Insights into the Effect of Soil pH on N₂O and N₂ Emissions and Denitrifier Community Size and Activity[▽]

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The objective of this study was to investigate how changes in soil pH affect the N₂O and N₂ emissions, denitrification activity, and size of a denitrifier community. We established a field experiment, situated in a grassland area, which consisted of three treatments which were repeatedly amended with a KOH solution (alkaline soil), an H₂SO₄ solution (acidic soil), or water (natural pH soil) over 10 months. At the site, we determined field N₂O and N₂ emissions using the ¹⁵N gas flux method and collected soil samples for the measurement of potential denitrification activity and quantification of the size of the denitrifying community by quantitative PCR of the *narG*, *napA*, *nirS*, *nirK*, and *nosZ* denitrification genes. Overall, our results indicate that soil pH is of importance in determining the nature of denitrification end products. Thus, we found that the N₂O/(N₂O + N₂) ratio increased with decreasing pH due to changes in the total denitrification activity, while no changes in N₂O production were observed. Denitrification activity and N₂O emissions measured under laboratory conditions were correlated with N fluxes *in situ* and therefore reflected treatment differences in the field. The size of the denitrifying community was uncoupled from *in situ* N fluxes, but potential denitrification was correlated with the count of NirS denitrifiers. Significant relationships were observed between *nirS*, *napA*, and *narG* gene copy numbers and the N₂O/(N₂O + N₂) ratio, which are difficult to explain. However, this highlights the need for further studies combining analysis of denitrifier ecology and quantification of denitrification end products for a comprehensive understanding of the regulation of N fluxes by denitrification.

Denitrification is the microbial reduction of NO₃[−] via NO₂[−] to gaseous NO, N₂O, and N₂, which are then lost into the atmosphere (36). It therefore results in considerable loss of nitrogen, one of the most limiting nutrients for crop production in agriculture (20). Denitrification is also of environmental concern since, together with nitrification, it is the main biological process responsible for N₂O emissions (7). N₂O is a potent greenhouse gas which has a global warming potential about 320 times greater than that of CO₂ and has a lifetime of approximately 120 years (32). In the stratosphere, N₂O can also react with O₂ to produce NO, which induces the destruction of stratospheric ozone (8). N₂O can be released into the atmosphere by incomplete denitrification due to the effect of environmental conditions on the regulation of the different denitrification reductases (14, 41, 51), but it has recently been suggested that it could also be due to lack of nitrous oxide reductase in some denitrifiers (19, 41). Since N₂O is an intermediate in the denitrification pathway, both the amount of N₂O produced and the N₂O/(N₂O + N₂) ratio are important in understanding and predicting N₂O fluxes from soils.

The main environmental factors known to influence the N₂O/(N₂O + N₂) ratio are pH, organic carbon and NO₃[−]

availability, water content, and O₂ partial pressure (50). Soil pH is one of the most important factors influencing both denitrification and N₂O production (43). In general, the denitrification rate increases with increasing pH values (up to the optimum pH) while, in contrast, the N₂O/(N₂O + N₂) ratio decreases (50). This relationship has been characterized in laboratory experiments (9, 45), but it is not clear whether the same relationships exist in the field because of methodological limitations of *in situ* measurement of N₂ emissions (16). Nevertheless, ¹⁵N tracing experiments based on the addition of a labeled denitrification substrate to soil offer a useful tool to quantify emissions of both N₂O and N₂ *in situ* (47, 49). Soil pH is also an important factor influencing denitrifier community composition (35, 39), which can be an important driver of denitrification activity and N₂O emissions (5, 21). A recent study reported a negative relationship between the proportion of bacteria genetically capable of reducing N₂O within the total bacterial community and the N₂O/(N₂O + N₂) ratio, with both being strongly correlated with soil pH (38).

The objective of the present study was to explore the effect of changes in soil pH on *in situ* N₂O and N₂ emissions, denitrifying enzyme activity (DEA), and potential N₂O production. In addition, we also investigated whether differences in N fluxes could be related to changes in the size of the microbial community possessing the different denitrification genes. A field experiment was conducted using replicated grassland plots in which the soil pH was modified by addition of either acid or hydroxide to the soil. A ¹⁵N tracer method was used to provide information on N emissions. In addition to measuring

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potential denitrification activity, the size of the denitrifier community was determined by real-time PCR quantification of the denitrification genes.

