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ORIGINAL PAPER

Contrasting response of two forest soils to nitrogen input: rapidly altered NO and N_2O emissions and *nirK* abundance

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Abstract Denitrification represents one of the main microbial processes producing the primary and secondary greenhouse gases nitrous oxide (N_2O) and nitric oxide (NO) in soils. It is well established that abiotic factors like the soil water content and the availability of nitrogen (N) are key parameters determining the activity of denitrifiers in soils. However, soils differing regarding their characteristics such as the content of C_{org}, the soil texture or the pH value may respond in specific manners to equivalent changes in soil moisture and N input. Thus, short-term incubation experiments were performed to test and compare the capacity of two contrasting Austrian forest soils to respond to mineral N application at increased soil water contents. Soils from the pristine Rothwald forest (rich in C_{org}) and the more acidic Schottenwald forest (poor in C_{org}) were amended with either NH_4^+-N or NO_3^--N and were incubated at 40% and 70% water-filled pore space for 4 days. Changes in mineral N pools, nitrite reductase activity and NO and N_2O emission rates were measured, and the abundance and structural community composition of the functional group involved in nitrite reduction were analysed via quantitative real-time polymerase chain reaction and terminal restriction fragment length polymorphism analysis of the *nirK* gene.

Rapid and distinct activity responses to increased soil moisture and altered mineral nitrogen availability were observed in two contrasting forest soils. In both soils, nitrogen oxide emission rates were stimulated by N inputs and, depending on the soil moisture status, either NO or N_2O emission was prevailing. However, different N cycling processes appeared to predominate in either soil under equivalent treatment. Nitrogen oxide emissions peaked following NO_3^- application in Schottenwald soils but were the highest after NH_4^+ application in Rothwald soils. Denitrifying (*nirK*) communities differed significantly in Rothwald and Schottenwald soils; however, changes in the community structure were marginal during the short-term incubation. Abundances of *nirK* genes remained unaffected by N application in either soil. The soil water content affected *nirK* gene abundances only in Rothwald soil, indicating a distinct reaction of nitrite reducing communities in the two soils.

Keywords Denitrification · Nitrite reductase · NO_3 · NH_4 · Nitrogen oxide emission · Soil moisture · Forest soils

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Introduction

The production and subsequent liberation of nitrogen oxides from soils are primarily driven by microbial processes such as denitrification and nitrification (Firestone and Davidson 1989). Nitric oxide (NO) and nitrous oxide (N_2O) represent potent greenhouse gases, which indirectly and directly affect global warming (Conrad 1996). Their emission rates depend on the availability of mineral N and are strong functions of climate as they vary with soil moisture and soil temperature (Davidson and Swank 1986; Smith et al. 1998; Pilegaard et al. 2006; Ciarlo et al. 2007).

Field and laboratory investigations evidenced a general increase in nitrogen oxide emissions from forest soils as a response to increased N availability (Butterbach-Bahl et al. 1998; Ambus et al. 2006). Nevertheless, soil characteristics such as the C_{org} content, the soil texture and the pH value are expected to have secondary effects on the availability of water and N in soils. For instance, Conrad (1996) and Smith et al. (2003) highlighted the significance of soil aeration determining N oxide emissions, and Simek and Cooper (2002) outlined the influence of soil pH on denitrification rates. Additionally, high C_{org} contents have been associated with high microbial biomass and activity (Kandeler et al. 2005). Thus, soils differing regarding their soil characteristics may respond specifically to equivalent changes in abiotic factors such as soil N concentrations and soil moisture.

The present study compares the potentials of two contrasting Austrian forest soils to respond to NH_4^+ or NO_3^- input at increased soil moisture. The C_{org}-rich, pristine *Rothwald* (R) spruce-fir-beech forest is located in a remote valley in Lower Austria with N deposition rates of less than 5 kg N ha^{-1} $year^{-1}$. In contrast, the more acidic *Schottenwald* (S) beech forest, situated in direct vicinity of Vienna (Wienerwald), is subject to N inputs of 20.2 kg N ha^{-1} $year^{-1}$ resulting from traffic and industry (Kitzler et al. 2006). The two forest soils were incubated with either NH_4^+ or NO_3^- amendment and increased soil moisture levels for 4 days. Emission rates of NO and N_2O were measured together with soil mineral nitrogen concentrations and the nitrate reductase activity. In order to investigate adaptations of the underlying denitrifying microorganisms, the abundance of the *nirK* gene coding for the enzyme nitrite reductase was determined via real-time polymerase chain reaction (PCR). To assess potential changes and differences in the structural composition of denitrifying communities, the two soils were monitored via terminal restriction fragment length polymorphism (T-RFLP) analysis of *nirK* gene amplicons.

