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Differences in activity and N demand between bacteria and fungi in a microcosm incubation experiment with selective inhibition

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ABSTRACT

Bacteria and fungi are important micro-organisms in the soil, but may differ in their impact on net N-mineralization. The hypothesis was tested that fungi are characterized by low microbial activity, but also low immobilization, and bacteria by high activity and high immobilization. A one-month laboratory incubation experiment with selective inhibition of fungi (cycloheximide) or bacteria (streptomycin) was conducted with samples of organic layer and mineral topsoil (0–10 cm) from neutral, bacteria-dominated and acidic, fungi-dominated Luxembourg beech forests. In the control treatment, respiration was higher in neutral than in acidic soil, but net N-mineralization was lower, due to higher immobilization. In the antibiotic treatments, differences in nitrification suggest that selective inhibition indeed occurred; in all soils and horizons, nitrification was especially limited by bactericide. Besides as inhibitor of the target group, antibiotics may also serve as source of C and N for the non-target group. For both bactericide and fungicide, acidic soils showed higher net recovery of C and N from antibiotics than neutral soil, which suggests that uptake or sorption of antibiotics is higher in the latter. Clear differences between neutral and acidic soils arose when the main micro-organisms were stimulated. In bacteria-dominated neutral soil, application of fungicide led to increased microbial respiration. In fungi-dominated acidic soil, however, application of bactericide did not lead to higher respiration, but to increased net N-mineralization per unit respiration, which supports a lower immobilization. Differences between antibiotics were consistent for organic layer and mineral topsoil, with increase in activity with fungicide, and lower immobilization with bactericide. The results provide correlative and experimental evidence that reduced immobilization by fungi compensates for their lower rates of activity with respect to N-availability to the vegetation.

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1. Introduction

Fungi and bacteria are different micro-organisms, which live in different places in the soil (Moore et al., 2005), and differ in morphology, physiology and lifestyle (Myrold and Posavatz, 2007). However, both are involved in mineralization of C and N in the soil (Swift et al., 1979). Fungi often dominate in acid soils (Blagodatskaya and Anderson, 1998; Bååth and Anderson, 2003), and are associated with slow and conservative nutrient cycling. Fungal-dominated soils are also thought to have low net N-mineralization, due to low biological activity (Aerts and Chapin, 2000; Ponge, 2003; Schimel and Bennett, 2004), and high immobilization in or through the extensive hyphal network (de Vries et al., 2011).

Bacteria are more common in so-called nutrient-rich soils, often with high pH and dominated by earthworms. Bacteria are associated with high biological activity and decomposition, and net N-mineralization in bacteria-dominated soils is thought to be high as well (Aerts and Chapin, 2000; Ponge, 2003; Schimel and Bennett, 2004).

Yet, there are some problems with this scenario. More than a few studies report higher instead of lower net N-mineralization in acidic, fungal-dominated systems, compared to neutral, bacteria-dominated soil (Zöttl, 1960; Davy and Taylor, 1974; Verhoeven et al., 1990; Kooijman and Besse, 2002; Kooijman et al., 2008; Kooijman and Smit, 2009; Mettrop et al., 2014). This may be explained by low immobilization in fungi, due to high C:N ratios and relatively slow life cycles (Hassink, 1994; Moore et al., 2005; Cleveland and Liptzin, 2007). In bacteria, microbial N-demand may be much higher, due to lower C:N ratios, fast life cycles, and use of

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amino acids as osmoregulators instead of carbohydrates (Measures, 1975; Kuehn et al., 1998).

Nevertheless, this scenario has not been experimentally tested, with e.g., selective inhibition. Selective inhibition of fungi and bacteria has been applied in many short-term experiments, often with substrate induced respiration (SIR), used to separate fungal and bacterial activity (Anderson and Domsch, 1973; Bååth and Anderson, 2003; Bailey et al., 2003; Myrold and Posavatz, 2007). In longer-term experiments, use of fungicides and bactericides is usually more complicated, because the antibiotics not only selectively kill fungi or bacteria, but also supply the remaining micro-organisms with extra dissolved organic C (DOC) and N (DON) (Chen et al., 2001). Also, often used antibiotics such as cycloheximide and streptomycin differ in C:N ratio, which may confound effects of selective inhibition. On the other hand, several methods exist to separate fungi and bacteria (e.g., Frostegård et al., 1991; Bloem et al., 1995; Bååth and Anderson, 2003; Bloem and Vos, 2004), but most of them are unsuitable to study functional differences in N-cycling.

In this study, we applied selective inhibition with the fungicide cycloheximide and the bactericide streptomycin in a one-month laboratory incubation experiment, to test differences between bacteria and fungi in C and N-cycling. Selective inhibition was applied to the ectorganic layer and mineral topsoil (0–10 cm) of two beech forest soils with different microbial community composition: a neutral soil on calcareous marl, dominated by bacteria, and an acidic soil on sandstone parent material, dominated by fungi (Kooijman et al., 2008, 2009).

