Nitrogen losses from two grassland soils with different fungal biomass

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ABSTRACT

Nitrogen losses from agricultural grasslands cause eutrophication of ground- and surface water and contribute to global warming and atmospheric pollution. It is widely assumed that soils with a higher fungal biomass have lower N losses, but this relationship has never been experimentally confirmed. With the increased interest in soil-based ecosystem services and sustainable management of soils, such a relationship would be relevant for agricultural management. Here we present a first attempt to test this relationship experimentally. We used intact soil columns from two plots from a field experiment that had consistent differences in fungal biomass (68 ± 8 vs. $111 \pm 9 \, \mu g \, C \, g^{-1}$) as a result of different fertilizer history (80 vs. $40 \, kg \, N \, ha^{-1} \, y^{-1}$ as farm yard manure), while other soil properties were very similar. We performed two greenhouse experiments: in the main experiment the columns received either mineral fertilizer N or no N (control). We measured N leaching, N_2O emission and denitrification from the columns during 4 weeks, after which we analyzed fungal and bacterial biomass and soil N pools. In the additional ^{15}N experiment we traced added N in leachates, soil, plants and microbial biomass. We found that in the main experiment, N_2O emission and denitrification were lower in the high fungal biomass soil, irrespective of the addition of fertilizer N. Higher ^{15}N recovery in the high fungal biomass soil also indicated lower N losses through dentrification. In the main experiment, N leaching after fertilizer addition showed a 3-fold increase compared to the control in low fungal biomass soil (11.9 ± 1.0 and $3.9 \pm 1.0 \, kg \, N \, ha^{-1}$, respectively), but did not increase in high fungal biomass soil (6.4 ± 0.9 after N addition vs. $4.5 \pm 0.8 \, kg \, N \, ha^{-1}$ in the control). Thus, in the high fungal biomass soil more N was immobilized. However, the ^{15}N experiment did not confirm these results; N leaching was higher in high fungal biomass soil, even though this soil showed higher immobilization of ^{15}N into microbial biomass. However, only 3% of total ^{15}N was found in the microbial biomass 2 weeks after the mineral fertilization. Most of the recovered ^{15}N was found in plants (approximately 25%) and soil organic matter (approximately 15%), and these amounts did not differ between the high and the low fungal biomass soil. Our main experiment confirmed the assumption of lower N losses in a soil with higher fungal biomass. The additional ^{15}N experiment showed that higher fungal biomass is probably not the direct cause of higher N retention, but rather the result of low nitrogen availability. Both experiments confirmed that higher fungal biomass can be considered as an indicator of higher nitrogen retention in soils.

1. Introduction

Fungi and bacteria are the main organisms decomposing organic matter in soils. They each support their own food chain of soil fauna, and soil ecosystems are often characterized by having a fungal-dominated or a bacterial-dominated decomposition pathway (Wardle et al., 2004a). 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 'slow' and highly conservative nutrient cycling (Van der Heijden et al., 2008; Wardle et al., 2004a). There are a number of studies that show that shifts from fungal toward bacterial-dominated microbial communities are associated with increased rates of nutrient cycling. For example, Bardgett et al. (2006) showed that the presence of the hemiparasite *Rhinathus minor* in grassland lead to a shift in the microbial community toward increasing dominance of bacteria, which was associated with increasing rates of N cycling in soil. Wardle et al. (2004b) found that ecosystem decline was associated with increasing

phosphorus limitation, which was paralleled by a shift in the microbial community toward fungal dominance. Also, De Vries et al. (2006) found that a higher fungal biomass in soil was associated with reduced N leaching in agricultural grasslands, whereas Gordon et al. (2008) found that, after drying and rewetting, soil from unimproved grasslands with a high fungal biomass retained nutrients better than soil from improved, more bacterial-rich grasslands. Repeated observations of decreasing fungal biomass with intensification of management have led to the inference that fungal dominance is indicative for ecosystems with low N losses (Bardgett and McAlister, 1999; De Vries et al., 2007; De Vries et al., 2006). However, although this inference is widespread in ecology, most studies mentioned above are correlative. The relationship between higher fungal biomass and lower N losses has never been tested experimentally. With the increasing interest in soil-based ecosystem services and the sustainable management of soils, the demonstration of such a relationship could have implications for agricultural management: promoting agricultural soils with higher fungal biomass would contribute to reduction of N losses, which are known to cause eutrophication of ground- and surface water as well as global warming and atmospheric pollution (Tilman, 1999).

Several mechanisms have been proposed to underlie this assumed negative relationship between fungal biomass and N losses. First, fungal-dominated food webs have been shown to have lower rates of gross N mineralization (Högberg et al., 2007). Fungi assimilate more carbon per unit substrate decomposed than bacteria (Holland and Coleman, 1987), although some recent papers question this idea (Six et al., 2006; Thiet et al., 2006). Carbon (C) and N assimilation are coupled stoichiometrically (Hessen et al., 2004), but because of their higher C/N ratio (Van Veen and Paul, 1979), even if they are more efficient at assimilating C, fungi need to assimilate less N than bacteria. On the other hand, fungi grow on more recalcitrant substrates (with higher C/N ratios) that contribute less to N mineralization, and are able to use them more efficiently than bacteria (Hunt et al., 1987). Furthermore, fungi can access spatially separated recalcitrant substrates and inorganic N simultaneously with their extensive hyphal networks and thus immobilize readily available N (Frey et al., 2003; Holland and Coleman, 1987). Although, in general, fungal-dominated systems are assumed to have higher immobilization of N than bacterial-dominated systems (Schimel and Bennett, 2004), experimental confirmation is mostly lacking. It has been shown, though, that microbes in extensively managed grasslands, with a (presumed) larger proportion of fungi relative to bacteria, immobilize more N than their more intensively managed, bacterial-rich, counterparts (Bardgett et al., 1993, 2003). Because fungal hyphae are more persistent in the soil than bacterial cells (Amelung et al., 2002; Martin and Haider, 1979; Solomon et al., 2001, but see De Vries et al., 2009), N captured in fungal hyphae forms a more stable N pool in the soil and is less easily remineralized than N in bacterial cells. Adding to this, 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, fungi can increase aggregate formation in the soil by entangling soil particles with their hyphae and by excreting extracellular polysaccharides and proteins that glue together soil particles (Ritz and Young, 2004). Especially the abundance of arbuscular mycorrhizal fungi (AMF), which (almost) exclusively produce glomalin, has been found to be closely correlated with soil aggregation and carbon sequestration (Wilson et al., 2009). As soil aggregation protects detritus from microbial degradation, increased aggregation results in lower carbon, and thus N, mineralization rates (Rillig et al., 2007). On top of this, it has been suggested that in N rich micro sites, mycorrhizal fungi may be the agents performing the first

