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Microclimate and forest management alter fungal-to-bacterial ratio and N_2O-emission during rewetting in the forest floor and mineral soil of mountainous beech forests

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Abstract The effects of site exposure (microclimate) and forest management (thinning) on fungal-to-bacterial (F:B) respiratory ratio and N_2O emission from forest floor and Ah layer samples were studied at untreated and thinned beech forests. Microclimate effects were studied by selecting sites facing north-east (NE) or south-west (SW). The F:B respiratory ratio was estimated using substrate-induced respiration in combination with inhibitors either affecting fungi or bacteria. N_2O production was evaluated after moistening samples initially pre-incubated at different moisture levels to 100% of the water holding capacity (WHC). F:B respiratory ratios were significantly affected by microclimate and thinning, with site exposure having the strongest effect on fungal-to-bacterial ratio and N_2O production both for the forest floor and the Ah layer. Significantly more N_2O was produced from soils pre-incubated under low (15% WHC) moisture conditions as compared to soils pre-incubated under air dry (5% WHC) or wet conditions (30–60% WHC). A positive correlation between N_2O emission and F:B respiratory ratio for Ah layer samples and a negative correlation between bacterial substrate induced respiration (SIR) and N_2O emission for both Ah layer and forest floor samples indicated that net N_2O production was the result of predominantly fungal N_2O production and predominantly bacterial N_2O consumption. The latter hypothesis was further supported by increased N_2O emission from samples treated with bacterial inhibitor.

Keywords Forest soils and management · Thinning · Fungal-to-bacterial ratio · Nitrous oxide emission · Streptomycin · Cycloheximide

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Introduction

Soil microbial processes account for 70% of the global source strength of the primarily radiatively active atmospheric trace gas nitrous oxide (Conrad 1996). Nitrous oxide (N_2O) is produced in soils by bacteria but also by fungi as end- or by-products of nitrification, denitrification and dissimilatory nitrate reduction to ammonium. These processes occur both

under aerobic and anaerobic conditions. The composition of the soil microbial community exerts a strong effect on the pedosphere-atmosphere exchange of N_2O (Bollag and Tung 1972; Smith and Tiedje 1979; Lloyd et al. 1987; Skiba et al. 1993; Conrad 1996). Soils which differ in the composition of denitrifier communities showed significant differences in the magnitude of N_2O production and consumption (Munch 1989; Cheneby et al. 1998; Cavigelli and Robertson 2001; Mei et al. 2004). It has been believed for many years that denitrification can be performed by many phylogenetically distant bacterial strains but only by a few fungi (Conrad 1996). Recent gene analyses, however, have revealed the genes encoding fungal denitrification system which is localized in the mitochondria and is widely distributed among fungi (Kobayashi et al. 1995; Kudo et al. 2001; Uchimura et al. 2002; Hayatsu et al. 2008). Fungal denitrification can dominate total denitrification in ecosystems, as was demonstrated for a temperate grassland soil in Northern Ireland (Laughlin and Stevens 2002) and for a forest nursery soil in Byromville, USA (Spokas et al. 2006). Potential role of fungi in the N_2O emission from soil was suggested in semiarid regions (McLain and Martens 2006), in woodland and arable soils (Castaldi and Smith 1998). However, knowledge on the role of fungal and bacterial communities in regulating the soil-atmosphere exchange of N_2O is still scarce (Hayatsu et al. 2008) since there are no simple quantitative methods to separate the fungal and bacterial biomass. The application of the most suitable approach based on selective inhibition of substrate-induced respiration of eu- and prokaryotes is not common due to the necessity of thorough, laborious preliminary optimization of inhibitor concentrations.

Varying microclimate conditions in ecosystem mosaics complicate the estimation of ecological processes at the ecosystem level (Conrad 1996). Both the structure of microbial communities and N_2O emissions may vary significantly at sites with different microclimate coexisting on small scales. The effect of microclimate on the composition of soil microbial communities and its possible importance for soil N_2O emissions has not been well studied yet. Differing exposure and forest management practices, involving significant reduction of the stand density, can significantly alter temperature, moisture and irradiation conditions (Holst et al. 2004b). These environmental factors are known to influence microbial N_2O production and consumption (Conrad 1996). To our knowledge there are no studies on the relationship between fungal-to-bacterial (F:B) ratio and N_2O emission as influenced by microclimate and forest management.

N_2O emissions from soils often show a distinct seasonal pattern driven by the changes in climatic conditions such as e.g., soil freezing–thawing events in winter or rainfall in summer (Papen and Butterbach-Bahl 1999; Müller et al. 2002; Davidson et al. 1993). The duration of an increase in N_2O emissions after the first rainfall event following a long lasting drought period can vary from several hours to several days (Butterbach-Bahl et al. 2004). Remarkably large fluxes of N_2O have been observed following wetting of dry soil (Davidson 1992; Fenn et al. 1996). The effect of the initial level of soil moisture before wetting usually was not taken into account. Hence, the investigation of rewetting effects on microbial N_2O production in soils is of major interest for improving our understanding of the observed variability of N_2O emissions under natural conditions.

The aim of the present study was to investigate the contribution of bacteria and fungi to N_2O emission from moistened Ah layer and forest floor samples at sites with different microclimate and forest management treatment histories. The following hypotheses were tested:

1. N_2O production as well as F:B ratio in the Ah layer and forest floor are affected by forest management and microclimate.
2. Net N_2O production is related to the relative contribution of bacteria and/or fungi to total microbial respiration.

Furthermore, we evaluated the effect of the initial soil moisture level during pre-incubation on N_2O production after rewetting to 100% WHC.

Methods

Experimental site

For investigating the effect of microclimate we choose two study sites located on the two opposite slopes of a narrow valley facing southwest (SW) and northeast (NE) in the Swabian Jura, a low mountain

range in Southern Germany. The sites are characterized by warm–dry microclimate (SW site) and cool–moist microclimate (NE site). At every site adjacent untreated control ("C") and thinning ("T") plots, where the basal area of the stands was evenly reduced from approx. 27 m^2 ha^{-1} (NE site) and approx. 21 m^2 ha^{-1} (SW site) to approx. 10 m^2 ha^{-1}, were established in 1999. Beech (*Fagus sylvatica* L.) is the dominant species at both sites and the average stand age is 70–80 years. The mean annual net irradiance balances are 240 W m^{-2} and 220 W m^{-2} for the SW and NE sites, respectively (Holst et al. 2005). The differences in soil temperatures in 3 cm depth between the SW and the NE sites amounted to 1.1 and 1.2°C for the control and thinned plots, respectively (Holst et al. 2004b). For both SW and NE sites the differences in soil temperature in 3 cm depth between control and thinned plots did not exceed 0.5°C in the vegetation period, since higher temperatures during the day were partly compensated by lower nighttime temperatures at the thinned plots (Holst et al. 2004b; Dannenmann et al. 2006). Although the average annual rainfall above canopy (860 mm) does not vary significantly across the valley (Geßler et al. 2001) the SW site is characterized by lower mean water content in both Ah horizon and forest floor as compared to the NE site (Dannenmann et al. 2006, 2007a). The soil profiles are characterized as Rendzic Leptosols derived from limestone and marls. Selected characteristics of the forest floor and Ah layer samples are presented in Tables 1 and 2. Soil characteristics were different between SW and NE site samples due to higher pedogenetical development of the NE site soils (e.g., lower stone content, higher decarbonization, lower pH values, less shallow soil profile, lower water holding capacity (WHC), lower C content, and lower C/N ratio) promoted by the different microclimate (Dannenmann et al. 2007b).