The effects of bactericide and fungicide on C and N-cycling may be complicated, because neutral and acidic soils are dominated by different microbial groups. Apart from selective inhibition of the target groups, we therefore also used side effects to the non-target groups such as extra supply of C and N (Chen et al., 2001), rather than consider them as complicating effects. The main microbial group in a particular soil may be stimulated by selective inhibition of the other group, but also by supply of C and N from antibiotics. We tested stimulation and inhibition of the main microbial group in particular soils separately. To stimulate the main microbial group, the other group was inhibited with fungicide in neutral soil, and bactericide in acidic soil. In this way, differences between (neutral) bacteria-dominated and (acidic) fungi-dominated soil could be maximized, and differences between bacteria and fungi in respiration, net N-mineralization and microbial immobilization better tested. In another set of experiments, the main microbial group was inhibited, and the other stimulated with bactericide in neutral soil, and fungicide in acidic soil. With inhibition of the main group, antibiotics probably at least partly lead to a shift in microbial communities. However, because part of the main group may still be present despite (relative) increase of the minor group, differences between soil types may be smaller than in the first set, where probably mainly the main group of micro-organisms survived.

2. Methods

2.1. Study sites

The study sites were selected in Luxembourg, south of Diekirch, with humid temperate climate and rainfall in all months, and consisted of two clearly different model ecosystems: neutral and acidic beech (*Fagus sylvatica* L.) forests. Although mainly studied qualitatively and with relative rather than absolute amounts, the two forests clearly differ in microbial community composition, with bacteria more prominent in neutral soil, and fungi in acidic soil in both organic layer and mineral topsoil (Table 1). To reduce differences in litter input as much as possible, sampling localities were selected in monospecific, mature beech stands, without substantial undergrowth. The neutral site was located on Mesozoic marl, had Ah-Bw-C soil profile and calcareous Cambisol soil type (IUSS WRB, 2006), Vermimull humus form (Green et al., 1993) and was covered with *Hordelymo-Fagetum* forest (Niemeijer et al., 2010). The acidic site was located on Mesozoic sandstone, had Ah-E-Bw-C soil profile with signs of recent podzolisation and brunis Arenosol soil type, Mormoder humus form, and *Luzulo-Fagetum* forest type. In each forest, samples were collected in five randomly selected plots in the beginning of October. The organic layer was sampled in 25 × 25 cm squares; fresh litter was separated and not included. The mineral topsoil was sampled in three metal rings of 10 cm depth, which comprised the Ah completely, and were combined to a composite sample. Fresh weight and gravimetric moisture content were determined, and dry weight and bulk density calculated. After drying (48 h at 70 °C for organic and 105 °C for mineral samples) and grinding of subsamples, C and N contents were determined with a CNS analyzer (Westerman, 1990).

2.2. Selective inhibition of bacteria and fungi

Bacteria and fungi were selectively inhibited with the bactericide streptomycin and the fungicide cycloheximide (Anderson and Domsch, 1973; Bååth and Anderson, 2003). Dosages were applied according to Bååth and Anderson (2003). The two antibiotics differ in C:N ratio, with values of 3 for streptomycin and 16 for cycloheximide. Streptomycin was applied as 4 mg g⁻¹ dry weight in the mineral topsoil of the neutral site, and 2 mg g⁻¹ in the acidic site, where bacteria were less abundant. Cycloheximide was applied as 6 mg g⁻¹ in acidic soil and 3 mg g⁻¹ in neutral soil, where fungi were less abundant. For the organic layer, the amount of antibiotics applied was the same as for mineral topsoil, but organic material is lighter, and sample fresh weights were approximately two times lower. To prevent regrowth, antibiotics were applied weekly during the one month incubation experiment described below. Fresh samples were measured 24 h after the first application of antibiotics, and incubated samples a few days after the last application.

Table 1
Soil and microbial characteristics in ectorganic layer and mineral topsoil (0–10 cm) of neutral and acidic beech forests in Luxembourg. Data are derived from Kooijman et al. (2008, 2009) and based on cultivation experiments and micromorphological analysis of thin sections. CFU = colony forming units; primary fungi feed on leaf fragments; secondary fungi feed on faecal pellets.

	Neutral organic layer	Neutral mineral topsoil	Acidic organic layer	Acidic mineral topsoil
pH _{H2O}	6.4 (0.7)	7.0 (0.5)	4.7 (0.4)	3.7 (0.1)
Litter input (kg C m ⁻² yr ⁻¹)	0.23 (0.04)	–	0.23 (0.05)	–
N in litter input (g N m ⁻² yr ⁻¹)	4.2 (0.3)	–	5.9 (1.0)	–
Bacteria colony forming units (10 ⁸ CFU m ⁻²)	86 (58)	276 (352)	573 (275)	52 (14)
Bacteria in micromorphological thin sections	High amount	High amount	Not detected	Not detected
Primary fungi in thin sections	Low amount	Not detected	High amount	Not detected
Secondary fungi in thin sections	Not detected	Not detected	High amount	High amount

2.3. Laboratory incubation experiments

Potential net N-mineralization was measured in a one-month laboratory incubation experiment. Samples were homogenized by hand, and roots were removed. Fresh samples were put into large petri dishes and brought to optimal gravimetric moisture levels, where microbial activity was not hampered by drought or oxygen stress (300% for organic and 50% for mineral soil samples; Tietema, 1992). Petri dishes were stored at 20 °C in the dark in a climate chamber, in slightly open polyethylene bags with moist paper; moisture content was checked and replenished when necessary. Ammonium and nitrate of fresh and incubated samples were extracted with 50 ml 0.5 M K₂SO₄ solution, and measured on a continuous-flow analyzer (Westerman, 1990). The amount of DOC and total N was also measured, and DON was calculated as the difference between total N and ammonium + nitrate. Net N-mineralization was calculated from differences in ammonium and nitrate concentrations between incubated and fresh samples. Nitrification was calculated as the amount of nitrate released over the incubation period, expressed as percentage of net N-mineralization. Nitrification was considered to be primarily a bacterial process, as the contribution of heterotrophic nitrification is usually not more than a few percent (Barraclough and Puri, 1995). In the fungicide and bactericide treatments, net recovery of the total amount of DOC and DON supplied was calculated as the net release of DOC and DON during the incubation period, compared to the total DOC and DON input from antibiotics. Net recovery of DOC and DON was expressed as percentage of the total input from antibiotics, and used as indicator of differences in (microbial) uptake and/or sorption of C and N from antibiotics in different soils.