step in mineralization: the release of dissolved organic N (DON) by depolymerization of organic matter (Schimel and Bennett, 2004).

A third mechanism concerns another role of AMF. Arbuscular mycorrizal fungi can, in addition to their effect on aggregate formation, also directly affect N cycling. Although their contribution to decomposition is much smaller than that of decomposer fungi (Hodge et al., 2001), they can enhance plant uptake of inorganic and organic N (Hodge et al., 2001; Mäder et al., 2000). This increased plant N uptake could hypothetically reduce leaching losses of N (Smith and Read, 1997). However, N addition often suppresses root biomass and percentage of root colonization by AMF (Staddon et al., 2004) and, unlike ectomycorrhizal fungi, AMF are thought to play a minor role in plant N uptake in N rich systems. Still, Van der Heijden (2010) showed that microcosms with AMF had slightly lower ammonium leaching than microcosms without fungi, although differences were much larger and more significant for phosphorus losses. In addition, Tu et al. (2006) showed that AMF increased plant biomass ^{15}N of wild oat by 125% and decreased soil inorganic N by 20%. In addition to increasing plant N uptake, AMF can also take up considerable amounts of N for their own use. However, in systems that are not limited by N and P, plants reduce their belowground C allocation, thus reducing AMF biomass (Johnson, 2010).

Each of these three mechanisms, or any combination, could underlie the suggested negative correlation between fungal biomass and N losses. However, because a high fungal biomass is a characteristic of systems with low nutrient availability, it is not only almost impossible, but also not realistic, to disentangle these mechanisms from the system that they are inherently linked to. Therefore, we used two soils from one field experiment in which we have monitored fungal and bacterial biomass for years. Although these two soils have had widely differing fungal biomass for years as a result of different inputs of farm yard manure, other soil properties were very similar. To test whether fungal-dominated soils indeed show lower N losses, and to explore the mechanisms involved, we performed a factorial experiment in which we used these two soils with two levels of fungal biomass and two levels of fertilization. Thus, we determined N losses from soils with high and low fungal biomass after addition of an equal amount of inorganic fertilizer, as well as from unfertilized controls. In addition, we did an experiment adding labeled N to the same soils, tracing ^{15}N in leachates, soil, plants and microbial biomass.

2. Materials and methods

2.1. Sampling and experimental design

We collected soil samples from two grass−clover plots differing in fungal biomass from an experimental field trial at Heino, the Netherlands (52°25′ north and 6°15′ east). The trial was established in 2001 and the humid sandy soil was classified as a gleyey sand with a semi-permeable loam horizon at 70−80 cm (for a detailed description of the field trial see De Vries et al. (2006)). The difference in fungal biomass between the two soils resulted from a long-term (six year) difference in fertilization rate (40 vs. 80 kg N ha^{-1} y^{-1} as farm yard manure) (De Vries et al., 2006). Both soils received additional P and K fertilization as P$_2$O$_5$ (107 kg ha^{-1} y^{-1}) and K$_2$O (372 kg ha^{-1} y^{-1}). Soil properties other than fungal and bacterial biomass, including concentrations of mineral N, did not differ significantly (Table 1). Differences in fungal biomass between the two soils were up to a factor two and had been consistent for more than five years (Fig. 1); both levels of fungal biomass were within the range commonly found in Dutch agricultural grasslands (De Vries et al., 2007). In an earlier (pilot) experiment, we found that after the addition of NH$_4$NO$_3$ (equivalent to 30 kg N ha^{-1}) the two soils differed in their N leaching (Fig. 2).

Table 1
Soil properties of the two soils used for the factorial experiment.

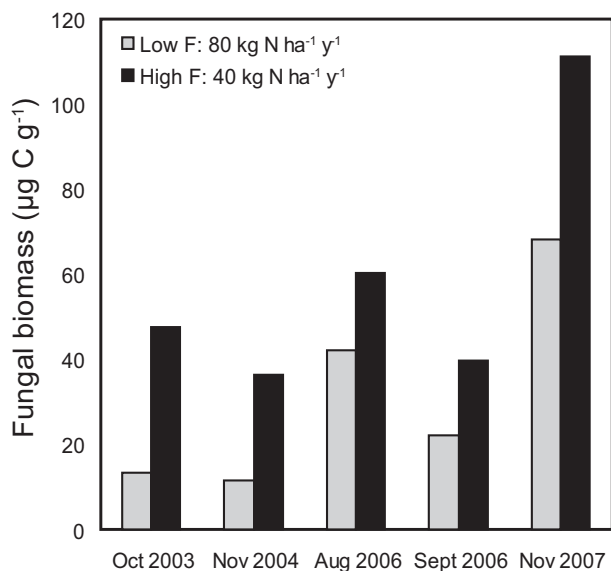|	Low fungal biomass (80 kg N ha^{-1})	High fungal biomass (40 kg N ha^{-1})
Production (kg N ha^{-1})[a]	332	317
Total production (t dm ha^{-1})[a]	11.4	10.2
Clover production (t dm ha^{-1})[a]	5.94	5.44
pH (KCl)[a]	4.5	4.3
Organic matter % (loss on ignition)[a]	5.3	5.5
Bulk density (g cm^{-3})	1.30 (0.01)	1.34 (0.01)
Total soil N (g kg^{-1})	2.46 (0.08)	2.66 (0.07)
Total soil carbon (g kg^{-1})	26.3 (0.9)	28.5 (0.7)
Soil mineral N Sept. 2006 (mg kg^{-1})	2.83 (0.05)	3.31 (0.19)
Soil mineral N Nov. 2007 (mg kg^{-1})	2.39 (0.05)	1.60 (0.05)

[a] Values represent single observations (bulk samples, 50 cores from each plot). Other values represent means (s.e.m.).