MATERIALS AND METHODS

Experimental field. The field experiment with manipulation of the soil pH was established in a grassland area at Borová Farm near Český Krumlov in South Bohemia, Czech Republic (48°52'N, 14°13'E; altitude, 630 m above sea level). The pasture site had been irregularly and occasionally grazed by cattle over the past 10 years. The soil at the site is classified as Haplic Phaeozem (arenic; World Reference Base system) containing 60 to 80% sand, 14 to 32% silt, and 6 to 14% clay (USDA classification system). The mean annual precipitation in the area is 650 mm, and the annual average temperature is 7°C. After the establishment of the experimental site in July 2007, it was fenced to prevent further access by cattle and the sward was periodically cut.

The experimental field (12 by 18 m) was divided into 24 plots (each 3 by 3 m), 12 of which were used in this study for manipulation of the soil pH as follows: (i) four random plots were amended with a KOH solution to increase the pH, (ii) four random plots were amended with an H₂SO₄ solution to decrease the pH, and (iii) four random plots were amended with water (control plots with a natural pH). The KOH and H₂SO₄ solutions were applied three times (11 July 2007, 13 September 2007, and 2 April 2008) before the experiment commenced in May 2008. The KOH (15 liters of a 0.97 M solution for each of the three subsequent amendments) and H₂SO₄ (15 liters of a 0.73 M solution for each of the three subsequent amendments) solutions were applied uniformly to the whole surface (3 by 3 m) of each plot using a sprinkling can, and the same volume of water was applied to the control plots. For the following assessment of N emissions and soil sampling, only the inner 2.5-by-2.5-m plots were used to ensure a 1-m protective zone between the treatments. The plant cover was a mixture of perennial grasses (mainly *Lolium perenne* and *Phleum pratense*), clover (*Trifolium hybridum*), and other dicotyledonous plants (e.g., *Achillea millefolium*, *Plantago* sp., *Rumex* sp., and *Vicia* sp.). The application of H₂SO₄ and KOH solutions led to an approximately 40% reduction in the vegetation cover (based on the covered soil surface). Moreover, clover was suppressed in both cases and *Echinochloa crus-galli* with *Elytrigia repens* appeared on the acidic plots while *Chenopodium album* with *Atriplex patula* appeared on the alkaline plots.

In situ gas emission measurements. Gas fluxes were determined using medium-size (basal area, 0.096 m²; volume, 10 dm³), nonvented static chambers consisting of a permanent base (bottom part) and top (lid with a rubber septum for gas sampling) (23). Three days before the measurements were started, the permanent bases were inserted 5 cm into the soil and the vegetation inside the bases was clipped to ca. 5 cm above the soil surface. There were 3 chambers in each plot and a total of 36 chambers (12 chambers for each pH treatment). One day before adding the ¹⁵N-labeled tracer, we measured the natural rates of N₂O and CO₂ emission in each chamber. For this purpose, headspace gas samples were collected using a hypodermic needle and a polypropylene syringe immediately (9:00 a.m.) and 2 h after closing the chambers (11:00 a.m.). Gas samples for N₂O analyses were collected using a 15-ml syringe and stored in pre-evacuated (<100 Pa), septum-capped, 12-ml vials (Labco), while gas samples for CO₂ analyses were collected using a 5-ml syringe and stored in pre-evacuated (<100 Pa), septum-capped, 3.5-ml vials (Venoject). N₂O and CO₂ were quantified using 6890N and 6850N gas chromatographs (Agilent Technologies) equipped with μ ECD and TCD detectors, respectively. Integration of measured peaks and N₂O and CO₂ quantification were conducted using GC ChemStation software (Rev. B.02.01.; Agilent Technologies).