During the first years after tree harvesting the thinned plots showed significantly lower plant area indices (Holst et al. 2004a) as compared to the untreated control plots and therefore, higher interception of photosynthetic active radiation (Mayer et al. 2002). This resulted in a fast development and establishment of dense understory vegetation at the thinned plots, which grew from heights <0.5 m in the year 1999 up to >3.5 m in 2003. During the vegetation period, thinning plots are on average characterized by higher mean gravimetric soil water content in the topsoil (52 and 45% of soil dry weight for NE and SW site, respectively) as compared to the adjacent control plots (45 and 40% of soil dry weight for NE and SW site, respectively, Dannenmann et al. 2007a). Thus, both the different exposures of the two sites and the differences in stand densities led to differences in microclimates at the plot scale.

Sampling of the forest floor and Ah layers

At each plot samples were taken the 1st of August 2003 from five randomly chosen replicate microsites, from the forest floor and from the 0–10 cm mineral soil (Ah) layer.

Field-fresh samples were stored in polyethylene bags sealed with cotton-wool plugs in the dark at 4°C. Prior to analysis, the soil and forest floor samples were homogenized manually, sieved (mesh size 2 and 4 mm, respectively) and moistened to 60% of the WHC. Plant debris and stones were removed from soil samples before analysis.

Determination of the fungal-to-bacterial ratio and inhibitor optimization

Fungal-to-bacterial respiratory ratios were determined using the initial rate of substrate-induced respiration (SIR, with glucose as the substrate) and

Table 1 Characteristics of the forest floors from NE and SW sites

Exposition	Variant	WHC (%)	pH	C (%)	N (%)	C:N ratio
North–East	Control	585 ± 29^{a}	5.6 ± 0.1^{a}	43.5 ± 0.8^{a}	1.64 ± 0.06^{a}	26.7 ± 1.0^{b}
	Thinning	483 ± 33^{a}	6.0 ± 0.3^{a}	41.3 ± 1.4^{a}	1.87 ± 0.05^{b}	22.1 ± 1.0^{c}
South–West	Control	618 ± 40^{a}	5.6 ± 0.3^{a}	46.0 ± 0.7^{a}	1.48 ± 0.03^{a}	31.3 ± 1.1^{a}
	Thinning	602 ± 37^{a}	5.7 ± 0.4^{a}	43.0 ± 1.0^{a}	1.49 ± 0.04^{a}	28.9 ± 0.7^{ab}

Values are means [in each column followed by different letters are significantly different ($p < 0.05$)] ±SE

Table 2 Selected physical and chemical properties (±SE) of mineral soils after removal of gravel and stones

Exposition	Variant	WHC (%)	pH	Gravimetric stone content (%)	C (%)	N (%)	NO_3^-, (mg N/kg)	NH_4^+, (mg N/kg)	C/N
North–East	Control	79.4 ± 0.5^b	6.0 ± 0.3^b	21.3 ± 3.3^c	7.85 ± 0.2^b	0.55 ± 0.003^b	0.46 ± 0.06^d	2.79 ± 0.18^b	14.3 ± 0.2^b
	Thinning	80.2 ± 0.6^b	5.8 ± 0.5^b	8.1 ± 3.3^d	7.8 ± 0.2^b	0.52 ± 0.03^b	0.74 ± 0.03^c	5.43 ± 0.5^a	15 ± 0.4^b
South–West	Control	106.4 ± 1.1^a	6.8 ± 0.3^a	52.3 ± 3.5^a	11.9 ± 1.5^a	0.67 ± 0.06^a	2.15 ± 0.12^a	1.25 ± 0.05^c	17.8 ± 0.6^a
	Thinning	105.7 ± 0.8^a	6.9 ± 0.1^a	32.0 ± 4.2^b	10.2 ± 1.5^a	0.61 ± 0.02^a	1.69 ± 0.3^b	0.5 ± 0.15^d	16.7 ± 0.7^a

Values in each column followed by different letters are significantly different ($p < 0.05$)

the selective inhibition method (SIRIN) according to Anderson and Domsch (1975, 1978) with modifications for litter samples according to Beare et al. (1990). Briefly, subsamples of soil (1 g) or forest floor (0.5 g; on oven dry basis) were weighed into 15 ml glass vials. The following four different treatments were applied to subsamples from each plot: (A) glucose addition only; (B) glucose + fungicide; (C) glucose + bactericide; (D) glucose + both fungicide and bactericide (for further details see below). After substrate and/or inhibitor addition the vials were sealed gas-tight for 3 h and the increase in CO_2-concentrations in the headspace of the vials was measured by syringe sampling and subsequent GC analysis.

Fungal and bacterial SIR rates were defined as (A−B) and (A−C), respectively. Treatment D served as a control, in order to quantify the effectiveness of the application of bactericide and fungicide to reduce microbial (bacterial plus fungal) activity.

Optimum incubation time and amount of glucose required for maximum respiratory response in soils and forest floors were determined in preliminary experiments by evaluating the following glucose concentrations: 0.1, 0.5, 1, 5, and 10 mg g^{-1} for Ah layer sample dry weight (sdw) and 2.5, 12.5, 25, 65, and 130 mg g^{-1} for forest floor sdw. Optimum glucose concentrations for the application of the SIR-method to our samples were 5 mg g^{-1} sdw glucose for Ah layer soil samples and 65 mg g^{-1} sdw glucose for forest floor samples, respectively, (data not shown).

The antibiotics, streptomycin sulfate and cycloheximide, prokaryotic and eukaryotic inhibitors, respectively, were chosen as inhibitors of de novo protein synthesis. Different concentrations of antibiotics were tested for their effectiveness to suppress microbial respiration. For streptomycin sulfate we tested additions of 1, 2, 4, 8, 16, 20, 30, and 40 mg g^{-1} sdw for Ah layer samples and 2.5, 5, 10, 20, 40, 50, 60, 80, and 100 mg g^{-1} sdw for forest floor samples. For cycloheximide we tested concentrations of 7.5, 15, 20, 30, and 35 mg g^{-1} sdw for Ah layer samples and 20, 40, 50, 70, 80, and 160 mg g^{-1} sdw for forest floor samples. In addition, different exposure times (1–20 h) with inhibitors prior to the addition of glucose were studied. In order to comply with the criteria of selective inhibition two indices were calculated: (1) the total combined

inhibition index (TCI = [(A−D)/A × 100])—which shows percent of microbial respiration inhibited by application of both antibiotics—TCI amounts to 100% if fungal as well as bacterial respiration is fully inhibited by treatment D; (2) the inhibitor additivity ratio index (IAR = [(A−B) + (A−C)]/(A–D)—which is a measure of inhibition of non-target organisms, e.g., fungi inhibited by bactericide or bacteria by fungicide (Scheu and Parkinson 1994). The IAR allows to indirectly assessing antibiotic selectivity and approaches 1.0 if there is no overlapping or non-target inhibition.

