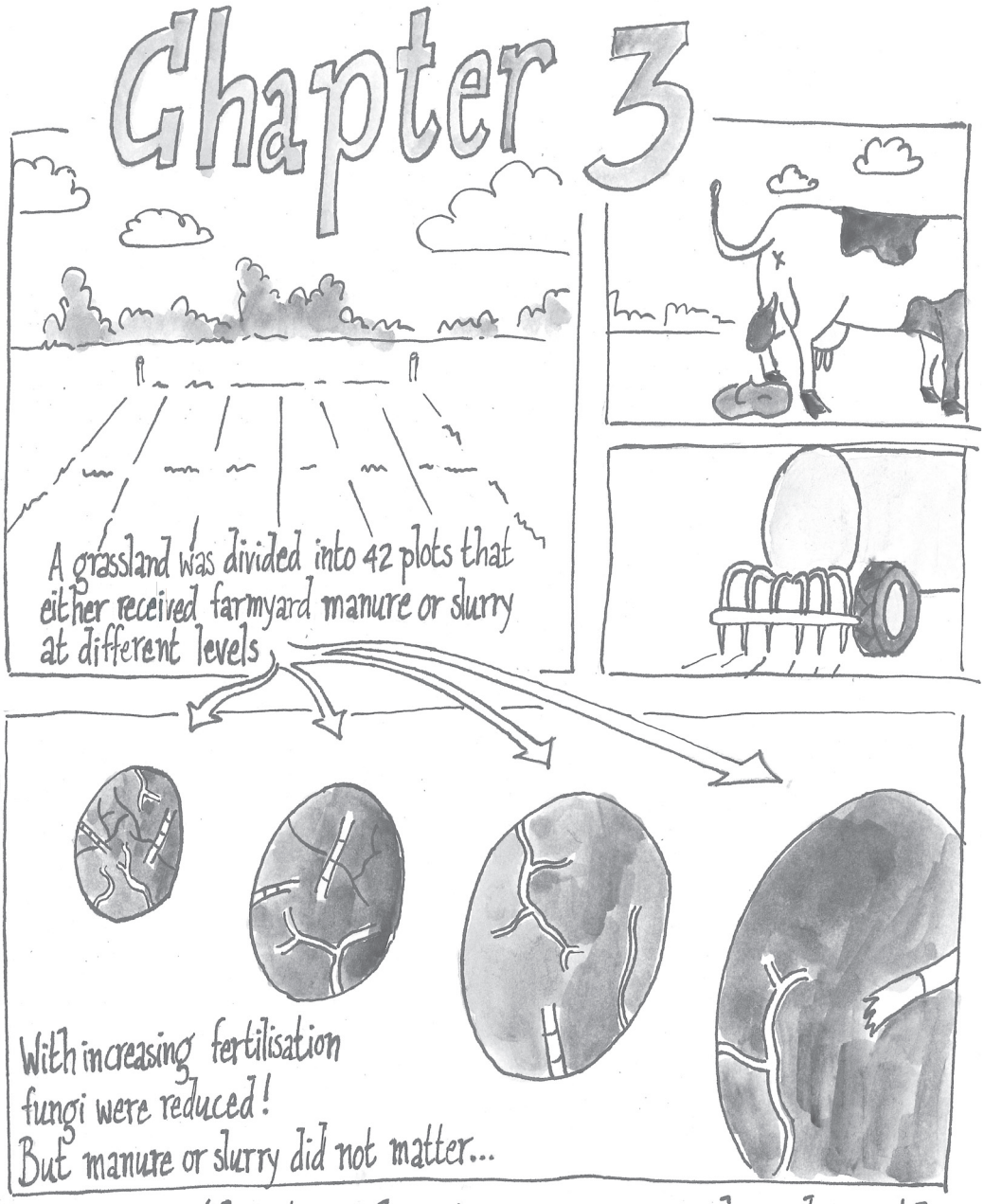

Fungal / bacterial ratios in grasslands with contrasting nitrogen management

3 Fungal/bacterial ratios in grasslands with contrasting nitrogen management

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Abstract

It is frequently hypothesised that high soil fungal/bacterial ratios are indicative for more sustainable agricultural systems. Increased F/B ratios have been reported in extensively managed grasslands. To determine the shifts in fungal/bacterial biomass ratio as influenced by grassland management and to find relations with nitrogen leaching potential, we sampled a two year old field experiment at an organic experimental farm in the eastern part of The Netherlands. The effect of crop (grass and grass/clover), N application rate (0, 40, 80, 120 kg N ha^{-1}) and manure type (no manure, farm yard manure and slurry) on the F/B ratio within three growing seasons was tested, as well as relations with soil and crop characteristics, nitrate leaching and partial N balance. Biomass of fungi and bacteria was calculated after direct counts using epifluorescence microscopy. Fungal and bacterial biomass and the F/B ratio were higher in grass than in grass/clover. The F/B ratio decreased with increasing N application rate and multiple regression analysis revealed a negative relationship with pH. Bacterial activity (measured as incorporation of [^{3}H]thymidine and [^{14}C]leucine into bacterial DNA and proteins) showed the exact opposite: an increase with N application rate and pH. Leaching increased with N application rate and was higher in grass/clover than in grass. Partial N balance was more positive at a higher N application rate and showed an inverse relationship with fungal biomass and F/B ratio. We conclude that the fungal/bacterial biomass ratio quickly responded to changes in management. Grasslands with higher N input showed lower F/B ratios. Grass/clover had a smaller fungal biomass and higher N leaching than grass. In general, a higher fungal biomass indicated a lower nitrogen leaching and a more negative partial N balance (or smaller N surplus), but more observations are needed to confirm the relationship between F/B ratio and sustainability.

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3.1 Introduction

Organic matter plays a key role in many soil processes because it affects, among others, soil structure, nutrient dynamics and soil life. Decomposition of soil organic matter is a highly important process. The rate of decomposition depends on environmental conditions (e.g. temperature, moisture conditions), on the quality (e.g. C/N ratio) of the substrate and on the characteristics of the decomposing organisms and their predators (i.e. C/N ratio, growth efficiency) (Bloem et al., 1997; Swift et al., 1979).

The main decomposition pathways in soil are either bacterial-based or fungal-based. Both bacteria and fungi support their own chain of soil fauna (De Ruiter et al., 1993; Wardle and Lavelle, 1997). Therefore, the biomass of fungi compared to bacteria can be considered as an indicator for the activity of two pathways of the soil food web, formed by fungivores or bacterivores and their predators, respectively.

Generally fungal biomass is found to be greater than bacterial biomass in agricultural soils (Anderson and Domsch, 1975; Sakamoto and Oba, 1994; Schnurer et al., 1986; Zelles et al., 1995). In the Netherlands, however, analyses of soil samples from conventionally managed arable as well as grassland soils have shown that the soil microbial biomass is usually strongly dominated by bacteria (Bloem et al., 1994; Hassink et al., 1993; Velvis, 1997). Less than 20% of the soil microbial biomass in these soils consists of fungi. Consequently, the soil fauna is dominated by bacterivores and their predators (De Ruiter et al., 1993). Recent analyses of more extensively managed Dutch grasslands show a much higher contribution of fungi to the microbial biomass (50-80%) (Bloem et al., 2004).

Increased fungal/bacterial (F/B) biomass ratios in extensively managed grasslands are consistent with recent other reports (Bailey et al., 2002; Bardgett and McAlister, 1999; Donnison et al., 2000; Grayston et al., 2001; Zeller et al., 2001). The mechanisms responsible for shifts in the soil microbial community remain largely unknown. Some studies have shown that on arable land soil management affects the F/B biomass ratio (Beare et al., 1997; Frey et al., 1999). In most cases bacteria dominate under conventional tillage, whereas fungi dominate under no-tillage. This has been attributed to a direct contact between bacteria and substrate under conventional tillage, encouraging bacterial growth (Beare et al., 1997). Also, mycelial networks are destroyed by tillage. The effect of "tillage" (cultivation or slit injection of slurry) on fungal/bacterial biomass ratios in grasslands has not been investigated. Grazing has been reported to have either a positive (Bardgett et al., 1997) or a negative impact (Ghani et al., 2003) on the F/B ratio. Shifts in the F/B ratio related to grassland management have so far been attributed to quantity (Mawdsley and Bardgett, 1997) and quality (Grayston et al., 2001) of root exudates, changes in quality and quantity of litter or input of animal faeces (Bardgett et al., 1996) and plant productivity and composition (Donnison et al., 2000). F/B ratios may also be affected by other factors e.g. toxic metals (Tobor-Kaplon et al., 2005). Most of these factors are related to nutrient availability. Bittman et al. (2005) found a decreasing fungal biomass as a consequence of application of manure and fertiliser. Inorganic nitrogen fertilisation has been reported to reduce the F/B biomass ratio (Bardgett et al., 1999b; Bloem et al., 2004), while organic matter with a high C/N ratio stimulates fungal growth and thus increases the F/B ratio (Alexander, 1977; Henriksen and Breland, 1999; Vinten et al., 2002). pH has been seen to have either a positive or a negative effect on F/B ratio (Bååth and Anderson, 2003; Blagodatskaya and Anderson, 1998).

Higher fungal/bacterial (F/B) biomass ratios are suggested to be indicative for a more sustainable agroecosystem with lower impact on the environment, in which organic matter decomposition and N mineralisation dominate the provision of plant nutrients for crop growth (Bardgett and McAlister, 1999; Bardgett et al., 1999a; Beare et al., 1992; Yeates et al., 1997). Because of the higher C/N ratio of fungi compared to bacteria (10 versus 4), grazing by fungivores results in a lower N mineralisation rate than grazing by bacterivores. In addition, fungal-feeding fauna generally have a smaller biomass and lower turnover rates than bacterial-feeding fauna (Didden et al., 1994; Zwart et al., 1994). A fungi-dominated food web may therefore result in a lower N-mineralisation rate. This, however, does not necessarily lead to a lower crop production. The biomass of mycorrhizal fungi probably increases at lower soil nutrient contents (Mäder et al., 2000; Smith and Read, 1997). Their contribution to nutrient uptake may counterbalance the negative effects of a low nutrient availability to the crop and thus reduce nutrient losses to the environment (Jeffries and Barea, 1994; Smith and Read, 1997).

If an increased F/B biomass ratio has a positive effect on crop nutrient uptake efficiency and nutrient retention it is desirable to get a handle on management practices and soil properties that increase this ratio. The aim of this study was therefore to find out which management practices and soil characteristics affect the F/B biomass ratio in a Dutch grassland agroecosystem, and whether the F/B ratio can be used as an indicator for a system with low nutrient losses.

We hypothesise an increased fungal biomass and/or F/B ratio: (1) at lower availability of inorganic N, (2) as a consequence of application of manure with higher C/N ratio, (3) when farmyard manure is applied superficially compared to slit injection of slurry. We furthermore hypothesise that a higher F/B ratio reduces N leaching potential. We evaluate factors related to fertiliser regime and management, i.e. plant species composition and organic matter characteristics together with pH as predictors for the F/B biomass ratio or fungal and bacterial biomass alone.

3.2 Materials and methods

3.2.1 Sampling site

A field trial was done in a pasture of the organic experimental farm "Aver Heino" at Heino in the eastern part of The Netherlands (52°25' north and 6°15' east), which was sown with a grass/clover mixture in 1997. After a period of grazing and mowing the field trial was established in 2001. The humid sandy soil was classified as a gleyey sand with a semi-permeable loam horizon at 70 to 80 cm.

The trial consisted of seven rows, corresponding to three manure treatments: no manure (one row), farm yard manure (three rows) and slurry (three rows). The three rows of farm yard manure and slurry received manure at three different N application rates: 40 kg N ha^{-1}, 80 kg N ha^{-1} and 120 kg N ha^{-1}. Each of the seven rows was subdivided into nine subplots which differed in crop: grass (*Lolium perenne* L.) or grass/clover (*Lolium perenne* L. and *Trifolium repens* L.). Dimensions of the subplots were 15 m x 2.7 m, bordered by a 0.3 m bufferzone. In each row a number of subplots was sampled. For a detailed overview of treatments see Table 3.1. Growth of white clover in grass plots was inhibited by the herbicide Starane.

The C/N ratio of the farm yard manure was 12.3 and of the slurry 6.8. All plots received potassium and phosphorus additionally at a rate of 107 kg P_2O_5 ha^{-1} and 372 kg K_2O ha^{-1} for

Table 3.1. Treatments of the field trial.

Crop	Treatment	n	Manure type and application	N total (kg $ha^{-1}y^{-1}$)	P_2O_5 total (kg $ha^{-1}y^{-1}$)	K_2O total (kg $ha^{-1}y^{-1}$)
Grass- clover	No manure	4	No	0	107	372
	FYM 40	4	Farm yard manure, superficial	40	107	372
	FYM 80	4	Farm yard manure, superficial	80	107	372
	FYM 120	4	Farm yard manure, superficial	120	107	372
	Slurry 40	4	Slurry, injection	40	107	372
	Slurry 80	4	Slurry, injection	80	107	372
	Slurry 120	4	Slurry, injection	120	107	372
Grass	No manure	4	No	0	122	487
	Slurry 40	2	Slurry, injection	40	122	487
	Slurry 80	2	Slurry, injection	80	122	487
	Slurry 120	2	Slurry, injection	120	122	487

grass/clover and 122 kg P_2O_5 ha^{-1} and 487 kg K_2O ha^{-1} for grass. Farm yard manure was applied superficially while slurry was applied through slit injection. Both were applied in spring.

3.2.2 Soil and crop characteristics

Soil samples were collected in October 2003, three growing seasons after establishment of the trial. For each plot a bulk sample of 30 cores (0-10 cm depth, 3.5 cm diam) was collected, sieved (5 mm mesh size), homogenised and stored at field moisture content overnight at 4 ºC before analysis(Bloem et al., 2006). An additional sample was taken for the determination of bulk density (Elliott et al., 1999).

Soil dry matter content was determined after oven-drying of approximately 30 g of the bulk sample (in duplicate) at 105 °C. Prior to further analysis bulk samples were oven-dried at 40 °C. Organic matter content was determined by loss-on-ignition (Ball, 1964) and pH of the 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). C/N ratio of the soil was calculated from total N and organic matter percentage, assuming 58% of organic matter to be C.

Water-extractable phosphorus (Pw) (Sissingh, 1971) and ammonium lactate-acetic acid-extractable P (P-Al) were determined (Schouwenburg and Walinga, 1967). Extracts were analysed by spectrophotometry.

Potassium content (K-HCl) was analysed by shaking approximately 10 g of soil with 100 ml of extraction solution (0.2 M HCl and 0.4 M oxalic acid). The suspension was shaken for 1 hour, filtered and measured by Eppendorf Elex 6361 Flame-AES.

The field trial was mown in 5 cuts: 5/13, 6/26, 8/2, 8/28 and 10/9/2003. Herbage was analysed for N, P, and K contents as described by Novozamsky et al. (1983) and measured by Segmented Flow Analysis. For every plot total production and clover production were calculated in tons dry matter per hectare.

3.2.3 Fungal and bacterial biomass

200 g soil was pre-incubated at 50% WHC (water holding capacity) at 12°C for four weeks to stabilise soil conditions and to avoid effects of temperature and moisture fluctuations in the field (Bloem et al., 2006). For each sample, 20 g of soil and 190 ml of demineralised water were homogenised 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). The water-repellent coating keeps the suspension in defined area of 113 mm^2. The hole was precleaned with 70% ethanol and washing-up liquid (Dreft). Slides with soil suspension were then 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 were assumed to be inactive or dead, red hyphae were assumed 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 can be 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 $1.3x10^{-13}$ g C μm^{-3} (Bakken and Olsen, 1983; Veen and Paul, 1979).

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).

Note that the specific carbon content used for bacteria is 2.4 times higher than that used for fungi. This means that our fungal to bacterial biomass ratios based on carbon are 2.4 times lower than ratios based on biovolume (Velvis, 1997).

3.2.4 Bacterial activity

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^{-1}) and L-[U-^{14}C]leucine (11.5 GBq mmol^{-1}) 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^{-1}). 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 (6500 rev min^{-1}, outer ring of a rotor for 16 x 25 ml tubes) 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 substracted 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.

3.2.5 N leaching

Two ceramic cup samplers were placed in each field plot at a depth of 30 cm below the soil surface. A weather station was situated on the farm and meteorological data were obtained daily from the KNMI (Royal Dutch Meteorological Institute) website. The cups were sampled after 50 mm of precipitation during January and February 2004. Pore water samples were analysed for NO_3^- and NH_4^+ using Segmented Flow Analysis (Houba et al., 2000).

N leaching was calculated by multiplying the average concentration of two sampling dates with the precipitation surplus in the period in between (De Vos and Assink, 2004; Smit et al., 2004).

3.2.6 Partial N balance

A partial N balance (IN – OUT) was calculated for each plot.

Inputs of the budget were: fertiliser, N_2 fixation and atmospheric deposition. Biological N_2 fixation by Rhizobium in symbiosis with white clover was estimated using the formula given by Carlsson and Huss-Danell (2003). Atmospheric deposition was assumed to be 50 kg ha^{-1} for the eastern part of The Netherlands (Aarts et al., 2000).

Output of the budget was crop yield. Unknown items were leaching and gaseous losses.

3.2.7 Statistical analysis

Statistical analysis was carried out using the statistical package SPSS (SPSS Inc., Chicago, Illinois). F/B ratio was transformed using the arcsine square root to meet the requirements of normality and homogeneity of variances. Stepwise forward multiple linear regression analysis was used to elucidate relationships of soil characteristics and management with microbial biomass and activity.

Treatment effects on microbial parameters were analysed by a two-way ANOVA, because the layout of the trial did not permit the comparison of the seven manure treatments. The factors were manure type (no manure, farmyard manure and slurry) and N application rate (0, 40, 80 and 120 kg N ha^{-1}). This was done for grass/clover and grass separately because degrees of freedom were insufficient for a three-way ANOVA with crop, manure type and N application rate. These analyses were followed by Tukey's test to detect differences between treatments. Differences between grass/clover and grass, and between the control and slurry for grass, were analysed using t-tests.

3.3 Results

3.3.1 Soil and crop characteristics

pH values in the trial ranged from 4.35 to 4.80, being significantly lower in grass/clover treatments than in grass (Table 3.2). In grass/clover pH increased with increasing N application rate and this tendency could also be seen in grass. Plots receiving farm yard manure had a higher pH than no manure in grass/clover; slurry had a higher pH than no manure in grass.

In grass the soil C/N ratio was higher in the plots with no manure than in the slurry treatments (Table 3.2). Though not significantly different, organic matter content was higher in grass/clover than in grass, and tended to increase with the application rate of (N) fertilisation. Although soil C/N ratios did not differ significantly between grass/clover and grass, N content in the removed crop (aboveground) did. Total production and N yield were higher in grass/clover than in grass, but showed no relation with N application rate and fertiliser type.

Soil potassium contents in the trial ranged from 71 mg K_2O 100 g^{-1} to 270 mg K_2O 100 g^{-1} and were classified as "sufficient" to "very high" according to Dutch standards (Evers et al., 2000). In grass/clover the potassium content was significantly affected by manure type and N application level. Slurry had lower potassium content than no manure and farm yard manure treatments. N application level did not have a consistent effect.

The average values of Pw and P-Al for all fields were, respectively, 19 ± 5 mg P_2O_5 100 g^{-1} and 43 ± 8 mg P_2O_5 100 g^{-1}, which are classified as "sufficient". Total soil N was 2.3 ± 0.3 mg kg^{-1}. Bulk density was higher in the no manure treatments than in the other treatments and ranged from 1.40 to 1.58 g cm^{-3}. Dry matter percentage of the entire field was 85 ± 2.