The ¹⁵N gas flux method was used to measure the N₂O and N₂ emissions, and nitrogen was added as ¹⁵N-labeled KNO₃ at a 60 atom% excess at a rate of 10 kg N ha⁻¹. The required amount of N was dissolved in 250 ml H₂O and dispensed uniformly over the soil surface inside the chamber using a small sprinkling can at 9:00 a.m. of the day after the measurement of natural N₂O and CO₂ emissions. Fluxes of N gases were determined five times following KNO₃ application (2 to 4, 6 to 8, 24 to 26, 48 to 50, and 72 to 74 h). At the time the lid of the chamber was fitted to the base section and after 2 h, headspace gas samples were collected using a 12-ml syringe and needle. The linearity of gas accumulation in the chambers was successfully tested in a preliminary experiment (intensive sample routine every 15 min over 2 h). The gas samples were stored in evacuated (<100 Pa), septum-capped, 12-ml vials (Labco), and the concentration and ¹⁵N content of N₂O and the ¹⁵N content of the N₂ in each 12-ml vial were determined by automated isotope ratio mass spectroscopy as described by Stevens et al. (48), using a Europa Scientific 20-20 stable-isotope analyzer interfaced with a Europa Scientific ANCA-TG trace gas preparation system (Europa

Scientific, Crewe, United Kingdom) with a Gilson autosampler (Anachem, Luton, United Kingdom). The ion currents (I) at *m/z* 44, 45, and 46 enabled concentrations and molecular ratios ⁴⁵R (⁴⁵I/⁴⁴I) and ⁴⁶R (⁴⁶I/⁴⁴I) to be calculated for N₂O. The sources of N₂O were then apportioned into the fraction (*d_D*) derived from the denitrifying pool of enrichment *a_D* and the fraction *d_N* = (1 - *d_D*) derived from the nitrifying pool or pools at natural abundance (2). For N₂, the ion currents at *m/z* 28, 29, and 30 enabled the ²⁹R (²⁹I/²⁸I) and ³⁰R (³⁰I/²⁸I) molecular ratios to be determined. Differences between the molecular ratios in enriched and normal atmospheres were calculated as $\Delta^{29}\text{R}$ and $\Delta^{30}\text{R}$. The flux of N₂ was calculated by using data for $\Delta^{29}\text{R}$ and $\Delta^{30}\text{R}$ to calculate the enrichment of the denitrifying pool (¹⁵X_N) and then the N₂ flux according to Mulvaney and Boast (31). The total N fluxes during the 74-h period after the application of ¹⁵N-labeled KNO₃ were calculated by integration, assuming a linear change in flux rates between observation times.

Soil sampling and analyses. On the day of labeled KNO₃ addition, topsoils (0 to 20 cm) were sampled next to all 36 chambers. Three soil samples were taken from a ca. 25-cm distance outside each chamber and combined to produce one composite sample associated with each chamber. Soils were sieved (5 mm) immediately after sampling and stored at field moisture content in plastic bags at 4°C until required (denitrifying activity plus mineral N concentrations and other soil characteristics were determined within 3 days and 2 weeks after the soil sampling, respectively). Subsamples for molecular analyses were stored in Eppendorf tubes at -80°C. Soil pH was measured using a combined electrode (SenTix 61; WTW GmbH, Weilheim, Germany) and pH meter (526/538 pH meter; WTW) in a 10-g/50-ml soil-to-H₂O mixture. Before the pH measurements, the soil slurries were shaken on an end-to-end shaker for 5 min and allowed to stand for 2 h. Soil moisture was determined gravimetrically by drying the soil at 105°C for 24 h, and all results were expressed per gram of dry soil. Soil mineral N (NH₄⁺, NO₃⁻) was measured colorimetrically in 1 M KCl extracts using a soil (fresh field moisture)/KCl solution ratio of 40 g/200 ml (55). Total organic carbon (C_{org}) was determined by wet oxidation with acid dichromate, and total nitrogen content (N_{tot}) was determined by Kjeldahl digestion (55). Microbial biomass C (C_{mic}) was determined by the chloroform fumigation extraction method as described by Vance et al. (53), followed by dichromate digestion of extractable C.