Fungal-to-bacterial SIR ratio was calculated by dividing the respiration inhibition caused by application of fungicide with the respiration inhibition caused by the application of bactericide (A−B)/(A−C). Bacterial and fungal SIR was calculated assuming that the F:B ratio in the uninhibited biomass was the same as indicated by the results of the SIRIN method (Lin and Brookes 1999; Laughlin and Stevens 2002).

Pre-incubation at different soil moisture

In order to evaluate the effect of initial soil moisture on N_2O production after wetting of the samples to 100% WHC, the samples were pre-incubated at 4°C for 48 h under different moisture conditions corresponding to: 5, 15, 30, 45, and 60% of the WHC. After 2 days pre-incubated samples were moistened to 100% WHC by application of distilled water and N_2O production was determined as described in following section. Additional soil and forest floor samples from each site were sterilized by triplicate autoclaving for 20 min at 120°C and then moistened to 100% WHC with sterile water.

In order to assess the real N_2O dynamics from soils and forest floors after moistening in our experimental setup no additional enrichment by available C and N was done. Since nitrate concentration in our samples prior to moistening (Table 2) was high enough to allow denitrification in the first hours, moistening was done by adding only distilled water to the samples.

Determination of N_2O production

Three different treatments per plot were used for the determination of total, bacterial and fungal N_2O production from Ah layer and forest floor samples during and following rewetting to 100% WHC:

(a) distilled water only
(b) distilled water + streptomycin
(c) distilled water + cycloheximide, and
(d) distilled water + both streptomycin and cycloheximide

The fungal or bacterial inhibitors were added to the samples at the same concentrations which were used for the determination of F:B respiratory ratios (Laughlin and Stevens 2002): 30 and 60 mg g^{-1} sdw streptomycin and 30 and 70 mg g^{-1} sdw cycloheximide for mineral soil and forest floor samples. Soil (1 g sdw) and forest floor (0.5 g sdw) samples were placed in 15 ml glass flasks, moistened to 100% of the WHC, immediately sealed with a rubber stopper and incubated at 28°C in a thermostatic rotary shaker. The increase in N_2O concentration in the headspace of the flasks was followed for 24 h by 4–6 syringe samplings (100 µl volumes each) and subsequent GC analysis.

Analytical methods

Analysis of total C content, carbonate content and N-content in soil and forest floor samples was performed according to DIN ISO 13878 and 10694; WHC was determined according to BS EN 13041; colorimetrical analysis of NH_4^+ and NO_3^- concentrations was performed according to the VD LUFA method A6141 (Hoffmann 1991) by a commercial laboratory (Dr. Janssen, Gillersheim, Germany). Organic C content was calculated by subtraction of carbonate-C from total C content.

Soil and forest floor pH was measured in 0.01 M $CaCl_2$ with a soil to solution ratio of 1:2

Gas analysis

The concentrations of CO_2 and N_2O in the headspace of the glass vials and glass flasks were determined by use of a gas chromatograph (Perkin–Elmer 8500) equipped with (a) a thermal conductivity detector (TCD) and (b) an electron capture detector (ECD). Measurements were conducted using a separation column GS-Q (30 m Megabore; 0.53 mm inner diameter) at following conditions: oven temperature

40°C; TCD: carrier gas He 5.0 at 20 ml min^{-1}, detector temperature 250°C; ECD: carrier gas 5% Methane 3.5 in Argon 4.8 at 40 ml min^{-1}, detector temperature 350°C. Chromatical software (APEX v2.12) was used for recording and integration of the chromatograms. Standard gases were used for internal calibration of the GC and for calculation of the headspace concentrations of CO_2 and N_2O.

Statistical analysis

All results are expressed as arithmetic means ± standard deviation (SD). A two-way ANOVA was performed for comparison of the effect of the two independent factors "site exposure" and "thinning" on F:B ratio and N_2O production from samples after moistening. In case significant effects were found, a multiple comparison was performed using Student–Newman–Keuls test ($p < 0.05$). Pearson's correlation coefficients (r) and regression coefficients (R^2) based on linear regression models were used to evaluate the relationship between N_2O emission and bacterial SIR or F:B respiratory ratio.

Results

Inhibitor optimization

Tests of optimum inhibitor concentrations revealed that for Ah layer samples the addition of at least 30 mg g^{-1} sdw of cycloheximide was required to effectively inhibit fungal respiration whereas, the critical concentration for the bacterial antibiotic streptomycin was 20–30 mg g^{-1} sdw (Fig. 1a, b). Essentially higher concentrations, i.e., at least 50 mg g^{-1} sdw for both cycloheximide and streptomycin, were required to inhibit bacterial or fungal respiration in forest floor samples (Fig. 1c, d). The prolongation of prior exposure to streptomycin from 1 to 4, 12, 16 and 20 h before the addition of glucose did not result in a significant increase of inhibition of the initial respiratory response in forest floor as well as in Ah layer samples. For some samples respiratory response to inhibition by streptomycin even decreased with pre-incubation time (data not shown). In contrast, prior exposure to cycloheximide had a well pronounced effect on SIR inhibition: for both forest floor