Microbial C and N were measured with chloroform fumigation and extraction (Brooks et al., 1985). Microbial C and N were only measured in the control treatment, because in the antibiotic treatments, DOC and DON concentrations in the unfumigated samples were too high to detect differences with the fumigated samples. Fumigated samples were flushed for 24 h with chloroform and extracted with 0.5 M K₂SO₄ immediately afterwards, to prevent microbial regrowth. Ammonium, nitrate, DON and DOC were measured in fumigated and non-fumigated samples, using a continuous-flow analyzer. Concentrations of DOC and total N (DON, ammonium and nitrate) were measured in fumigated and non-fumigated samples, both from fresh and incubated samples. Microbial C and N concentrations were calculated as differences between fumigated and non-fumigated samples. To characterize the two soil types, microbial C and N of fresh samples were used. However, net N-mineralization per unit microbial C or N was based on mean values of microbial C in fresh and incubated samples.

Respiration was measured at the start and end of the incubation experiments, as preliminary experiments indicated that CO₂-production during the experiment was more or less constant (Kooijman and Smit, 2009). Fresh or incubated material was placed in open glass jars during one night, with the equivalent of 5 g dry weight for organic and 10–15 g for mineral samples. During measurements, the jars were closed and air samples were extracted by needle. CO₂-concentrations were measured three times (after approximately 10, 13 and 16 h) by injecting air samples into a Carlo Erba Varian gas chromatograph (Tietema, 1992). CO₂-production rates were calculated from the increase in CO₂-concentration during the day, the volume of the head space and sample dry weight. Total CO₂-production over the incubation period was calculated, based on its duration and CO₂-production at start and end of the experiment.

Efficiency of N-mineralization was calculated as net N-mineralization per unit C respired, which can be used as proxy for microbial immobilization, and the relative amount of N

available to the vegetation (Kooijman et al., 2008; Kooijman and Smit, 2009). In this case, high N mineralization per unit respiration indicates low immobilization by micro-organisms, and a high efficiency of N-mineralization for the vegetation. For the control treatment, efficiency of N-mineralization was also expressed as net N-mineralization per unit soil C and microbial C and N.

2.4. Modelled immobilization

Microbial immobilization could only be calculated for the control treatment, in which microbial C and N were measured at the start and end of the incubation experiment. Immobilization was calculated with a theoretical model, which must be seen as indicative rather than absolute. The model is based on existing theoretical equations of C and N dynamics (Tietema and Wessel, 1992), which were reformulated in Kooijman et al. (2008). When respiration, net N-mineralization and N:C ratios of substrate and microbes are known, microbial growth efficiency (e_c) can be estimated. Microbial growth efficiency is the fraction of gross C-release used for microbial assimilation, and a key parameter in the allocation of C and N from organic matter to microbes (Schimel, 1988; Tietema and Wessel, 1992). Microbial growth efficiency was calculated with measured values of respiration (Q), net N-mineralization (NM) and N:C ratios of substrate (NC_S) and microbes (NC_M) as:

$$e_c = \frac{(NC_S \times Q) - NM}{(NC_M \times Q) - NM}$$

Using the estimated e_c values, immobilization (I) was calculated as:

$$I = \left(\frac{e_c}{1 - e_c} \right) \times NC_M \times Q$$

Substrate C:N ratios were derived from fresh samples. Microbial C:N ratios were derived from mean values, over the entire incubation period. Microbial C:N ratios used in acidic soils were 6.6 ± 1.4 for the organic layer and 9.7 ± 3.3 for the mineral topsoil, and in neutral soil 5.8 ± 2.0 for the organic layer and 7.8 ± 2.3 for the mineral topsoil. In acidic soil, one microbial C:N ratio of the mineral topsoil was discarded, due to the extreme value of 41.8. Mean values and standard deviations of modelled immobilization were calculated for different sites and horizons, based on full four-factor perturbation tests (Henderson-Sellers and Henderson-Sellers, 1993). Each perturbation test was based on 16 runs with mean values of the four input parameters (respiration, net N-mineralization and N:C ratios of substrate and microbes), plus or minus one standard deviation.

2.5. Statistical analysis

Differences between soil types in soil and microbial characteristics in fresh soil were tested with two-way analysis of variance, with soil type (neutral and acidic) and horizon (organic layer and mineral topsoil) as independent factors (Cody and Smith, 1987). Differences between individual mean values were tested with post hoc LS-means tests.

Potential effects of antibiotics on nitrification and net recovery of C and N from antibiotics were tested with three-way analysis of variance, with soil type, horizon and treatment (control, bactericide and fungicide) as independent factors, and post hoc LS-means tests.