Materials and methods

Soil sampling

Experimental sites were set up in the pristine spruce-fir-beech forest Rothwald, situated at the eastern border of the Austrian Alps (Lower Austria), and in the Schottenwald beech forest, located in close vicinity of the Vienna city area. Soil samples from the Rothwald forest and the Schottenwald forest were collected in June 2005. Soil and site characteristics are listed in Table 1. At each site, ten soil samples were taken from the mineral layer (0–10 cm depth) at 5 m intervals along transects of 50 m length. Soil samples were sieved to 5 mm, and the ten individual samples from each site were combined to a composite sample by mixing thoroughly.

Table 1 Site characteristics and soil properties of Rothwald and Schottenwald

	Rothwald[a]	Schottenwald[b]
Soil type	Chromic cambisol	Dystric cambisol
Geology	Dolomite	Sandstone
pH ($CaCl_2$)	5.3	4.4
C_{org} (%)	16.0	3.8
N_{tot} (%)	0.94	0.24
C/N	17.1	16.0
Initial WFPS (%)	35	22
Elevation (m a.s.l.)	1,035	370
Average annual Temp. (°C)	5.5	9.0
Mean annual Ppt. (mm)	1,759	465

Temp. temperature, *Ppt.* precipitation, *a.s.l.* above sea level

[a] Data from Hackl et al. (2004)

[b] Data from Kitzler et al. (2006)

Incubation

Thirty stainless steel cylinders of 7.5 cm diameter and 5 cm height each were filled with approximately 120 g homogenised fresh Rothwald and Schottenwald soil, respectively. The water contents of 15 soil cores each were adjusted to either 40% ('40', 'moist') or 70% water-filled pore space (WFPS; '70', 'wet') by wetting with a syringe. The bulk density was 0.78 g cm^{-3}±0.04 for Schottenwald soil and 0.62 g cm^{-3}±0.03 for Rothwald soil. Of 15 cores adjusted to a specific water level, five soil cores were amended with 0.1 mg $(NH_4)_2SO_4$-N g^{-1} dry soil (NH_4), five soil cores were amended with 0.1 mg KNO_3-N g^{-1} dry soil (NO_3) and five control cores were treated with distilled water only (oN). The amended soil cores and the control cores were incubated for 4 days at 20°C with periodical re-adjustment of the water content. After 4 days, one soil core of each treatment was sampled destructively for replicated determination of NH_4^+-N and NO_3^--N concentrations and measurement of nitrate reductase activity. The other four replicate soil cores of each treatment were used for measurement of nitrogen trace gas fluxes (NO, N_2O). Following process measurements, soils were stored at −20°C for subsequent DNA extraction.

Soil chemical analyses

Nitrate reductase activity was measured using KNO_3 as a substrate (final concentration 0.0625 M) and dinitrophenol (DNP) as inhibitor for nitrite reductase (Schinner et al.

1996). Preliminary tests showed optimal inhibition at a concentration of 7×10^{-3} mg DNP g^{-1} dry soil (data not shown). For maintaining the adjusted WFPS, the substrate together with DNP was applied in 400 µl aqueous solution to 2.5 g soil aliquots of each treatment (n=6) with a micro-syringe followed by mixing. This volume corresponded well to the amount of water evaporating during 24 h of incubation (data not shown). Four replicates were kept at 20°C for 24 h, and two samples, serving as blanks, were immediately frozen at −20°C. Total NO_2^- from samples and blanks was extracted with 3 M KCl, analysed photometrically (µQuant, BIO TEC Instruments, Inc.), and nitrate reductase activities were calculated (Schinner et al. 1996).

Soil mineral nitrogen contents were measured in four sub-samples of each treatment, following extraction of 5 g soil aliquots in 30 ml 0.0125 M $CaCl_2$ with shaking for 30 min. In filtered soil extracts, NH_4^+-N was detected photometrically at 660 nm while NO_3^--N was determined as NO_2^- after overnight reduction with copper sheathed granulated zinc at 210 nm (Schinner et al. 1996).

Analysis of nitrogen gas emissions

For N_2O emission measurements, soil cores (n=4) were incubated in closed, gas-tight jars (headspace volume 450 ml) at 20°C. Headspace gas samples of 15 ml were taken with a syringe immediately after closure and after 3 and 6 h of incubation. Gas samples were transferred into evacuated 10 ml glass vials, sealed with silicone grease and stored under water at 4°C until measurement. Nitrous oxide concentrations were analysed by gas chromatography (Hewlett-Packard 5890 Series II; injector, 120°C; detector, 330°C; oven, 120°C; carrier gas, N_2) using a ^{63}Ni electron capture detector. Nitrous oxide fluxes were calculated from the linear increase in concentration over the incubation time and corrected for air temperature and air pressure.