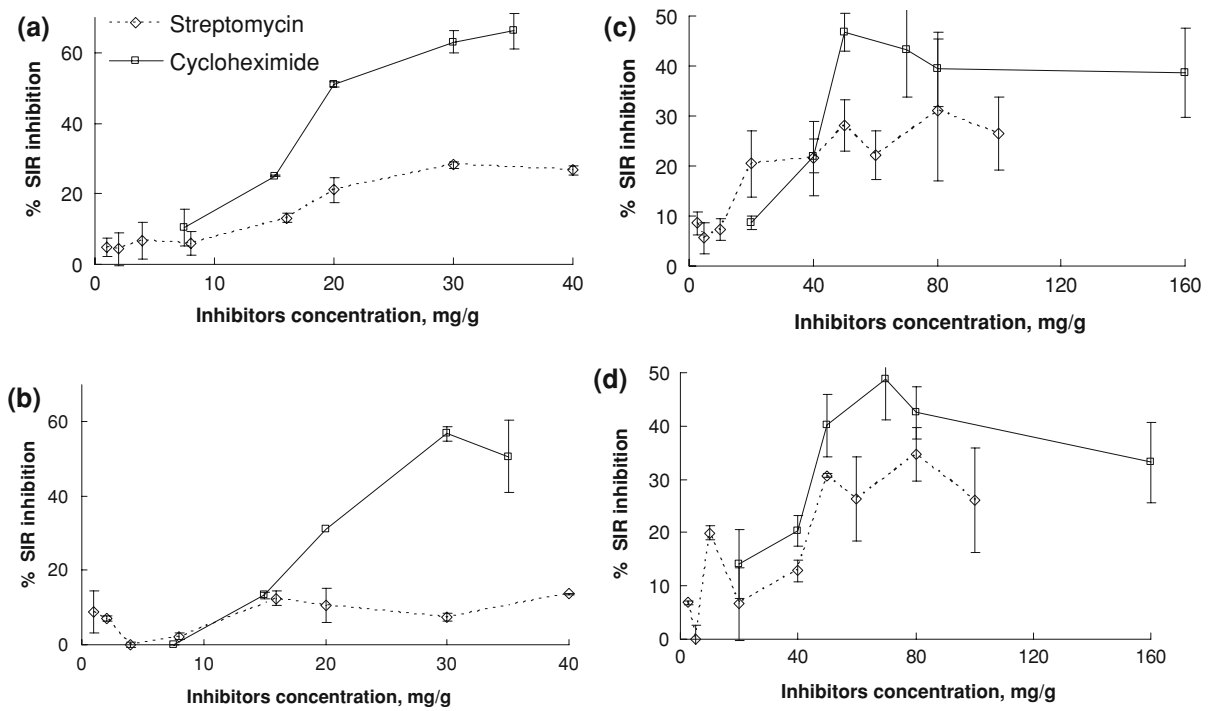

Fig. 1 Percentage of inhibition of substrate induced respiration (*SIR*) after addition of streptomycin or cycloheximide to soils (**a**, **b**) and forest floors (**c**, **d**) taken from plots of the north-east (**a**, **c**) and south-west (**b**, **d**) sites. *Error bars* represent standard deviations

samples and Ah layer samples 12–20 h prior exposure doubled SIR inhibition as compared to 1–4 h exposure (data not shown). Therefore, for the determination of F:B ratios, samples were exposed to streptomycin for 1 h and exposed to cycloheximide for 16–20 h (overnight) before glucose addition. In order to optimize the inhibitory effect of the antibiotics the maximum selective coupled concentrations of streptomycin and cycloheximide were chosen to adjust the IAR index to 1.0 and to maximize the TCI index. In Ah layer samples the optimum values for IAR and TCI were found for an antibiotic application rate of 30 mg g^{-1} sdw streptomycin and 30 mg g^{-1} sdw cycloheximide. For forest floor samples the optimum selective combined concentrations were 60 mg g^{-1} sdw for streptomycin and 70 mg g^{-1} sdw for cycloheximide. Inhibitors applied at optimum combined concentrations provided stable reduction of microbial respiratory activity in a range of 43–72% at least during the 24 h incubation period (Table 3). The TCI in forest floor samples was at maximum 65% while IAR, with the exception of the SWT plot, where non-target inhibition was negligible, ranged from 1.19 to 1.25 indicating that the amount of non-target inhibition was 19–25% (Table 3). For Ah layer samples the inhibitory effect of both antibiotics was higher than for forest floors samples and amounted to 60–72% whereas, unspecific inhibition was relatively low (Table 3).

Fungal-to-bacterial ratio

F:B ratio with regard to substrate induced respiration (SIR) was found to be >1 for all samples investigated within this study. For forest floor samples the fungal contribution to SIR was higher for plots located at the NE site as compared to the plots of the SW site (Fig. 2a). However, for Ah layer samples the opposite effect, i.e., higher F:B SIR ratios, were observed for the SW slope (Table 3; Fig. 2a). The maximum (3.74, $LSD_{0.05}$ 0.43) and minimum (1.33, $LSD_{0.05}$ 0.29) average F:B respiratory ratios were found at the SW site in Ah layer and in forest floors samples, respectively (Fig. 2a). Two-way ANOVA revealed that the exposure of the plots, i.e., microclimate, was the dominating factor for explaining variations of F:B ratios in forest floor (74%) and in Ah (91%) layer samples (Fig. 3).

The effect of forest management on F:B respiratory ratio was less pronounced than the effect of site exposure. Only 26 and 4% of the variation in F:B respiratory ratio could be explained by thinning for forest floor samples and Ah layer samples, respectively (Fig. 3). The ratio of F:B respiration was higher for Ah layer and forest floor samples from the thinned plots as compared to control plots, except for the Ah layer of the NE site (Table 3).

Table 3 Substrate induced respiration rates (V_{SIR}), indexes characterizing the effect of optimum combined inhibitors concentrations on SIR (Inhibitor additivity ratios and percentage of total combined inhibition); fungal-to-bacterial ratios of SIR and the corresponding rates of N_2O production rates in 5 h after moistening of mineral soil and forest floor samples

Sample	Plot	V_{SIR} (μg CO_2-C g^{-1} h^{-1})	Inhibitor additivity ratio	Total combined inhibition in 5 and 24 h after glucose addition (%)		Fungal-to-bacterial-SIR ratio	N_2O-flux (μg N_2O-N g^{-1} h^{-1})
				5 h	24 h		
Forest floor	NEC	566 ± 33[b]	1.22 ± 0.1	51.3 ± 5	54.8 ± 7.2	1.84 ± 0.15[b]	0.273 ± 0.011[a]
	NET	569 ± 5[b]	1.25 ± 0.08	65.2 ± 3.7	65.8 ± ± 5.9	2.31 ± 0.03[a]	0.272 ± 0.001[a]
	SWC	471 ± 3[c]	1.19 ± 0.08	47.1 ± 1.1	45.4 ± 9.6	1.13 ± 0.01[d]	0.241 ± 0.023[b]
	SWT	772 ± 17[a]	0.97 ± 0.05	43.8 ± 1.6	44.1 ± 1.8	1.54 ± 0.05[c]	0.161 ± 0.035[c]
Ah layer	NEC	26 ± 1[b]	1.02 ± 0.06	60.6 ± 4.8	63.1 ± 7.6	2.4 ± 0.15[c]	0.064 ± 0.004[c]
	NET	31 ± 2[a]	1.06 ± 0.09	71.8 ± 10	70.9 ± 6.3	1.35 ± 0.28[d]	0.030 ± 0.002[d]
	SWC	25 ± 1[b]	1.01 ± 0.07	67.1 ± 3.8	66.1 ± 2.4	3.43 ± 0.23[b]	0.119 ± 0.009[b]
	SWT	19 ± 0.6[c]	0.87 ± 0.11	70.6 ± 13	69.4 ± 8.9	4.04 ± 0.18[a]	0.155 ± 0.012[a]