Table 3.2. Soil characteristics and yield data. Values denoted with the same letter are not significantly different (Tukey's post-hoc test, $P < 0.05$ or t-test, $P < 0.05$). Comparisons were made for treatments between horizontal lines. Asterisks in the crop effect row indicate significant differences (t-test, * $P < 0.05$, ** $P < 0.01$) between grass-clover and grass.

Crop	Treatment	n	pH	Soil C/N ratio	Organic matter %	Clover production (tons dm ha^{-1})	Total production (tons dm ha^{-1})	N yield in crop (kg ha^{-1})
Grass/clover	0	4	4.4^{a}	12.8	5.2^{ab}	5.5	10.7	314
	40	8	4.4^{a}	13.1	5.1^{a}	5.8	11.0	319
	80	8	4.5^{ab}	13.3	5.2^{ab}	5.5	10.7	314
	120	8	4.6^{b}	13.0	5.4^{b}	5.9	11.4	331
	No manure	4	4.4^{a}	12.8	5.2	5.5	10.9	314
	FYM	12	4.5^{b}	12.9	5.3	5.7	10.9	321
	Slurry	12	4.5^{ab}	13.3	5.1	5.8	11.2	322
Grass	0	4	4.6	14.8	5.0	1.1	6.3	136
	40	2	4.8	12.0	5.0	1.0	7.3	150
	80	2	4.8	14.0	5.1	1.4	8.1	169
	120	2	4.8	13.0	5.1	0.1	6.6	130
	No manure	4	4.6^{a}	14.8^{a}	5.0	1.1	6.3	135
	Slurry	6	4.8^{b}	13.0^{b}	5.1	0.8	7.4	150
Crop effect			**			**	**	**

3.3.2 Microbial biomass and activity

On average, fungal biomass constituted 25 ± 7% of the total microbial biomass in the field trial and ranged from 19.2 µg C g^{-1} to 46.0 µg C g^{-1}. Bacterial biomass was 81.0 ± 16.1 µg C g^{-1} for the entire field and thymidine and leucine incorporation averaged, respectively, 14.4 ± 4.9 pmol g^{-1} h^{-1} and 277.6 ± 55.9 pmol g^{-1} h^{-1} for all treatments together.

The F/B biomass ratio varied between 0.25 and 0.46.

Fungal and bacterial biomass, bacterial activity and the F/B ratio were significantly affected by management and soil properties.

Management effects

Crop

The differences between grass and grass/clover treatments were large and highly significant. Fungal biomass in grass treatments was almost two-fold higher than in grass/clover treatments (38.6 ± 9.8 µg C g^{-1} vs. 25.2 ± 7.2 µg C g^{-1}, t-test, $P < 0.001$) (Fig. 3.1). Also bacterial biomass, which was 94.4 ± 14.2 µg C g^{-1} in grass and 79.1 ± 14.2 µg C g^{-1} in grass/clover, and total microbial biomass were significantly higher in grass (t-test, $P = 0.007$ and $P < 0.001$, respectively). This could also been seen for the F/B biomass ratio, which was 0.34 overall for grass/clover and 0.42 for grass (t-test, $P = 0.022$).

Regression of the F/B ratio with clover production resulted in a weak but significant negative relationship ($R^2 = 0.12$, $P = 0.035$).

Table 3.3. Thymidine and leucine incorporation for crop, N application rate and manure type. Values denoted with the same letter are not significantly different (Tukey's post-hoc test, $P < 0.05$ or t-test, $P < 0.05$). Comparisons were made for treatments between horizontal lines.

Crop	Treatment	n	Thymidine incorporation (pmol g^{-1} h^{-1})	Leucine incorporation (pmol g^{-1} h^{-1})
Grass/clover	0	4	10^{a}	236^{a}
	40	8	11^{ab}	237^{a}
	80	8	14^{ab}	268^{ab}
	120	8	16^{b}	307^{b}
	No manure	4	10^{a}	236
	FYM	12	13^{ab}	260
	Slurry	12	15^{b}	281
Grass	0	4	14	266
	40	2	15	295
	80	2	19	340
	120	2	25	387
	No manure	4	15	266^{a}
	Slurry	6	19	340^{b}

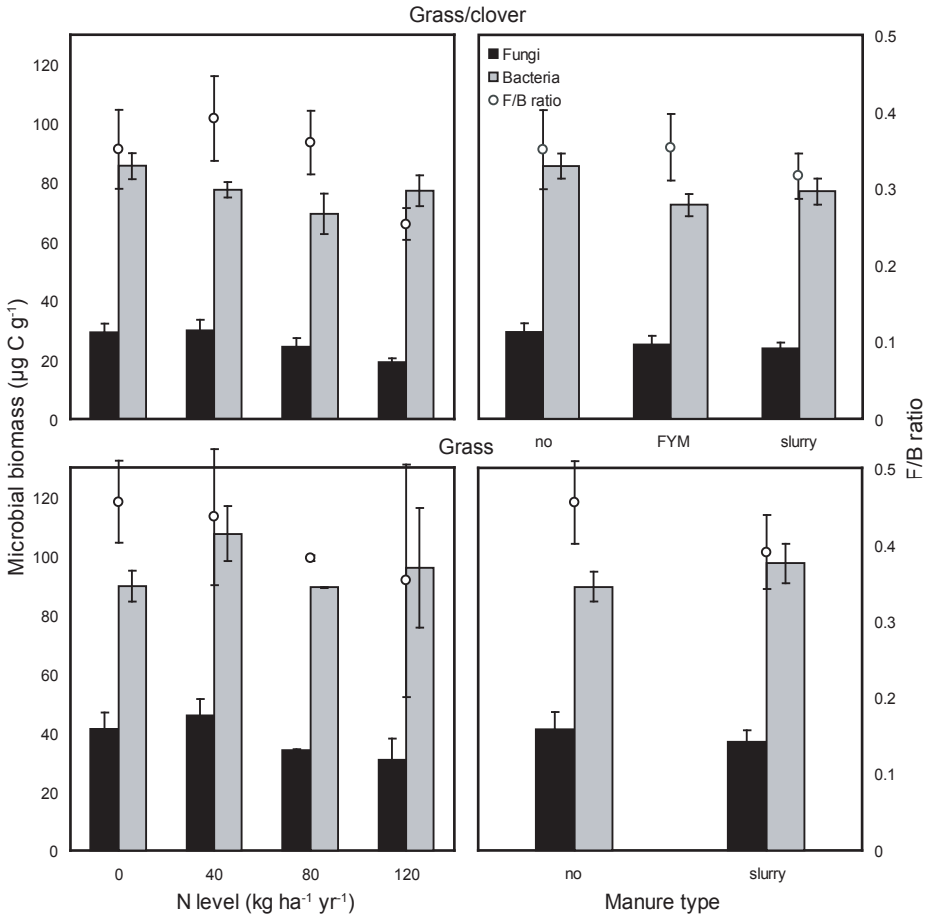


Fig. 3.1. Fungal and bacterial biomass and F/B ratio for nitrogen level and manure type (No = no manure, FYM = farm yard manure) in grass-clover (top) and grass (bottom). Mean values ± SE are shown.

N application rate

Increasing N application rates reduced the total amount of fungi in the grass/clover treatments (ANOVA, $P = 0.014$; Figure 3.1). The 0 and 40 kg N ha^{-1} treatments had a significantly higher amount of fungi than the 120 kg N ha^{-1} treatment. In the grass treatments this trend could also be seen but was not statistically significant (Fig. 3.1). The amount of active fungi followed the same trend (data not shown), but without significant differences. No significant effect of N application rate was found on bacterial biomass. For grass/clover thymidine and leucine incorporation were highest in the 120 kg N ha^{-1} treatment (ANOVA, $P = 0.027$ and $P = 0.007$ respectively, Table 3.3).

N application rate had a significant negative main effect on the F/B biomass ratio (P = 0.034).

Manure type

There was no effect of manure type on fungal and bacterial biomass (Fig. 3.1). For grass/clover an interaction-effect was seen of N application rate and manure type on fungal biomass and F/B ratio (P = 0.025).

Relations with soil characteristics

pH

The F/B biomass ratio decreased with increasing pH for grass/clover and grass separately (Fig. 3.2).

Thymidine and leucine incorporation both had a significant positive relationship with pH. When these regressions were separated for grass/clover and grass significant relationships were found for thymidine incorporation (R^2 = 0.42, P < 0.001 and R^2 = 0.60, P = 0.008) as well as for leucine incorporation (R^2 = 0.47, P < 0.001 and R^2 = 0.74, P = 0.001, Fig. 3.2).

Organic matter quality and quantity

No significant relationship with microbial biomass or F/B ratio was found for C/N ratio and organic matter percentage. Neither thymidine nor leucine incorporation and CO_2 evolution showed any relationship with organic matter characteristics.

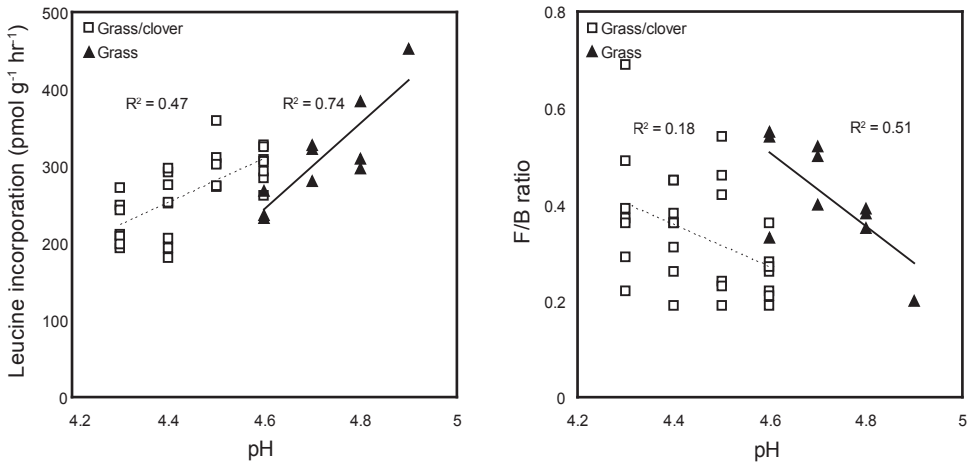


Fig. 3.2. Relationship between leucine incorporation, F/B ratio and pH for grass-clover and grass. Markers represent observations, lines represent regressions, for grass-clover (dashed, P = 0.025) and for grass (solid, P = 0.021).

3.3.3 N leaching

The N leaching in grass/clover treatments was significantly higher than in grass (t-test, $P < 0.001$, Fig. 3.3). Multiple regression analysis with management and soil characteristics (N application rate, manure type, clover production, C/N ratio, pH, organic matter percentage, fungal and bacterial biomass) pointed out that pH and N application rate determined leaching ($R^2 = 0.53$, $P < 0.001$). Leaching increased with higher N application rates and decreasing pH-values. No correlation with fungal biomass or F/B ratio was found when all single observations were used separately. This was caused by the large variation of the fungal biomass. However, when regression was performed with the means of treatments a negative relationship with fungal biomass was revealed (Fig. 3.4).

3.3.4 Partial N balance

N application rate had a significant main effect on the partial N balance (ANOVA, $P < 0.001$). The partial N balance was negative at the lowest N application rates (0 and 40 kg ha^{-1}), and positive at high N application rates (80 and 120 kg ha^{-1}). Stepwise multiple regression analysis (dependent variables see paragraph 3.4) included N application rate and pH ($R^2 = 0.82$, $P <$

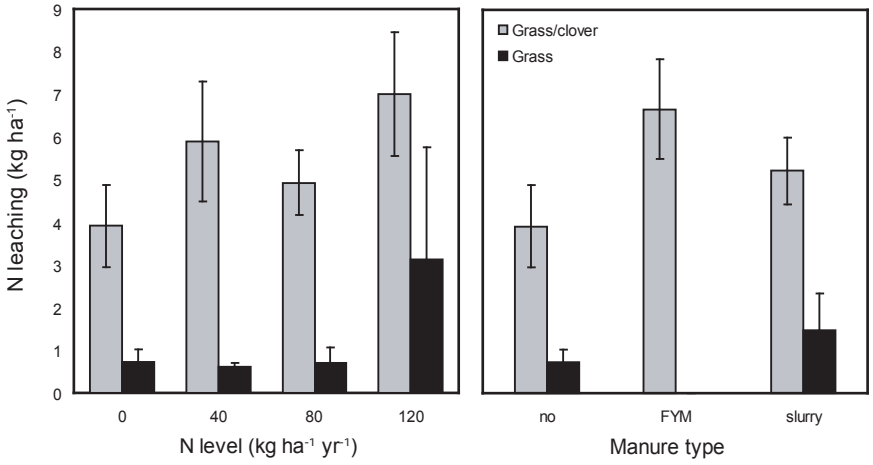


Fig. 3.3. Nitrogen leaching between January 16 and February 17 at a depth of 30 cm in grass-clover and grass, for manure type (No = no manure, FYM = farm yard manure) and nitrogen level. Mean values ± SE are shown.

0.001), in which the partial N balance was higher with increasing N application rate and pH. No correlation was found for partial N balance with N leaching. Regressions with fungal biomass and F/B ratio both resulted in a weak, significant negative relationship ($R^2 = 0.12$, $P = 0.034$ and $R^2 = 0.11$, $P = 0.040$ respectively). When regression was done with means for treatments of the partial N balance with fungal biomass a significant negative relationship with a higher correlation coefficient was the result (Fig. 3.4). Here treatments were not split up for grass/clover and grass as no crop effect was present on the N balance.

3.4 Discussion

The bacterial biomasses we found were somewhat lower than Bloem and Breure (2003) found in grasslands, but twice as high as the biomasses Van der Wal et al. (2006) found in agricultural sites, ex-arable fields and heathlands. The fungal biomasses we found were comparable with biomasses found by Van der Wal et al., which, in combination with our higher bacterial biomasses, resulted in lower F/B biomass ratios in our fields. Bittman et al. (2005) found higher F/B ratios in grass

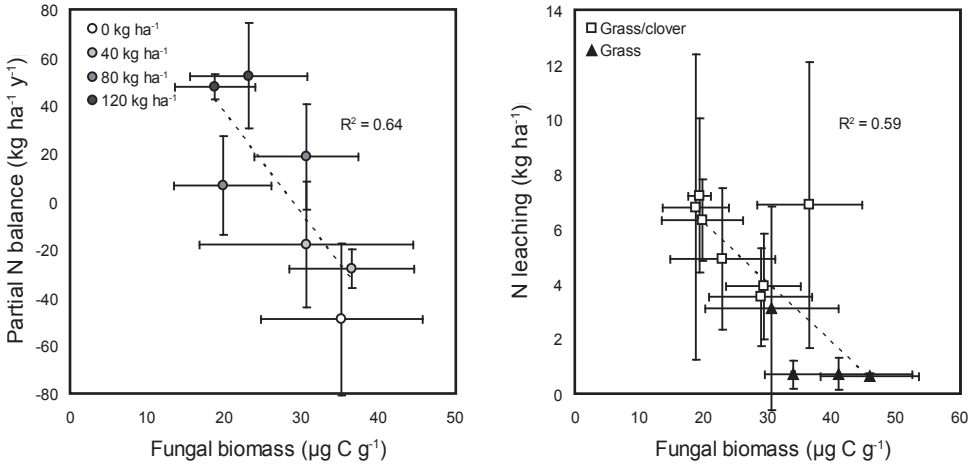


Fig. 3.4. Relationship between means of leaching and partial N balance and fungal biomass. Markers indicate means per treatment (see Table 1). Bars represent standard deviations, dashed lines represent regression with means (upper graph P = 0.006, lower graph P = 0.03)

swards, which was mainly caused by higher fungal biomass. It should be noted that factors used for converting bacterial numbers and hyphal lengths into biomass-values differed from ours. Often measures of fungal and bacterial biomass are made using phospholipid fatty acid analysis (Bardgett et al., 1996; Frostegard and Bååth, 1996). F/B ratios are then expressed as fungal PLFA/bacterial PLFA. Because of all the different methods and conversion factors for calculating F/B ratios, it is questionable to compare different studies.

In this study, fungal and bacterial biomass and the F/B ratio showed remarkably quick responses to changes in management and fertiliser regime. Already within three growing seasons after the start of the experiment fungi responded to crop and to N application rates.

In line with our first hypothesis, the F/B ratio decreased with increasing N application rate. This was mainly caused by a decrease of fungal biomass, while bacterial biomass remained approximately constant with increasing N application rate (Fig. 3.1). Both fungal and bacterial biomasses were lower under grass/clover than under grass.

In contrast to our hypothesis, manure type did not seem to be of much importance for determining F/B ratio and microbial biomass. Probably, the C/N ratios of the two manure types,

6.8 for slurry vs. 12.3 for farmyard manure, were not high enough to enhance fungi. Organic matter that has been reported to stimulate fungal growth had C/N ratios of 20 or higher (Vinten et al., 2002). Moreover, it often takes much longer than two years (decades) before changes in manure type have clear effects on soil organic matter quality and soil organisms (Bloem et al., 1997; Mäder et al., 2000; Bloem et al., 2004). Furthermore the knives of the slit-injector may have been to far apart (20 cm) to have a detrimental effect on mycelial networks.

The presence of white clover had a negative effect on fungal biomass and F/B ratio. This result seems to be in contrast with reports on a higher arbuscular mycorrhizal colonisation in clover than in grass (Ryan and Ash, 1999; Zhu et al., 2000). This was attributed to the finer branched root system and root hairs of grass, which makes it less dependent on mycorrhizal fungi. Gamper et al (2004), however, found higher levels of colonisation in rye grass compared to white clover. It should be recognised that a higher level of colonisation (expressed as percentage of root length infected) does not necessarily mean also a higher biomass of mycorrhizal hyphae per gram of soil. Moreover, we do not know which fraction of the total fungal biomass consists of mycorrhizas and which fraction consists of saprophytic fungi. Not only fungal biomass but also bacterial biomass and activity were greater under grass than under grass/clover (Fig. 3.1, Table 3.3). This might be a consequence simply of the denser root system, and thus higher rhizosphere/bulk soil ratio, of perennial ryegrass compared to white clover (Schortemeyer et al., 1997). However, white clover has a much higher ammonium exudation than perennial ryegrass (Paynel and Cliquet, 2003) which theoretically might affect fungal abundance negatively and bacterial biomass positively. However, Breland and Bakken (1991) did not find any profound effect of plant species on microbial immobilisation of carbon and nitrate in the rhizosphere.

Although grass/clover mixtures have been reported to have a higher aboveground production (Elgersma and Hassink, 1997), as could also be seen in this study, this did not increase the amount of soil organic matter in the two years the experiment had lasted. Clover can change the C/N ratio of the soil organic matter because the litter has a lower C/N ratio compared to grass (Neergaard et al., 2002). We did not find a lower C/N ratio of the soil organic matter in grass/clover treatments compared to grass treatments, but the N content of the removed crop was higher in grass/clover than in grass. This implies that the difference in fungal biomass between grass and grass/clover might be attributed to the difference in litter quality.

If more nitrogen in root exudates or litter from white clover suppresses the amount of fungi, this does not explain why bacteria were less abundant in grass/clover than in grass. Some unknown effects of exudates of grass or clover might be involved.

Despite the narrow pH range in our experiment, we found that the F/B biomass ratio decreased with increasing pH (Fig 3.2). This was caused by an increasing bacterial biomass rather than a decreasing fungal biomass. These results were consistent with other reports. Fungi can stand low pH better than bacteria (Swift et al., 1979). Bardgett et al. (1993) reported an increase in the amount of total mycelium with increasing acidity. Also effects of pH on the fungal-to-bacterial substrate induced respiration were reported: the respiratory activity of fungi increased with acidification, while that of bacteria decreased (Blagodatskaya and Anderson, 1998). Bååth and Anderson (2003) reported a decreasing F/B respiration ratio with increasing pH, but a slightly increasing F/B biomass ratio (using PLFA technique). Bacterial activity increased with increasing pH, as has been reported several times (Bååth, 1996; Bååth, 1998; Bååth et al., 1995; Pennanen et al., 1998).