2.1.1. Main experiment

In November 2007, 48 intact soil columns (12 cm diam., 30 cm depth) with herbage were collected from both fields and incubated in the greenhouse for 4 weeks (20 °C, 16 h daylight). After clipping the herbage and covering the bottom of the columns with a 1 mm nylon mesh, half of the columns received fertilizer as NH_4NO_3 solution (34 mg N, equivalent to 30 kg N ha^{-1}). At the start of the experiment, each treatment had 12 replicates. Columns were arranged in a factorial randomized complete block design and placed on top of a container to allow for the collection of leachates. After 3 and 29 days of incubation, three columns of each treatment combination were harvested to determine actual denitrification (see below). All columns were watered weekly (350 ml, equivalent to 30 mm rainfall) with demineralized water for 4 weeks, and N leaching and N_2O emissions were measured at regular intervals. At the end of the 4-week experiment, the six remaining columns of each treatment were harvested destructively and analyzed for fungal and bacterial biomass and N pools.

2.1.2. ^{15}N experiment

In March 2008, we took an additional 12 columns (6 from each plot) to determine the fate of added fertilizer N. Columns were

Fig. 1. Fungal biomass throughout the years in the two soils used for the factorial experiment. Bars represent single observations (bulk samples, 50 cores from each plot). Abbreviations: Low F, low fungal biomass soil; High F, high fungal biomass soil.
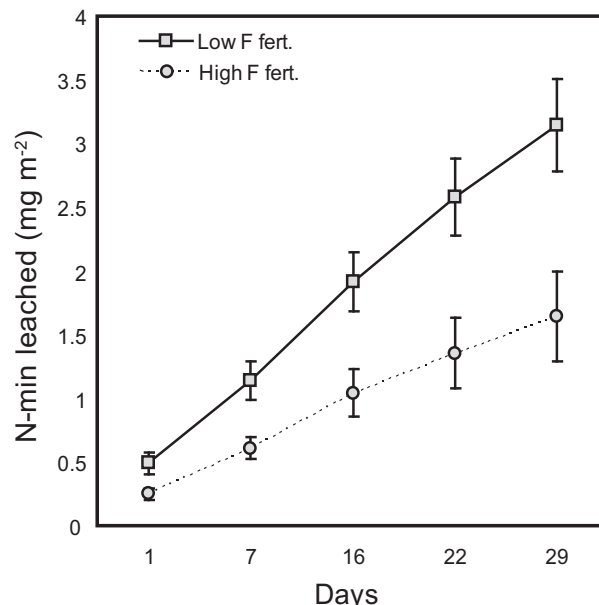
Fig. 2. Pilot experiment: cumulative amounts of N lost after N fertilization in low vs. high fungal biomass soil, using intact soil columns. Symbols represent means ± 1 s.e.m. ($n = 6$). Abbreviations: Low F fert., low fungal biomass fertilized; High F fert., high fungal biomass fertilized.

treated similarly as the main experiment. After addition of $^{15}NH_4^{15}NO_3$ (98.24% enriched, equivalent to 30 kg ha^{-1}), columns were leached weekly and harvested destructively 2 weeks after N addition, when N pools and their ^{15}N enrichment were measured.

2.2. Fungal and bacterial biomass

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, $C_{40}H_{42}N_{120}O_{10}S_2$ Na$_2$ (FW 960.9, Fluostain I, cat no. F0386, Sigma Chemical Co., St LouisMD, USA) in 50% ethanol). Hyphal length was measured using an epifluorescence microscope at 400× 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 1.3×10^{-13} g C μm^{-3} (Bakken and Olsen, 1983; Van Veen and Paul, 1979).

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. 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).

2.3. Aggregate-size distribution

After gently passing moist soil through an 8 mm sieve soil samples were air dried. Water-stable aggregates were isolated using the wet-sieving method as described by Elliott (1986). Briefly, 100 g

air dried soil was submerged for 5 min in deionized water on top of a 2000 μm-mesh sieve. Subsequently, the sieve was moved in an up-and-down motion 50 times within 2 min, after which the >2000 μm aggregates were backwashed, oven dried at 50 °C until no water remained, and weighed. Soil and water that passed the sieve were transferred onto the next sieve with a finer mesh. This procedure was repeated with a 250 μm and a 53 μm sieve to yield aggregate fractions of >2000 μm, 250–2000 μm, 53–250 μm, and <53 μm.

2.4. N losses and pools

Leachates were collected one day after irrigation and colorimetrically analyzed for NO_3^- and NH_4^+. After oxidation with $K_2S_2O_8$, total soluble N was analyzed and dissolved organic N (DON) was calculated by subtracting the amount of inorganic N from total soluble N. Total amounts of NO_3^-, NH_4^+ and DON leached per column were calculated by multiplying concentrations with volumes of leachates.

During the first week, N_2O emissions were measured daily, while from the second week on, N_2O emissions were measured three times a week. Columns were closed with an air-tight PVC lid. After 30 min, N_2O concentrations in the headspace of the columns were measured with a photo-acoustic infra-red gas analyzer (Innova1312). The gas analyzer was equipped with filters to minimize interference by CO_2 (a soda-lime scrubbing filter), and N_2O concentrations were internally corrected for measured CO_2 concentrations and water vapor. The assumption of linear increase during the measurement period under our experimental conditions was checked several times.