Values are means [separately for forest floors and soils values in each column followed by different letters are significantly different ($p < 0.05$)] ± SD for V_{SIR}, Fungal-to-bacterial-SIR ratio and for N_2O-flux; for the indexes inhibitor additivity ratio and total combined inhibition standard errors are given after ±

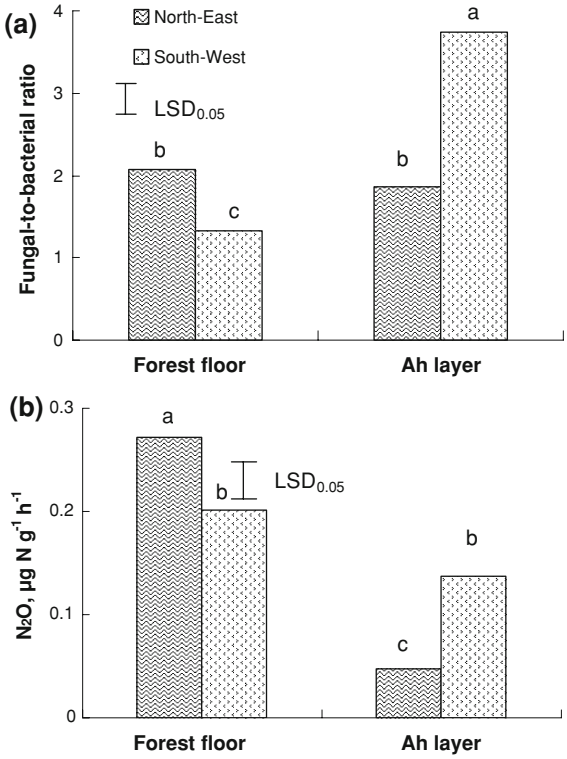

Fig. 2 Effect of exposure of the site on fungal-to-bacterial ratio (**a**) and on N_2O production (**b**) in samples taken from the forest floor layer and from the uppermost mineral soil (Ah layer). Different *letters* indicate significant differences in the samples ($p < 0.05$). *Bars* represent least significant difference at $p = 0.05$

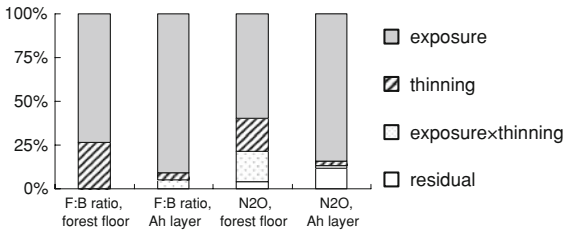

Fig. 3 Contribution of independent single factors "exposure", "thinning" and the combination of both "exposure + thinning" to variation of fungal-to-bacterial ratio and rate of N_2O emission estimated by application of two-way ANOVA test

Effect of pre-incubation at different water contents on N_2O production

For both NE and SW site samples maximum N_2O production occurred in soils pre-incubated at 15% WHC (Fig. 4a, b). Total N_2O efflux during the first

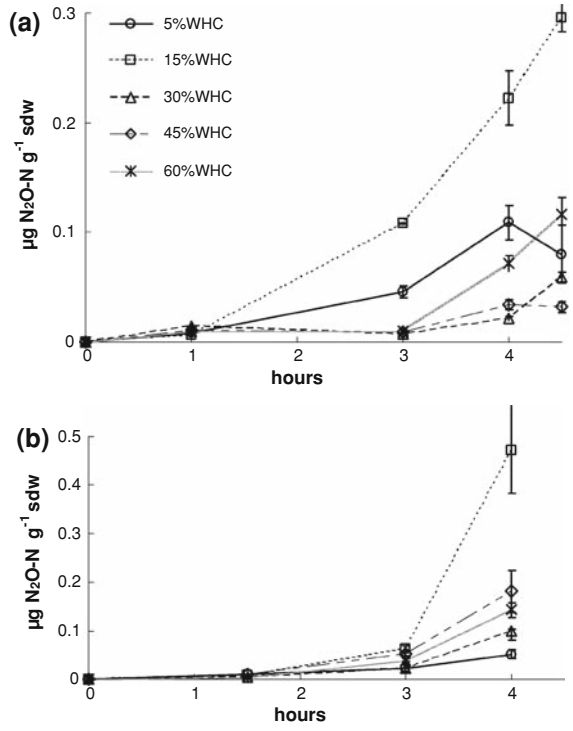

Fig. 4 Effect of initial moistening of samples taken from the Ah layer of the plots of the north-east (**a**) and south-west (**b**) sites on the dynamic of N_2O production rate after soil moistening to 100% of the WHC. *Error bars* represent standard deviations

4 h after rewetting was at least 2.5 times higher than in other variants. The effect of other moisture contents during pre-incubation was in general inconsistent across the sites (Fig. 4a, b). Relatively higher N_2O effluxes were observed from samples pre-incubated at 60% as compared to samples pre-incubated at 30% WHC (Fig. 4a, b). As a consequence, 15% WHC was chosen for pre-incubation of samples in subsequent experiments where N_2O production was evaluated after moistening to 100% WHC. The optimized procedure of soil moistening consisted in pre-incubation of soils and forest floors at 15% WHC, with following water addition up to 100% WHC. This optimized procedure simulates the conditions of microbial metabolism with low oxygen availability in the soil micro sites at field conditions when heavy rainfalls moisten the almost dry soil to 100% WHC. During 24 h of incubation no significant emission of N_2O as well as CO_2 from sterile samples of soils and forest floors were detected.

N_2O emission after moistening to 100% WHC

Application of the two-way ANOVA revealed that 60 and 84% of the variance in N_2O emission can be explained by the site exposure for forest floor and Ah layer samples, respectively (Fig. 3). The effect of forest management on N_2O emission was comparably low (Fig. 3), i.e., 19 and only 2.5% for forest floor and Ah layer samples, respectively. Both differences in N_2O emission and F:B SIR ratio across the four investigated plots showed the same pattern (Fig. 2a, b). Mean N_2O emission from NE site forest floor samples was 40% higher as compared to forest floor samples taken from the SW site (Fig. 2b). In contrast, N_2O emissions from the Ah layer of the SW site were 3.3 times higher as compared to samples from the NE site (Table 3; Fig. 2b). Compared to the Ah layer samples, N_2O emission rates found for forest floor samples were approx. 2–9 times higher when related on a sdw basis (exception SWT plot: insignificant differences).

Relationship between F:B respiratory ratio and N_2O production

Fungal-to-bacterial respiratory ratios and N_2O productions in soil samples were significantly positively correlated for Ah layer samples ($r = 0.97$; $p < 0.01$, Fig. 5a) while there was an insignificant tendency towards higher N_2O emissions with increasing F:B respiratory ratio ($r = 0.46$, Fig. 5a) for forest floor samples. Furthermore, significant negative correlations between bacterial SIR and N_2O emissions were found for both Ah layer ($p < 0.01$) and forest floor ($p < 0.05$) samples. Thus, elevated N_2O emissions consistently coincided with high F:B ratios and decreasing bacterial SIR. Both N_2O emissions and F:B respiratory ratios in the Ah layer were generally higher for SW site samples as compared to NE site samples.