DEA measurement. DEA was determined by the phase I assay of Smith and Tiedje (46), which was slightly modified as described by Šimek and Hopkins (44). Briefly, soil slurries were made by mixing 25-g field-moist soil samples in 120-ml serum bottles with 25 ml of a solution containing 1 mM glucose and 1 mM KNO₃. Bottles were capped with rubber stoppers and metal holders, evacuated, and flushed four times with 99.99% He. The slurries were then incubated either with or without acetylene (10%, vol/vol) on an end-to-end shaker at 25°C for measurements of DEA and potential N₂O emission, respectively. After 30 and 60 min, 0.5-ml headspace samples were taken with a gas-tight syringe and N₂O was immediately quantified using gas chromatography (see above).

DNA extraction and quantification of the denitrifier community. For each of the 36 samples, DNA was extracted from 250 mg of soil by the method of Martin-Laurent et al. (27). Briefly, samples were homogenized in 1 ml of extraction buffer for 30 s at 1,600 rpm in a Mini-Beadbeater cell disrupter (Mikro-Dismembrator S; B. Braun Biotech International). Soil and cell debris were eliminated by centrifugation (14,000 × *g* for 5 min at 4°C). After precipitation with ice-cold isopropanol, nucleic acids were purified using both polyvinylpyrrolidone and Sepharose 4B spin columns. Soil DNA quality and size were checked by electrophoresis on 1% agarose. DNA was also quantified by spectrophotometry at 260 nm using a BioPhotometer (Eppendorf, Hamburg, Germany).

The size of the denitrifier community was estimated by quantitative PCR (qPCR) of genes encoding the catalytic subunit of the key enzymes of the denitrification pathway. Fragments of the *narG*, *napA*, *nirK*, *nirS*, and *nosZ* genes encoding the membrane and periplasmic nitrate reductases, the *cd1* and copper nitrite reductases, and the nitrous oxide reductase, respectively, were amplified using primers and thermal cycling conditions described previously (4, 18, 19). The total bacterial community was quantified using 16S rRNA as a molecular marker (25). Reactions were carried out in an ABI Prism 7900 sequence detection system (Applied Biosystems). Quantification was based on the fluorescence intensity of the SYBR green dye, which binds to double-stranded DNA. The 20- μ l PCR mixture contained 12.5 μ l of SYBR green PCR Master Mix (Absolute QPCR SYBR green Rox ABgen), 1 μ M of each primer, 100 ng of T4 gp32 (Qbiogene), and 12.5 ng of DNA. Standard curves were obtained using serial dilutions of linearized plasmids containing cloned *narG*, *napA*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes amplified from denitrifying strains. No-template controls gave null or negligible values. The presence of PCR inhibitors in DNA extracted from soil was estimated by (i) diluting soil DNA extract and (ii) mixing a known

TABLE 1. Selected properties of soils from experimental plots differing in pH management

Parameter	Value for indicated type of soil ^a		
	Acidic pH	Natural pH	Alkaline pH
pH	5.52 ± 0.48 (a) ^a	6.82 ± 0.40 (b)	7.67 ± 0.17 (c)
Moisture (g H ₂ O g ⁻¹)	0.23 ± 0.02 (a)	0.24 ± 0.04 (ab)	0.26 ± 0.04 (b)
NH ₄ ⁺ (μg N g ⁻¹)	2.74 ± 2.65 (a)	0.56 ± 0.27 (b)	0.68 ± 0.31 (b)
NO ₃ ⁻ (μg N g ⁻¹)	1.88 ± 0.82 (a)	3.77 ± 1.41 (b)	5.03 ± 2.03 (c)
N _{tot} (mg N g ⁻¹)	3.31 ± 0.71 (a)	2.90 ± 1.26 (ab)	2.51 ± 1.21 (b)
C _{org} (mg C g ⁻¹)	20.9 ± 6.5 (a)	20.5 ± 4.4 (a)	17.5 ± 8.5 (b)
C _{mic} (μg C g ⁻¹)	587.1 ± 170.6 (a)	951.8 ± 354.8 (b)	1,249.7 ± 331.0 (c)
CO ₂ emission ^b (μg C m ⁻² h ⁻¹)	118.3 ± 35.1 (a)	182.4 ± 23.6 (b)	122.1 ± 19.6 (a)
N ₂ O emission ^b (μg N m ⁻² h ⁻¹)	12.3 ± 3.8 (a)	31.5 ± 17.4 (b)	36.1 ± 26.5 (b)

^a Mean values ± standard deviations ($n = 12$) are shown. The different letters in parentheses indicate significant differences between the specific pH treatments ($P < 0.05$).