Actual denitrification at the start and at the end of the incubation was measured by acetylene inhibition (Robertson and Tiedje, 1987). Briefly, intact soil columns were divided into three equal parts of 10 cm height, and put into air-tight containers (7.5 L) after which acetylene was added to a 5% concentration (v/v). Twenty-four and 48 h after acetylene addition, N_2O concentrations in containers were measured with a photo-acoustic infra-red gas monitor as described above. We calculated cumulative denitrification as follows: we calculated N_2O/N_2 ratios for each of the four treatments separately, using N_2O production rates and actual denitrification rates on the same day at the start and at the end of the experiment. We assumed that for each treatment, these N_2O/N_2 ratios changed linearly over the course of the experiment. Thus, for each treatment, we interpolated these N_2O/N_2 ratios, resulting in a linear function that predicted N_2O/N_2 ratios at every date we measured N_2O production. We then used those ratios combined with actual N_2O measurements to calculate total denitrification at each sampling date, which we then used to calculate cumulative denitrification.

Microbial biomass N and ^{15}N were determined using the fumigation-extraction method (Brookes et al., 1985). Briefly, 5 g of soil was fumigated with $CHCl_3$ for 24 h at 20 °C. After removal of $CHCl_3$, soluble N was extracted from fumigated and unfumigated samples with 0.5 M K_2SO_4 for 30 min on an orbital shaker. DON in the extracts was converted to NH_4^+ and NO_3^- by persulfate digestion, and the microbial N flush was converted to microbial biomass N using a k_{EN} factor of 0.55 (Brookes et al., 1985). In the second experiment, after persulphate digestion, microbial biomass ^{15}N was determined by diffusing ammonium and nitrate onto an acid treated filter wrapped in teflon tape. The sample was subsequently analyzed for ^{15}N enrichment on an isotope ratio mass spectrometer (ANCA-IRMS, Europa Scientific Integra, UK) interfaced with a CN sample converter at the UC Davis Stable Isotope Facility, with atmospheric N_2 as a standard (0.3663% atomic excess).

Herbage was clipped and removed from the columns at weekly intervals during the 4-week incubation. Total plant herbage was

dried at 70 °C, weighed, milled, and analyzed for total N and ^{15}N content using a C/N analyzer as described above. In the ^{15}N experiment, in addition to above ground plant material, roots were washed, dried, milled and analyzed for total and ^{15}N content.

Total N content in the aggregate-size fractions obtained by wet-sieving were also analyzed using a C/N analyzer. Soil inorganic N was extracted by 1 M KCl and measured using Skalar segmented flow analysis (Breda, the Netherlands). Soil organic matter N was calculated as total N minus inorganic N and microbial N.

2.5. Statistical analysis

All data were checked for normality and homogeneity of variance, 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 fertilized, high fungal biomass treatment. Main and interaction effects of fungal biomass and fertilizer addition were assessed by two-way analysis of variance, with the exception of N leaching and N_2O fluxes, which were assessed by two-way repeated measures ANOVA. All statistical tests were done using the statistical package SPSS (SPSS Inc., Chicago, IL).

3. Results

3.1. Fungal and bacterial biomass in main experiment

Fungal biomass increased in all treatments during incubation (Fig. 3A). The increase was highest in the high fungal biomass soil ($F_{1,19} = 56.29$, $P < 0.001$), and was reduced by fertilization ($F_{1,19} = 36.93, P < 0.001$). The negative effect of fertilization on fungal growth was stronger in the high fungal biomass soil than in the low fungal biomass soil (fungal biomass × fertilization interaction $F_{1,19} = 8.81$, $P = 0.008$), which resulted in convergence of fungal biomass in the unfertilized low fungal biomass soil and the fertilized high fungal biomass soil (Fig. 3A). Contrastingly, bacterial biomass decreased during incubation (Fig. 3B). Bacterial biomass did not differ significantly between the low and high fungal biomass soil at the start of the experiment, nor after 4 weeks of incubation. Fertilization differentially affected bacterial biomass in the low and in the high fungal biomass soil (fungal biomass × fertilization interaction $F_{1,19} = 4.79$, $P = 0.041$). The change in fungal/bacterial biomass ratio (F/B ratio; data not shown) was largely driven by changes in fungal biomass. The F/B ratio was highest in the high fungal biomass soil ($F_{1,19} = 16.08$, $P = 0.001$), was reduced by fertilization ($F_{1,19} = 9.17, P = 0.007$), and the negative effect of fertilization on the F/B ratio was greatest in the high fungal biomass soil (fungal biomass × fertilization interaction $F_{1,19} = 7.63, P = 0.012$).

3.2. N losses in main experiment

N_2O emission ($F_{1,30} = 7.36$, $P = 0.011$) (Fig. 4A, Table 2) and cumulative denitrification ($F_{1,31} = 6.47$, $P = 0.016$, Table 2) were lowest in the high fungal biomass soil. Fertilizer addition increased N_2O emission ($F_{1,30} = 5.60$, $P = 0.025$) (Fig. 4A, Table 2) and denitrification ($F_{1,31} = 21.56$, $P < 0.001$, Table 2) in both soils. We found a negative relationship between fungal biomass and N_2O emission in the unfertilized treatments, but not in the fertilized treatments (Fig. 5A). Over the whole experimental period, N_2O emissions were positively correlated to leachate NO_3^- concentrations ($R^2 = 0.147$, $P < 0.001$).