For potential effects of antibiotics on C and N-cycling, net N-mineralization and respiration were calculated per kg OM, to allow a direct comparison of neutral and acidic soils, as well as organic layers and mineral topsoils. In addition, the dataset was separated

into two parts: one with stimulation of the main group of micro-organisms, to enlarge differences between bacteria and fungi as much as possible, and the second with inhibition of the main group, to reduce differences in microbial community composition and/or shift this towards the other group. In the first set, fungicide was used in neutral soil, and bactericide in acidic soil, to inhibit the minor and stimulate the main microbial group in both organic layer and mineral topsoil. In the second set, bactericide was used in neutral soil, and fungicide in acidic soil, to inhibit the main and stimulate the minor microbial group in both organic layer and mineral topsoil. Differences in C and N-cycling between soil types, horizons and antibiotic treatments were tested for the stimulation and inhibition set separately. In each set, three-way analysis of variance was applied with soil type, horizon and treatment (control and stimulation, or control and inhibition of the main group) as independent factors. Because values of the control treatment were used in both sets, Bonferroni-corrections were applied, and differences were significant below 0.025 instead of 0.05. Differences between individual mean values were tested with post hoc LS-means tests.

The relative contribution to total C and N-cycling of soil types and horizons largely differed due to differences in amount of OM, and respiration and net N-mineralization of the upper 10 cm were also calculated as total per m². To test potential overall effects of bactericide and fungicide on total respiration, net N-mineralization and net N-mineralization per unit C respired, one-way analysis of variance was applied for each soil layer separately, with treatment (control, bactericide and fungicide) as independent factor. Differences between individual mean values were tested with post hoc LS-means tests.

3. Results

3.1. Soil and microbial characteristics

As expected, neutral and acidic soils clearly differed in soil characteristics (Table 2). Soil pH was indeed higher in neutral than in acidic soil, with values of 6.8–7.1 and 3.8–4.4, respectively.

Neutral soil had approximately 1.5 times higher amounts of soil organic matter in organic layer and mineral topsoil combined than acidic soils. In neutral soil, the mineral topsoil was more important than the organic layer, and accounted for 95% of total soil organic matter. In acidic soils, the organic layer was also important, and accounted for 38% of the total. The amount of N varied accordingly, but differences in C:N ratio between the sites were rather small.

Neutral and acidic soils also clearly differed in microbial C and N. Microbial biomass per m² was largest in the neutral mineral topsoil with 34 g m⁻², although lowest in its organic layer, with only 2 g m⁻². In acidic soil, microbial biomass was with values of 8–12 g m⁻² more evenly distributed between mineral topsoil and organic layer, due to high mass of the latter. When expressed per unit OM, however, differences between neutral and acidic soil in microbial C and N were much smaller, and only slightly higher in neutral soil.

Microbial behaviour also differed between neutral and acidic soil in both organic layer and mineral topsoil. Respiration, based on CO₂-measurements, was significantly higher per kg OM in the organic layer than in the mineral topsoil, possibly due to the fresher substrate, but also showed 1.6–1.9 times higher values in neutral than in acidic soil. Total respiration per m² was 1.7 times higher in neutral than in acidic soil. Respiration quotient, i.e., respiration per unit microbial C, did however not differ, although values were higher for the organic layer than for the mineral soil.

With respect to soil type, net N-mineralization showed opposite results. Net N-mineralization per kg OM was 5.5 and 2.3 times lower in neutral than in acidic soil for organic layer and mineral topsoil, respectively. Low values for net N-mineralization in neutral soil were also measured when expressed per m², unit microbial C or N, or unit C respired. In neutral soil, which was dominated by bacteria (see Table 1), low net N-mineralization may be related to higher microbial immobilization. Modelled values suggest that immobilization in neutral soil was very high, with values of 86% or more. In contrast, in acidic, fungi-dominated soil, N-immobilization values only reached 5–22%.

Table 2

Soil and microbial characteristics related to C and N cycling of organic layer and mineral topsoil (0–10 cm) in neutral and acidic beech forests in Luxembourg. OM = organic matter; NM = net N-mineralization. Significant differences between soil type, horizons and interactions between them are indicated (two-way Anova; ns = not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001). Mean values (*n* = 5) and standard deviations; different letters indicate significant differences (*p* < 0.05) between sites and/or horizons for a particular parameter.

	Soil type	Horizon	Soil type × horizon	Neutral organic layer	Neutral mineral topsoil	Acidic organic layer	Acidic mineral topsoil
pH	***	ns	*	6.8 (0.2) ^b	7.1 (0.5) ^b	4.4 (0.2) ^a	3.8 (0.1) ^a
soil OM (kg m ⁻²)	*	***	***	0.6 (0.1) ^a	12.0 (3.7) ^c	3.1 (0.9) ^b	5.0 (0.8) ^b
Soil N (g m ⁻²)	**	***	***	12 (2) ^a	377 (107) ^d	69 (21) ^b	131 (30) ^c
C:N ratio (g g ⁻¹)	ns	***	***	25 (1) ^d	16 (2) ^a	22 (1) ^c	19 (2) ^b
Microbial C (g m ⁻²)	**	***	***	2 (1) ^a	34 (16) ^c	8 (3) ^b	12 (6) ^b
Microbial N (g m ⁻²)	***	***	***	0.3 (0.1) ^a	6.5 (1.4) ^c	1.2 (0.5) ^{ab}	1.7 (1.1) ^b
Microbial C (g kg ⁻¹ OM)	*	ns	ns	3.5 (0.8) ^b	3.0 (1.0) ^{ab}	2.6 (0.4) ^{ab}	2.2 (1.0) ^a
Microbial N (mg kg ⁻¹ OM)	*	ns	ns	455 (90) ^{ab}	576 (149) ^b	401 (99) ^{ab}	323 (184) ^a
Microbial C:N ratio (g g ⁻¹)	ns	ns	ns	7.7 (0.4) ^{ab}	5.2 (0.4) ^a	6.8 (1.2) ^{ab}	8.6 (4.8) ^b
Respiration (g kg ⁻¹ OM)	***	***	*	22 (3) ^d	9 (2) ^b	13 (1) ^c	5 (1) ^a
Respiration (g m ⁻²)	**	***	***	13 (4) ^a	100 (24) ^c	41 (15) ^b	24 (5) ^{ab}
Respiration quotient (g g ⁻¹)	ns	***	ns	5.1 (0.6) ^b	2.4 (1.0) ^a	6.4 (1.7) ^b	3.0 (0.7) ^a
Net N-mineralization (mg kg ⁻¹ OM)	***	*	*	90 (104) ^a	112 (70) ^{ab}	494 (165) ^c	253 (92) ^b
Net N-mineralization (g m ⁻²)	**	ns	*	0.1 (0.1) ^a	1.2 (0.4) ^b	1.5 (0.8) ^b	1.3 (0.6) ^b
NM per unit microbial C (mg g ⁻¹)	***	*	*	37 (49) ^a	32 (16) ^a	258 (83) ^c	121 (24) ^b
NM per unit microbial N (mg g ⁻¹)	*	ns	ns	179 (220) ^a	264 (182) ^a	1645 (396) ^b	1840 (1543) ^b
NM per unit respiration (mg g ⁻¹)	***	**	ns	5 (6) ^a	13 (5) ^b	37 (10) ^b	52 (13) ^c
Modelled immobilization (%)	***	ns	ns	91 (12) ^b	86 (6) ^b	22 (26) ^a	5 (32) ^a