^b Natural emission determined before KNO₃ addition.

amount of standard DNA with soil DNA extract prior to qPCR. No inhibition was detected.

Statistical analyses. All data obtained were first analyzed using descriptive statistical methods, and the Kolmogorov-Smirnov test was used to check the normality of the data. Differences in means of characteristics among the three pH treatments were tested by hierarchical analysis of variance, where the factor “plot” was nested in the factor “pH treatment,” followed by a *post hoc* test of comparison (Tukey honestly significant difference [HSD] test). To test possible relationships among N emissions, DEA, abundance of denitrification genes, and soil parameters, Pearson's correlation coefficients (r) were calculated for data from all 36 soil samples and chambers. All statistical tests were performed in the program package STATISTICA (v7.1., StatSoft, Inc., 2005). Significance was accepted at a probability (P) level of <0.05 .

RESULTS

Soil pH and other chemical and biological parameters. Table 1 shows means and standard deviations of selected soil properties and microbial activities in soils from the experimental plots differing in pH management. Manipulation of soil pH via the application of acid or alkali solutions during the 10 months before the gas flux measurements resulted in a significant change in the soil reaction. The pH values were statistically significantly different (Tukey HSD test, $P < 0.05$) with a difference of 0.85 to 2.15 pH units between the treatments. Several soil properties other than pH were also affected by the addition of acid or alkali solutions. For example, the NH₄⁺ concentration was higher in the acidic soil than in the natural pH and alkaline soils, while the NO₃⁻ concentration was higher in the soils with alkaline pH and decreased significantly with decreasing pH (Tukey HSD test, $P < 0.05$). The microbial biomass determined by the fumigation extraction method (C_{mic}) was lowest in the acidic soils and highest in the alkaline soils (Tukey HSD test, $P < 0.05$). Emissions of CO₂ and N₂O determined before the application of ¹⁵N-labeled KNO₃ were also affected by the treatments. Thus, significantly higher CO₂ flux occurred in the control plots with natural pH soil (182 μg C m⁻² h⁻¹) than in the other plots (118 and 122 μg C m⁻² h⁻¹ in the acidic and alkaline soils, respectively). In contrast, N₂O emissions were similar from the natural pH and alkaline soils (32 to 36 μg N m⁻² h⁻¹) but significantly lower from the acidic soils (12 μg N m⁻² h⁻¹).

In situ N₂O and N₂ emissions after the application of ¹⁵N-labeled NO₃⁻. Figure 1 shows the time course of the total N fluxes (N₂O + N₂) and of the N₂O fluxes from the soils with different pH treatment during the 74 h after the ¹⁵N-labeled KNO₃ amendment. Higher N gas emissions were observed in

FIG. 1. Dynamics of total N (N₂O + N₂) and N₂O *in situ* fluxes after the addition of ¹⁵N-labeled NO₃⁻ to acidic, natural pH, and alkaline soils. Mean values ± standard errors of the means are shown ($n = 12$).