The addition of fertilizer had a differential effect on mineral N leaching in the low and high fungal biomass soil (fungal biomass × fertilization interaction $F_{1,31} = 11.47, P = 0.002$). In the high fungal biomass soil, fertilization did not increase leaching of mineral
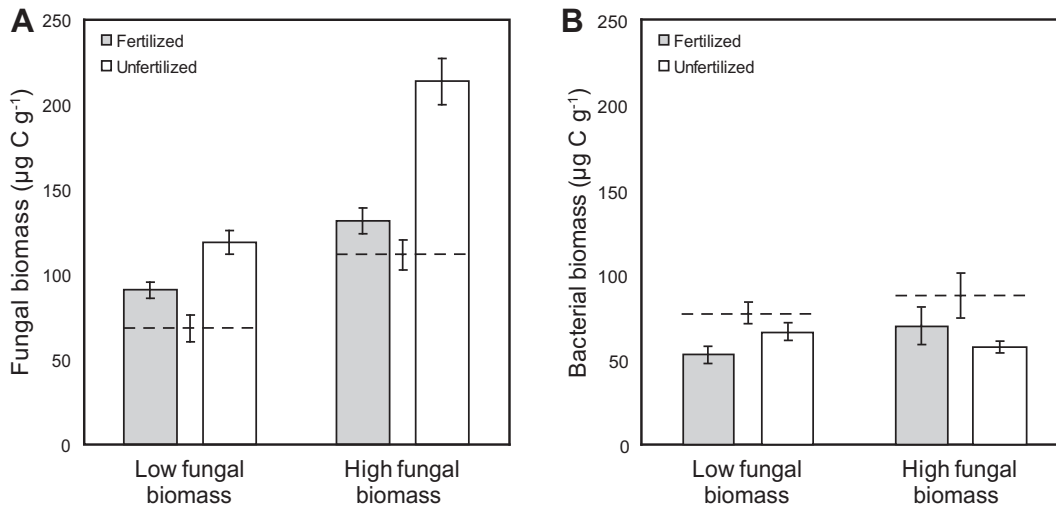
Fig. 3. Effects of N fertilization after 4 weeks on fungal (A) and bacterial (B) biomass in low vs. high fungal biomass soil. Dashed lines indicate fungal and bacterial biomass at the start of the experiment (mean $\pm$ 1 s.e.m., $n = 3$). Fungal biomass differed significantly between the two soils at the start (t-test, $P = 0.011$), bacterial biomass did not (t-test, $P = 0.5$). Bars denote fungal and bacterial biomass at the end of the experiment (mean $\pm$ 1 s.e.m., $n = 6$).

N, whereas in the low fungal biomass soil, leaching was increased more than threefold (Fig. 4B). N leaching did not differ between the unfertilized controls of both soils (Fig. 4B, Table 2). There was a negative correlation between fungal biomass and N leaching in the fertilized treatments, but not in the unfertilized treatments (Fig. 5B). Organic N leached was higher in the high fungal biomass soil than in the low fungal biomass soil, but was not affected by fertilization (Table 2). Organic N leaching only formed a small part of the total amount of N lost (Table 2). Apart from day 1, volumes of leachates did not differ between the two soils (data not shown).

Total N lost was highest in the fertilized treatments. After the addition of fertilizer, the amount of N lost was greatest in the low fungal biomass soil (Table 2).

3.3. N pools

3.3.1. Main experiment

Plant N uptake in shoots did not differ among the treatments (Table 2); also mineral N in the soil columns at the time of sampling

did not differ between the low and the high fungal biomass soil, nor was it affected by fertilization. There was, however, an interaction effect of fungal biomass and fertilization on soil mineral N: the positive effect of fertilization on mineral N was more pronounced in the high fungal biomass soil than in the low fungal biomass soil (Table 2). Microbial biomass N at the end of the incubation was significantly higher in the low fungal biomass soil than in the high fungal biomass soil (Table 2), and was not affected by fertilization.

The two soils neither differed in their micro- or macro-aggregate-size distribution, nor in their aggregate-associated carbon and N (data not shown).

3.3.2. ^{15}N experiment

Microbial biomass N was generally lower in this additional experiment (Table 3) than in the main experiment, probably because soil columns were taken in late winter instead of late autumn. Fungal biomass was 55 ± 6 and 92 ± 10 µg C g^{-1} for the low and high fungal biomass soil, respectively; bacterial biomass was 36 ± 4 and 28 ± 4 for low and high fungal biomass soil. Leaching was
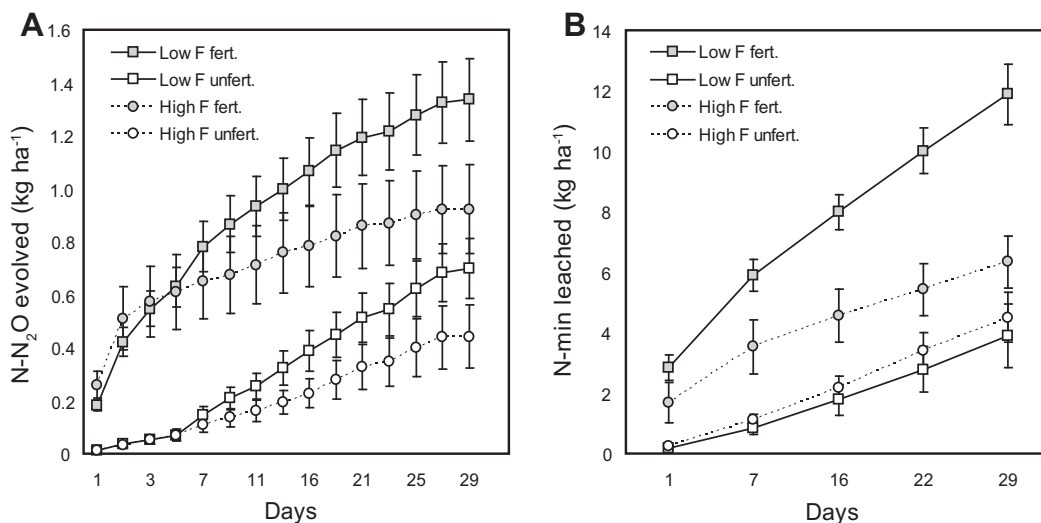
Fig. 4. Cumulative amounts of N lost following different N fertilization treatments in low vs. high fungal biomass soil. A, N$_2$O evolved. B, mineral N leached. Symbols represent means $\pm$ 1 s.e.m. ($n = 9$). Abbreviations: Low F fert., low fungal biomass fertilized; Low F unfert., low fungal biomass unfertilized; High F fert., high fungal biomass fertilized; High F unfert., high fungal biomass unfertilized.