Following N_2O measurements, NO gas fluxes from the same soil cores (n=4) were measured with a chemo-luminescence nitrogen oxide analyser (HORIBA-APNA-360) connected with a fully automated incubation system (Schindlbacher and Zechmeister-Boltenstern 2004). Soil cores were placed in individual temperature-controlled glass chambers, and NO concentrations in the flow-through stream of each vessel were analysed separately at intervals of 8 min.

DNA extraction and quantification by real-time PCR

DNA was extracted from 0.5 g bulk soil using the UltraClean soil DNA Extraction Kit (MoBio Laboratories, Inc.). Three replicate DNA extractions per soil core used for chemical process analysis were pooled and subjected to PCR amplification.

The functional marker gene encoding nitrite reductase (*nirK*) was quantified by real-time PCR using an iCycler IQ (Biorad). The 25-µl PCR reaction mix contained 15 µg bovine serum albumin, 0.675 µl dimethyl sulfoxide, 12.5 µl of Q Mix (Biorad, 100 mM KCl, 40 mM Tris-HCl, 6 mM $MgCl_2$, 0.4 mM each of deoxyribonucleotide triphosphate, 50U/ml iTaq DNA Polymerase, SybrGreen I, 20 mM fluorescein) and 25 ng template. Fluorescence acquisition was performed at 77°C where all primer dimers had melted but specific products had not. For the quantification of *nirK* copies, 1 µl (of a 10-µM solution) of each PCR primer nirK1F (GGMATGGTKCCSTGGCA) and nirK5R (GCCTCGATCAGRTTRTGG) amplifying a 514-bp product (Braker et al. 1998) was added to the PCR mix. The cycling conditions were 95°C for 3 min, six touch-down cycles of 15 s at 95°C, 30 s 63°C, 30 s at 72°C, decreasing 1°C per cycle, followed by 40 cycles of 15 s at 95°C, then 58°C, 72°C and 77°C for 30 s each.

Abundances of *nirK* genes refer to copy numbers per gram dry soil. Standards for quantitative real-time polymerase chain reaction (qPCR) were generated by serial dilution of stocks containing a known number of plasmids carrying the respective functional gene as an insert. The reaction efficiencies of qPCRs were 90% (±2.6), and R^2 values were 0.99 for all runs. High concentrations of template showed reduced PCR efficiency indicative of PCR inhibition. For minimising these effects, minimal amounts of template DNA (25 ng) were used in PCR reactions. Analysis of real-time PCR raw data demonstrated that the log phase of amplification curves of the samples and standard had the same slope, indicating an accurate quantification of gene copy numbers in the soil samples.

Community profiling by T-RFLP analysis

For T-RFLP analysis, fluorescently end-labelled forward primers (carboxyfluorescein phosphoramidite, FAM) were used for the amplification of *nirK* genes. PCR amplifications were performed in T1 thermocyclers (Biometra). The *nirK* PCR using the primers nirK1F and nirK5R was performed with the PuRe Taq Ready-to-go beads (GE healthcare) adding 0.175 µl of each primer (100 µM) and 25–50 ng DNA template. The PCR involved an initial denaturation step at 95°C for 5 min, followed by 34 cycles of 30 s at 95°C, 40 s at 54°C (Rothwald) or 58°C (Schottenwald), 30 s at 72°C, followed by a final elongation step of 72°C for 7 min. Temperature-gradient PCRs demonstrated better amplification with 54°C and 58°C annealing temperature for Rothwald and Schottenwald soil samples, respectively.

Amplification of *nirS* using the primer pair Cd3aF and R3cd failed (Throbäck et al. 2004) and no distinct *nirS* gene product was obtained using the primer set nirS 1F and nirS 1R (Braker et al. 1998). For generating T-RFLP profiles, at

least three replicate PCR reactions from each sample were pooled and purified with Invisorb Spin PCRapid Kit (Invitek) before digest as recommended previously (Hartmann et al. 2007). A 10-µl reaction volume containing 150 ng FAM marked *nirK* products was digested with *TaqI* (Promega) enzyme at 65°C for 3 h. Digests were purified using Sephadex columns. HIDI loading buffer (Formamide) and Rox 500 bp length standard (Applied Biosystems) were added to the digests and denatured at 92°C for 2 min prior to analysis on an ABI Prism 3100 Genetic Analyser (Applied Biosystems). Lengths of T-RFs were determined by comparing with internal standards using GeneScan 3.1 software (2001, Applied Biosystems). Terminal fragments smaller than 50 bases were excluded from the analysis. Electropherograms were analysed using the Molecular Fingerprint Analysis Software by SequentiX (Digital DNA Processing, Klein Raden, Germany).