TABLE 2. Causes of N₂O flux in acidic, natural pH, and alkaline soils^a

Parameter	Soil	Mean value $\pm$ SD at following time after KNO ₃ addition:				
		4 h	8 h	26 h	50 h	74 h
d_D (fraction)	Acidic	ND ^b	0.48 $\pm$ 0.22	0.73 $\pm$ 0.22	ND	ND
	Natural pH	0.75 $\pm$ 0.15	0.83 $\pm$ 0.12	0.61 $\pm$ 0.24	ND	ND
	Alkaline	0.83 $\pm$ 0.14	0.87 $\pm$ 0.13	0.63 $\pm$ 0.26	ND	ND
$d_N = (1 - d_D)$ (fraction)	Acidic	ND	0.52 $\pm$ 0.22	0.27 $\pm$ 0.22	ND	ND
	Natural pH	0.25 $\pm$ 0.15	0.17 $\pm$ 0.12	0.39 $\pm$ 0.24	ND	ND
	Alkaline	0.17 $\pm$ 0.14	0.13 $\pm$ 0.13	0.37 $\pm$ 0.26	ND	ND
a_D (enrichment; atom%)	Acidic	ND	0.56 $\pm$ 0.02	0.56 $\pm$ 0.03	ND	ND
	Natural pH	0.54 $\pm$ 0.03	0.55 $\pm$ 0.03	0.50 $\pm$ 0.06	ND	ND
	Alkaline	0.54 $\pm$ 0.05	0.55 $\pm$ 0.04	0.51 $\pm$ 0.07	ND	ND

^a The fractions of the N₂O flux which were derived from the NO₃⁻ (denitrification) pool (d_D), from the NH₄⁺ (nitrification) pool (d_N), and the enrichment (a_D) of the NO₃⁻ (denitrifying) pool (atom%) in acidic, natural pH, and alkaline soils are shown ($n = 12$).

^b ND, not determined due to low N₂O fluxes.

the alkaline plots than in the control (natural pH) and acidic plots. Different total N fluxes were observed among the three treatments during the first 24 h after the application of KNO₃. However, 50 to 74 h after the KNO₃ amendment, the total N fluxes were around 1 mg N m⁻² h⁻¹ in all treatments. In the control and alkaline plots, the N₂O emissions reached a maximum of ca. 1.1 mg N m⁻² h⁻¹ within 8 h after KNO₃ application and then decreased to ca. 0.4 mg N m⁻² h⁻¹. In the acidic soil, the N₂O emissions increased up to 26 h before decreasing. In all three pH treatments, N₂ was the only gas product at the end of the experimental period.

Table 2 shows the fraction of the N₂O flux which was derived from the NO₃⁻ (denitrification) pool (d_D) together with the enrichment (a_D) of this pool and also the fraction from the NH₄⁺ (nitrification) pool (d_N). Valid calculations could only be done, however, whenever there was a significant N₂O flux. Therefore, the values were not determined at 50 and 74 h after the KNO₃ addition for all three soils and 4 h after the KNO₃ addition for the acidic soil. In general, denitrification was the major process responsible for N₂O emissions in all three soils, accounting for ca. 61 to 87%. The exception was the acidic soil 8 h after KNO₃ addition, where the N₂O emissions were split equally between nitrification and denitrification. The enrichment of the denitrify-

ing pool (a_D ; on average, 54 atom%) was close to the enrichment of the added KNO₃ (60 atom%).

Cumulative fluxes of N₂O + N₂ over the experimental period (74 h) were 50.7, 91.2, and 177.1 mg N m⁻² from the acidic, natural pH, and alkaline soils, respectively (Fig. 2A). The N₂ emissions were mostly responsible for the differences in the total N fluxes between the pH treatments, as the N₂O emissions were not significantly different (Tukey HSD test, $P > 0.24$). The large differences in N₂ emissions between acidic and control plots were not significant (although marginally: Tukey HSD test, $P = 0.055$), probably due to the large variation in fluxes between individual chambers (Fig. 2A). Figure 2B shows the relative contribution of N₂O to the total cumulative N₂O + N₂ fluxes expressed as the N₂O/(N₂O + N₂) molar ratio. The N₂O/(N₂O + N₂) ratio increased with decreasing soil pH. The total losses of added NO₃-N (10 kg N ha⁻¹) as N₂O and N₂ (emission factors) were 5.1, 9.1, and 17.7% during the experimental period for the acidic, natural pH, and alkaline soils, respectively. The emission factors for N₂O only were 1.8, 2.1, and 2.8% for the acidic, natural pH, and alkaline soils, respectively.