3.2. Input of C and N with antibiotics

Before they can employ their inhibitive effects, antibiotics should first be taken up by the micro-organisms. In most cases, only part of the C and N supplied with antibiotics was recovered, for both bactericide and fungicide (Fig. 1). For neutral soils, recovery values were even below 50%. This suggests that uptake by micro-organisms and/or sorption to soil particles occurred in all sites and horizons. Differences between bactericide and fungicide treatments were generally significant (Table 3), but relatively small. The percentage of DOC recovered from the total amount supplied with antibiotics did not consistently differ between bactericide and fungicide. Recovery of DON was lower for bactericide than for fungicide treatments, despite the higher N-content, but only 1.3 times or less.

Recovery of DOC and DON clearly differed between neutral and acidic soils, for both organic layer and mineral topsoil. In neutral soil, recovery of DOC and DON was on average 1.8 times lower than in acidic soil, and microbial uptake and/or sorption to soil particles presumably higher. Recovery of DOC varied between 37–49% in neutral soil, compared to 73–108% in acidic soil. For DON, recovery was even lower and ranged from 25 to 36% in neutral soil to 46–62% in acidic soil, independent of the type of antibiotic.

3.3. Effects of selective inhibition on nitrification

In the control treatment, the amount of nitrate net produced was lower in neutral than in acidic soil for both organic layer and mineral topsoil, due to the overall low net N-mineralization (Fig. 2). However, in relative terms, both neutral and acidic soils showed high nitrification, with values ranging between 78 and 109% of total net N-mineralization. With bactericide, the absolute amount of nitrification did not change in neutral soil, because values in the control treatment were already low. However, in relative terms, nitrification, which is a bacterial process, dropped with bactericide to values below 21% in both organic layer and mineral topsoil. In contrast, with fungicide, nitrification increased in absolute amounts in neutral soil compared to the control treatment, due to higher net N-mineralization when fungi were inhibited, and the dominant bacteria stimulated by extra C and N (Fig. 3). In relative terms, with fungicide, nitrification was as high as in the control treatment in the mineral topsoil, and accounted for 47% in the organic layer. The clear drop in nitrification in neutral soil with bactericide, but not with fungicide, suggests that at least some selective inhibition had occurred.

In acidic soils, nitrification in the control treatment was high for both organic layer and mineral topsoil. This was the case in absolute amounts, due to high overall net N-mineralization, but also for the relative amounts, which amounted to approximately 100% of total net N-mineralization in both layers. In acidic soils, like in neutral soils, application of bactericide led to a clear drop in nitrification, in both absolute and relative amounts. This suggests that the bacterial process was negatively affected also in acidic soil. However, in contrast to neutral soil, nitrification also decreased with fungicide, when the dominant fungi were negatively affected and bacteria stimulated. This was the case for both absolute and relative amounts.

3.4. Stimulation and inhibition of the main group of micro-organisms

In neutral soil, net N-mineralization significantly increased compared to the control treatment in both antibiotic treatments (Fig. 3 and Table 4). This increase occurred in both organic layer and mineral topsoil, probably due to the extra supply of N from antibiotics or dead fungal biomass. In acidic soil, however, net N-mineralization did not increase in antibiotic treatments at all.

In neutral, bacteria-dominated soil, respiration significantly increased when the main group of micro-organisms was stimulated with fungicide, but not at all when inhibited with bactericide. Net N-mineralization per unit C respired did not change when bacteria were stimulated. However, when bacteria were inhibited, net N-mineralization per unit C respired significantly increased, which points to lower immobilization. The increase in net N-mineralization in neutral soil with antibiotics may thus have different causes: increased respiration and activity when bacteria are stimulated, and lower immobilization when bacteria are inhibited, and fungi stimulated.