Statistical analysis

Analysis of variance (ANOVA) of nitrogen concentrations, process data and real-time PCR data (one-way ANOVA, multiple range test) was performed using the Statgraphics Plus 5.0 package. A least significant difference test was used for comparison of equal sample size numbers, while the Scheffé's test was used for samples with varying numbers of replicates.

Prior to statistical analysis, T-RFLP profiles were normalised by dividing individual peak height values of a profile by the sum of all peak height values in the corresponding profile (Dunbar et al. 2001). Subsequent statistical analysis was performed on square-root transformed data using Primer 6 software (PRIMER-E, Plymouth, UK). Non-metric multidimensional scaling (nmMDS) using Bray–Curtis similarity calculation was used for demonstrating the relatedness of individual samples under different treatments (Kenkel and Orloci 1986; Minchin 1987). Similarity between treatments on the basis of T-RFLP profiles was calculated using the one-way univariate analysis of similarity (ANOSIM) including 10,000 permutations with significant differences considered as $p<0.01$ (Clarke 1993; Clarke and Gorley 2001). A similarity percentage analysis was used to identify the major T-RFs responsible for observed differences within and between treatments. Permutational multivariate ANOVA (PERMANOVA) including a Monte Carlo test was used to test for effects of factors, interactions and pair-wise comparisons. Significant differences in T-RF abundances were investigated performing ANOVA using a Tukey's honestly significant difference test (Statgraphics Plus 5.0).

Relationships among mineral nitrogen pools, nitrate reductase activity and the abundance of the *nirK* gene were assessed with principal component analysis PCA using Primer 6 software (PRIMER-E, Plymouth, UK). Analysed parameters represented by vectors with parallel orientations were considered related, and correlations between parameters were calculated at a 95% confidence level ($p<0.05$) by means of multiple-variable analysis (Statgraphics 5.0).

Results

Mineral nitrogen pools

The soil water content showed a more pronounced effect on NO_3^- pools than on NH_4^+ pools (Table 2). In R and S soil, NO_3^- concentrations were lower at 70% WFPS than at 40% WFPS. In contrast, NH_4^+ concentrations were higher in R soils at 70% WFPS than at 40% WFPS in N-amended R soils.

Nitrate reductase activity

In R soil, nitrate reductase activities ranged from 60 to 600 ng $N g^{-1} 24\ h^{-1}$ and were mostly higher at 70% WFPS than at 40% WFPS (Table 3). Nitrate reductase activity in S soil was close to the detection limit of the assay. Nitrogen amendment did not significantly contribute to changes in nitrate reductase activity.

Nitrogen gas emission rates

Nitric oxide emission from R and S soils varied according to the water content and mineral nitrogen amendment (Fig. 1). Mean NO emissions were mostly higher in R than in S soil for corresponding treatments. The soil water content affected

Table 2 Mean ($n=4$) nitrate and ammonium concentrations in Schottenwald and Rothwald soils at 40% WFPS and 70% WFPS after NO_3^- and NH_4^+ treatment and without N addition, expressed as microgram NO_3-N per gram soil and microgram NH_4-N per gram soil

	oN	NO_3	NH_4
NO_3-N (µg g^{-1} soil)			
S40	17.34 c	127.13 h	17.77 c
S70	1.34 a	75.70 g	4.09 ab
R40	32.70 d	162.13 i	43.07 e
R70	6.54 b	67.12 f	4.22 ab
NH_4-N (µg g^{-1} soil)			
S40	3.27 a	5.70 ab	168.09 f
S70	8.07 ab	12.03 ab	154.79 f
R40	8.72 ab	11.32 ab	79.18 d
R70	19.93 bc	29.79 c	110.31 e

Different lowercase characters indicate significant differences at the 95% confidence level, $p<0.05$

S Schottenwald, *R* Rothwald, *40* 40% WFPS, *70* 70% WFPS, *oN* without N addition

Table 3 Mean nitrate reductase activity (microgram N per gram per 24 h) in the Schottenwald and Rothwald soils at 40% WFPS and 70% WFPS treated with NO_3^- and NH_4^+ and without N addition

WFPS (%)	R		S	
	40	70	40	70
NO_3^-	0.06 ab	0.21 b	−0.05 a	−0.05 a
NH_4^+	0.2 b	0.41 c	0 a	−0.03 a
oN	0.17 b	0.57 c	−0.06 a	−0.04 a

Different lowercase characters indicate significant differences at the 95% confidence level, $p<0.05$

S Schottenwald, *R* Rothwald, *40* 40% WFPS, *70* 70% WFPS, *oN* without N addition

the NO emission when nitrogen was applied to the soils. Four to seven times higher NO emissions were measured at 40% WFPS compared to 70% WFPS in nitrogen-amended R and S soils. In oN soils, the emission rates of NO were not affected by the soil water content. Ammonium and nitrate amendment affected the two investigated soils differently. At 40% WFPS, the application of NO_3^- led to a more pronounced increase in NO emissions from S soils, while NH_4^+ addition resulted in peak emissions in R soils.