Effect of selective inhibitors on N_2O emission from soils after moistening to 100% WHC

Nitrous oxide emissions from moistened Ah layer samples of the SW site were always significantly lower for soil samples with additions of the fungicide cycloheximide as compared to soil samples which only received additions of water (Fig. 6). However, for soil samples taken from the NE site no consistent trend was observed.

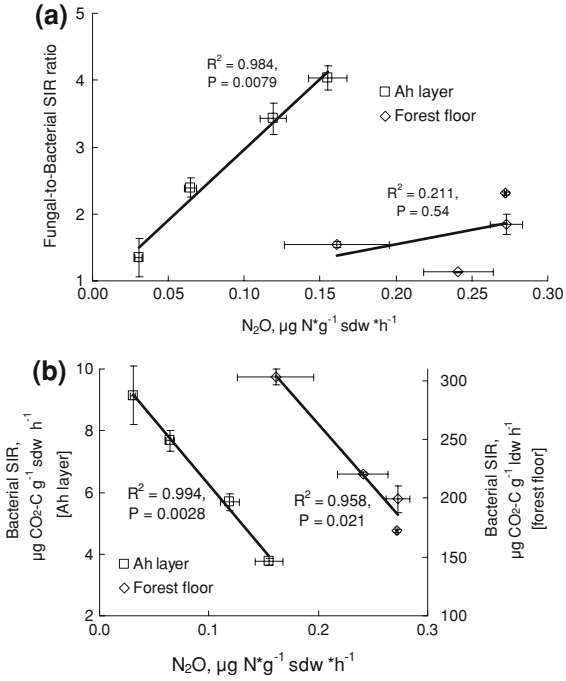

Fig. 5 Results of regression analysis between N_2O production from soils and forest floors after moistening and F:B respiratory ratio (**a**) and bacterial SIR (**b**). *Error bars* represent standard deviations

Application of the bactericide streptomycin, per contra, did never result in a significant decrease of N_2O emission as compared to soil samples with water only additions (Fig. 6). In contrast, at the NE site N_2O emissions were consistently significantly increased (4.4–12.6 times) after bactericide application, while at the SW site in most cases no significant response of N_2O emissions to bactericide application was observed (Fig. 6). Furthermore, the application of both inhibitors regularly significantly increased N_2O production at the NE site (Fig. 6).

Discussion

Optimization of inhibitors concentrations and fungal-to-bacterial ratio in forest floors and Ah layer soils

In our experiments approx. the same concentration of cycloheximide and 2.5–3.7 times higher concentrations

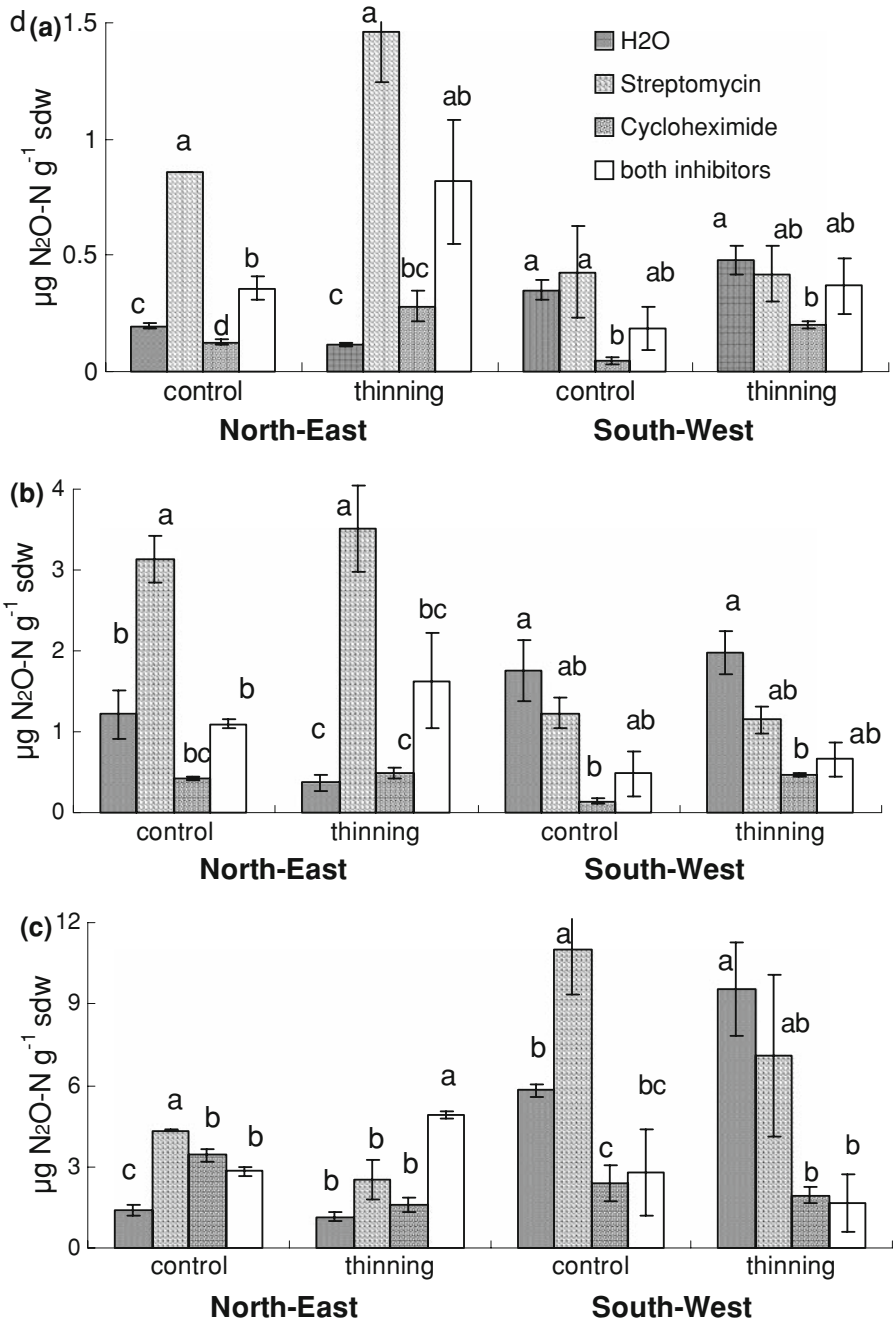

Fig. 6 Effect of selective inhibitors applied on N_2O production from samples taken from the Ah soil layers of the control and the thinned plots of the NE and the SW sites during 4 h (**a**), 8 h (**b**) and 24 h (**c**) of incubation after moistening to 100% WHC of the soil samples. *Error bars* represent standard deviations. Different *letters* indicate significant differences in the samples ($p < 0.05$)