N leaching was higher in grass/clover than in grass, while fungal biomass was higher in

grass. No clear relationship was found for leaching and fungal biomass when single observations were used, but regression analysis with means of treatments resulted in a negative relationship. Also, when analysed in the same way, means of partial N balance showed a relationship with means of fungal biomass (Fig. 3.4). A higher fungal biomass thus indicates a system with lower N fertilisation, which results in a more negative partial N balance (or smaller N surplus) and a lower N leaching potential.

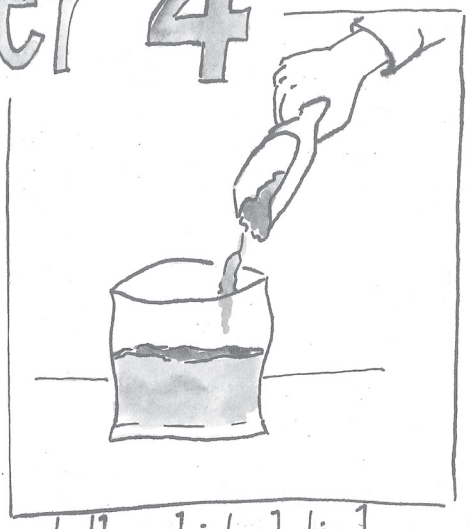
However, a causal relationship cannot be drawn from these results. A field with a higher fungal biomass does not necessarily coincide with a low N leaching potential. Leaching and the partial N balance were not correlated indicating that processes unaccounted for, such as volatilisation and denitrification were of differential importance across the treatments. A complete N balance is needed to explain the relationships between fungal biomass, F/B ratio and N leaching. Our results in general support the suggestion that the F/B ratio is indicative for a sustainable system with lower N losses, but more observations are needed to confirm the relationship between F/B ratio and sustainability.

Acknowledgements

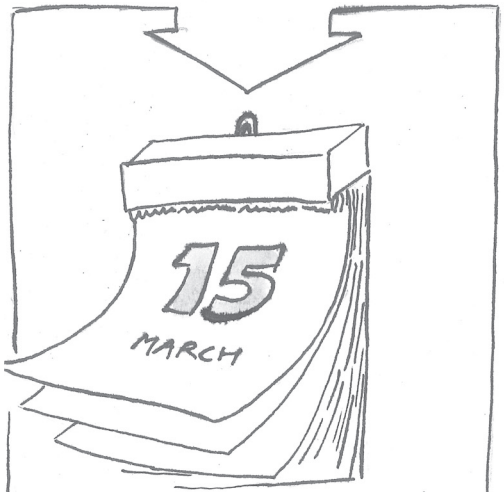
We thank An Vos, Meint Veninga and Popko Bolhuis for technical assistance. We thank Thom Kuyper, Ron de Goede, Kor Zwart and Annemieke Smit for support and helpful discussions. Ellen Heeres and the staff of Aver Heino made it possible to use the field trial.

Chapter 4

I took soil samples, sieved them,

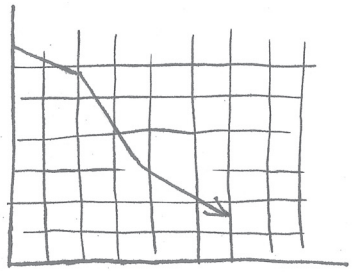


put the soil into plastic bags...



incubated them for 4 weeks...

Already after 2 weeks fungal biomass decreased!



(...this almost looks like...)

High turnover of fungal hyphae in incubation experiments

4 High turnover of fungal hyphae in incubation experiments

Franciska T. de Vries, Erland Bååth, Thom W. Kuyper and Jaap Bloem

Abstract

Soil biological studies are often conducted on sieved soils without the presence of plants. However, soil fungi build delicate mycelial networks, often symbiotically associated with plant roots (mycorrhizal fungi). We hypothesised that as a result of sieving and incubating without plants total fungal biomass decreases. To test this, we conducted three incubation experiments. We expected total and arbuscular mycorrhizal (AM) fungal biomass to be higher in less fertilised soils than in fertilised soils, and thus to decrease more during incubation. Indeed, we found that fungal biomass decreased rapidly in the less fertilised soils. A shift towards thicker hyphae occurred, and the fraction of septate hyphae increased. However, analyses of PLFAs and NLFAs could not clarify which fungal groups were decreasing. We propose that in our soils, there was a fraction of fungal biomass that was sensitive to fertilisation and disturbance (sieving followed by incubation without plants) with a very high turnover (possibly composed of fine hyphae of AM and saprotrophic fungi), and a fraction that was much less vulnerable with a low turnover (composed of saprotrophic fungi and runner hyphae of AMF). Furthermore, PLFAs might not be as sensitive to detect changes in fungal biomass as previously thought.

Based on: De Vries, F.T., E. Bååth, T.W. Kuyper, and J. Bloem. 2009. High turnover of fungal hyphae in incubation experiments. FEMS Microbiology Ecology, in press.

4.1 Introduction

Fungi are important soil organisms. In agricultural soils, saprotrophic fungi are—together with bacteria—the main decomposing organisms and thereby form the base of the food web; mycorrhizal fungi play an important role in plant nutrition. Although much research has been done on the ecology of fungi and their impact on soil structure and ecosystem processes, they remain an elusive group of microorganisms because of their filamentous life form (Klein and Paschke, 2004). Field studies have shown that fungi are sensitive to physical disturbance and fertilisation (Beare et al., 1997; De Vries et al., 2006; De Vries et al., 2007; Frey et al., 1999; Johnson et al., 2005). Laboratory experiments are a useful tool to investigate the impacts of fungi on ecosystem processes. However, because of the filamentous life form and high sensitivity to physical disturbance of fungi, these experiments might not represent the situation in the field, especially when the soil is sieved and incubated without plants. More specifically, sieving might destroy hyphal networks and thereby kill the most sensitive fungi, and mycorrhizal fungi might not survive during incubation without a host plant.

Arbuscular mycorrhizal fungi (AMF) can make up a substantial amount of the total fungal biomass in agricultural soils (Gosling et al., 2006). It has been suggested that AMF are more susceptible to disturbance, fertilisation and disruption by soil invertebrates than saprotrophic fungi (Bradley et al., 2006; Johnson et al., 2005; Kabir et al., 1997). Ectomycorrhizal (EM) fungi, which constitute a large part of the total fungal biomass in forest soils, have been shown to decrease in the absence of a host plant (Bååth et al., 2004; Frostegård et al., 1996; Siira-Pietikäinen et al., 2001). Similarly, AMF might decrease in the absence of plants. Therefore, soil biological incubation studies are—as a result of sieving and incubating without plants—likely to induce changes in the relative contribution of saprotrophic and AM fungal biomass. Especially if soils from different systems are compared, for instance from more and less fertilised grasslands that differ in biomass of saprotrophic and AM fungi, incubation will have a differential effect on fungal biomass in these soils.

Changes in fungal biomass as a result of sieving or incubation might take place rapidly, even within the first weeks of incubation. AM fungal hyphae have been observed to die off within 5-6 days (Staddon et al., 2003), although this has been criticised by Zhu and Miller (2003), stating that the main components of AM fungal hyphae, chitin and glomalin, have much longer residence times in the soil. Olsson and Johnson (2005) observed that AM runner hyphae were persistent, whereas average longevity of the finely branched absorbing hyphae was only 5.3 days, which corresponds with earlier findings of Friese and Allen (1991) and Bago et al. (1998). Less information is available on longevity of saprotrophic fungal hyphae. Schmidt et al. (2007) reported microbial turnover times in soil in between 9 and 18 days, but they did not distinguish between bacteria and fungi. In their paper, they stress that turnover of microbial communities is an important missing piece in our understanding of nutrient cycling in terrestrial ecosystems.

Traditionally, total fungal biomass in soil has been quantified microscopically. AM fungal hyphae can be distinguished visually from hyphae of other fungi—in contrast to saprotrophic fungi, AM fungal hyphae generally have non-septate hyphae and irregular cell walls and show angular, unilateral branching (Bonfante-Fasolo, 1986). However, distinguishing AMF from saprotrophic fungi on the basis of these morphological structures is a highly time-consuming method.

Most recent studies measure saprotrophic fungal biomass using ergosterol, which is a compound of the membrane of EM and saprotrophic fungi, or the phospholipid fatty acid (PLFA) 18:2ω6,9, which is only present in the cell membranes of EM and saprotrophic fungi. PLFA 16:1ω5 has been used to quantify AM fungal biomass, but this fatty acid is present also in bacteria. Therefore, the neutral lipid fatty acid (NLFA) 16:1ω5, which is mainly present in AM fungal spores and therefore does not represent the active fraction, is a more specific indicator for the presence, but not always the biomass, of AMF (Olsson, 1999). If AM fungi would—like EM fungi—die off during incubation without plants, the decline of total fungal biomass could be used to estimate the fraction of AM fungal biomass, in a similar way as used for EM fungal biomass (Bååth, 2001).

The aim of the present study was to follow the dynamics of fungal hyphae during short-term incubation without plants. We hypothesised that as a result of sieving and incubating without plants total fungal biomass decreases, and we expected this decrease to be caused primarily by AMF dying off. To test this hypothesis, we conducted three incubation experiments. In all three experiments, we compared a less or unfertilised soil with a (more) fertilised soil. We expected total and AM fungal biomass to be higher in the less or unfertilised soils. Thus, we expected fungal biomass in the less or unfertilised soils to decrease more than in the (more) fertilised soils.

4.2 Methods and materials

4.2.1 Experiment 1

Two plots from an experimental field trial at Heino in the eastern part of the Netherlands (52°25′ north and 6°15′ east) were sampled. Both plots were sown with a grass-clover mixture (*Lolium perenne* L. and *Trifolium repens* L.) in 2001 and have since been fertilised with farm yard manure: one plot at a rate of 40 kg N ha^{-1} y^{-1}, the other plot at a rate of 80 kg N ha^{-1} y^{-1}. The two plots were chosen on the basis of their widely differing fungal/bacterial (F/B) biomass ratio (determined by microscopy): 0.19 for the plot fertilised with 80 kg N ha^{-1} y^{-1}, versus 0.69 for the plot fertilised with 40 kg N ha^{-1} y^{-1}. The field trial was situated on a sandy soil, for a detailed description of the field trial, see De Vries et al. (2006).

A bulk soil sample, consisting of 100 cores (0-10 cm depth, 3.5 cm diam.), was taken from each of the two plots in November 2004. The two samples were sieved (5 mm), homogenised and stored at 4° C for one week. The equivalent of 50 g of dry soil was weighed into a 250 ml serum bottle and adjusted to a moisture content of 25% (w/w). Flasks were closed with a cotton wool plug to prevent drying of the soil but to allow gaseous exchange. Flasks were weighed and rewetted to the initial moisture content weekly.

Of each soil, a control treatment without the addition of straw was incubated, as well as a treatment that received 2.5 mg milled wheat straw (C/N = 137) per gram of dry soil (soil C content was 18.5 mg C g^{-1} and 20.5 mg C g^{-1} for the 80 kg N ha^{-1} y^{-1} plot and 40 kg N ha^{-1} y^{-1} plot, respectively). The addition of the wheat straw was carried out for each experimental unit separately. The contents of the flasks were thoroughly mixed and incubated at 20° C. The flasks were sampled destructively and fungal biomass was determined after 0, 1 and 8 weeks of incubation. Each treatment was replicated 4 times. We assessed the dynamics of fungal biomass using microscopy.

4.2.2 Experiment 2

We conducted this experiment to test whether the observed decrease in fungal biomass in Experiment 1 would also occur in another soil type. Soil samples were taken in March 2005 from four plots of the Ossekampen Fertiliser Experiment in Wageningen. This trial was established in 1958 on a heavy riverine clay soil. Two of the four plots have been unfertilised ever since (plots 13O and 16O: pH-KCl 4.0, 20.0% organic matter in the top 5 cm soil layer), the other two plots have received 160 kg N, 74 kg P_2O_5 and 375 kg K_2O ha^{-1} y^{-1} (plots 11NPK and 19NPK: pH-KCl 3.8, 23.7% organic matter in the top 5 cm soil layer) (for details, see Elberse et al. (1983)).

From each plot, 50 cores (10 cm depth, 3.5 cm diam.) were bulked into one sample. After sieving and homogenising, moisture content was adjusted to 50% WHC. Triplicate samples from each plot (100 g soil) were incubated in polyethylene bags at 12°C (average soil temperature). Bags were sampled destructively and samples were analysed for fungal biomass after 0, 1, 2, and 4 weeks. As in Experiment 1, we assessed dynamics of fungal biomass using microscopy.

4.2.3 Experiment 3

This experiment was conducted to assess which fungal groups were causing the changes in fungal biomass observed in Experiment 2. The soil used for this experiment also came from the Ossekampen Fertiliser Experiment. In September 2005, we sampled the same plots as in Experiment 2. This time, duplicate samples from each of the four plots were incubated under the same conditions as in Experiment 2, and sampled destructively after 0, 1, 2, and 4 weeks. To assess the dynamics of total fungal biomass and AM fungi, we analysed fungal biomarkers and, as in the previous two experiments, we used microscopy. We measured hyphal diameters in the unfertilised samples before and after incubation.

4.2.4 Measurements of fungal biomass, fungal biomarkers and hyphal diameters

Microscopic slides were prepared and hyphal length was measured by epifluorescence microscopy as described by Bloem and Vos (2004). In addition, using transmitted light, hyphae were categorised into blue-stained and melanised hyphae. Fungal biomass (C) was estimated from hyphal lengths and biovolume using the calculations described by Bloem and Vos (2004). Hyphal diameters were measured at 1000x magnification. Of each slide, diameters of 100 fragments of blue-stained hyphae were measured and classified as septate or non-septate.

PLFA and NLFA biomarkers for AMF and saprotrophic fungi were analysed as described by Frostegård et al. (1993). PLFA 18:2ω6,9 was used to indicate saprotrophic fungi, NLFA 16:1ω5 was used to indicate AM fungal storage structures, and PLFA 16:1ω5 was used to indicate AM fungal hyphae (Frostegård and Bååth, 1996; Olsson and Johansen, 2000; Olsson et al., 1995), although the PLFA 16:1ω5 is also present in bacteria.

4.2.5 Statistical analyses

Fungal biomass data of Experiment 1 were analysed by a three-way ANOVA with factors fertiliser level (40 and 80 kg N ha^{-1} y^{-1}), straw amendment (straw and no straw) and incubation time (0, 1 and 8 weeks). Data of fungal and bacterial biomass and fungal biomarkers of Experiments 2 and 3 were analysed by a two-way ANOVA with factors fertiliser level (unfertilised and fertilised) and incubation time (0, 1, 2, 4 weeks). Hyphal diameters were divided into classes and the differences were analysed by t-tests. All statistical tests were done with the statistical package SPSS (SPSS Inc., Chicago, USA).

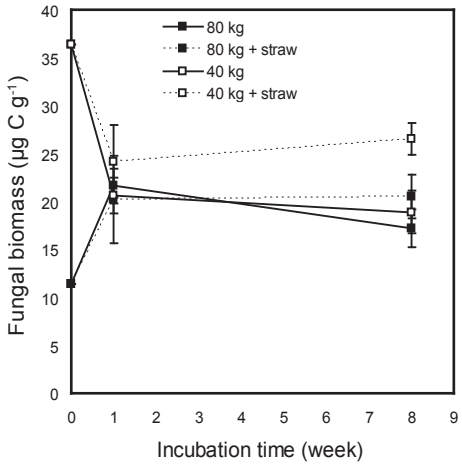


Fig. 4.1. Fungal biomass in Experiment 1, determined by microscopy. Symbols represent means ± 1 SE.

4.3 Results

4.3.1 Experiment 1

At the start of the incubation, fungal biomass in the 40 kg N ha^{-1} y^{-1} field was three times higher than in the 80 kg N ha^{-1} y^{-1} field. Fungal biomass in the two soils converged rapidly within one week of incubation (Fig 4.1.). Fungal biomass was significantly affected by fertilisation level and straw amendment ($P < 0.001$ and $P = 0.05$, respectively), but not by week ($P = 0.52$). However, fungal biomass in the 40 kg N ha^{-1} fertilised soil responded differently to incubation than fungal biomass in the 80 kg N ha^{-1} fertilised soil ($P = 0.017$ for the field x week interaction effect).

4.3.2 Experiment 2

Fungal biomass in the heavy riverine clay soil from the Ossekampen Fertiliser Experiment was 10 times higher than fungal biomass in the sandy loam soil of Experiment 1. At the start of the incubation, fungal biomass in the unfertilised treatments (13O and 16O) was two times higher than in the fertilised treatments (11NPK and 19NPK) (Fig. 4.2A). Fertilisation level and time had a significant effect on fungal biomass ($P < 0.001$ for both fertilisation level and time). An

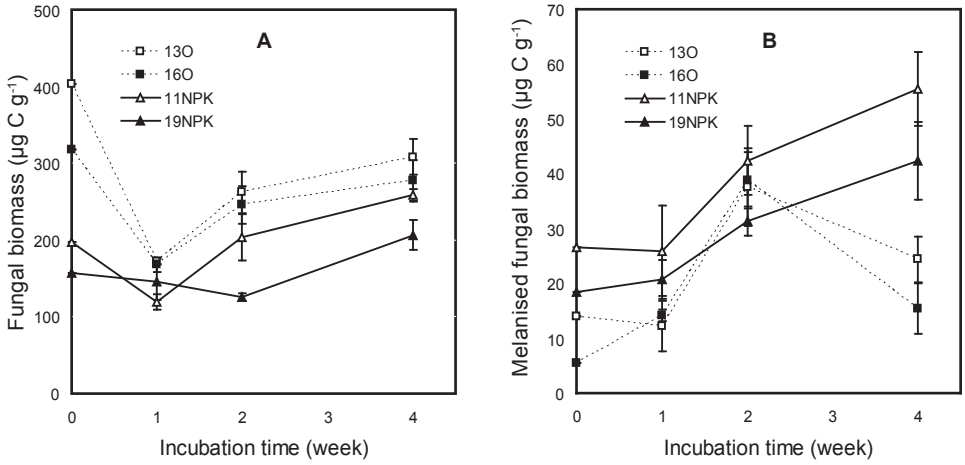


Fig. 4.2. Total fungal biomass (A) and melanised fungal biomass (B) in Experiment 2, determined by microscopy in unfertilised treatments (13O and 16O) and fertilised treatments (11NPK and 19NPK). Symbols represent means ± 1 SE.

interaction effect of fertilisation level and time was present (P = 0.02), indicating that fungal biomass in the unfertilised treatments responded differently to incubation than in the fertilised treatments. Already after one week of incubation, fungal biomass in the unfertilised treatments decreased, whereas fungal biomass in the fertilised soil hardly decreased or remained constant. The effect of time on fungal biomass was significant in the unfertilised treatments (P = 0.029),

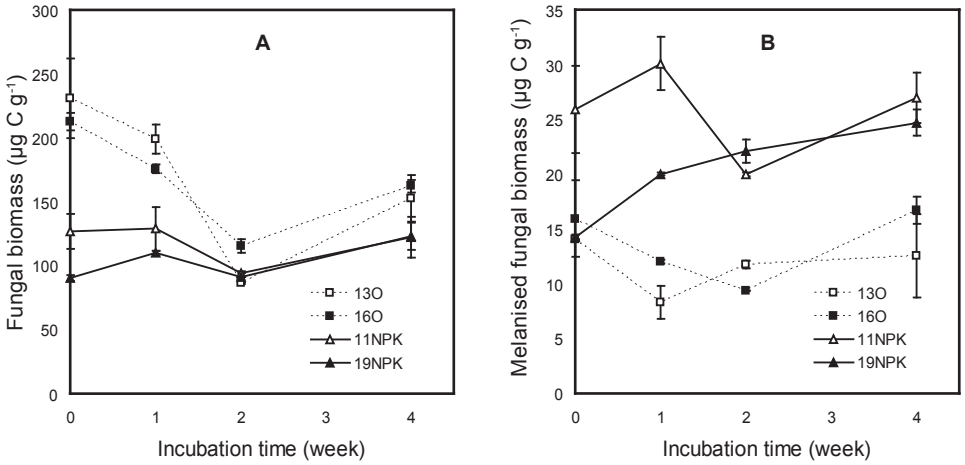


Fig. 4.3. Total fungal biomass (A) and melanised fungal biomass (B) in Experiment 3, determined by microscopy in unfertilised treatments (13O and 16O) and fertilised treatments (11NPK and 19NPK). Symbols represent means ± 1 SE.

but only marginally so in the fertilised treatments ($P = 0.077$).