Nitrous oxide emission rates in N-amended soils were generally higher at 70% WFPS than at 40% WFPS; however, the two forest soils responded differently to the addition of NH_4^+ and NO_3^- (Fig. 2). Peak N_2O emissions occurred following NO_3^- treatment in S soil at 70% WFPS and after NH_4^+ application in R soils at 70% WFPS. Apart from these peak fluxes at high water contents, N_2O emissions from both soils were comparably low.

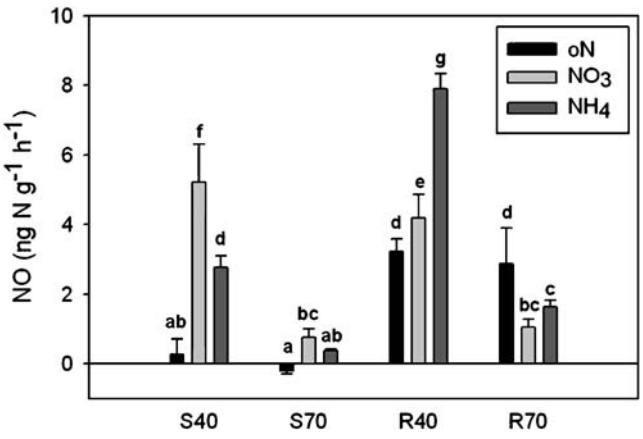

Fig. 1 Mean NO emissions (nanograms N per gram per hour) under NH_4^+ and NO_3^- treatment and without N addition (*oN*) of Rothwald (*R*) and Schottenwald (*S*) soil at 40% WFPS (*40*) and 70% WFPS (*70*). *Error bars* represent the standard deviation. *Different lowercase characters* represent significant differences at the 95% confidence level, $p<0.05$, Scheffé's test

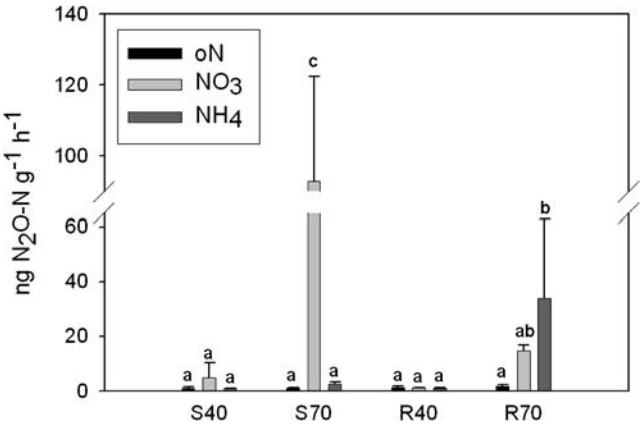

Fig. 2 Mean N_2O emissions (nanograms N_2O-N per gram per hour) of Schottenwald (*S*) and Rothwald (*R*) soil at 40% WFPS and 70% WFPS with NH_4^+ and NO_3^- treatment and without N addition (*oN*). *Error bars* represent the standard deviation. *Different lowercase characters* represent significant differences at the 95% confidence level, $p<0.05$

The ratio of NO to N_2O emission was higher in R soils than in S soils for equivalent treatments. The highest NO/N_2O ratios occurred in R soils at 40% WFPS.

Community profile

Community profiles of the *nirK* gene recorded a total of 102 T-RFs. The majority of T-RFs showed low abundances within the profile with four and three T-RFs having an abundance of more than 5% in R (100, 178, 181, 236 bp) and S (109, 236, 409 bp) soils, respectively. Community profiles of the *nirK* gene analysis revealed a significant dissimilarity between *nirK* communities of R and S soils ($R=0.7$, $p<0.001$; Fig. 3). The difference between the soils could not be attributed to a finite number of specific peaks but rather to a large number of smaller peaks contributing with ≤5% to the dissimilarity. Beside a significant site effect (p(MC)=0.0001), the structure of the *nirK* community was also affected by the N treatment (p(MC)=0.0092). In R soils, NO_3^- and NH_4^+ treatment effects on the *nirK* community composition were close to the significance level ($R=0.156$, $p=0.057$) when analysed with ANOSIM. A pair-wise PERMANOVA test, however, delivered a weak but significant difference between oN- and NO_3^--treated *nirK* communities in R soils (p(MC)=0.026). In S soils, the *nirK* community structure was affected by N treatment ($R=0.242$, $p=0.03$) and a pair-wise comparison demonstrated a significant difference between oN and NH_4^+ treatment (p(MC)=0.0248).