In acidic, fungi-dominated soils, respiration increased in the organic layer, but only when fungi were inhibited by fungicide, and bacteria stimulated. When fungi were stimulated, respiration did not change. However, when fungi were stimulated, net N-mineralization per unit C respired increased, especially in the mineral topsoil. This points to lower immobilization when fungi are stimulated. As already indicated, extra supply of N from antibiotics and dead micro-organisms did not lead to increased net N-mineralization in acidic, fungi-dominated soils. Nevertheless, when fungi were stimulated, immobilization, which was already low in this soil type, further decreased. In contrast, when fungi were inhibited, respiration increased, which suggests higher microbial activity when fungi decreased and bacteria increased.

Fig. 1. Net recovery of DOC and DON derived from antibiotics in a one-month incubation experiment with selective inhibition of fungi and bacteria in the organic layer and mineral topsoil (0–10 cm) of neutral and acidic Luxembourg beech forests. Fungicide = *cycloheximide*; bactericide = *streptomycin*. Mean values ($n = 5$) and standard deviations; different letters indicate significant differences ($p < 0.05$) between treatments, soil types and/or horizons for a particular parameter.

Table 3

Statistical analysis of the effects of treatment (fungicide or bactericide), soil type, horizon and their interactions on the net recovery of DOC and DON derived from antibiotics and on nitrification in a one-month incubation experiment with selective inhibition of fungi and bacteria in the organic layer and mineral topsoil (0–10 cm) of neutral and acidic Luxembourg beech forests. Significant differences are indicated with asterisks (three-way Anova; ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

	Treatment	Soil type	Horizon	Treatment × soil type	Treatment × horizon	Soil type × horizon	Treatment × soil type × horizon
Net recovery DOC (% input)	*	***	**	***	***	ns	*
Net recovery DON (% input)	***	***	ns	*	ns	ns	ns
Nitrification (% N-mineralization)	***	**	ns	***	ns	***	ns

3.5. Effects of bactericide and fungicide on total C and N-cycling

In the control treatment, net N-mineralization per m^2 was generally lower in neutral than in acidic soil (Fig. 4 and Table 5). Total net N-mineralization however increased with antibiotics, especially with fungicide, when fungi were inhibited and the dominant bacteria further stimulated. Respiration per m^2 was already high in neutral soil in the control treatment, especially in the mineral topsoil, which may be due to higher SOM and

microbial biomass than in the other soil horizons. Respiration per m^2 further increased when bacteria were stimulated by fungicide in both organic layer and mineral topsoil. However, respiration values did not change in neutral soil when bactericide was applied and bacteria were inhibited.

In acidic soil, net N-mineralization per m^2 was higher than in neutral soil in the control treatment for both organic layer and mineral topsoil. Net N-mineralization per m^2 did not change at all with application of bactericide, which stimulated the resident fungi.

Fig. 2. Nitrification in different antibiotic treatments in the organic layer and mineral topsoil (0–10 cm) of neutral and acidic beech forests in Luxembourg. Bactericide = streptomycin and fungicide = cycloheximide. Mean values and standard deviations ($n=5$). Significant differences from the control treatment in a particular horizon are indicated with an asterisk ($p < 0.05$).

Fig. 3. Increase in (A) net N-mineralization, (B), respiration and (C) net N-mineralization per unit C respired in antibiotic treatments compared to the control treatment, in the organic layer and mineral topsoil (0–10 cm) of neutral and acidic soils in Luxembourg beech forests. Effects of bactericide and fungicide are expressed as effects of stimulation or inhibition of the main group of micro-organisms in each soil type, i.e., bacteria in lime-rich soil and fungi in lime-poor soil. Values given are mean values ($n=5$) and standard deviations. Significant differences from the control treatment are given with as asterisk ($p < 0.025$, due to multiple comparisons with control treatment).

Table 4

Statistical analysis of stimulation or inhibition of the main group of micro-organisms in a one-month incubation experiment in the organic layer and mineral topsoil (0–10 cm) of neutral and acidic Luxembourg beech forests. The effects of treatment, soil type, horizon and their interactions on net N-mineralization ($\text{mg kg}^{-1} \text{OM}$), respiration ($\text{g kg}^{-1} \text{OM}$) and net N-mineralization per unit C respired ($\text{mg Ng}^{-1} \text{C}$) were tested with three-way anova. Differences were significant at $p < 0.025$, due to bonferroni-corrections for double use of the control treatment (ns = not significant, * $p < 0.025$, ** $p < 0.01$, *** $p < 0.001$).

	Treatment	Soil type	Horizon	Treatment × soil type	Treatment × horizon	Soil type × horizon	Treatment × soil type × horizon
Stimulation of the main group of micro-organisms							
Net N-mineralization	***	ns	ns	**	ns	*	ns
Respiration	***	****	***	ns	ns	ns	ns
Net N-mineralization per unit C respired	***	****	****	***	ns	ns	ns
Inhibition of the main group of micro-organisms							
Net N-mineralization	***	ns	**	**	ns	ns	ns
Respiration	***	**	***	**	ns	ns	**
Net N-mineralization per unit C respired	**	***	**	***	ns	*	ns

With application of fungicide, which may have stimulated bacteria, net N-mineralization per m^2 increased only in the mineral topsoil. Respiration per m^2 was relatively low in acidic soil in the control treatment for both organic layer and mineral topsoil, and did not increase when the already dominant fungi were further stimulated with bactericide. However, with fungicide, when the resident fungi were inhibited and the supposedly less abundant bacteria stimulated, respiration per m^2 clearly increased in both organic layer and mineral topsoil, although not as much as in neutral soil.