Table 2
Fertilization effects after 4 weeks on N pools and loss pathways (kg N ha^{-1}) in low vs. high fungal biomass soil.

	Mineral N leached	Organic N leached	N–N$_2$O evolved	Estimated denitrification	Total N lost[a]	Shoot N uptake	Soil mineral N	Microbial biomass N
Low fungal biomass								
Fertilized	11.9 (1.0)	1.5 (0.1)	1.3 (0.2)	8.7 (1.2)	22.0 (0.8)	31.4 (2.5)	16.3 (1.4)	243 (8.3)
Unfertilized	3.9 (1.0)	1.3 (0.1)	0.7 (0.1)	3.4 (0.5)	8.6 (1.5)	29.4 (5.9)	18.1 (1.7)	248 (13.3)
High fungal biomass								
Fertilized	6.4 (0.9)	2.2 (0.2)	0.9 (0.2)	6.1 (1.6)	14.6 (2.3)	30.6 (4.3)	19.3 (3.0)	218 (15.4)
Unfertilized	4.5 (0.8)	2.3 (0.1)	0.4 (0.1)	1.9 (0.5)	8.8 (1.2)	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 (0.098)	0.02 (0.9)	0.78 (0.4)	5.36 (0.032)
Fertilization	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 × fertilization	7.90 (0.008)	1.48 (0.2)	0.05 (0.8)	0.12 (0.7)	4.12 (0.033)	0.32 (0.6)	4.74 (0.042)	0.05 (0.9)

Values in the upper part of the table represent means (s.e.m.; *n* = 6).
[a] Total N lost is the sum of mineral N leached, organic N leached, and estimated denitrification.

higher in the high fungal biomass soil: Two weeks after the addition of 30 kg ha^{-1} ^{15}N-labeled fertilizer, the amount of mineral N leached was almost 3-fold higher in the high fungal biomass soil than in the low fungal biomass soil (Table 3). Leaching of ^{15}N was 4-fold higher.

Microbial biomass N was 19% higher in the high fungal biomass soil, while ^{15}N immobilized in this pool was twice as high in the high fungal biomass soil. However, the amounts of ^{15}N immobilized were small relative to the amounts found in plants and soil organic matter (Table 3). Thus, microbial biomass was not a major sink of ^{15}N. ^{15}N recovery in microbial biomass was 2.9%, vs. 22.5 and 16.3% in the plants and the soil organic matter, respectively, in the high fungal biomass soil. In this soil, due to much lower root biomass N, total plant N was much lower (171 vs. 269 kg N ha^{-1}) but the ^{15}N percentage tended to be higher. The result was a lower ^{15}N immobilization in the plants in the high fungal biomass soil (6.8 vs. 7.4 kg ^{15}N ha^{-1}). Total ^{15}N recovery was considerably higher in the high fungal biomass soil (59 vs. 45%). This suggests that in the low fungal biomass soil, which showed lower N leaching, more ^{15}N was lost by denitrification (which was not measured in the additional experiment). When leaching was subtracted, ^{15}N recovery in soil plus plant was similar in both soils: 44 and 41% in the high and low fungi soil, respectively.

4. Discussion

Our main experiment, and our pilot experiment, showed that N losses were lower in the soil characterized by a higher fungal biomass. The key finding of the main experiment is that the addition of inorganic N did not increase N leaching in the high fungal biomass soil, whilst in the low fungal biomass soil N leaching increased threefold compared to the unfertilized control (Fig. 4A). N$_2$O emission and total denitrification were lower in the high fungal biomass soil irrespective of the addition of inorganic N (Fig. 4B). However, when we did an additional experiment using ^{15}N to unravel the mechanism behind these lower N losses in the high fungal biomass soil, we found contrasting results: N leaching tended to be higher in the high fungal biomass soil.

In the introduction we listed three potential mechanisms which might explain the negative relationship between fungal biomass and N losses: fungal-dominated soils could have (1) lower gross N mineralization and higher immobilization of inorganic N into microbial biomass; (2) increased protection of organic matter because of increased aggregate formation, and (3) increased plant N uptake through arbuscular mycorrhizal fungi. Because aggregate-size distribution did not differ between treatments, potential
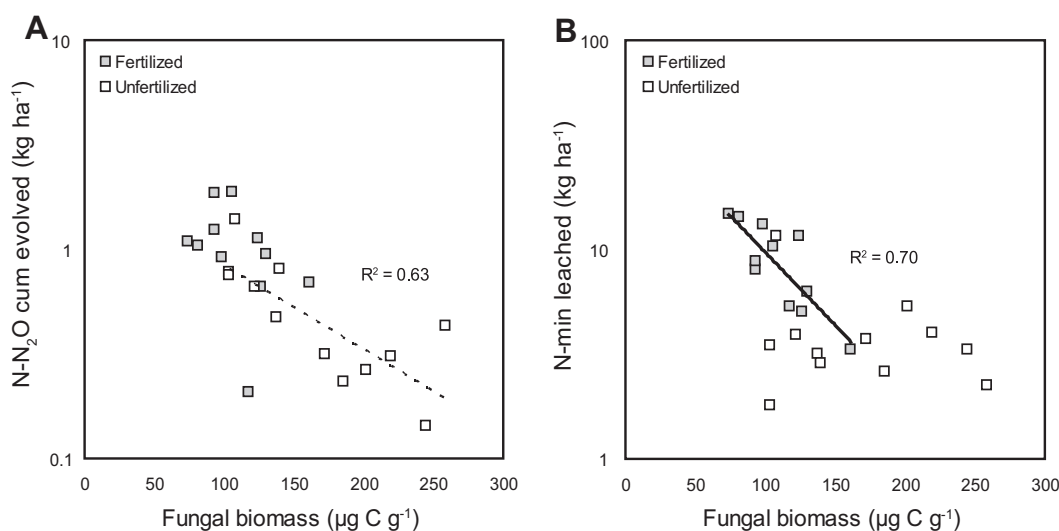
Fig. 5. Relationships between fungal biomass and total amounts of N lost. A, relationship between fungal biomass and total N$_2$O emissions in fertilized and unfertilized treatments. The correlation was significant for the unfertilized treatments (dashed line, $F_{1,10}$ = 16.79, P = 0.002). B, relationship between fungal biomass and mineral N leaching in fertilized and unfertilized treatments. The correlation was significant for the fertilized treatments (solid line, $F_{1,9}$ = 20.59, P = 0.001). Symbols represent single observations.