Abundance of *nirK* genes

No significant treatment effects on the abundance of *nirK* genes were observed in S soil (Fig. 4). In R soil, a soil

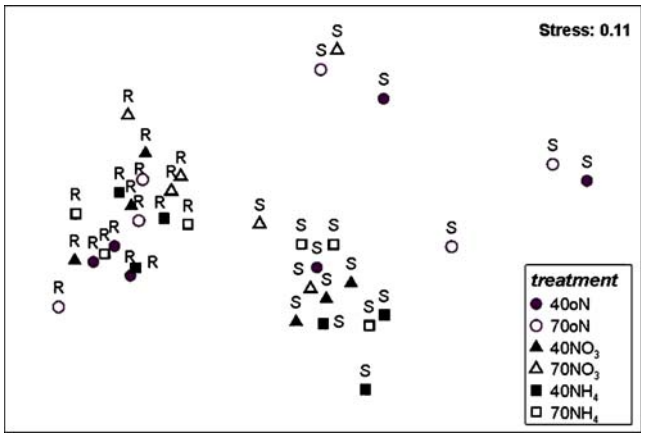

Fig. 3 Plot (nmMDS) based on Bray–Curtis similarities showing the relative similarities of the *nirK* community structure based on T-RFLP profiles of Schottenwald (*S*) and Rothwald (*R*) soil at 40% WFPS (*40*) and 70% WFPS (*70*) under treatment with NO_3^- and NH_4^+ and without N addition (*oN*). The stress value is given in the plot

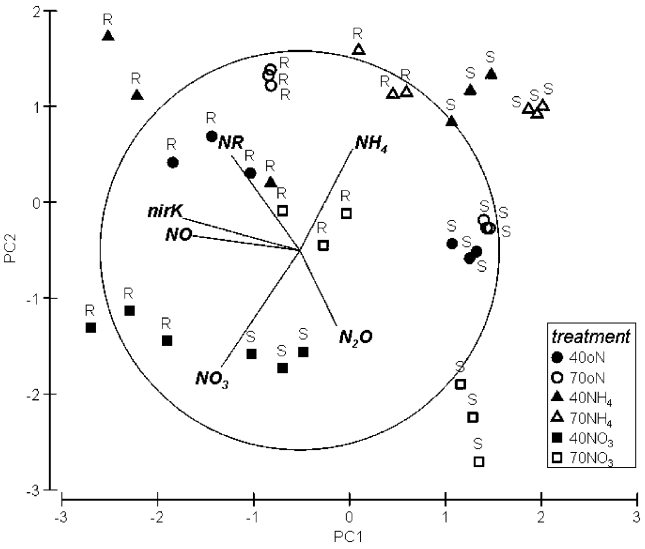

Fig. 5 2D PCA plot of Schottenwald (*S*) and Rothwald (*R*) soil at 40% WFPS (*40*) and 70% WFPS (*70*) under treatment with NO_3^- and NH_4^+ and without N addition (*oN*). Vectors represent the *nirK* gene abundance, the nitrate reductase activity (*NR*), concentrations of ammonium (NH_4) and nitrate (NO_3) and emissions of nitric oxide (*NO*) and nitrous oxide (N_2O)

moisture effect was evident, with 40% WFPS soil containing twice as many *nirK* copies as 70% WFPS soil. A definite effect of the nitrogen treatment on the *nirK* gene abundance could not be observed.

Principal component analysis

Principal component analysis delivered three components with Eigenvalues>1, which together accounted for 76% of the variability within the data (Fig. 5). A two-dimensional PC plot (Fig. 5) revealed separable clusters for R and S soils with individual soil samples clustering according to the treatment. The R soil was characterised by higher nitrate reductase activities, higher NO emission rates and higher *nirK* gene abundances compared to the S soil. The vector representing the soil NO_3^- concentration was negatively related to the NH_4^+ concentration (R^2 −0.42, $p<0.05$) and correlated positively with the NO emission rate (R^2 0.43, $p<0.05$). The abundance of the *nirK* gene was related to NO emission rates (R^2 0.43, $p<0.05$) and to the nitrate reductase activity (R^2 0.5, $p<0.05$).