Correlation analysis, which included data from all 36 experi-

FIG. 2. *In situ* cumulative losses of N (separately as N₂O and N₂) (A) and relative N₂O production expressed as the N₂O/(N₂O + N₂) molar ratio (B) over the 74 h after the addition of ¹⁵N-labeled KNO₃ to acidic, natural pH, and alkaline soils. Mean values and $\pm$ standard deviations are shown ($n = 12$). The different letters next to the bars indicate significant differences between the specific pH treatments ($P < 0.05$).

TABLE 3. Pearson's correlation coefficients for relationships between soil parameters (including abundance of denitrification genes and their ratios) and N gas fluxes estimated *in situ* using ^{15}N and under laboratory conditions using DEA with the respective $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ molar ratio

Parameter	P value			
	<i>In situ</i>		In laboratory	
	$\text{N}_2\text{O} + \text{N}_2$	$\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio	$\text{N}_2\text{O} + \text{N}_2$	$\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio
pH	0.540 ^a	-0.490 ^b	0.637 ^a	-0.848 ^a
Moisture	0.195	-0.209	0.835 ^a	-0.349 ^c
NO_3^-	0.353 ^c	-0.458 ^b	0.825 ^a	-0.578 ^a
NH_4^+	-0.266	0.153	-0.299	0.567 ^a
C_{mic}	0.465 ^b	-0.269	0.940 ^a	-0.599 ^a
N_{tot}	-0.525 ^b	0.215	-0.293	0.309
C_{org}	-0.500 ^b	0.066	-0.119	0.143
16S rRNA gene copy no.	-0.040	-0.006	-0.096	-0.268
<i>narG</i> gene copy no.	-0.156	-0.339 ^c	0.243	-0.452 ^b
<i>napA</i> gene copy no.	0.122	-0.130	0.175	-0.470 ^b
<i>nirS</i> gene copy no.	-0.012	-0.415 ^c	0.441 ^b	-0.602 ^a
<i>nirK</i> gene copy no.	0.080	-0.005	-0.121	-0.186
<i>nosZ</i> gene copy no.	-0.062	-0.095	0.191	-0.261
<i>narG</i> /16S rRNA ratio	-0.139	-0.481 ^b	0.544 ^a	-0.386 ^c
<i>napA</i> /16S rRNA ratio	0.387 ^c	-0.304	0.657 ^a	-0.686 ^a
<i>nirS</i> /16S rRNA ratio	0.063	-0.493 ^b	0.666 ^a	-0.612 ^a
<i>nirK</i> /16S rRNA ratio	0.101	-0.022	-0.120	-0.043
<i>nosZ</i> /16S rRNA ratio	-0.062	-0.167	0.409 ^c	0.015
<i>nosZ</i> / <i>nirS</i> ratio	-0.275	0.433 ^b	-0.491 ^b	0.706 ^a
<i>nosZ</i> / <i>nirK</i> ratio	-0.066	-0.124	0.349	0.005
<i>nosZ</i> /(<i>nirS</i> + <i>nirK</i>) ratio	-0.239	0.381 ^c	-0.359 ^c	0.662 ^a

^a $P < 0.001$ ($n = 36$).

^b $P < 0.01$.

^c $P < 0.05$.

mental points (both soil samples and chambers for emission measurements), showed significant negative correlations between the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio and soil pH ($r = -0.493$, $P = 0.002$) and NO_3^- concentration ($r = -0.458$, $P = 0.005$) (Table 3). We also found significant correlations between total N fluxes ($\text{N}_2\text{O} + \text{N}_2$) and pH ($r = 0.540$, $P < 0.001$) but also N_{tot} , C_{org} , C_{mic} , and NO_3^- concentration (Table 3). There were no other statistically significant correlations with soil properties (Table 3).