Table 3
Total N pools, their ^{15}N percentage, ^{15}N (kg N ha^{-1}), and ^{15}N recoveries (%), 2 weeks after N addition in low vs. high fungal biomass soil. Values represent means (s.e.m.; $n = 6$).

	Mineral N leached	Soil mineral N	Microbial biomass N	Shoot N	Root N	Total plant N	Org. matter N
Low fungal biomass							
Total N (kg N ha^{-1})	2.2 (0.8)	30.9 (8.3)	106.6 (6.7)	37.9 (5.2)	230.7 (28.7)	268.5 (31.6)	8032 (497)
^{15}N (% of total N)	25.2 (10.9)	0.9 (0.1)	0.4 (0.1)	8.4 (0.8)	1.6 (0.2)	2.6 (0.3)	0.04 (0.01)
^{15}N (kg N ha^{-1})	1.1 (0.5)	0.3 (0.1)	0.5 (0.1)	3.4 (0.3)	4.0 (0.3)	7.5 (0.4)	4.09 (1.05)
^{15}N Recovery (%)	3.5 (1.8)	1.2 (0.4)	1.6 (0.4)	11.5 (1.1)	13.4 (0.9)	24.9 (1.3)	13.6 (3.5)
High fungal biomass							
Total N (kg N ha^{-1})	5.8 (2.0)	30.5 (13.2)	127.3 (9.1)	53.4 (15.8)	117.6 (23.2)	171.1 (38.6)	8749 (366)
^{15}N (% of total N)	48.5 (12.8)	2.0 (0.3)	0.6 (0.1)	10.4 (3.0)	3.0 (1.0)	4.8 (1.3)	0.05 (0.00)
^{15}N (kg N ha^{-1})	4.5 (1.7)	0.7 (0.3)	0.9 (0.2)	3.9 (0.4)	2.9 (0.2)	6.8 (0.5)	4.90 (0.54)
^{15}N Recovery (%)	15.1 (5.8)	2.3 (1.0)	2.9 (0.5)	12.9 (1.5)	9.6 (0.8)	22.5 (1.8)	16.3 (1.8)
P-value (t-test)							
Total N	0.07	0.49	0.05	0.19	0.006	0.04	0.14
^{15}N %	0.10	0.006	0.08	0.27	0.12	0.08	0.25
^{15}N	0.05	0.16	0.04	0.22	0.006	0.04	0.26
^{15}N Recovery %	0.04	0.15	0.04	0.22	0.005	0.16	0.25

mechanism (2) can be rejected. This leaves mechanisms (1) and (3) to be considered.

Some of our observations support mechanism (1). The lower N$_2$O emission (Fig. 4A) and total denitrification (Table 2) in the high fungal biomass soil, both with and without fertilizer addition, might be explained by a lower NO$_3^-$ availability in the high fungal biomass soil due to lower gross N mineralization. This would also explain lower NO$_3^-$ leaching (Fig. 4B). There was a negative relationship between fungal biomass and N$_2$O production in the unfertilized treatments, which is in sharp contrast with findings of fungi dominating denitrification (Crenshaw et al., 2008; Laughlin and Stevens, 2002; McLain and Martens, 2006). However, this negative relationship between fungal biomass and N$_2$O production was blurred after fertilizer addition (Fig. 5A). This shows that NO$_3^-$ availability was a larger control on N$_2$O emissions than microbial community composition (Cavigelli and Robertson, 2000). Although we did not measure denitrification in the ^{15}N experiment, we found a lower recovery of ^{15}N in the low fungal biomass soil than in the high fungal biomass soil (45 vs. 59%, respectively), which indicates higher denitrification in the low fungal biomass soil. Recovery of ^{15}N was low in both low and high fungal biomass soil, indicating high N losses through denitrification in this experiment. A possible explanation for high denitrification rates might be that columns were collected at the end of the winter when the soil was partly frozen. This resulted in high moisture content and slow water infiltration rates during the two-week experiment, probably creating anaerobic conditions favoring denitrification.

The addition of mineral fertilizer in the main experiment had a differential effect on mineral N leaching in the low and high fungal biomass soil: in the high fungal biomass soil, fertilization did not increase leaching of mineral N, whereas in the low fungal biomass soil, leaching increased more than threefold (Fig. 4B). This suggests that added N was either immobilized into microbial biomass—which was supported by the negative relationship between mineral N leaching and fungal biomass in the fertilized treatments (Fig. 5B)—or taken up by plants in the high fungal biomass soil. There was no relationship between microbial biomass N and N leaching however, nor was microbial biomass N affected by fertilization (Table 2). In the main experiment, the amount of mineral N added (30 kg N ha^{-1}) and the amounts of total N lost were small compared to the amount of N in microbial biomass (over 200 kg N ha^{-1}). Therefore immobilization in microbial biomass was hard to measure. Shoot N uptake equaled 30 kg N ha^{-1} but was the same in all treatments and could not explain the difference in N loss between the low and high fungal biomass soil.