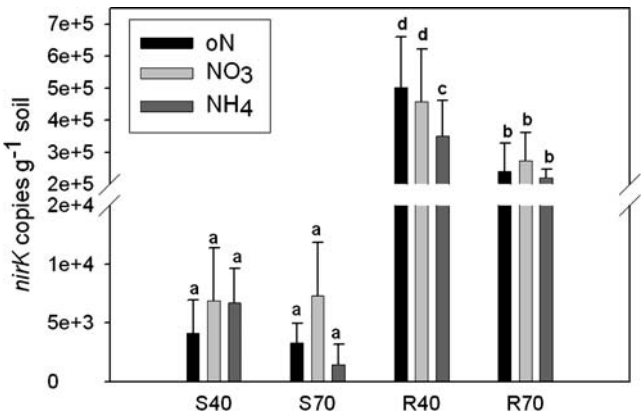

Fig. 4 Mean abundances of *nirK* copies (copies per gram soil) in the Schottenwald (*S*) and Rothwald (*R*) soil at 40% WFPS (*40*) and 70% WFPS (*70*) under treatment with NO_3^- and NH_4^+ and without N addition (*oN*). *Error bars* represent the standard deviation. *Different lowercase characters* represent significant differences at the 95% confidence level, $p<0.05$

Discussion

Source of N oxide emissions

Microbial communities in Rothwald and Schottenwald soils responded specifically to N treatment, indicating that different N cycling processes prevailed at equivalent conditions in the two soils. In the Rothwald soil, N gas emissions peaked following NH_4^+ amendment, whereas the Schottenwald soil showed peak emissions after NO_3^- application. The rate of O_2 supply, influenced by the soil water content, determines whether aerobic processes such as nitrification or anaerobic processes such as denitrification dominate within the soil (Smith et al. 2003). Although previous studies reported that denitrification was the main source for N_2O under wet conditions (Firestone and Davidson 1989; Pilegaard et al. 2006), in the Rothwald soil, N_2O emissions peaked at high soil water contents

following NH_4^+ application, suggesting nitrification as the main source for N_2O. Denitrification and nitrification have optima under different environmental conditions, but it is well established that both soil processes may occur simultaneously (Davidson et al. 2000). Soils rich in C_{org}, such as the Rothwald soil, permit a better aeration and may have a higher number of aerobic micro-sites, where nitrification can be maintained. However, the silty loam in Schottenwald may have become anoxic at lower soil moisture contents (Bollmann and Conrad 1998; Groffman and Tiedje 1991). Ambus et al. (2006) stated that N_2O emissions can be driven by nitrification activity, although N_2O per se is produced mainly through denitrification. Consequently, we suggest that high N_2O emissions observed in wet Rothwald soils upon NH_4^+ application may derive from nitrification and denitrification, rather than one of these processes alone. The Rothwald soil has previously been characterised by strong internal N cycling (Hackl et al. 2004), and hence, a continuous production of NO_3^- through the oxidation of NH_4^+ in aerobic micro-sites may have delivered additional substrate for denitrification.

Comparing 11 European forest soils, Ambus et al. (2006) showed that a high contribution (up to 100%) of $^{15}NH_4^+$ to N_2O emissions was observed only in the Tyrolian Achenkirch forest soil. Similar to the Rothwald soil, the Achenkirch soil is a chromic cambisol, located on limestone bedrock, exhibiting a high C_{org} content of 14.1% (Ambus et al. 2006). Both forest soils show high microbial activity and are exposed to a similar mountainous climate. Particularly the high C_{org} content, resulting in a higher water-holding capacity, may explain a similar response of Rothwald and Achenkirch soils to NH_4^+ treatment. In contrast, the Schottenwald soil represented the other extreme, where 100% of the N_2O emissions derived from $^{15}NO_3$ (Ambus et al. 2006). A similarly high contribution of denitrification to N_2O emissions from Schottenwald soil is suggested by the results of our study.

Treatment effect on N oxide emission rates

Under nitrogen amendment, NO was consistently produced under drier conditions (40% WFPS), whereas N_2O was released under wetter conditions (70% WFPS). This was observed for both soils and was in good accordance with microbiological theories raised by Firestone and Davidson (1989). In agricultural soils, easily mineralisable N from manure application together with high water contents (80% WFPS) resulted in peak N_2O emissions within 2–5 days (D'Haene et al. 2008). A 12- to 30-fold increase in N_2O emission was observed in grassland and arable soils when water contents were raised from 60% to 80% WFPS with water containing NH_4NO_3 (Dobbie and Smith 2001). In the Rothwald soil, a combination of increased nitrogen supply and high soil water content led to a 33-fold increase in N_2O fluxes at 70% relative to 40% WFPS when NH_4^+ was added. Nitrous oxide emissions from the Schottenwald soil were 19 times higher at 70% WFPS relative to 40% WFPS when amended with NO_3^-. Decreased NO_3^- concentrations at 70% WFPS compared to 40% WFPS indicated enhanced reduction of NO_3^- in wet compared to moist soils. These results demonstrate that N inputs have the potential of rapidly stimulating N trace gas emissions from forest soils in dependence of the prevailing soil moisture.