The above results suggest that the two antibiotics evoked specific responses in microbial activity in all horizons, independent of soil type and initial microbial composition. In almost all horizons, net N-mineralization per m^2 was stimulated by one or two of the antibiotics, probably due to extra supply of N. However, in all soil horizons, even in acidic soil, respiration per m^2 was only stimulated by fungicide, which inhibited fungi and stimulated bacteria. Respiration per m^2 did however not increase at all with bactericide, not even in acidic soil. In contrast, bactericide led to a clear increase in net N-mineralization per unit C respired in both neutral and acidic soil, and both organic layer and mineral topsoil, which suggests lower microbial immobilization and higher efficiency of N-mineralization to the vegetation. When fungicide was applied and bacteria were stimulated, net N-mineralization per unit C respired did not increase, except in the organic layer of neutral soil. As indicated before, it is not likely that inhibition of the main group of micro-organisms could lead to a complete shift in microbial communities. Nevertheless, the results suggest that a shift towards bacteria may lead to increased respiration and microbial activity, while a shift towards fungi seems accompanied by lower microbial immobilization and higher efficiency of the N-mineralization process to the vegetation.

4. Discussion

4.1. Selective inhibition

In this study, we further explored the paradox that neutral soils often have lower net N-mineralization than acidic soils, despite higher biological activity. We tested with selective inhibition whether differences in net N-mineralization could be related to differences in activity and microbial immobilization between bacteria and fungi. Changes in microbial community from neutral to acidic soil are naturally more complex than just a shift from bacteria to fungi (e.g., Blagodatskaya and Anderson, 1998; Bååth and Anderson, 2003). Also, microbial communities may be affected by other factors than pH, such as soil C:N ratio (Högberg et al., 2007) or texture and water supply (Hassink, 1994; Williams and Rice, 2007).

Unfortunately, bacterial and fungal populations could not be measured during the experiment. However, earlier research in previous years showed that the neutral and acidic soils of this study consistently differed in dominance of bacteria and fungi (Kooijman et al., 2008, 2009). Also, the use of antibiotics such as streptomycin and cycloheximide is a well established method to study differences in functioning between bacteria and fungi, which in short term experiments leads to selective inhibition of the target group (Anderson and Domsch, 1973; Schmidt et al., 2000; Chen et al., 2001; Bååth and Anderson, 2003; Bailey et al., 2003; Myrold and Posavatz, 2007). In this one-month experiment, antibiotics were applied weekly to prevent regrowth. To which extend this happened was unfortunately not measured, but we tried to maximize differences between neutral and acidic soil by use of fungicide in neutral soil, and bactericide in acidic soil. Fungal: bacterial ratios can change rather rapidly when different substrates are supplied (Thiet et al., 2006). In this way, the minor microbial group in each soil type was repressed, but the main group stimulated by extra C and N from antibiotics and dead micro-organisms, and soils were created with even higher dominance of bacteria or fungi than in the control treatment. Differences in C and N-cycling between neutral and acidic soil were indeed largest when the minor microbial group (fungi in neutral and bacteria in acidic soil) in each soil type was repressed, and the main group (bacteria in neutral and fungi in acidic soil) stimulated. Also, differences between neutral and acidic soils largely disappeared when the main microbial group was suppressed, which suggests that the microbial community composition had become more mixed.

Selective inhibition was also supported by the differential effects of bactericide and fungicide on nitrification, especially in neutral soil. Nitrification is basically a bacterial process (Booth et al., 2005; Myrold and Posavatz, 2007), which clearly dropped in all four bactericide applications, but remained high with fungicide in neutral soil, where nitrifiers are common. In acidic soil, however, nitrification was also reduced with fungicide. Possibly, fungicide had a negative impact on nitrifying bacteria (Chen et al., 2001), or heterotrophic nitrification was larger than expected (Barraclough and Puri, 1995). Also, reduced nitrification with fungicide in acidic soil may reflect more complicated changes in microbial behaviour. Nitrifying bacteria are relatively weak competitors, which only increase when ammonium demand of other micro-organisms has been satisfied (Hart et al., 1994; Schimel and Bennett, 2004). In the control treatment, high nitrification was perhaps possible in acidic soil because N-mineralization and availability of ammonium were also high, and fungal N-demand relatively low. With fungicide, however, which repressed fungi and stimulated bacteria, microbial N-demand probably increased, as suggested by the increase in

Fig. 4. Effect of bactericide and fungicide treatments on total net N-mineralization per m², total respiration per m² and net N-mineralization per unit C respired in the organic layer and mineral topsoil (0–10 cm) of neutral and acidic Luxembourg beech forests. Each soil type and horizon was tested separately, due to large differences in total net N-mineralization and respiration. Significant differences compared to the control treatment are indicated with an asterisk ($p < 0.05$).

Table 5

Effect of antibiotic treatments (control, bactericide and fungicide) on total net N-mineralization per m², total respiration per m² and net N-mineralization per unit C respired in the organic layer and mineral topsoil (0–10 cm) of neutral and acidic Luxembourg beech forests. One-way anova was applied for each soil type and horizon separately, due to large differences in total net N-mineralization and respiration. Significant effects are indicated with asterisks (ns = not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

		Net N-mineralization (g m ⁻²)	Respiration (g m ⁻²)	Net N-mineralization per unit C respired (mg N g ⁻¹ C)
Neutral	Organic layer	**	*	**
	Mineral topsoil	*	**	ns
Acidic	Organic layer	ns	ns	*
	Mineral topsoil	ns	ns	*

respiration. Possibly, the extra N from antibiotics and dead fungi was used for bacterial growth, rather than for nitrification.