In contrast to total fungal biomass, melanised fungal biomass was higher in the fertilised treatment than in the unfertilised treatment (Fig. 4.2B, $P = 0.001$). In both treatments, melanised fungal biomass was affected by time ($P < 0.001$). The effect of time differed between the treatments ($P = 0.005$ for the fertilisation level x time interaction).

4.3.3 Experiment 3

Microscopically measured fungal biomass (Fig. 4.3A, Table 1) and all fungal biomarkers were higher in the unfertilised treatments than in the fertilised treatments (Fig. 4.4A,B,C, Table 1).

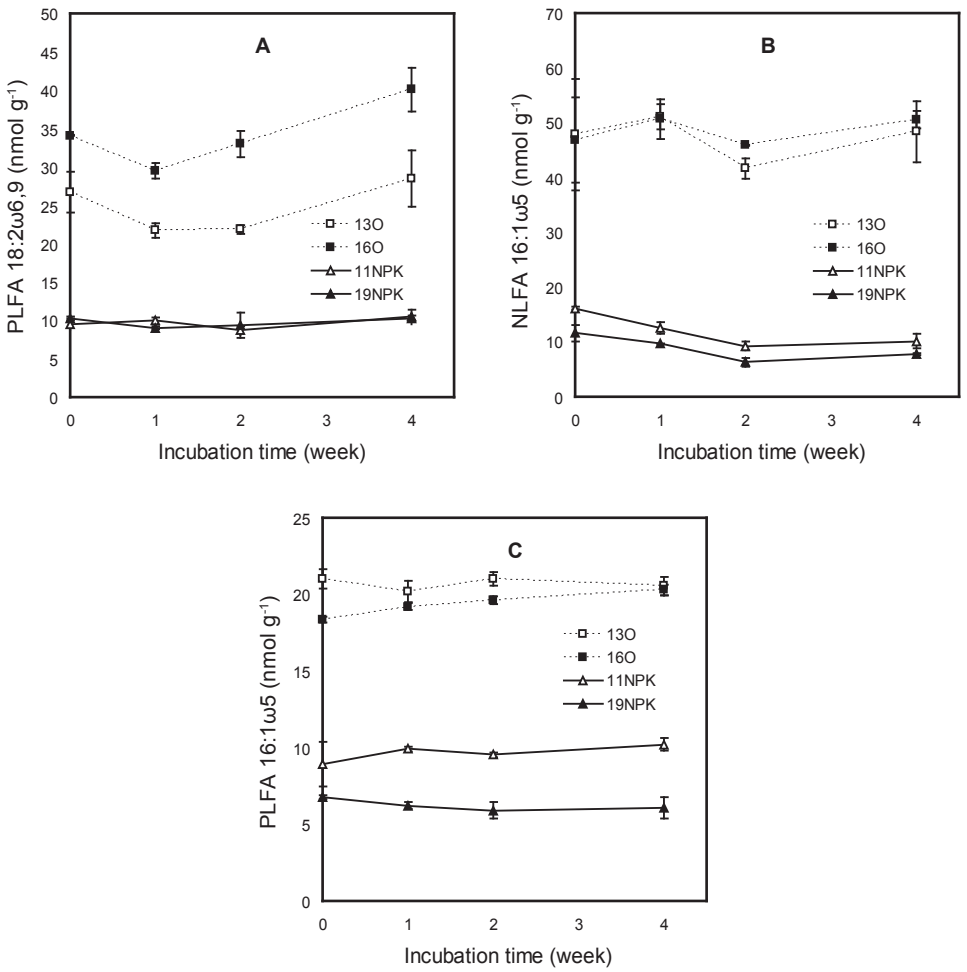


Fig. 4.4. Biomarkers for saprotrophic fungi (A), AM fungal spores (B), and AM fungal hyphae (C) in Experiment 3, in unfertilised treatments (13O and 16O) and fertilised treatments (11NPK and 19NPK). Symbols represent means ± 1 SE.

Table 1. Two-way ANOVA summary statistics of the effects of fertilisation level and incubation time on fungal biomass and biomarkers, in Experiment 3.

		Fungal C		Melanised fungal C		PLFA 18:2ω6,9		NLFA 16:1ω5		PLFA 16:1ω5	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Fertilisation level	1	73.1	<0.001	50.8	<0.001	165	<0.001	522	<0.001	413	<0.001
Incubation time	3	20.8	<0.001	1.6	0.22	1.9	0.15	2.1	0.13	0.14	0.93
Fertilisation x time	3	11.8	<0.001	1.9	0.16	1.2	0.34	1.0	0.41	0.14	0.94

Abbreviations: Fungal C, microscopically determined fungal biomass; Melanised fungal C, microscopically determined biomass of melanised fungi; PLFA 18:2ω6,9, biomarker for saprotrophic fungi; NLFA 16:1ω5, biomarker for AM fungal spores; PLFA 16:1ω5, biomarker for AM fungal hyphae.

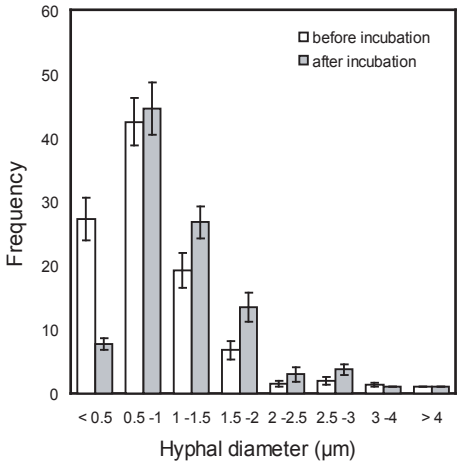


Fig. 4.5. Hyphal diameters in unfertilised treatments before and after four-week incubation in Experiment 3. Bars represent mean frequencies ± 1 SE.

However, microscopically measured fungal biomass decreased after two weeks of incubation, whereas fungal biomarkers remained constant during the whole incubation period. Incubation time affected fungal biomass more strongly in the unfertilised treatments than in the fertilised treatments (Fig. 4.3A, P < 0.001 for the interaction effect of fertilisation level x time, Table 1). This difference in the effect of time between unfertilised and fertilised treatments was not present in any of the fungal biomarkers (Table 1). Biomass of melanised fungi was—unlike total fungal biomass—higher in the fertilised treatments than in the unfertilised treatments (Fig. 4.3B, Table 1). In contrast to the previous experiment, biomass of melanised fungi was not affected by incubation time (Table 1).

The distribution of hyphal diameters in the unfertilised treatments had shifted as a result of the incubation (Fig. 4.5). After 4 weeks of incubation, frequency of hyphae with diameters smaller than 0.5 µm had decreased ($P < 0.001$), while frequency of thicker hyphae with diameters between 1 and 1.5 µm and between 1.5 and 2 µm had increased ($P = 0.045$ and $P = 0.025$,

respectively). Percentage of septate hyphae was higher as a result of incubation, 27.5 ± 1.73 after incubation vs. 18.7 ± 5.68 before incubation ($P = 0.013$).

4.4 Discussion

We set out to monitor the dynamics of fungal hyphae in short-term incubation experiments without plants. We hypothesized that total fungal biomass would decrease as a result of sieving and incubating, and that total and AM fungal biomass would be higher in the less or unfertilised treatments than in the fertilised treatments. This was the case for microscopically measured fungal biomass in all three experiments. Also, biomarkers for saprotrophic and AM fungi were highest in the unfertilised treatments of Experiment 3. As we hypothesized, fungal biomass decreased during the incubation, but only in the less or unfertilised treatments. This decrease set in rapidly: either after one week (Experiments 1 and 2) or after two weeks of incubation (Experiment 3).

We observed a large difference in fungal biomass between the sandy soil and the riverine clay soil. Fungal biomass in the clay soil was 10 times higher than in the sandy soil. As organic matter and clay content have been reported to be positively correlated to (saprotrophic) fungal biomass (e.g. Van der Wal et al., 2006), these factors may explain the difference in fungal biomass between the two soils.

We expected AM fungal hyphae to decrease more than saprotrophic fungal hyphae. To test this, we measured AM fungal biomarkers and saprotrophic fungal biomarkers. However, all fungal biomarkers remained constant during the incubation. Nonetheless, percentage of septate hyphae increased during the experiment, indicating that this group of fungi was less affected by the incubation than non-septate hyphae. Since AM fungi are non-septate (Bonfante-Fasolo, 1986), this could signify that AM fungi were dying off during the first weeks of incubation. This quick decline of (presumably AM) fungal biomass is remarkable, since Bååth et al. (2004) incubated for ten months to estimate EM fungal biomass by the decline in total fungal biomass (measured by ergosterol and PLFA 18:2ω6,9, which are both present in EM fungi and in saprotrophic fungi).

Melanised fungal hyphae did not decrease during the experiment. Melanins in fungal hyphae are associated with protection from environmental stress and microbial degradation (Bell and Wheeler, 1986; Butler and Day, 1998). Either melanised fungi were not affected by the incubation, or their hyphae were too recalcitrant to decompose during the incubation. Although AM fungal spores can be melanised (Purin and Rillig, 2008), AM fungal hyphae are not. From the distribution of hyphal diameters before and after incubation, we can conclude that after four weeks, almost all blue-stained hyphae with a diameter smaller than 0.5 µm had disappeared (Fig. 4.5). These thin hyphae might represent branched absorbing structures (BAS) of the extraradical mycelium of AM fungi (Bago et al., 1998). Third order BAS branches can be very thin and are known to have a lifespan between 2.5 and 10 days (Bago et al., 1998). However, the hyphae that disappeared in our experiment are much thinner than the 2 µm that Bago et al. reported. The observed shift towards thicker hyphae could just denote the higher decomposability of thinner hyphae and might not indicate the dying off of a specific group of thin fungi.

The discrepancy between microscopic measurements and concentrations of fatty acids is noteworthy. Although both methods showed comparable differences between treatments at the start of the incubation, microscopy indicated decreasing fungal biomass while PLFAs remained

constant throughout the incubation. This is surprising, because PLFAs are assumed to decompose rapidly after cell death—although actually there is only one reference for this (White et al., 1979). However, in a study on heating effects on peat, PLFAs degraded more slowly at low temperatures, which was attributed to slower enzymatic reactions (Ranneklev and Bååth, 2003). Therefore, the low incubation temperature of Experiment 3 (12°C, which is the average annual soil temperature) might explain why PLFAs did not disappear in the current experiment.

Another explanation for the discrepancy between microscopy and PLFAs could be that the decrease in fungal biomass was mainly caused by the disappearance of thin hyphae. Since PLFAs are a compound of the cell membrane, the amount of PLFAs would be linearly related to hyphal surface, and thus increase with a factor four if hyphal diameter increases with a factor two. The high contribution of thick hyphae to PLFAs could camouflage the decrease of thin hyphae. Especially melanised hyphae, which did not disappear during the incubation and which can be up to 10 times thicker than blue-stained hyphae, might have had a relatively high contribution to the amount of PLFAs. In addition, much of the carbon present in thin hyphae can be translocated into storage structures and thick hyphae (Bago et al., 2002; Olsson and Johnson, 2005). This translocation might—apart from the apparent low degradability—explain why PLFAs did not disappear at the same rate as fungal hyphae.

Fungal PLFAs have been proven to adequately indicate differences in fungal biomass between treatments or systems under stable field conditions, and to reflect fungal growth under laboratory conditions (e.g. Balser et al., 2005; Gordon et al., 2008; Meidute et al., 2008; Rousk and Bååth, 2007). Compared to the use of ergosterol, the use of PLFAs is a relatively recent method. The decomposability of ergosterol under laboratory conditions has been under discussion (Mille-Lindblom et al., 2004; Shand et al., 1995; Zhao et al., 2005) and PLFAs have been suggested to better reflect negative treatment effects (Högberg, 2006). The current experiment shows that also PLFAs can fail to detect negative treatment effects on fungal biomass.

Microscopic counts have been criticised, because they do not accurately represent the living fraction of fungal biomass (Frankland, 1975). Because hitherto fungal hyphae have been assumed to degrade slowly in soil, microscopic counts would not be suitable for detecting negative treatment effects. In the current experiment, we demonstrated that microscopically determined hyphal lengths can reflect changes in fungal biomass even quicker than analysis of fungal PLFAs.

The rapid degradation of fungal hyphae we found has never been observed before. The rapid decrease of fungal hyphae is in line with a recent—though criticized (Olsson and Johnson, 2005; Zhu and Miller, 2003)—study by Staddon et al. (2003), but is in contrast with the general assumption that fungal hyphae degrade slowly in soil. This assumption is based on the relatively recalcitrant components present in the fungal cell wall (Kassim et al., 1981; Martin and Haider, 1979), and on protection from degradation through interaction of fungal hyphae and soil aggregates (Simpson et al., 2004). As we discussed above, non-melanised fungal hyphae might degrade more rapidly than melanised fungal hyphae. In addition, after sieving the soil, as we did in the current incubation experiments, the aggregate structures that protect fungal hyphae from degradation will be disrupted.

In summary, microscopic counts showed that during short-term incubation, part of the fungal biomass died off. As we hypothesised, fungal biomass was higher in the less or unfertilised treatments, and these treatments also showed the largest decrease in fungal biomass. Although there were some indications, we could not confirm that AMF were decreasing more than

saprotrophic fungi. We propose that in our soils, there was a fraction of fungal biomass that was sensitive to disturbance and fertilisation with a very high turnover, and a fraction that was much less vulnerable with a low turnover. The first fraction possibly consisted of fine hyphae of both AMF and saprotrophic fungi, the second fraction possibly consisted of saprotrophic fungi and runner hyphae of AMF. Our results show that incubation or pre-treatment of soil samples can rapidly nullify long-lasting differences in fungal biomass between unfertilised and fertilised soils. Furthermore, PLFAs might not be as sensitive to detect changes in fungal biomass as previously thought.

Acknowledgements

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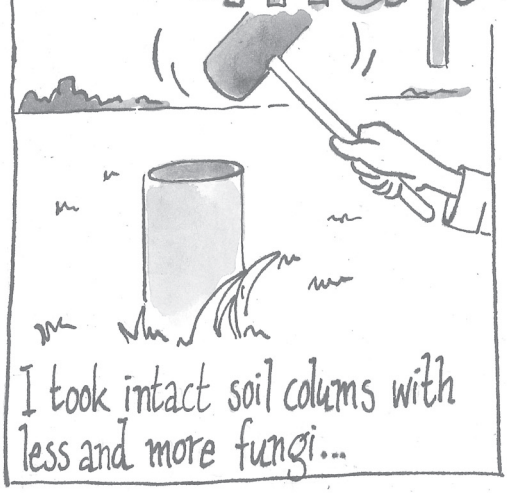
Chapter 5



Soil fungi reduce nitrogen losses from grasslands

5 Soil fungi reduce nitrogen losses from grasslands

Franciska T. de Vries, Jan Willem van Groenigen, Ellis Hoffland and Jaap Bloem

Abstract

Nitrogen losses from grasslands cause eutrophication of ground- and surface water and contribute to global warming and atmospheric pollution (Tilman, 1999). High soil fungal biomass is widely assumed to reduce nitrogen losses (Bardgett, 2005; Bardgett and McAlister, 1999; Van der Heijden et al., 2008; Wardle et al., 2004). However, this assumption is only based on the dominance of fungi in systems with low nutrient availability (Coleman et al., 1983), without nitrogen losses from such systems ever having been quantified. Here we report the first experimental evidence that soil fungi decrease nitrogen losses. Nitrous oxide emission and denitrification were lower in soil with relatively high fungal biomass than in soil with relatively low fungal biomass. After addition of fertiliser, nitrogen leaching increased in the low fungal biomass soil but not in the high fungal biomass soil, which we attribute to the higher immobilisation of nitrogen into microbial biomass in the high fungal biomass soil. We conclude that higher immobilisation and lower mineralisation rates reduce nitrate availability in soil with higher fungal biomass, which in turn reduces nitrogen leaching and denitrification. Our results provide a mechanistic basis for the widespread assumption that fungi reduce nitrogen losses. In the face of increasing attention for soil-based ecosystem services such as nutrient and carbon retention, this research is an important step in understanding the role of soil microbes in nutrient cycling.

Fungi and bacteria are the main organisms decomposing organic matter in soils. They both support their own food chain of soil fauna, and soil ecosystems are often characterized by having a fungal-dominated or bacterial-dominated decomposition pathway (Wardle et al., 2004). Fungal-dominated food webs occur in relatively undisturbed, late-successional sites with a high organic matter content and low resource quality (Coleman et al., 1983). Because of the low nutrient availability in these systems, fungi are associated with low nitrogen losses to groundwater and atmosphere. Repeated observations of decreasing fungal biomass with intensification of management have led to the inference that fungal dominance is indicative for ecosystems with low nitrogen losses (Bardgett and McAlister, 1999; De Vries et al., 2006; De Vries et al., 2007). Although this inference is widespread in ecology, it has never been properly tested. Several mechanisms have been proposed. First, fungal-dominated food webs have been proposed to have lower nitrogen mineralisation rates. Fungi have higher carbon assimilation efficiencies than bacteria (Holland and Coleman, 1987). Because carbon (C) and nitrogen (N) mineralisation are coupled stoichiometrically (Hessen et al., 2004), fungi should mineralise less nitrogen per unit biomass than bacteria. In addition, because of the higher C/N ratio of fungi coupled with the smaller biomass and lower turnover rates of fungal-feeding fauna than their bacterial-feeding counterparts, grazing on fungi releases less N than grazing on bacteria (Chen and Ferris, 2000; De Ruiter et al., 1993). Second, it has been proposed that fungi immobilise readily available N in their biomass, especially under nutrient-limiting conditions (Holland and Coleman, 1987). In that case, N that is captured in fungal hyphae will be less easily remineralised than N in bacterial cells, as fungal hyphae are more persistent in the soil than bacterial cells (Martin and Haider, 1979). Third, it has been proposed that fungal hyphae increase aggregate formation in soil (Ritz and Young, 2004) and thereby protect organic matter from mineralisation. Finally, the higher abundance of arbuscular mycorrhizal fungi (AMF) in fungal-dominated soils might increase plant N uptake, which should reduce N leaching losses (Smith and Read, 1997).