Previous field measurements in the Schottenwald soil revealed high N losses through N leaching and N_2O emissions within the upper range for temperate forest soils (Zechmeister-Boltenstern et al. 2002). Thus, the Schottenwald soil, receiving N loads of 20.2 kg $Nha^{-1}year^{-1}$ (Kitzler et al. 2006), was considered N saturated. By comparing European forest soils, Kesik et al. (2005) found correlations of the NO/N_2O ratio with C_{org}, acidity of the mineral soil and atmospheric N deposition. In fact, for equivalent treatments, NO/N_2O ratios were consistently lower in the more acidic Schottenwald soil with high N deposition compared to the Rothwald soil, which contrasted in these parameters.

Treatment effect on the *nirK* gene abundance

Activity responses to changing soil conditions have been reported to occur very rapidly, and soils have been attributed a potential of metabolising inputs of mineral nitrogen to N_2O within several days (Hynst et al. 2007). In Rothwald and Schottenwald soils, the short-term burst of microbial activity, measured 4 days after N amendment, was, however, not reflected in the abundance of the underlying microbial communities. Since denitrifiers, as heterotrophs, rely on the availability of C_{org}, increased denitrification activity is not necessarily coupled to population growth. Consequently, nitrogen treatment increased NO gas emissions but did not affect the *nirK* gene abundance in the Rothwald and Schottenwald soil. However, long-term fertilised forests even showed decreased *nirK* abundances, possibly as a response to secondary changes in C availability, moisture or pH value (Wallenstein 2004). In contrast, the single N pulse unlikely altered C_{org} availability and pH value in Rothwald and Schottenwald soils after 4 days of incubation.

Generally, higher *nirK* gene copy numbers and higher NO emission rates in the Rothwald soil compared to the Schottenwald soil resulted in a positive correlation between *nirK* abundance and NO emissions. This may be due to the four times higher C_{org} content of the Rothwald soil, favouring growth of the mostly heterotrophic nitrite reducers. The significance of C availability for denitrifiers was highlighted by Dandie et al. (2007), who reported an increase in *norB* populations proportionally with the amount of glucose-C added.

At increased soil water content, the abundance of the nitrite reducing bacteria was reduced in the Rothwald soil, whereas the Schottenwald soil remained unaffected. In both soils, total denitrification appeared to be higher at increased soil moisture, where reduced NO_3^- concentrations indicated nitrate consumption. Nevertheless, *nirK* genes in the Rothwald soil were more abundant at 40% WFPS than at 70% WFPS, where conditions generally were more supportive to denitrification. As denitrification represents a facultative respiratory pathway, bacteria carrying *nirK* genes switch to energetically less favourable nitrite reduction only under oxygen limited conditions. Therefore, even if *nirK* genes were more abundant at 40% WFPS, the respective organisms may respire oxygen instead of nitrate.

Treatment effect on the *nirK* community composition

While microbial activities respond rapidly to changes in environmental factors, previous studies have shown that the structure and composition of microbial communities required more time for adaptation (Avrahami et al. 2002; Stres et al. 2008). The composition of the nitrite reducing community differed significantly in Rothwald and Schottenwald soils and small but diverging effects of N treatment were observed. Small variations in the *nirK* community composition observed at higher NO_3^- concentrations in R soils and at altered NH_4^+ concentrations in S soils may indicate a beginning adaptation of the nitrite reducing community to N treatment after 4 days of incubation. Stres et al. (2008) reported a lacking effect of the soil water content on *nosZ* denitrifiers, performing nitric oxide reduction, in drained fen grassland soils after 12 weeks of incubation. Forest soils, in particular the Rothwald soil with its high microbial biomass and diversity (Hackl et al. 2004), may also harbour a high quantity and/or diversity of facultative denitrifiers enabling an accelerated ecological response compared to other soils. According to the diversity–stability hypothesis, a large functional diversity, including ecotypes capable of adaptation to changing environmental conditions, may allow ecosystem functioning to be maintained under perturbance (Chapin et al. 2000; McCann 2000).

Fingerprints of denitrifying communities targeting *nosZ* and *narG* genes showed some changes due to application of organic and inorganic fertilisers over 46 years (Enwall et al. 2005). These long-term fertilisation effects have been ascribed to indirect implications for denitrifying communities, e.g. through soil acidification (Enwall et al. 2005). Due to the short incubation time in our experiment, only marginal shifts in the *nirK* community structure were observed, which nevertheless provide evidence for the capacity of nitrite reducing bacteria to adapt to changes in the environment within short time spans.

In summary, the results of the study demonstrate a rapid and distinct response of the two contrasting forest soils to NO_3^--N and NH_4^+-N treatment at increased soil moisture, which included an increase in nitrogen oxide emissions and altered *nirK* gene abundances within a short incubation period of 4 days.

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