4.2. Different roles of bacteria and fungi

Our results suggest that bacteria and fungi really differ in cycling of C and N, especially with respect to microbial activity and N-demand. In the control treatment, the results further support the hypothesis that neutral soils have higher biological activity than acidic soils, but that net N-mineralization may nevertheless be lower due to higher microbial N-demand and immobilization. This could explain why net N-mineralization was lower in neutral than in acid soil in e.g., Zöttl (1960), Davy and Taylor (1974), Verhoeven et al. (1990), Kooijman and Besse (2002), Kooijman et al. (2008), Kooijman and Smit (2009) and Mettrop et al. (2014).

In the antibiotic treatments, microbial activity and N-demand were also higher for bacteria than for fungi. This was most obvious in the most extreme soils when the main micro-organisms were stimulated: neutral soil with fungicide, and acidic soil with bactericide. In neutral soil with fungicide, respiration not only increased compared to the control treatment, but was also much higher than in fungi-dominated acidic soil. Net N-mineralization per unit C respired however showed the opposite response, with increased values in acidic soil with application of bactericide, and higher values than in neutral soils in any case.

Higher N-demand by bacteria than by fungi may to some extent be supported by the lower recovery of DOC and DON in neutral than in acidic soil. Lower recovery could be partly due to higher sorption of DOC and DON to soil particles, especially since the neutral soil is also loamy, and stabilization of organic matter by clay and silt should be more pronounced than in the sandy, acidic soil (Plante et al., 2006). However, recovery was also lower in neutral soil in the organic layer, in which mineral soil particles are absent. Differences in microbial communities could also play a role. In the control treatment, microbial biomass was higher in bacteria-dominated neutral soil than in fungi-dominated acidic soil. Also, if microbial activity is higher for bacteria than for fungi, uptake of C and N from antibiotics may be higher as well. This should be further tested with e.g., stable isotopes.

Differences between bacteria and fungi in microbial activity and N-demand even emerged when the main group of micro-organisms was inhibited, and microbial communities probably more mixed. Despite differences in soil types and initial microbial community structure, application of fungicide showed surprisingly similar responses in neutral and acidic soil, and in organic layer and mineral topsoil, with a general increase of respiration when bacteria were stimulated. For bactericide, the response was also more or less similar for all soil horizons, with increase of net N-mineralization per unit C respired, which points to lower immobilization when fungi are stimulated. The differences in response to the two antibiotics cannot be explained by differences in dosage. For example, DOC-input from antibiotics was the same for fungicide and bactericide in neutral soil, but respiration only increased with fungicide, in both organic layer and mineral topsoil.

The results thus further support the evidence that bacteria and fungi really differ in microbial activity and immobilization.

4.3. Ecological implications

The different strategies for N-cycling of bacteria and fungi are important to ecosystem functioning. In accord with Aerts and Chapin (2000) and Schimel and Bennett (2004), fungi are characterized by low activity, but, in contrast to de Vries et al. (2011), also by low immobilization. Low fungal immobilization may lead to higher net N-mineralization than expected from the low activity, and may explain the higher values often found in acidic than in neutral soil (Zöttl, 1960; Davy and Taylor, 1974; Verhoeven et al., 1990; Kooijman and Besse, 2002; Kooijman et al., 2008; Kooijman and Smit, 2009; Mettrop et al., 2014). Bacteria, in contrast, are characterized by high respiration, but also by high immobilization, which may explain why neutral soils often have lower net N-mineralization than expected from high activity. The laboratory incubation experiment does not give values of actual net N-mineralization in the field. Nevertheless, in the neutral site of this study, N-availability to the vegetation is probably actually lower than in the acidic site. Litter input was the same in both beech forests, but litter N-content was significantly lower in the neutral site (Kooijman et al., 2008).

High bacterial immobilization in lime-rich ecosystems may be compensated to some extent. Earthworms, excluded from most incubation experiments, are prominent in neutral soil and may increase net N-mineralization by excretion of N in mucoproteins and dead tissue (Scheu, 1997). In addition, calcicole plant species usually have relatively high N-content (Ellenberg et al., 1974; Scheu, 1997; Kooijman and Hedenäs, 2009), which may increase input of N-rich litter to the soil. As suggested by the use of fungicide in neutral soil, increase of N-supply may largely increase bacterial activity and net N-mineralization. Also, N-availability may be increased by grazing of bacteria by protozoa such as amoeba and flagellates (Kuikman and van Veen, 1989). In addition, plants may take up DON in addition to ammonium and nitrate (Schimel and Bennett, 2004). In neutral soil, most microbial N may even become available again, because bacteria have short life cycles. However, Phoenix et al. (2003) suggested that N-storage is higher in neutral than in acidic soil. At least part of the microbial N becomes part of the stable soil organic matter (Sjöberg and Persson, 1998). In neutral soil, stable soil organic matter consists of a high proportion of microbial-derived compounds, stabilized by interactions with mineral surfaces, which is supported by high calcium contents (Schmidt et al., 2011).

4.4. Concluding remarks

This study provides correlative and experimental evidence for higher activity, but also higher N-demand in bacteria than in fungi. The assumption that neutral, bacteria-dominated soils have high net N-mineralization and high N-availability to the vegetation due to high microbial activity and gross N-release is thus too simple,

because microbial N-demand is not taken into account. In neutral soil, where microbial N-demand is high, ecosystem N-fertility is sustained by high gross N-release. However, in acidic soil, net N-mineralization may be substantial despite low gross N-release, because fungal N-demand is low as well.

Ethical statement

The authors declare that they have no conflict of interest.

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