of streptomycin were required for the quantitative inhibition of fungal or bacterial respiration as compared to previous studies with forest floors or plant residues (Beare et al. 1990; Scheu and Parkinson 1994). Required inhibitor concentrations of both streptomycin and cycloheximide for Ah layer

samples were 2.5 times higher in our study as compared to other results gained for mineral forest soils (West 1986; Scheu and Parkinson 1994). The reason that in this study higher inhibitor concentrations were needed as compared to previous studies may be due to an antibiotic deactivation effect of the in situ environment as described by Beare et al. (1990). Application of low inhibitor concentrations to soils with high antibiotic deactivation capacity may result in low total inhibition and nevertheless in high overlapping effects. The optimum concentrations of both streptomycin and cycloheximide ranged from 2 to 16 mg g^{-1} sdw for a range of forest floors and mineral soils in the USA and Canada, which were different in terms of texture and C content (Scheu and Parkinson 1994; Bailey et al. 2002). The values of the TCI index in these soils, however, were comparably low—22.1% (Scheu and Parkinson 1994) and 31.4–43.1% (Bailey et al. 2002), whereas, the overlapping inhibition amounted to 40% in the latter study. The inhibitor concentrations used in our study ensured a higher percentage of total combined inhibition of SIR (44–65% and 61–72% in forest floor and in Ah layer samples, respectively), and a comparatively low antibiotic overlap (Table 3). Thus, in spite of the requirement of high inhibitor concentrations the application of SIRIN technique seems to be more reliable for the soils investigated within this study as compared to soils of other studies which reported lower optimum inhibitor concentrations.

For all investigated samples in this study the fungal SIR was higher than bacterial SIR (Table 3). This is consistent with other studies (Anderson and Domsch 1975; Ingham and Horton 1987; Neely et al. 1991) suggesting that fungal mineralization activity is the predominant process in the forest floor and in the uppermost mineral soil horizons. F:B ratios with regard to SIR determined in our study for samples taken from the forest floor (1.1–2.3) were close to those found for decaying plant residues by Beare et al. (1990), which were in a range of 1.6–2.5 and to those (approx. 1–2.5) found for Of and Oh forest floor material (Scheu and Parkinson 1994). Furthermore, the F:B respiratory ratio for soil samples determined at our sites (1.4–4.0) were in good agreement with those (averaged 3.0) reported for 10 soils of neutral soil pH and under beech forest (Blagodatskaya and Anderson 1998).

Effect of site exposure and forest management on fungal-to-bacterial ratio

Compared to site exposure, the silvicultural treatment had only a minor influence on F:B respiratory ratio both in the forest floor and mineral soil (Fig. 3). This may be related to comparably low differences in both meteorological conditions (Dannenmann et al. 2006, 2007a,b) and physical and chemical soil parameters (Tables 1, 2) between control and thinned plots. In contrast, site effects may indeed be caused by differences in microclimatic conditions or chemical and physical characteristics of the soil and litter samples (Tables 1, 2).

At the NE site, characterized by lower impact of solar radiation but also higher mean soil moisture values (Dannenmann et al. 2006), mean F:B respiratory ratio was not significantly different between forest floor and Ah layer (Fig. 2a). In contrast, at the SW site, with comparable lower mean soil moisture values, F:B respiratory ratios were significantly lower in the forest floor as compared to the mineral soil (Fig. 2a). This comparison should be conducted with caution considering the considerably lower total combined inhibition in the forest floor than in the Ah layer. However, the basic and commonly accepted assumption of the SIRIN method suggests the same respiratory F:B ratios for inhibited and uninhibited microbial biomass (see e.g., Lin and Brookes 1999; Laughlin and Stevens 2002, Joergensen and Wichern 2008). If this assumption was valid, the differences in F:B respiratory ratios between forest floor and Ah layer found in our study might be either related to physicochemical soil parameters and soil moisture differences or by a direct effect of solar radiation on fungal activity, causing a relocation of fungal activity at the SW site from the forest floor to the Ah layer. A radiation-triggered redistribution of fungal activity from the forest floor to the Ah layer might be promoted by the high competitive ability of filamentous fungi in the occupation of favorable ecological niches (Duguay and Klironomos 2000; Moody et al. 1999). Bailey et al. (2002), investigating a climate gradient of 6°C and 90 mm annual precipitation in desert mountains, found higher fungal activity at the site characterized by lower temperatures and higher precipitation. This is in agreement with our findings for the forest floor only. However, for the Ah layer we found an opposite effect: F:B respiratory ratio was

found to be higher in the Ah layer of the warm–dry SW site as compared to the Ah layer of the cool–moist NE site. This inverse effect might be related to a radiation-triggered redistribution of fungal activity to deeper soil layers like hypothesized above or by the sensitivity of fungal activity and colonization to the frequency and severeness of drying–wetting cycles, which are most pronounced for the forest floor at SW site as compared to all other layers and sites in this study (Dannenmann et al. 2007a).

Fungal-to-bacterial respiratory ratio in the Ah layer is supposed to rather be affected by physical and chemical soil properties. Due to higher pedogenetical development the Ah layer of the NE site is characterized by lower content of SOC and total N as well as lower C/N ratios and pH values than the Ah layer of the SW site. (Table 2). Bailey et al. (2002) reported a significant correlation between fungal activity and SOC content which is in agreement with our observation, that relative to the bacterial activity fungal activity is higher in the Ah layer of the SW site as compared to the Ah layer of the NE site.

Besides SOC content, soil texture might have influenced the observed variation in the F:B respiratory ratio in the Ah layer of the investigated sites (Frey et al. 1999). Higher stone and gravel content at the SW site might favor aeration conditions for aerobic fungal microorganisms and thus, might have contributed to the difference in F:B respiratory ratio between the SW and NE sites.

Effect of pre-incubation at different soil moisture on N_2O emission

In this study, we measured N_2O emissions at 100% of the WHC, i.e., for conditions when all soil macropores were water-filled. Since samples were preincubated at different water content, the experimental design imitated heavy precipitation events at field conditions following periods of soil drying of different length. Dendooven et al. (1996) found differences in N_2O emissions from permanent pasture soils, which either have been antecedently submerged in water for a short or for an extended period and then drained. In our study significantly more N_2O was produced from soils pre-incubated under low moisture conditions (15% WHC) than from almost dry soils (5% WHC; Fig. 4). This indicates that even in spite of the lack of water, microorganisms seem to maintain an active state at low soil moisture and quickly increase their activity after water addition. Air dried soils showed a quick but much lower response to moistening as compared to soils pre-incubated under 15% WHC moisture conditions (Fig. 4). These results support the conclusion of Davidson (1992) that nitrifying and denitrifying microorganisms appear to be well adapted to surviving extreme drought and are capable of becoming active within minutes after wetting of dry soil. Lower N_2O production in air-dried soils as compared to soils still containing low amounts of water demonstrated within this study implicates that the physiological state of an essential part of microorganisms in air-dried soil seems to change into inactive resistant forms (such as spores and cysts) and as a consequence more time until complete recovering of the microbial activity after moistening is necessary. Stevens et al. (1997) investigated the effect of soil moisture on N_2O emission from fresh soil without pre-incubation and revealed that more N_2O was produced at 60% WHC and 50% WHC than at 40% WHC. A comparable tendency was found within this study for soil moisture during pre-incubation: N_2O emissions were significantly higher from soils pre-incubated at 60 than at 30% WHC (Fig. 4). During pre-incubation under optimum (60% WHC) or slightly deficit moisture conditions (45–30% WHC) the supply of available nutrients is likely to be consumed by active microflora and, as a consequence, denitrification after moistening to 100% WHC is supposed to be substrate (C and N) limited as compared to samples pre-incubated at low moisture content (15% WHC), where inorganic N accumulation during pre-incubation coincides with microorganisms quickly increasing activity after moistening.