To investigate whether fungal-dominated soils indeed show lower N losses, and to establish the mechanisms involved, we did a factorial experiment with two levels of fungal biomass and two levels of fertilisation. We sampled intact soil columns from two grass-clover plots differing in fungal biomass (De Vries et al., 2006) and took them to the greenhouse. Half of them were fertilised with ammonium nitrate (34 mg N, equivalent to 30 kg N ha^{-1}). All columns were watered weekly (350 ml, equivalent to 30 mm rainfall) for four weeks, and nitrogen leaching and nitrous oxide emission were measured at regular intervals. To determine the fate of fertiliser nitrogen, we added ^{15}N-labelled nitrogen as $^{15}NH_4^{15}NO_3$ in a second experiment with a comparable set up. The difference in fungal biomass between the two soils we used resulted from a long-term difference in fertilisation rate (40 vs. 80 kg N ha^{-1} y^{-1} as farm yard manure) (De Vries et al., 2006). Other soil properties, including mineral N, were very similar (see Supplementary Table 5.1). Differences in fungal biomass had been consistent for more than 5 years (see Supplementary Figure 5.1) and levels of fungal biomass were representative for Dutch agricultural grasslands (De Vries et al., 2007). For a truly factorial experiment, we should have modified fungal biomass ourselves instead of using soils with different fertilisation history. However, modifying the microbial community without affecting other soil properties is impossible, which makes our set-up the best alternative.

During the experiment, fungal biomass increased in all treatments (Fig. 5.1). It remained highest in the high fungal biomass soil ($F_{1,19} = 56.29$, $P < 0.001$), and was reduced by fertilisation

($F_{1,19}$ = 36.93, P < 0.001). The reducing effect of fertilisation on fungal growth was stronger in the high fungal biomass soil than in the low fungal biomass soil (fungal biomass x fertilisation interaction $F_{1,19}$ = 8.81, P = 0.008), which resulted in convergence of fungal biomass in the unfertilised low fungal biomass soil and the fertilised high fungal biomass soil (Fig. 5.1). This indicates that although differences in fungal biomass had been consistent for 5 years in the field, these differences were neutralized within 4 weeks after addition of inorganic fertiliser. Negative effects of inorganic nitrogen on fungi are mostly attributed to changes in vegetation and organic matter characteristics(Bardgett et al., 1999), but our results suggest a direct inhibiting effect on fungal growth, possibly by repression of enzyme activity or formation of toxic substances(Fog, 1988).

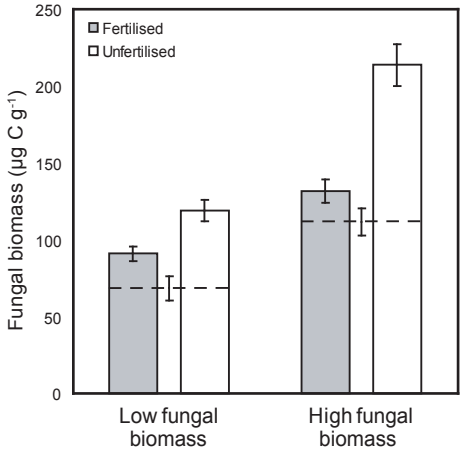


Fig. 5.1. Effects after 4 weeks of N fertilisation on fungal biomass in low vs. high fungal biomass soil. Dashed lines indicate fungal biomass at the start of the experiment (mean ± 1 s.e.m., n = 3), which differed significantly between the two soils (t-test, P = 0.011). Bars denote fungal biomass at the end of the experiment (mean ± 1 s.e.m., n = 6).

Nitrous oxide emission ($F_{1,30}$ = 7.36, P = 0.011) (Fig 5.2B, Table 5.1) as well as cumulative denitrification (Table 5.1) was lowest in the high fungal biomass soil. Because denitrification rates are driven by nitrate availability (Cavigelli and Robertson, 2000), these results indicate that nitrate availability was lower in the high fungal biomass soil. Fertiliser addition increased nitrous oxide emission ($F_{1,30}$ = 5.60, P = 0.025) (Fig 5.2B, Table 5.1) and total denitrification (Table 5.1) in both soils. We found a negative relationship between fungal biomass and nitrous oxide emission in the unfertilised treatments, but not in the fertilised treatments (Fig. 5.3B). These results highlight the fact that nitrate availability was a stronger control on denitrification than microbial community composition, because the dramatically increased nitrous oxide emission after fertiliser addition masked the relationship with fungal biomass. The negative relationship we found between fungal biomass and nitrous oxide emission and total denitrification conflicts with findings of fungi dominating denitrification in a grassland soil (Laughlin and Stevens, 2002).

Table 5.1. Fertilisation effects on nitrogen pools and loss pathways in low vs. high fungal biomass soil.

	Mineral N leached	Organic N leached	$N\text{-}N_2O$ evolved	Estimated total denitrification	Total N lost	Plant N uptake	Soil mineral N	Microbial biomass N
Low fungal biomass								
Fertilised	11.9(1.0)	1.5(0.1)	1.3(0.2)	8.7(1.2)	23.3(0.9)	31.4(2.5)	16.3(1.4)	243(8.3)
Unfertilised	3.9(1.0)	1.3(0.1)	0.7(0.1)	3.4(0.5)	9.3(1.6)	29.4(5.9)	18.1(1.7)	248(13.3)
High fungal biomass								
Fertilised	6.4(0.9)	2.2(0.2)	0.9(0.2)	6.1(1.6)	15.5(2.4)	30.6(4.3)	19.3(3.0)	218(15.4)
Unfertilised	4.5(0.8)	2.3(0.1)	0.4(0.1)	1.9(0.5)	9.2(1.3)	32.1(5.6)	13.0(1.2)	217(10.8)
***F* (*P*-value)**								
Fungal biomass	1.34(0.3)	36.79(<0.001)	5.81(0.022)	6.47(0.016)	3.23(0.079)	0.02(0.9)	0.78(0.4)	5.36(0.032)
Fertilisation	27.10(<0.001)	0.07(0.8)	14.05(0.001)	21.56(<0.001)	38.66(<0.001)	0.47(0.5)	1.55(0.2)	0.02(0.9)
Fungal biomass x fertilisation	7.90(0.008)	1.48(0.2)	0.05(0.8)	0.12(0.7)	4.12(0.049)	0.32(0.6)	4.74(0.042)	0.05(0.9)

Values represent means (s.e.m.), all values are in kg N ha^{-1}. Estimated total denitrification was calculated using interpolated N_2O/N_2 ratios. Total nitrogen lost is the sum of mineral N leached, organic N leached, nitrous oxide evolved and estimated total denitrification.

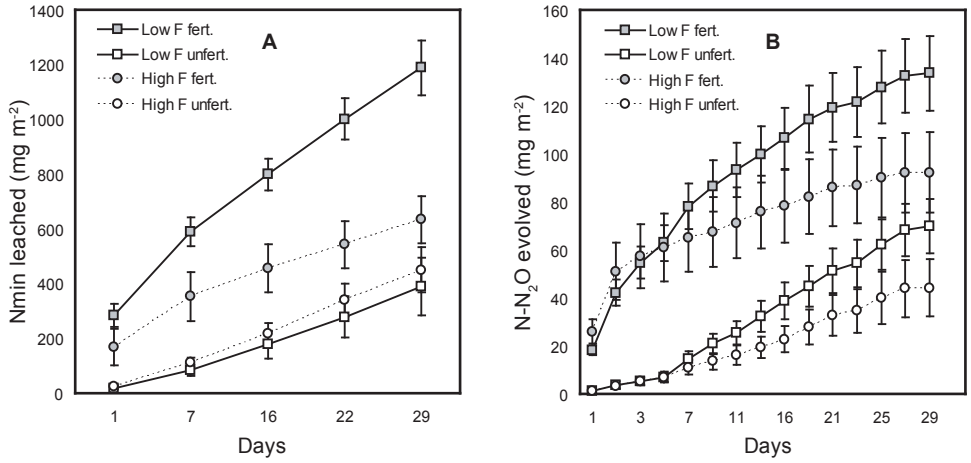


Fig. 5.2. Cumulative amounts of nitrogen lost following different N fertilisation treatments in low vs. high fungal biomass soil. A, mineral nitrogen leached. B, nitrous oxide evolved. Symbols represent means ± 1 s.e.m. (n = 9). Abbreviations: Low F fert., low fungal biomass fertilised; Low F unfert., low fungal biomass unfertilised; High F fert., high fungal biomass fertilised; High F unfert., high fungal biomass unfertilised.

Surprisingly, the addition of fertiliser had a differential effect on mineral N leaching in the low and high fungal biomass soil (fungal biomass x fertilisation interaction $F_{1,31}$ = 11.47, P = 0.002). In the high fungal biomass soil, fertilisation did not increase leaching of mineral N, whereas in the low fungal biomass soil, leaching was increased more than threefold (Fig. 5.2A). This is in accordance with results from an earlier experiment (see supplementary Fig. 5.2) and with trends observed in the field (De Vries et al., 2006). Nitrogen leaching did not differ between the unfertilised controls of both systems (Fig. 5.2A, Table 5.1) and neither did plant N uptake (Table 5.1), which suggests that mineralisation rates did not differ between the two soils. However, we already saw that as a result of lower nitrate availability, nitrous oxide emission and denitrification were lower in the high fungal biomass soil. Combined, our denitrification and leaching data suggest that denitrification is a more sensitive proxy for nitrate availability than leaching.

Because plant N uptake did not differ between treatments (Table 5.1), we can reject the fourth proposed mechanism of AMF-enhanced plant nitrogen uptake. In addition, the two soils did not differ in their micro- or macro-aggregate size distribution (data not shown), so we can discard the third mechanism as well. This leaves open the first and the second mechanism: lower mineralisation rates in fungal-based food webs and immobilisation of inorganic nitrogen into fungal biomass. The mechanism of lower mineralisation rates is supported by the lower nitrous oxide emission and total denitrification in high fungal biomass soil, which was the result of lower nitrate availability. Furthermore, fertiliser addition did not increase leaching in the high fungal biomass soil (Fig. 5.2A), which favours the second mechanism of immobilisation of added fertiliser nitrogen.

The mechanism of fungal immobilisation of fertiliser nitrogen is further supported by the negative correlation between fungal biomass and nitrogen leaching in the fertilised treatments (Fig. 5.3B). Although microbial biomass nitrogen did not differ between treatments, in the

additional experiment microbial immobilisation of fertiliser nitrogen was higher in the high fungal biomass soil. Two weeks after addition of ^{15}N-labelled fertiliser (equivalent to 30 kg ^{15}N ha^{-1}) 0.86 ± 0.16 kg ^{15}N ha^{-1} was immobilised in the fertilised high fungal biomass soil, compared with 0.48 ± 0.12 kg ^{15}N ha^{-1} in the low fungal biomass soil (t-test, P = 0.045, n = 6). This is a relatively small difference compared to the difference in N losses (Table 5.1). However, the peak of microbial immobilisation in soil generally occurs within the first few days after nitrogen

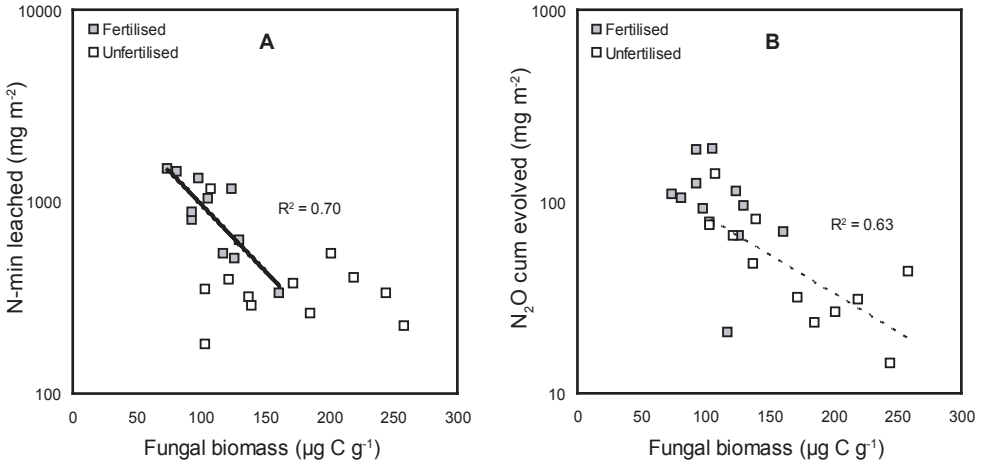


Fig 5.3. Relationships between fungal biomass and total amounts of nitrogen lost. A, relationship between fungal biomass and mineral nitrogen leaching in fertilised and unfertilised treatments. The correlation was significant for the fertilised treatments (solid line, $F_{1,9}$ = 20.59, P = 0.001). B, relationship between fungal biomass and total nitrous oxide emissions in fertilised and unfertilised treatments. The correlation was significant for the unfertilised treatments (dashed line, $F_{1,10}$ = 16.79, P = 0.002). Symbols represent single observations.

addition (Dunn et al., 2006; Recous et al., 1990). Since we measured after two weeks to certify that differences in nitrogen losses were significant, dilution and remineralisation are likely to have reduced the microbial ^{15}N recovery in our experiment.

These results illustrate that microbial immobilisation of ammonium can reduce nitrogen losses from agricultural grassland soils. Moreover, they indicate that the role of microbial immobilisation of nitrogen in productive, not nitrogen-limited grassland soils has been underestimated so far(Bardgett et al., 2003). Nitrification and immobilisation are the main competing pathways for ammonium in acid and neutral soils, and it has been shown that the ratio between the two correlates to nitrate leaching (Stockdale et al., 2002). In grassland soils, the dominant sink for ammonium is nitrification (Cookson et al., 2007), and here we show that immobilisation of ammonium by microbes can reduce nitrification. It is well accepted that heterotrophic microorganisms preferentially take up ammonium over nitrate (Recous et al., 1990). Although it is not possible to distinguish between immobilisation by bacteria and fungi, it has been suggested that fungi can access, and therefore immobilise, the non-mobile ammonium better than bacteria, because of their filamentous growth form (Myrold and Posavatz, 2007).

Our findings point out that the lower nitrogen losses in the high fungal biomass soil were

caused by a combination of the first and the second mechanism: first, nitrate availability was lower as a result of lower mineralisation and nitrification rates. These, in turn, could be caused by higher fungal carbon assimilation efficiencies (Holland and Coleman, 1987), slower degradation of fungal hyphae (Martin and Haider, 1979), and/or lower nitrogen excretion by fungal-feeding fauna (Chen and Ferris, 2000). This lower nitrate availability reduced nitrous oxide emission and total denitrification. Second, after addition of fertiliser nitrogen, ammonium was immobilised by fungi, which reduced nitrogen leaching in the high fungal biomass soil. Mineralisation and immobilisation of ammonium occur simultaneously in soil and describe opposite directions of the same process. Therefore, in essence, by adding fertiliser nitrogen we have shown that fungal-dominated systems have a lower availability of ammonium.

In conclusion, we provide the first experimental evidence that soil fungi reduce nitrogen losses, and we offer a mechanistic basis for this phenomenon. We have shown that a minor reduction in fertilisation in the field can create a soil with higher fungal biomass that retains nitrogen better than a soil with lower fungal biomass, while maintaining production. This offers perspectives for stimulating fungal biomass in agricultural soils to reduce nitrogen leaching and greenhouse gas emissions. To do so, further knowledge is required on what determines fungal biomass in soils, and fungal species composition has to be linked to nitrogen cycling. In an era where, in the face of climate and land use change, soil-based ecosystem services such as nutrient and carbon retention are receiving more and more attention, this research is one of the many steps in understanding the role of soil microbes in nutrient cycling.

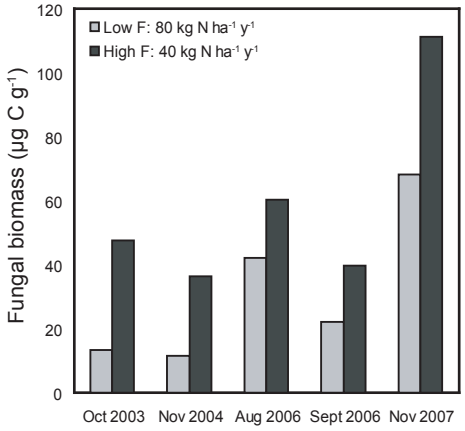
Methods summary

The factorial design of treatments with two levels of fungal biomass and two fertilisation levels allowed us to test the influence of fungal biomass on nitrogen losses with and without the addition of inorganic fertiliser. In November 2007, intact soil columns (PVC cylinders, 12 cm diam., 30 cm depth) were collected and incubated in the greenhouse for four weeks (16h daylight). After cutting the herbage and covering the bottom with mesh (1 mm), columns were placed on top of a container. Leachates were collected one day after watering and analysed for nitrate, ammonium and dissolved organic nitrogen (DON). Nitrous oxide emissions were measured daily during the first week and three times a week later, and total denitrification was measured by acetylene inhibition (Robertson and Tiedje, 1987) at the start and the end of the experiment. Fungal and bacterial biomass were analysed by fluorescence microscopy (Bloem and Vos, 2004), and microbial biomass nitrogen was determined by the fumigation-extraction method (Brookes et al., 1985). To determine microbial biomass ^{15}N, DON was converted to NH_4^+ and NO_3^- by persulfate digestion. Ammonium and nitrate were diffused onto an acid treated filter wrapped in teflon tape, which was analyzed by mass-spectrometry. All data were checked for normality and log-transformed if necessary. Outliers were determined using Cook's distance and deleted if there were *a priori* reasons to do so—this was the case for one sample from the fertilised, high fungal biomass treatment. Main and interaction effects of fungal biomass and fertiliser addition were assessed by two-way analysis of variance, with the exception of nitrogen leaching and nitrous oxide fluxes, which were assessed by two-way repeated measures ANOVA. All statistical tests were done using the statistical package SPSS (SPSS Inc., Chicago, IL).

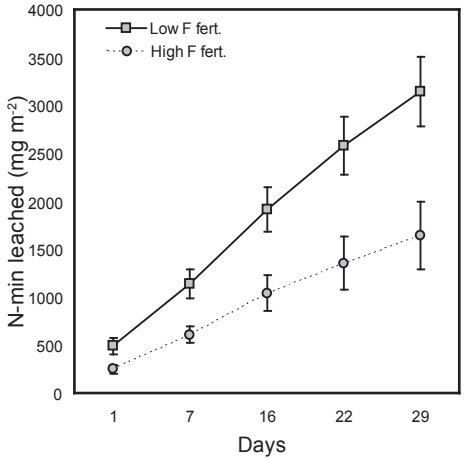
Supplementary figures and tables

Supplementary table 5.1. Soil properties of the two soils used for the factorial experiment.

	Low fungal biomass (80 N kg ha^{-1})	High fungal biomass (40 kg N ha^{-1})
Production (kg N ha^{-1})	332	317
Total production (t dm ha^{-1})	11.37	10.24
Clover production (t dm ha^{-1})	5.94	5.44
pH (KCl)	4.5	4.3
Total soil nitrogen (g kg^{-1})	2.46	2.66
Total soil carbon (g kg^{-1})	26.25	28.45
Soil mineral nitrogen Sept. 2006 (mg kg^{-1})	2.83	3.31
Soil mineral nitrogen Nov. 2007 (mg kg^{-1})	2.39	1.60



Supplementary fig. 5.1. Fungal biomass throughout the years in the two soils used for the factorial experiment. Bars represent single observations.



Supplementary fig. 5.2. Cumulative mineral nitrogen leaching from the two soils after addition of mineral fertiliser (equal to 30 kg N ha^{-1}). Symbols represent means ± 1 s.e.m. (n = 6). Abbreviations: Low F fert., low fungal biomass fertilised; High F fert., high fungal biomass fertilised.