In contrast to both the pilot and the main experiment, lower N leaching was found in the low fungal biomass soil after addition of mineral N in the ^{15}N experiment (Table 3). In fact, mineral N leached in the high fungal biomass soil was similar to that in the main experiment (6 kg N ha^{-1}) whereas mineral N leaching in the low fungal biomass soil was lower than in the main experiment (2 vs. 8 kg N ha^{-1} in 2 weeks) (Fig. 4B and Table 3). Although a 2-fold higher immobilization of ^{15}N into microbial biomass was found in the high fungal biomass soil, only 3% of total ^{15}N was found in the microbial biomass 2 weeks after adding N. Microbial biomass has been shown to be a significant short-term sink for added N, but the peak of immobilization occurs within days after N addition (Bardgett et al., 2003; Dunn et al., 2006; and reviewed by Kaye and Hart, 1997; Recous et al., 1990). Since we measured two weeks after N addition, the N immobilized in microbial biomass might have been remineralized and incorporated into other soil N pools. In any case, our addition experiment did not support microbial N immobilization as an important mechanism for lower N losses. Also, the mechanism of improved plant N uptake through AMF (mechanism 3) is not supported by the results of the additional experiment because there was no improved plant N uptake in the high fungal biomass soil. Due to the lower root biomass, even less ^{15}N was recovered in plant material in the high fungal biomass soil (6.8 vs. 7.5 kg ^{15}N ha^{-1}). In addition, the role of AMF was probably limited because both soils received farm yard manure and additional P and K fertilizer.

One possible explanation for the discrepancies between the results from our main and pilot experiment on the one hand, and the ^{15}N experiment on the other hand, might be the different experimental conditions due to different field sampling times (end of autumn in main and pilot experiment, vs. end of winter in ^{15}N experiment). Biomass of fungi and bacteria generally increases toward autumn, and declines toward the end of winter (Zwart et al., 1994), which is supported by the lower biomass of fungi and bacteria in the ^{15}N experiment. Moreover, fungal biomass has been shown to remain low well into spring, whereas bacterial biomass has been shown to increase again in February (Bloem et al., 1994; Zwart et al., 1994). Thus, presumably, fungi (including AMF) would have been relatively more active in our main and pilot experiment than in our ^{15}N experiment, whereas bacterial activity would have been more important in the ^{15}N experiment. In addition, the shorter duration might have prevented fungi to grow and exert an influence on N cycling to the same extent as in the main experiment.

In the main experiment, fungal biomass increased during the incubation, whereas bacterial biomass decreased (Fig. 3). Apparently,

conditions were in favor of fungi, supporting our (in the previous paragraph) proposed idea that fungi were relatively influential in the main experiment. Fertilizer addition caused fungal biomass in the high fungal biomass soil to converge with fungal biomass in the unfertilized low fungal biomass soil (Fig. 3A). So, although differences in fungal biomass between the two soils had been consistent for 5 years in the field (Fig. 1), these differences were neutralized within 4 weeks after a modest (30 kg N ha^{-1}) mineral fertilizer addition. Negative effects of inorganic N on fungi have been mostly attributed to changes in vegetation and organic matter characteristics (Bardgett et al., 1999; Donnison et al., 2000). Because of the short time course of the negative effect on fungal growth of inorganic N addition in this experiment, our results rather suggest a direct negative effect on fungal growth. It is not clear why inorganic N would have such an effect on fungal growth. It has been suggested that inorganic N represses enzyme activity (Fog, 1988).

N addition increased neither plant biomass nor fungal and bacterial biomass, which indicates that our grassland soils were not N limited. Still, in the main experiment, N retention was higher in the high fungal biomass soil than in the low fungal biomass soil. Even though the two soils did not differ in most parameters relevant for N cycling, the lower farm yard manure inputs of the high fungal biomass soil inevitably will have changed organic matter quality, reduced N availability and thus selected for a more fungal-dominated microbial community. This fungal-dominated microbial community might in turn have reduced N availability further (Wardle et al., 2004a). In addition, physical properties might have affected leaching rates, although we found no differences in aggregate-size distribution, bulk densities, and volumes leached. Thus, we propose that the lower manure inputs of the high fungal biomass soil have created a set of ecosystem properties that retain N better. Summarized, our results show that higher fungal biomass is probably not the direct cause of higher N retention, but rather an indication of low nitrogen availability.

In conclusion, we are offering a first attempt to answer the key question whether fungal-dominated systems have lower N losses. Our results confirm that a higher fungal biomass can be considered as an indicator of higher nutrient retention in soils. In our grassland soils the higher nitrogen retention was probably the result of lower farm yard manure inputs (40 vs. 80 kg N ha^{-1} yr^{-1}) in the past six years. Interestingly, in our soils with grass-clover, above ground production was maintained, not only in our 4-week experiment (Table 2, plant N uptake), but also in the field (De Vries et al., 2006). The clover in the sward may have compensated for the reduced fertilization; it is well documented that N fixation by legumes decreases with increasing N fertilization (Carlsson and Huss-Danell, 2003). Thus, increased nutrient retention does not necessarily result in lower crop production.

More research is needed to understand why soils with a higher fungal biomass can reduce N losses. If this is a general phenomenon as has been widely assumed so far, testing the relationship between fungal biomass and N losses on longer timescales, and across more pairs of soils or across a gradient of soils varying in fungal biomass would be a way forward. In addition, more mechanistic studies using sterilized soils reinoculated with different microbial communities might provide more insight in the mechanisms involved.

Acknowledgments

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