N_2O emission after moistening to 100% WHC

We found a sharp increase in N_2O production from Ah layer samples and forest floor samples after moistening to 100% WHC. Such an increase in N_2O production after moistening was also described for a laboratory study with soil from permanent pasture, Devon, UK (Dendooven and Anderson 1994). These authors explained the induction of increased N_2O production with a shift from prevailing aerobic to prevailing anaerobic incubation conditions. Such

pronounced changes in the magnitude in N_2O production were also observed in field studies, e.g., when first rains after long-lasting drought periods have moistened the soil (Davidson et al. 1993; Butterbach-Bahl et al. 2004). Both processes nitrification and denitrification simultaneously can be the sources of N_2O production after such quick moistening. Denitrification was found to be dominant during 2 days after moistening of partially air-dried bulk soil to 50–60% WHC (Stevens et al. 1997). However, in investigations with woodland soil it was demonstrated that denitrification was the dominant N_2O source only when soil water was above field capacity (Davidson 1992). A possible chemical N_2O production can be excluded in our study, since no N_2O as well as no CO_2 were detected in triple-autoclaved samples of soils and forest floors during 24 h of incubation after moistening with sterile water.

The rates of potential N_2O production in Ah layer samples after soil moistening found in our study (Table 3) were comparable with other laboratory studies performed with intact soil cores taken from a permanent pasture in England (0.11–0.21 μg N g^{-1} h^{-1}, Dendooven et al. 1996) or soils of a rice-growing regions in China (0.179–0.279 μg N g^{-1} h^{-1}, Mei et al. 2004).

Linkage between fungal-to-bacterial ratio and N_2O emission after moistening

A significant correlation between the F:B ratio (the relative contribution of fungi and bacteria to total SIR) and N_2O emission was found only for Ah layer samples but not for forest floor samples (Fig. 5a). Furthermore, a highly significant negative relationship between N_2O emission and absolute values of bacterial SIR was found for both Ah layer and forest floor (Fig. 5b). Thus, as was also concluded by Bailey et al. (2002), our results demonstrate that the relative proportions of each group of organisms with regard to total biomass may mask their actual activity in the soil. The negative correlation between N_2O production and bacterial respiratory activity provides further indication that N_2 is the most expected product of bacterial denitrification since N_2O-reductase is predominantly produced by bacteria while many fungal microorganisms are lacking N_2O-reductase (Shoun et al. 1992; Hayatsu et al. 2008).

Inhibitor effect on N_2O emission

In our study we frequently found an unexpected increase in N_2O production after moistening of soil samples amended with antibiotics (especially streptomycin) as compared to control experiments where only water was added (Fig. 6). Similar patterns were observed by McLain and Martens (2006) in a semiarid soil, where streptomycin additions caused a 100% increase in N_2O flux. These observations may be explained by: (1) a disturbance of the balance of N_2O production and consumption in the microbial community by streptomycin; (2) the antibiotics may have served as an additional easily available substrate for microbial metabolism and thus also for N_2O production.

Dendooven et al. (1994) found increased N_2O and CO_2 production after adding chloramphenicol, another inhibitor of protein synthesis of prokaryotic microorganisms (Dendooven et al. 1994; Dendooven and Anderson 1994), to mineral soil samples and hypothesized that chloramphenicol served as a C substrate for denitrification and thus contributed to the increase in N_2O production. If this hypothesis may be the case, streptomycin and cycloheximide may also be decomposed by microbes, thus contributing to increased N_2O emission after inhibitor addition as observed within this study.

However, in view of the short incubation periods of our experiments an inhibitor-triggered disturbance of the balance of N_2O production and consumption appears to be more plausible to explain increased N_2O emission after inhibitor addition. N_2O reduction to N_2 by denitrifying microorganisms is a crucial process in the investigated soil (Dannenmann et al. 2008), especially after soil moistening to 100% WHC (e.g., Conrad 1996), and thus, this reduction step may have been inhibited by streptomycin in our experiments. Mei et al. (2004) reported that the rates of N_2O consumption in soil slurries were 4.2–8.4 times higher compared to the rates of N_2O release during denitrification. Hence, net N_2O emission is the result of two dynamic processes: N_2O production by NO-reductase and N_2O consumption by N_2O-reductase. NO-reductase can be produced by both bacteria and fungi whereas, bacteria are largely responsible for N_2O-reductase activity which was not found for fungal species (Shoun et al. 1992; Spokas et al. 2006). The results found in our study may be explained as follows: streptomycin application, i.e., a prokaryotic inhibitor,

may have inhibited the bacterial synthesis of N_2O-reductase while fungal denitrification with N_2O as endproduct remained unaffected. In consequence this would lead to the observed high rates of N_2O production found within this study after addition of streptomycin. The high significance of fungi for N_2O production in the investigated soils is clearly shown by N_2O production being decreased by up to 87% due to cycloheximide addition which is consistent with the results of combined ^{15}N labeling and inhibitor experiments performed in meadow soils by Laughlin and Stevens (2002). These authors reported that N_2O production decreased by 89% after cycoloheximide additions. Together with the observation that low bacterial contribution to SIR coincides with high N_2O production (Fig. 5b) these findings strongly suggest that pedosphere-atmosphere fluxes of N_2O at our sites are mediated by both N_2O production predominantly by fungi and N_2O consumption predominantly by bacteria.

Conclusions

The SIRIN method proved to be a valuable but elaborate tool for investigating F:B respiratory ratios in the forest floor and uppermost mineral soil of the investigated forest ecosystem. N_2O production and F:B respiratory ratio for both Ah layer and forest floor were mainly affected by microclimate, whereas, thinning of the forest was of minor importance for the investigated parameters. Net N_2O production at 100% WHC seems to be a result of N_2O production predominantly by fungi and N_2O consumption predominantly by bacteria. Thus, varying N_2O emissions under different microclimate appear to be triggered by variations in the relative contribution of bacteria and fungi to the total microbial activity.

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