Acknowledgements

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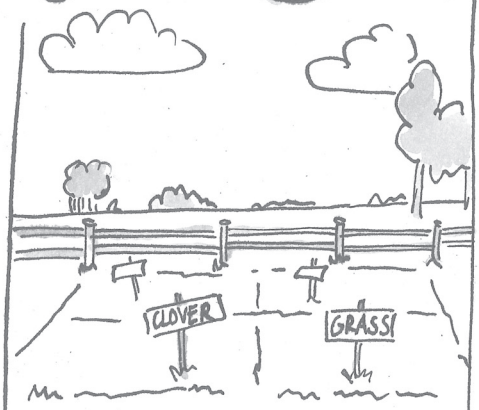
Chapter 6

Can clover stimulate fungi? (in soil)



To test this, I sampled 2 experiments with grass & clover treatments...

... but instead of being increased, fungi were reduced!!!

Legumes reduce fungal biomass in soil

6 Legumes reduce fungal biomass in soil

Franciska T. de Vries, Nick van Eekeren, Anjo Elgersma

Abstract

Seeding of legumes in agricultural grasslands has been proposed to stimulate fungal-based food webs to the effect that the nitrogen cycle becomes more closed. However, also negative correlations between legumes and fungi have been found in grasslands. We tested the effect of legumes on fungal biomass in two field experiments on two different soil types. In both soil types, fungal biomass was reduced by the presence of legumes, while potential nitrogen mineralisation was higher in grasslands with legumes than in those without. We propose that the negative effect of legumes on fungi is due to their higher nitrogen content. We question the use of seeding of legumes to stimulate fungal-based food webs.

It has often been suggested that agricultural grasslands with a soil community that bears the closest resemblance to natural ecosystems are most likely to rely on ecosystem self-regulation (Altieri, 1991; Wardle et al., 1995; Yeates et al., 1997). A key feature of natural grasslands is a soil community that is dominated by fungal pathways of decomposition (Bardgett et al., 1996). Because natural, mature systems have more closed nutrient cycles (Coleman et al., 1983), and because fungal biomass in grasslands has repeatedly been shown to decrease with fertiliser addition (De Vries et al., 2006; De Vries et al., 2007), it has been suggested that a high soil fungal biomass is indicative for agricultural systems with low nitrogen losses to the environment (Bardgett and McAlister, 1999; De Vries et al., 2006). If a higher fungal biomass is not only a consequence of sustainable management, but also a cause of lower nitrogen losses (see Chapter 5 of this thesis), it is desirable to increase fungal biomass in agricultural systems.

Fungal biomass in agricultural grasslands can be increased by reducing fertiliser inputs or ploughing (e.g. De Vries et al., 2006; De Vries et al., 2007). Not only does the increase result from reduced direct detrimental effects of nitrogen and tillage on fungi, but also from changes in organic matter quality and quantity as a result of changing plant species composition (Donnison et al., 2000). Several researchers have tried to directly relate plant species composition to soil fungal biomass (Bardgett et al., 1999; Innes et al., 2004; Wardle et al., 1999), but so far with little success. One of the few clear relationships between plant species composition and fungal biomass was found by Smith et al. (2003; 2008): in a long-term seed addition experiment saprotrophic fungal biomass (measured as PLFA 18:2ω6,9) was positively correlated to percentage of cover of both red and white clover (*Trifolium pratense* L and *Trifolium repens* L.). They inferred that the introduction of legumes would enhance the restoration of species-rich grasslands, via stimulation of a below-ground food web that has high resemblance to natural grasslands (i.e. a fungal-based food web).

In contrast, in a factorial field experiment, we found lower total fungal biomass in perennial ryegrass/white clover (*Lolium perenne* L./*Trifolium repens* L.) compared to grass only (De Vries et al., 2006). In another experiment on 48 grassland sites at 8 farms, we found a negative correlation between percentage of white clover and fungal biomass (De Vries et al., 2007). We attributed this to the higher litter quality of clover (Neergaard et al., 2002), which favors the bacterial rather than the fungal decomposition pathway (Wardle et al., 2004), and to the denser root system of grass compared to clover (Schortemeyer et al., 1997).

Because of these findings, we hypothesise that in grass/legume mixtures, fungal biomass will be lower than in grass only swards. We hypothesise that the negative effect of legumes on fungal biomass is caused by an increased input of nitrogen into the soil because of their higher litter quality. Therefore, we expect soil potential nitrogen mineralisation in grass/legume mixtures to be higher than in grass only treatments.

We tested the effect of legumes on fungal biomass in two experimental field trials on two different soil types. The first experiment was established in 2004 on a sandy loam soil (pH-KCl = 7.3, N total = 1.8 g kg^{-1}, C total = 20.0 g kg^{-1}) in the east of the Netherlands (52°26'N, 6°08E) and consisted of the following treatments: perennial ryegrass (*Lolium perenne* L.) unfertilised, perennial ryegrass fertilised, grass/white clover (*L. perenne*/*Trifolium repens*) unfertilised and white clover (*T. repens*) unfertilised, each laid out in six replicates. The second experiment was situated on a heavy clay soil (pH-KCl = 6.4, N total = 0.8 g kg^{-1}, C total = 7.3 g kg^{-1}) near Wageningen (51°58'N, 5°40'E) and comprised perennial ryegrass (*L. perenne*) monoculture, grass/lucerne (*L. perenne*/*Medicago sativa*), grass/red clover (*L. perenne*/*T. pratense*), and grass/white clover (*L.*

perennel T. repens) treatments, all unfertilised, in triplicate. This trial was established in 2003. Of each field of the two experiments, a bulk sample was collected, sieved and homogenised, and analysed for fungal biomass by epifluorescence microscopy as described by Bloem and Vos (2004). In the sandy soil experiment, potential nitrogen mineralisation was determined by incubating 200 g soil for five weeks at 50% water holding capacity (six weeks in total with a subtraction

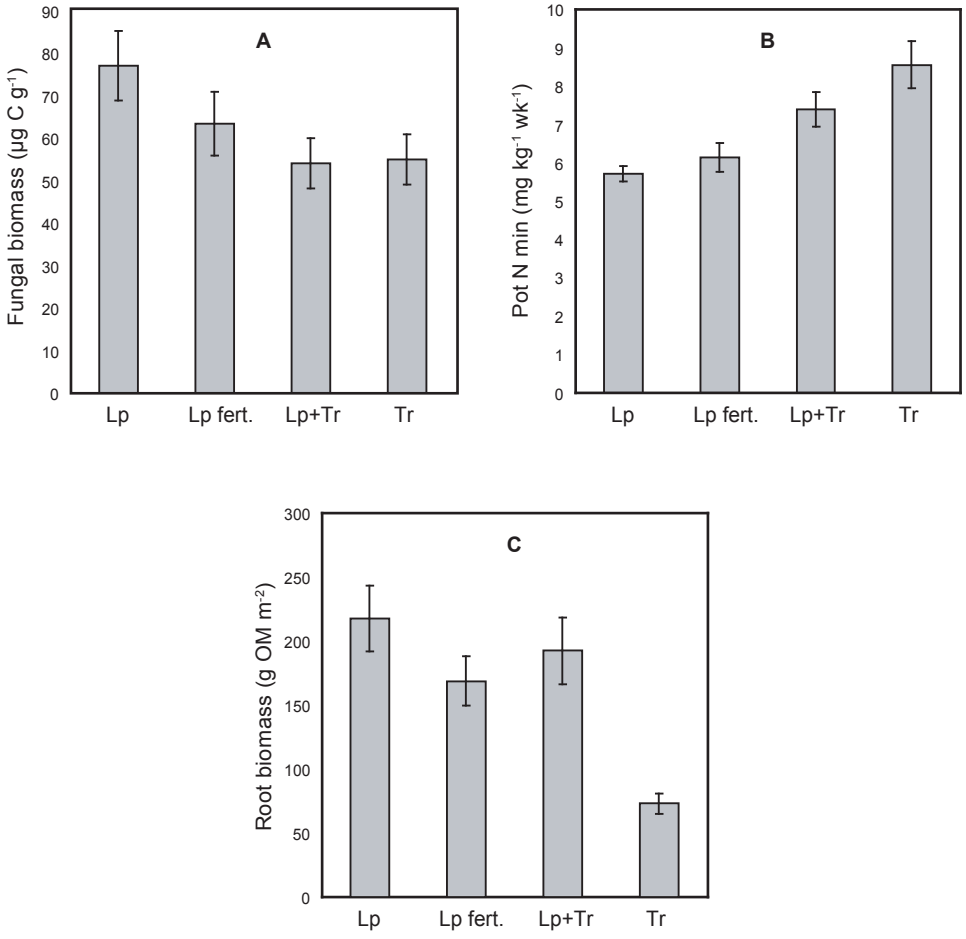


Fig 6.1. Effects of grass and clover treatments on fungal biomass (A), potential nitrogen mineralisation (B), and root biomass (C) in the sandy soil experiment. Bars represent means ± 1 s.e.m. (n = 6). Abbreviations: Lp: grass monoculture; Lp fert.: grass monoculture fertilised; Lp + Tr: grass/white clover; Tr: white clover monoculture.

of the first week to correct for the initial flush of mineralisation/ immobilisation) at 20°C. In the clay soil experiment, potential nitrogen mineralisation was determined by incubating for six weeks under the same conditions, but without subtracting the first week.

In the sandy loam soil experiment, fungal biomass was affected by treatment (one-way ANOVA, $F_{3,15} = 4.22$, $P = 0.024$, Fig. 6.1A). It was highest in the grass treatment, and declined with fertilisation and the presence of clover, although fungal biomass did not differ between the grass/clover and the clover only treatment. Potential nitrogen mineralisation showed the opposite (one-way ANOVA, $F_{3,15} = 9.90$, $P = 0.002$, Fig. 6.1B) and was highest in the clover only treatment. Total root biomass was also affected by treatment (one-way ANOVA, $F_{3,15} = 8.96$, $P = 0.001$, Fig. 6.1C) and lowest in the clover only treatment.

The clay soil experiment showed the same trends. Although fungal biomass was not significantly affected by treatment (one-way ANOVA, $F_{3,8} = 2.41$, $P = 0.142$), it was highest in the grass monoculture and was reduced in legume treatments. Potential nitrogen mineralisation

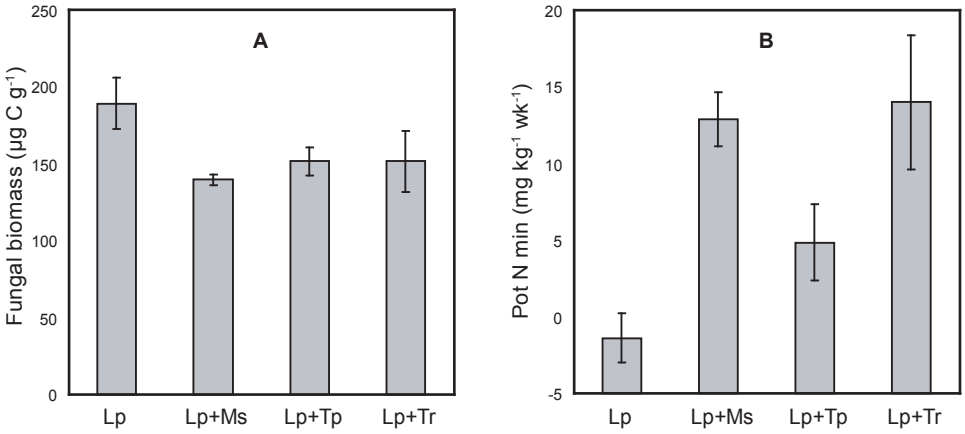


Fig. 6.2. Effects of grass and clover treatments on fungal biomass (A) and potential nitrogen mineralisation (B) in the clay soil experiment. Bars represent mean ± 1 s.e.m. (n = 3). Abbreviations: Lp: grass monoculture; Lp + Ms: grass/lucerne; Lp + Tp: grass/red clover; Lp + Tr: grass/white clover.

was affected by treatment (one-way ANOVA, $F_{3,8} = 6.72$, $P = 0.014$, Fig. 6.2B) and was highest in the legume treatments. The grass monoculture treatment showed immobilisation of nitrogen. Potential nitrogen mineralisation in the grass/lucerne and grass/white clover treatments was higher than in the grass/red clover treatment. Root biomass did not differ between any of the treatments (data not shown).

The results confirm our hypothesis that legumes reduce fungal biomass, at least in the two intensively managed, fertilised grassland soils we used. The present findings of decreasing fungal biomass and increasing potential nitrogen mineralisation with legumes, support our hypothesis that the higher litter quality of legumes might reduce fungal biomass. Furthermore, these results are in line with our earlier observations that fungal biomass is negatively correlated to the presence of legumes (De Vries et al., 2006; De Vries et al., 2007). Moreover, litter of poorer quality with high C/N ratio stimulates fungal biomass in soil (Rousk and Bååth, 2007; Vinten et al., 2002),

and addition of fertiliser nitrogen has repeatedly been shown to reduce fungal biomass (Bardgett et al., 1999; Bittman et al., 2005; De Vries et al., 2006).

Our root biomass data did not fully support the idea that negative effects of clover on fungal biomass are a consequence of the less dense root system of clover compared to grass (Evans, 1977; Schortemeyer et al., 1997). In the sandy loam soil experiment, fungal biomass did not differ between the grass/white clover and the pure white clover treatment, whereas total root biomass was much lower in the pure clover treatment (Fig. 6.1C). Furthermore, the level of fungal biomass in the fertilised grass treatment was in between the unfertilised grass and the two clover treatments (Fig. 6.1A), whereas root biomass in this treatment was in between unfertilized grass and grass/clover (Fig. 6.1C). In the clay soil experiment, although potential nitrogen mineralisation was lower in the grass/red clover treatment than in the other grass/legume mixtures (Fig. 6.2B), fungal biomass did not differ between these treatments (Fig. 6.2A). These findings indicate that the clover effect is probably not only due to root density and nitrogen. Other mechanisms might be involved, such as a rhizodeposition effect that is not linearly related to the amount of clover in a sward, or increased levels of AMF colonization of clover.

Root colonisation of clover by arbuscular mycorrhizal fungi (AMF) is generally found to be higher than that of grass (Ryan and Ash, 1999; Tisdall and Oades, 1979), but the effect on total AMF biomass per unit of soil might be neutralized by the lower root biomass of clover. Since we measured total fungal biomass microscopically, we could not distinguish between AM and saprotrophic fungal biomass. It has been shown that AMF can be more sensitive to fertilisation and disturbance than saprotrophic fungi (Bradley et al., 2006; Johnson et al., 2005; Kabir et al., 1997; see also Chapter 4 of this thesis). Furthermore, because they are less important in decomposition than saprotrophic fungi and can transport nitrogen directly to the host plant (Hodge et al., 2001), their effect on nitrogen cycling differs from that of saprotrophic fungi. However, their contribution to closing nitrogen cycles in grasslands by enhancing plant nitrogen uptake has been shown to be of minor importance (see Chapter 5 of this thesis).

In productive grasslands, such as the ones we studied, microbial growth is not limited by nitrogen and, thus, any potential positive effects of legumes on fungal biomass might not be expressed. For instance, Innes et al. (2004) found that, when *Trifolium pratense* was planted on unimproved (unfertilised, low-productivity) soil, it affected the F/B ratio positively relative to bare soil, while when it was planted on improved (fertilised, high-productivity) soil, it affected the F/B ratio negatively. In addition, Bardgett et al. (1999) found that when *Lolium perenne* was planted on unimproved soil, it affected fungal biomass negatively. However, as in our field experiments, Smith et al. (2003; 2008) started off with improved soils, and still found a positive relationship between legumes and fungal biomass. In their experiment, also correlations with other species were present, like *Rhinanthus minor*. This combination of multiple species might affect fungal biomass differently from our two-species grass-legume combinations.

According to the results from our clay soil experiment, different species of legumes did not have differential effects on fungal biomass. To our knowledge, fungal biomass as affected by different leguminous species has never been examined before. Because Smith et al. (2003; 2008) found the strongest positive correlations between fungal biomass and red clover, and we found negative correlations with white clover (De Vries et al., 2006; De Vries et al., 2007), we expected these two leguminous species to have a differential effect on fungal biomass. Although this differential effect was absent, potential nitrogen mineralisation was higher in the grass/white clover treatment than in the grass/red clover treatment. This contrasts with the higher nitrogen

content, rhizodeposition (Høgh-Jensen and Schjoerring, 2001), and higher N-fixation of red clover compared to white clover (Carlsson and Huss-Danell, 2003).

To conclude, our results show that leguminous species reduce fungal biomass in soil and that this negative effect can probably be attributed to the higher nitrogen content of legumes because of N-fixation. Some mechanisms might be unaccounted for, like the effect of rhizodeposition and possible interactions with other plant species, but still we question the use of seeding of legumes to stimulate fungal-based food webs to close the nitrogen cycle.

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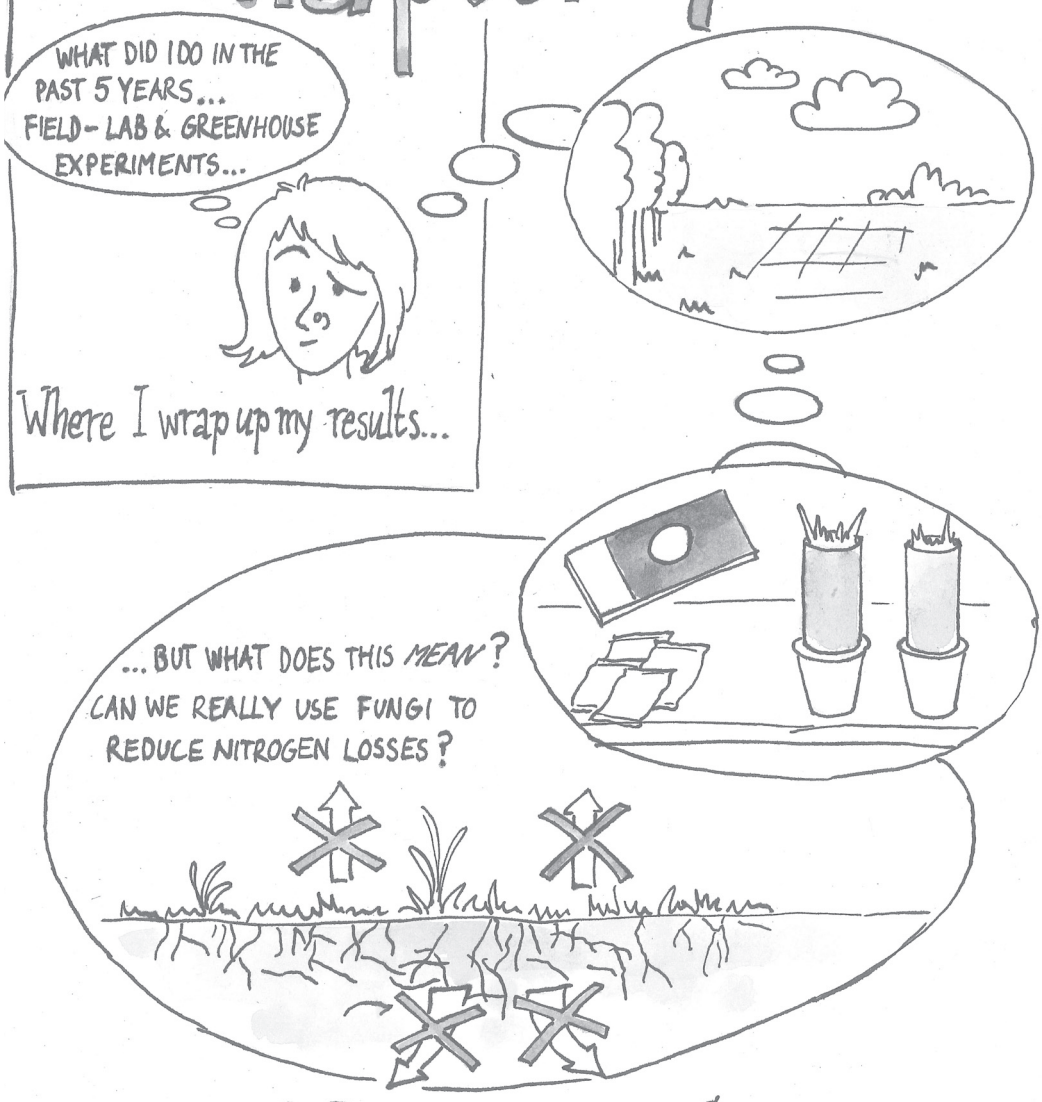
Chapter 7



General Discussion & Conclusions

7 General discussion and conclusions

7.1 Introduction

In the introduction of this thesis, I pointed out that a key feature of natural grassland systems is a soil community dominated by fungal pathways of decomposition, whereas in intensive agriculture the bacterial-dominated pathway is more prominent. It has been suggested that an increase in the fungal to bacterial biomass ratio (F/B ratio) is indicative for conversion from an intensive system to a low-input system. Not only is fungal biomass increased by extensification of management, such as cessation of fertilisation, grazing or tillage; fungi have also been proposed to reduce nitrogen losses to the environment. The latter however, was based on correlative observations and assumptions but has never been experimentally tested.

Thus, the key question of this thesis is: is an increased fungal biomass in soil not only a consequence of reduced fertilisation of grasslands, but can it also reduce nitrogen losses to the environment? To evaluate the use of a high F/B ratio as an indicator for grasslands with low nitrogen losses, I set out to elucidate further the causes of changing fungal biomass in grassland soils, and to determine the significance of these changes for nitrogen cycling.

To begin with, I explored the factors that determine fungal biomass in agricultural grasslands in two field studies. The factors that I included not only comprised soil characteristics such as pH and organic matter, but also management practices as they are carried out by real farmers, such as slit injection of cattle manure slurry. Since saprotrophic and arbuscular mycorrhizal (AM) fungi are supposed to respond differently to changes in management and can have different effects on nitrogen cycling, I quantified the contribution of these two groups to total fungal biomass in soil. I did this in a series of incubation experiments. In three greenhouse experiments, I was able to relate a higher soil fungal biomass to lower nitrogen losses to the environment. I quantified nitrogen leaching and nitrous oxide and denitrification losses from soils with relatively low and high fungal biomass, and investigated the mechanisms responsible. Finally, in two field experiments on different soil types, I evaluated whether seeding of legumes can be used to increase fungal biomass in soil. To synthesise and discuss the main findings of my work, I will now return to the hypotheses I stated in Chapter 1.4 one by one.

7.2 Testing hypotheses

Hypothesis 1: Fungal biomass increases with reduced fertilisation, superficial application of manure instead of injection of slurry, and grassland age

Fungal biomass has repeatedly been shown to decrease with increasing application of fertiliser or manure (Bardgett et al., 1999; Bittman et al., 2005; Bloem et al., 2004), mostly in field studies of different grassland types ranging from intensively managed to unfertilized natural grasslands. To assess whether fungal biomass can be increased by reducing fertiliser application in grasslands used for dairy framing in the Netherlands, we sampled 48 Dutch agricultural grasslands. The grasslands were all situated on sandy soil and received manure slurry at rates representative for Dutch practice. They did not just differ in management such as fertiliser application, but also in age. This allowed us to test whether fungal biomass does not only increase with age in natural chronosequences following cessation of fertilization or abandoned fields (Allison et al., 2005; Klein et al., 1995; Van der Wal et al., 2006), but also in grasslands that are still in production. Furthermore, to assess how quickly fungal biomass can be changed by differences in fertilisation rate, we sampled experimental fields at organic farm "Aver Heino". The experiment consisted of a wide range of fertilisation level treatments, applied as farm yard manure or slit-injected cattle manure slurry. This set-up allowed us to distinguish between the effects of fertilisation rate, manure type and application method on fungal biomass and the F/B ratio.

From the results in Chapters 2 and 3, it becomes clear that the application of farm yard manure and manure slurry both reduce fungal biomass in agricultural grasslands. I did not only demonstrate this negative effect of fertilisation in a factorial field experiment with a range of relatively low fertilisation levels (0-120 kg N/ha), but also in a range of grasslands of commercial farmers with higher fertilisation levels (60-250 kg N/ha). In the field experiment of Chapter 3, the effect of different fertilisation levels on fungal biomass and the F/B ratio was expressed already within three growing seasons since establishment of the trial, which is remarkably quick. Several earlier studies found that cessation of fertiliser applications had no effect on the F/B ratio or fungal biomass within 6 years (Bardgett and Leemans, 1995; Bardgett and McAlister, 1999; Perrott et al., 1992). Since the `trial field had been grazed before establishment, effectively, the 0 kg N ha^{-1} y^{-1} treatment can be considered a cessation of fertiliser application treatment. Thus, I show that already within three years, a fungal-based microbial community had developed that resembles natural grasslands (Altieri, 1991; Bardgett et al., 1996; Wardle et al., 1995; Yeates et al., 1997) and has been proposed to be more retentive of nitrogen (Bardgett and McAlister, 1999; Bardgett et al., 2003).

Although I confirmed the negative effect of fertilisation on fungal biomass and the F/B ratio, still the exact mechanisms for this remain unclear. The negative effects of nitrogen addition on fungal biomass have been mostly attributed to changes in plant species composition (Donnison et al., 2000b), resulting in changes in organic matter quality and quantity (Bardgett et al., 1996), changes in plant-specific exudates (Grayston et al., 2001; Mawdsley and Bardgett, 1997) and alterations in nutrient competition between plants and rhizosphere micro-organisms (Bardgett et al., 2003). The results from Chapter 2 seem to confirm this, since fungal biomass was positively correlated, and N-application rate was negatively correlated to organic matter percentage. However, in the greenhouse experiment described in Chapter 5, fungal growth was reduced

within four weeks as a result of application of inorganic nitrogen. This rapid negative effect of nitrogen on fungi indicates a direct toxic or inhibiting effect rather than an effect through changes in organic matter quality and quantity.

Superficial application of farm yard manure did not reduce fungal biomass less compared to slit injection of slurry, as I found out in Chapter 3. Detrimental effects of tillage on fungi have been attributed to the disruption of mycelial networks (Beare et al., 1997; Frey et al., 1999). In the case of slit injection of slurry, the knives might simply have been spaced too wide (20 cm) to have a negative effect on fungal hyphae. Furthermore, although application of organic material with a high C/N ratio has been shown to stimulate fungal biomass (Rousk and Bååth, 2007; Vinten et al., 2002), farm yard manure treatments did not show higher fungal biomass compared to slurry treatments. Either the difference in C/N ratio between farm yard manure and slurry was not large enough to cause differences in fungal biomass, or the effect just could not establish yet in the two years since the start of the experiment.

In line with observations of increasing fungal biomass in chronosequences of abandoned agricultural fields (Klein et al., 1995; Ohtonen et al., 1999; Van der Wal et al., 2006; Williamson et al., 2005), I found that grasslands older than 10 years had higher fungal biomass than younger grasslands. This cannot only be attributed to an increasing soil organic matter content or changing vegetation characteristics, but is probably also a result of reduced disturbance. In such old grasslands, fungal biomass has simply had more time to recover from ploughing and reseeding, which is commonly practiced once every 5-10 years in Dutch grasslands (Velthof et al., 2002).

Another factor that appeared to affect fungal biomass in the two field experiments was percentage of white clover. In Chapter 2, fungal biomass was negatively correlated to clover percentage, while in Chapter 3 fungal biomass was lower in grass/clover treatments compared to grass treatments. Although we saw no effect of grass/clover on soil C/N ratio, we attributed this negative effect to the higher nitrogen content of clover litter resulting from nitrogen fixation (Neergaard et al., 2002).

To summarize, fungal biomass in agricultural grasslands is reduced by nitrogen inputs to the soil, not only in the form of inorganic fertiliser, but also as farm yard manure or manure slurry and even through N fixation by legumes. Consequently, fungal biomass can be increased by reducing nitrogen inputs, but also by reducing tillage or ploughing and possibly, by managing the plant species composition or grassland abandonment. Although I found relationships between the F/B ratio and fertilisation level, species composition and grassland age, these were mainly caused by changes in fungal biomass, while bacterial biomass was hardly affected by management. Moreover, relationships with fungal biomass were stronger than relationships with the F/B ratio. Therefore, I conclude that it is more accurate to consider soil fungal biomass than the ratio between fungal and bacterial biomass as related to grassland management.

Hypothesis 2: Increased fungal biomass is predominantly the result of increased densities of arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) have been suggested to be more susceptible to disturbance and fertilisation than saprotrophic fungi (Bradley et al., 2006; Kabir et al., 1997a). Because they can affect nitrogen cycling via other mechanisms than saprotrophic fungi, it is important to

distinguish between the two in order to assess the effect of changes in fungal biomass on nitrogen cycling.

From my attempts in Chapter 4 to quantify AMF biomass in samples from different soil types and with different fertilisation rates, it becomes clear that this is easier said than done. Because the known biomarkers that can distinguish between AMF and saprotrophic fungi cannot be used to quantify their amount relative to total fungal biomass, we incubated soil samples without plants: it has been shown that ectomycorrhizal fungi die off during incubation without plants (Bååth et al., 2004). Therefore we hypothesised that the decrease in total fungal biomass, as determined by microscopy, would be due to the decrease of the initial biomass of AMF in the soil. In the third incubation experiment that I described in Chapter 4, I compared fungal biomass and biomarkers for AM and saprotrophic fungi in an unfertilised and a fertilised soil. Initially, fungal biomass in the unfertilised treatments was twice as high as in the fertilised treatments. After two weeks, in the unfertilised soil, total fungal biomass had decreased towards the level in the fertilised soil, which was thought to imply that the two-fold higher fungal biomass in the unfertilised soil was caused by a higher AM fungal biomass.

However, phospholidid fatty acid (PLFA) fungal biomarkers did not reflect the decreasing fungal biomass we found in our four-week incubations. Therefore, I was not able to show that it was AMF that died off during incubation without plants. Because towards the end of the incubation, fine hyphae decreased and the fraction of septate hyphae increased, I propose that there was a fraction of fungal biomass that was sensitive to disturbance and fertilisation and had a very high turnover. This fraction is possibly composed of fine hyphae of both AM and saprotrophic fungi.

Although I failed to quantify the amount of AMF relative to the total amount of fungi, it is possible to say something about my hypothesis that an increased fungal biomass is predominantly caused by an increase in AMF. If we compare the fertilised and the unfertilised soil in the third experiment, we see that at the start of the incubation, microscopically measured fungal biomass was twice as high in the unfertilised soil. The saprotrophic biomarker was three times higher, while biomarkers for AM hyphae and storage structures were three and five times higher, respectively. Thus, differences in biomarkers between the two soils were greater than differences in microscopically measured fungal biomass. In the unfertilised soil, AM fungal hyphae were not, but AM fungal storage structures were increased more than saprotrophic fungal biomass. Therefore, I conclude that increased fungal biomass in unfertilised soils compared to fertilised soils is not only the result of increased biomass of AMF.

Hypothesis 3: The higher the fungal biomass in soil, the lower the nitrogen losses to the environment and the higher the crop nitrogen uptake efficiency

Fungal-dominated food webs occur in undisturbed, late-successional sites with low nutrient availability (Coleman et al., 1983). This, in combination with observations of decreasing fungal biomass with fertilisation, has led to the inference that fungal dominance is indicative for ecosystems with low nitrogen losses (Bardgett, 2005; Bardgett and McAlister, 1999; Van der Heijden et al., 2008; Wardle et al., 2004). Although several mechanisms have been proposed of how fungi can affect nitrogen cycling, this relationship has never been tested. Therefore, it remains unclear whether these presumed lower nitrogen losses simply are a consequence of lower fertilisation rates, or whether fungi are actually able to reduce nitrogen losses. In this thesis, I

aimed to test the supposed relationship between fungi and nitrogen losses in the field and in greenhouse experiments.

Although I found lower nitrogen leaching with higher fungal biomass in the field experiment described in Chapter 3, this is not necessarily a causal relationship. After all, higher fungal biomasses in this experiment were a consequence of lower fertilisation, and therefore it is obvious that the treatments highest in fungal biomass would have the lowest nitrogen leaching. The partial N balance was also negatively correlated to fungal biomass. Because crop production and N in yield were not affected by fertilisation, this implies higher crop nitrogen uptake efficiency in soils with higher fungal biomass.

The results in Chapter 5 confirm that soils with higher fungal biomass have lower nitrogen losses. I did three successive greenhouse experiments with intact soil columns from two fields of the experimental field trial described in Chapter 3. As a result of a relatively small difference in fertilisation, the two soils differed in fungal biomass but not in other soil properties. By adding inorganic fertiliser to these two soils, I showed that the soil with higher fungal biomass had higher nitrogen retention. Although without addition of fertiliser leaching did not differ between the two soils, nitrous oxide emission and total denitrification were lower in the high fungal biomass soil. This resulted from a lower nitrate availability, which, in turn, was caused by lower mineralisation and nitrification rates. This difference in nitrate availability was too small to result in lower leaching losses. However, after addition of fertiliser nitrogen, leaching from the low fungal biomass soil was increased more than threefold, whereas in the high fungal biomass soil, leaching did not increase. This difference in nitrogen leaching was caused by a higher immobilisation of added ammonium in the high fungal biomass soil.

Therefore, my results corroborate experimental and model-based findings that fungal-based food webs have lower mineralisation rates than their bacterial-based counterparts (Berg et al., 2001; Chen and Ferris, 1999; De Ruiter et al., 1993; De Ruiter et al., 1994; Hunt et al., 1987). From my results it was not possible to deduce whether these lower mineralisation rates were due to higher carbon assimilation efficiencies, slower degradation of fungal hyphae or lower nitrogen excretion by fungal-feeding fauna. Since in Chapter 4 I show that fungal hyphae rapidly degrade during incubation, a combination of higher carbon assimilation efficiencies of fungi and lower nitrogen mineralisation by grazing on fungi seems the most plausible explanation.

Not only did the high fungal biomass soil have lower nitrogen mineralisation, immobilisation of added fertiliser nitrogen into microbial biomass was also higher. Until now, immobilisation of nitrogen into microbial biomass has been assumed to occur predominantly in nitrogen-limited systems (Bardgett et al., 2003), but here I show that it can also be a mechanism of importance in productive grassland soils. Although it was not possible to show that it was fungi that immobilised nitrogen in my experiments, fungi have been suggested to access, and thus immobilise, the non-mobile ammonium better than bacteria, because of their filamentous growth form (Myrold and Posavatz, 2007).

Although some studies have shown that AMF can enhance nitrogen acquisition (Hodge et al., 2001; Mäder et al., 2000b), I found no indications that this mechanism played a role in the two soils I used. AMF-enhanced plant nitrogen uptake might only play a role in nitrogen-limited systems, whereas the soils I used were productive grassland soils. I visualize the processes that play a role in reducing nitrogen losses in my experiments in figures 7.1A and B, which are modified versions of figure 1.2.

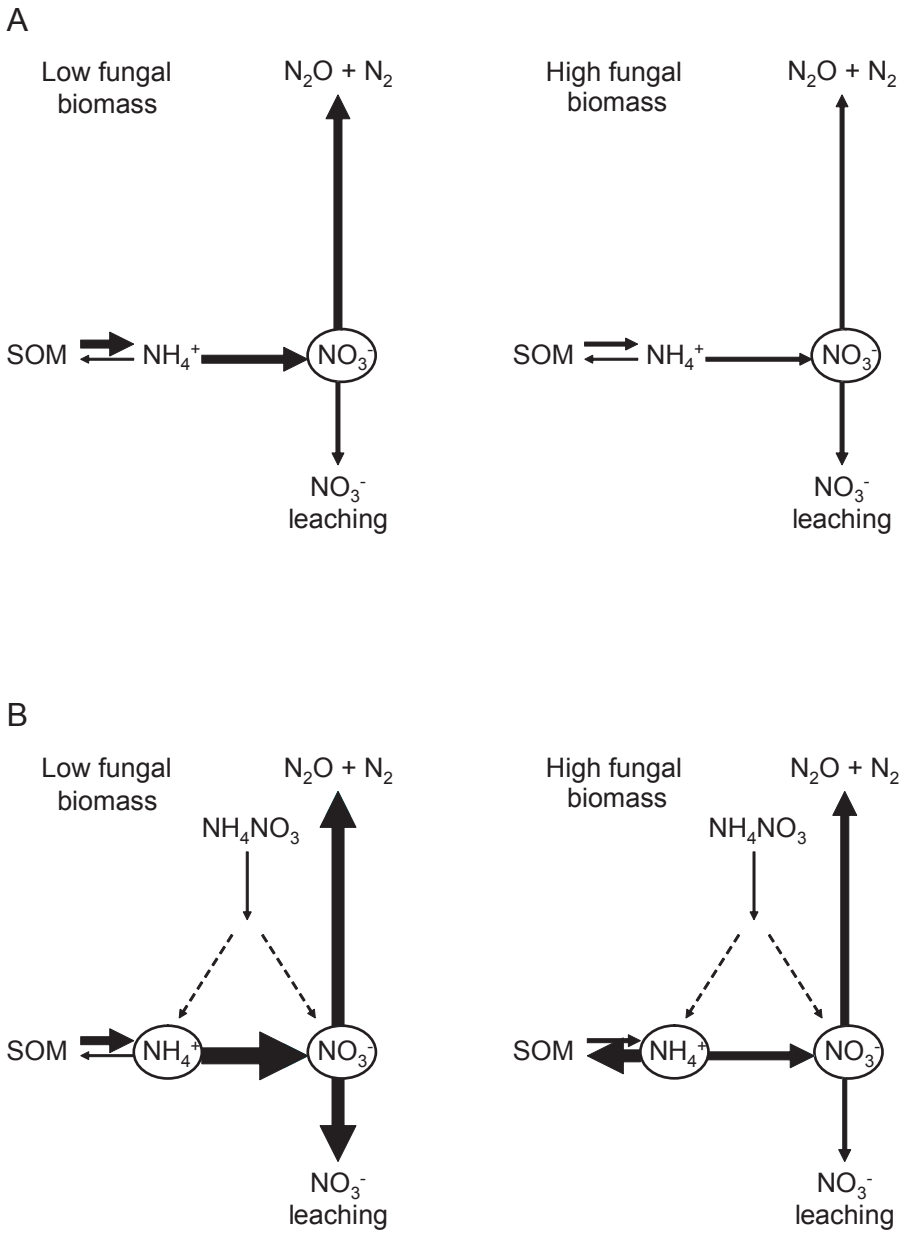


Fig. 7.1. Processes in the nitrogen cycle that can be affected by the amount of soil fungi present. A; Without addition of fertiliser, mineralisation rates are lower in high fungal biomass soil. This results in lower nitrate availability and thus lower denitrification rates. B; After addition of fertiliser N, ammonium is immobilised more in the high fungal biomass soil. This results in lower nitrification rates and thus lower nitrate availability, which reduces denitrification and leaching.

Although nitrogen losses differed between the high fungal biomass and the low fungal biomass soil, plant nitrogen uptake was not affected by fungal biomass or by the addition of nitrogen. So, a moderate reduction in fertilisation in the field can create a soil with higher fungal biomass that retains nitrogen better than a soil with lower fungal biomass while maintaining production. However, although this higher fungal biomass only requires a minor reduction in fertilisation, it takes several years for it to establish. Moreover, I also showed that fungal growth is reduced rapidly after fertilisation, so the higher nitrogen retention of these high fungal biomass soils would be a temporal effect that is cancelled out by fertilisation.

Hypothesis 4: Fungal biomass in soil can be increased by seeding of legumes

If a higher soil fungal biomass can reduce nitrogen losses in the field, it is desirable to increase fungal biomass in agricultural systems. Seeding of legumes has been proposed to stimulate fungal-based food webs with relatively closed nutrient cycles; Smith et al. (2003; 2008) found positive correlations between white and red clover and fungal biomass in the field. In contrast to their findings, I found negative correlations between white clover and fungal biomass in Chapters 2 and 3.

In Chapter 6, I clarified the relationship between legumes and fungal biomass, and tested whether fungal biomass can be increased by seeding of legumes. I sampled two field experiments on two different soil types that consisted of grass (*L. perenne* L.) monoculture treatments and treatments of grass combined with different legumes. In line with my observations in Chapters 2 and 3, in both experiments, fungal biomass was reduced by legumes. Parallel to this decreasing fungal biomass and AM fungal root infection, potential nitrogen mineralisation increased, which implies that the higher litter quality of legumes, related to their N-fixing ability might have reduced fungal biomass. These results show that leguminous species decrease fungal biomass in soil instead of increase. Therefore, I question the use of seeding of legumes to stimulate fungal-based food webs with closed nutrient cycles.

7.3 Conclusions

By testing hypotheses 1 to 4, I aimed to answer the question whether an increased fungal biomass in soil is not only a consequence of reduced fertilisation, but can also reduce nitrogen losses to the environment. By answering this question, I wanted to evaluate the use of the F/B ratio as an indicator for nitrogen retention.

My research confirms that fungal biomass in agricultural grasslands is reduced by nitrogen inputs to the soil. I found that this not only the case when nitrogen is added as inorganic fertiliser, but also when added as farm yard manure or manure slurry and even as a result of N-fixation by legumes. Consequently, fungal biomass can be increased by reducing nitrogen inputs. Grassland soils with higher fungal biomass as a consequence of reduced nitrogen inputs retain nitrogen better than comparable soils with lower fungal biomass, because they mineralise less nitrogen from organic matter and microbial biomass, and immobilise readily available nitrogen. Thus, an increased fungal biomass is not only a consequence of reduced fertilisation but also a cause of lower nitrogen losses.

Total fungal biomass correlated better to fertilisation levels, grassland age and nitrogen losses than F/B ratio, and plant uptake by AMF did not play a role in reducing nitrogen losses. These findings pave the way for total fungal biomass to be used as an indicator for nitrogen retention. When fungal biomass is higher, risks of nitrogen losses are lower. The challenge for a more sustainable agriculture is to combine an optimum nutrient retention with a reasonable crop yield.

An interesting question is whether it would be possible to stimulate soil fungal biomass in order to reduce nitrogen losses, while maintaining production. We have seen in Chapters 2 and 3 that reducing fertilisation stimulates fungal biomass, but this might also, at least on the long term, reduce grassland production. Therefore, it would be attractive to stimulate fungal biomass

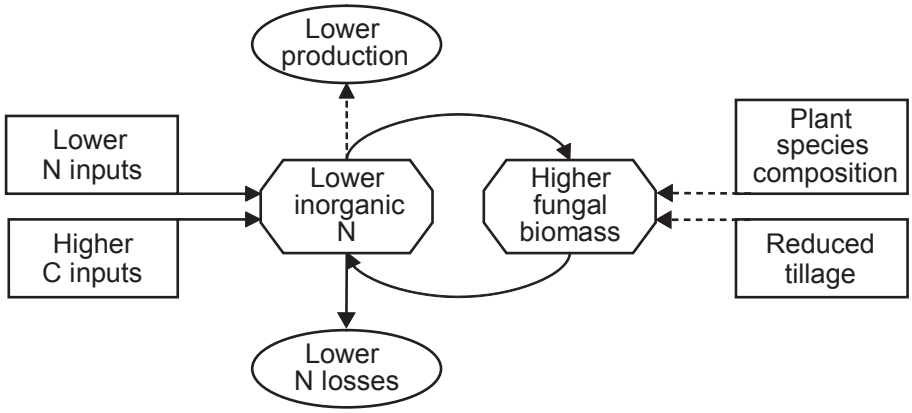


Fig. 7.2. Conceptual diagram of the relationship between nitrogen availability and fungal biomass. Solid arrows indicate relations with a known effect; dashed arrows indicate relations of which the impact is unknown. Reducing nitrogen inputs or increasing carbon inputs reduces inorganic nitrogen, which increases fungal biomass. The increased fungal biomass in turn reduces inorganic nitrogen and thus nitrogen losses. However, production might also be reduced.

not by decreasing nitrogen availability, but via direct measures, for instance seeding of plant species that promote fungal biomass. If that would be possible, fungal biomass could actually be used as a tool to reduce nitrogen losses.

Figure 7.2 conceptualises the interaction between inorganic nitrogen and fungal biomass, and shows the possibilities of intervention. Fungal biomass can be stimulated by reducing inorganic nitrogen, either by adding recalcitrant substrates such as straw or by reducing fertilisation. This reduces nitrogen losses but probably also production. When nitrogen is applied to maintain production, fungal biomass will decrease and its nitrogen-retentive effect will diminish. On the other hand, fungal biomass might be stimulated directly, by reduced tillage or by seeding of plant species that promote fungal biomass. In this way, nitrogen availability will be decreased by increasing fungal biomass. In the ideal situation, the positive effect of the measure should be strong enough to prevent a decrease in fungal biomass as a result of nitrogen addition, which is

necessary to maintain production.

Although extensive research has been done on what determines fungal biomass in soil, such measures are not known at this time. In this thesis I show that fungal biomass cannot be stimulated by seeding of legumes, but possibly other plant species or combinations of plant species might promote fungal biomass. For instance, Bardgett et al. (2006) showed that the hemi-parasitic plant *Rhinanthus minor* can strongly affect below-ground properties in grasslands ecosystems. Reduced tillage has been shown to increase soil fungal biomass, but this is preventing a detrimental effect on fungi rather than stimulating fungal biomass. The real challenge would be to find a measure that stimulates fungal biomass. To achieve this, research has to be done further on what determines fungal biomass in soils, for instance on plant species that can stimulate fungal biomass. Furthermore, to use fungal biomass as a tool to reduce nitrogen losses, the relationship between fungi and nitrogen cycling has to be specified more. This is where molecular methods become important to link fungal species composition and abundance, functional genes and gene expression to biogeochemical processes.

The findings of this thesis are relevant for maintaining soil quality in the face of the increasing demand for sustainable agricultural production of food, fibre and biofuel. Insight is needed in how changes in land use will affect soil-based ecosystem services such as nutrient retention. These developments have resulted in a renewed interest in the functional diversity of the soil microbial community, and how it affects biogeochemical processes. This thesis is one of the many steps necessary for understanding the role of soil microbes in nitrogen cycling, and using the soil microbial community to optimize ecosystem services.

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Summary

Agricultural intensification has increased crop yields but also poses severe environmental problems. Nitrogen losses from agricultural soils have been increasing, causing eutrophication of aquatic and terrestrial systems and contributing to atmospheric pollution and the greenhouse effect. Because of these problems, there is an increasing demand for sustainable agriculture. Agricultural systems in which mineralisation provides nutrients for crop growth at the right time and in the right amount, with a low potential for nutrient losses, can be considered sustainable. It is becoming evident that agricultural systems with a soil life that bears close resemblance to related natural systems come closest to this ideal.

A key feature of natural grasslands is a soil community that is dominated by fungal pathways of decomposition. It has been suggested that a high fungal biomass is indicative for successful conversion from an intensive system to a low-input system. Not only has it been shown that fungi increase with extensification of management, they have also been suggested to reduce nitrogen losses themselves. However, the factors that determine fungal biomass in soil, and the relationship between soil fungi and nitrogen cycling, remain largely unexplored.

In this thesis, my aim was to identify the factors that determine fungal biomass in grassland soil, and to find out whether an increased fungal biomass is not only a consequence of reduced fertilisation, but can also reduce nitrogen losses to the environment. To do so, I performed several field, laboratory and greenhouse experiments.

In the first two chapters, I describe two field studies in which I identified management factors and soil characteristics that determine fungal biomass in agricultural grasslands. In Chapter 2, I studied 48 Dutch grassland sites of differing age and management. I found out that fungal biomass was higher in older pastures and was increased by reduced fertilisation. The most important soil characteristic that was positively correlated to fungal biomass was organic matter percentage. Percentage of clover cover was negatively correlated to fungal biomass. In this chapter I show that even within a narrow range of management intensities, fungal biomass increases with grassland age and reduced nitrogen input.

In Chapter 3, I studied the effect of fertilisation on fungal biomass in depth in a field trial with different fertilisation levels and manure types and application methods. Here, I found out that fungal biomass responded to differences in fertilisation level already within three growing seasons, and was not affected by manure type or application method. Besides the rapid negative effect of fertilisation on fungal biomass, fungal biomass correlated negatively to nitrogen leaching. However, this is not necessarily a causal relationship, since the fields with the highest fungal biomass and the lowest nitrogen leaching also had the lowest fertilisation.

Because of the differential effects that arbuscular mycorrhizal fungi (AMF) and saprotrophic fungi can have on nutrient cycling, and because they respond differently to changes in management, I tried to quantify the contribution of AMF to total fungal biomass in Chapter 4. By incubating soil samples without plants (the carbon source for AMF), I aimed to quantify AM fungal biomass by measuring the decrease in total fungal biomass. I found that as a result of

incubation, fungal biomass was halved within two weeks in unfertilised soil, whereas in fertilised soil fungal biomass remained at the original level. This suggests that the increased fungal biomass in unfertilised soils is predominantly the result of the increase in biomass of AMF. However, by measuring specific biomarkers for AM and saprotrophic fungi, I could conclude that the difference between the unfertilised and fertilised soil was not only caused by AMF.

One of the major aims of this thesis was to establish the relationship between fungi and nitrogen cycling, and, specifically, to test whether an increased soil fungal biomass reduces nitrogen losses. In Chapter 5 I describe three greenhouse experiments with intact soil columns from fields with high and low fungal biomass. I measured both gaseous and aqueous nitrogen losses from the two soils, and found that the high fungal biomass soil had lower nitrogen losses than the low fungal biomass soil. This was due to lower nitrate availability because of lower mineralisation rates in the high fungal biomass soil, and higher immobilisation of ammonium into microbial biomass.

If a higher soil fungal biomass can reduce nitrogen losses in the field, it is desirable to increase fungal biomass in agricultural systems. It has been suggested that seeding of legumes would increase fungal biomass in grasslands. In Chapter 6, I clarified the relationship between legumes and fungal biomass in two field experiments. I found that in contrast to what has been suggested in the literature, legumes decrease fungal biomass. Therefore, I question the use of seeding of legumes to stimulate fungal biomass in grasslands.

In Chapter 7 I summarise my results. I conclude that an increased fungal biomass is not only a consequence of reduced fertilisation, but also reduces nitrogen losses to the environment. Therefore, total fungal biomass in soil can be used as an indicator for nitrogen retention. The next challenge is to find measures that stimulate fungal biomass to reduce nitrogen losses from agricultural soils, and at the same time, maintain production.

Samenvatting

Met de komst van de intensieve landbouw zijn de opbrengsten, maar ook de milieuproblemen, toegenomen. Stikstofverliezen veroorzaken eutrofiëring van grond- en oppervlaktewater, en dragen bij aan atmosferische verontreiniging en het broeikaseffect. Door deze problemen neemt de vraag naar een duurzamere landbouw toe. Landbouwsystemen waar mineralisatie op het juiste tijdstip en in de juiste hoeveelheid nutriënten levert aan het gewas, met een lage kans op stikstofverliezen, kunnen beschouwd worden als duurzaam. Het bodemleven in deze duurzame systemen vertoont vaak grote gelijkenis met dat in verwante natuurlijke systemen.

Het bodemleven van natuurlijke graslanden kenmerkt zich door een schimmelgedomineerde decompositie van organisch materiaal. Daarom wordt een hoge schimmelbiomassa gezien als indicatie voor een succesvolle overgang van een intensief beheerd systeem naar een meer extensief beheerd systeem. Het is niet alleen aangetoond dat schimmels toenemen naarmate er minder bemest wordt, er wordt ook gesuggereerd dat ze stikstofverliezen daadwerkelijk kunnen verminderen. Hoewel dit vaak beweerd wordt, is de interactie tussen schimmels en de stikstofkringloop nog nooit onderzocht.

In dit proefschrift probeer ik te achterhalen door welke factoren de schimmelbiomassa in grasland bepaald wordt, en of een toename in de schimmelbiomassa niet alleen het gevolg is van lagere bemesting, maar ook stikstofverliezen vermindert. Ik heb dit onderzocht in experimenten in veld, laboratorium en kas.

In de eerste twee hoofdstukken beschrijf ik twee veldstudies waarin ik onderzocht welk management en welke bodemkarakteristieken de schimmelbiomassa in graslanden bepalen. In hoofdstuk 2 heb ik 48 graslanden van verschillende leeftijd en met verschillend beheer bemonsterd. Hier vond ik dat de schimmelbiomassa hoger was naarmate het grasland ouder was. Ik vond ook dat de schimmelbiomassa toenam naarmate de bemesting lager was. De hoeveelheid organisch materiaal in de bodem was positief gecorreleerd met de schimmelbiomassa, en het percentage klaver juist negatief. In dit hoofdstuk laat ik zien dat, ook al waren de verschillen in beheer tussen de graslanden klein, de schimmelbiomassa toch toeneemt met de leeftijd van het grasland en met lagere bemesting.

In hoofdstuk 3 bestudeerde ik het effect van bemesting op de schimmelbiomassa in meer detail in een veldexperiment met verschillende bemestingshoeveelheden, en verschillende mestsoorten en toedieningsmethoden. Ik vond dat de schimmelbiomassa al binnen drie groeiseizoenen op verschillen in bemesting reageerde, en dat mestsoort en –toediening geen invloed hadden op de schimmelbiomassa. Naast dit snelle effect van bemesting op de schimmelbiomassa was er ook een negatieve correlatie tussen schimmelbiomassa en stikstofuitspoeling. Dit hoeft echter geen causaal verband te zijn, omdat de hoogste schimmelbiomassa en de laagste uitspoeling voorkwamen in de laagst bemeste veldjes.

Omdat arbusculaire mycorrhizavormende schimmels (AMF) en saprotrofe schimmels de stikstofkringloop op een verschillende manier beïnvloeden, en omdat ze verschillend reageren op beheer, wilde ik de bijdrage van deze groepen aan de totale schimmelbiomassa kwantificeren.

Door grondmonsters zonder plant te incuberen wilde ik de bijdrage van AMF meten als de afname van de totale schimmelbiomassa. Ik vond dat de totale schimmelbiomassa binnen twee weken halveerde in grond uit onbemeste veldjes, maar niet in grond uit bemeste veldjes. Dit suggereert dat de hogere schimmelbiomassa in de onbemeste veldjes vooral een gevolg was van meer AMF. Toch gaven specifieke signaalstoffen voor AMF aan dat het verschil tussen bemeste en onbemeste monsters niet alleen werd veroorzaakt door AMF.

Een van de belangrijkste doelstellingen van dit proefschrift was om de relatie tussen schimmels en de stikstofkringloop te onderzoeken, en, specifieker, om te testen of een hogere schimmelbiomassa stikstofverliezen kan verminderen. In hoofdstuk 5 beschrijf ik drie kasexperimenten met intacte grondkolommen met hoge en lage schimmelbiomassa. Ik kwantificeerde stikstofverliezen in de vorm van N_2O en N_2 in de lucht en nitraatuitspoeling, en ik vond dat de grond met veel schimmels lagere stikstofverliezen had dan de grond met minder schimmels. Dit was het gevolg van lagere nitraatbeschikbaarheid en hogere immobilisatie van ammonium in microbiële biomassa in de grond met veel schimmels.

Als een hogere schimmelbiomassa stikstofverliezen kan verminderen, is het wenselijk om de schimmelbiomassa te stimuleren in landbouwsystemen. Het zaaien van klavers is voorgesteld als maatregel om de schimmelbiomassa in grasland te stimuleren. In hoofdstuk 6 onderzocht ik of klavers inderdaad de schimmelbiomassa laten toenemen, maar ik vond het tegenovergestelde: de schimmelbiomassa nam af in de aanwezigheid van klavers. Daarom zijn klavers niet (altijd) geschikt om de schimmelbiomassa in grasland te verhogen.

In hoofdstuk 7 vat ik mijn resultaten samen. Ik concludeer dat een hogere schimmelbiomassa niet alleen een gevolg is van lagere bemesting, maar er ook voor kan zorgen dat stikstofverliezen verminderen. Daarom kan de totale schimmelbiomassa gebruikt worden als indicator voor stikstofretentie in de bodem. De volgende uitdaging is nu om maatregelen te vinden die de schimmelbiomassa stimuleren, zodanig dat stikstofverliezen beperkt worden, terwijl de productie op peil blijft.

Curriculum vitae

Franciska Trijntje de Vries was born on the 21st of June 1978 in Leeuwarden, the Netherlands. From 1990 to 1996 she followed secondary education on the Christelijk Gymnasium in Leeuwarden. In September 1996, Franciska started her study on Environmental Sciences at Wageningen University. After obtaining the propadeutic exam, she chose the specialisation of soil science/ nature conservation. Before she started her MSc theses, she was a member of the board of student's/ youth club Unitas for one year. In her first MSc thesis, she studied the effect of within-trophic group nematode diversity on nitrogen mineralisation at the Soil Quality group at Wageningen University. She did her second thesis at Radboud University, Nijmegen, on the risk of bioaccumulation of heavy metals in voles and shrews recolonising floodplains. Meanwhile, she worked in the cinema Molenstraattheater in Wageningen. Finally, Franciska modeled the hydrology of a peat swamp forest during her MSc internship at Alterra/ CIMTROP in Kalimantan, Indonesia. After her graduation in September 2003, she started her PhD research at the Soil Quality group (Wageningen University) and Alterra. Parallel to her scientific work, Franciska became a fanatic, semi-professional mountain biker, participating in national and international races. After finishing her PhD research in November 2008, she started a postdoc at Lancaster University, United Kingdom, in Januari 2009.

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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)

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- General introduction and general discussion and conclusions (2008)

Laboratory Training and Working Visits (3.5 ECTS)

- Ergosterol analysis; NIOO, Heteren
- Working visit; Lancaster University, UK
- Working visit to Lancaster; Lancaster University, UK

Post-Graduate Courses (5.4 ECTS)

- Advanced statistics; PE&RC (2004)
- Microbial ecology; FE (2004)
- Soil quality; Louis Bolk Institute (2005)
- Soil ecology; PE&RC (2007)

Deficiency, Refresh, Brush-up Courses (3.5 ECTS)

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- Information literacy; WUR (2006)
- Nutrient management; Soil Biology, WUR (2007)

Competence Strengthening / Skills Courses (4.5 ECTS)

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- Writing grant proposals; Language Centre (2008)
- Career perspectives; WGS (2007)

Discussion Groups / Local Seminars and Other Meetings (5.7 ECTS)

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- Multitrophic interactions seminar (2005)
- Bodembreed symposium (2007)
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- PE&RC symposium (2007)
- PE&RC day (2008)

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- 14th N workshop (2006)
- Soil & Water (2006)
- High Value Grassland Conference (2007)
- 15th N workshop (2007)
- NERN (2008)
- EURECO-GFOE (2008)

Supervision of MSc Students

- Effect of F/B ratios on mineralization; Effect of plant species on earthworms, fungi & bacteria (1.5 students supervised; 20 days)