DEA. DEA, expressed as the overall production of both denitrification products, N_2O and N_2 , was significantly affected by the pH treatment (189, 692, and 1,169 $\text{ng N g}^{-1} \text{h}^{-1}$ for the acidic,

natural pH, and alkaline soils, respectively; Tukey HSD test, $P < 0.01$; Fig. 3A). Similar to cumulative N emissions *in situ*, the N_2 production was responsible for the differences in DEA between the pH treatments, since N_2O production was not affected by pH (Tukey HSD test, $P > 0.1$). Correlation analysis showed a positive relationship between DEA and pH (Table 3). However, the best predictors of DEA were C_{mic} ($r = 0.940$, $P < 0.001$), soil moisture ($r = 0.835$, $P < 0.001$), and NO_3^- concentration ($r = 0.825$, $P < 0.001$). The proportion of N_2O produced as the terminal product of denitrification and calculated as the N_2O molar ratio ($\text{N}_2\text{O}/[\text{N}_2\text{O} + \text{N}_2]$) was also highly dependent on the pH treatment,

FIG. 3. DEA (separately as N_2O and N_2 production) of soils with different pH treatments (A) and relative N_2O production during DEA determination expressed as the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ molar ratio (B). DEA (A) was also estimated and is expressed as $\text{g N m}^{-2} 74 \text{ h}^{-1}$ (right vertical axis). Mean values $\pm$ standard deviations are shown ($n = 12$). The different letters next to the bars indicate significant differences between the specific pH treatments ($P < 0.05$).

being 79.2% in acidic soil, 38.5% in natural pH soil, and 10.5% in alkaline soil (Fig. 3B). In contrast to the overall N_2O and N_2 production (DEA), soil pH was the best predictor of the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ molar ratio ($r = -0.848$, $P < 0.001$) (Table 3). In addition, correlation analysis showed weak but significant relationships between *in situ* total N fluxes and DEA ($r = 0.393$, $P = 0.018$) and between the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ molar ratio from the field measurements and that from the DEA assay ($r = 0.379$, $P = 0.023$) (data not shown).

Abundance of 16S rRNA and denitrification genes. The highest denitrification gene copy numbers were observed in the natural pH plots, with significantly lower gene copy numbers in the acidic soil (Fig. 4A). A decrease compared to the natural pH plots was also observed in the alkaline plots, which was significant only for the *napA* and *nosZ* genes. The abundance of total bacteria estimated using 16S rRNA gene copy numbers was also affected by the H_2SO_4 and KOH amendments (4.4×10^5 , 6.4×10^5 , and 5.1×10^5 gene copies ng^{-1} DNA for the acidic, natural pH, and alkaline soils, respectively), similar to the denitrification genes. To better understand how the denitrifier community was influenced by soil pH, we also estimated the relative abundance of denitrifiers potentially capable of performing different steps in the denitrification cascade by calculating the ratios of different denitrification genes to the 16S rRNA gene (e.g., the *narG*/16S rRNA gene ratio) (Fig. 4B). The *nirS*/16S rRNA gene ratio was the only one which differed significantly among the three pH treatments (Tukey HSD test, $P < 0.05$), with the lowest and highest ratios in the acidic and alkaline soils, respectively. Calculation of the *nosZ*/*nirK* and *nosZ*/*nirS* gene ratios revealed that only the latter was affected by pH, with a significantly higher ratio in the acidic soils (Fig. 4C).

We also performed an analysis of the correlation between N fluxes (*in situ* N emissions, DEA, and $\text{N}_2\text{O}/[\text{N}_2\text{O} + \text{N}_2]$ ratio) and the size of the denitrifying community (expressed as absolute values of denitrification gene abundance, as the ratios of different denitrification genes to the 16S rRNA gene, and as ratios of the *nosZ* gene to genes encoding nitrite reductase) to identify possible linkages between them (Table 3). We found no correlation between *in situ* total N fluxes and the abundance of the denitrification genes, and only the abundance of the *nirS* gene was correlated with DEA. The abundance of this gene also correlated with the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ molar ratio from the field measurements and from the DEA assay. Significant correlations between the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio and the abundances of the genes *narG* and *napA* (*napA* was correlated only with the relative N_2O production determined during the DEA assay) and the *nirS*/16S rRNA gene, *napA*/16S rRNA gene, *nosZ*/*nirS*, and *nosZ*/*(nirS + nirK)* ratios were also observed (Table 3).

DISCUSSION

Manipulation of soil pH *in situ* is difficult to achieve because of the high buffering capacity of most soils. However, since soils having different natural pHs also differ in many other properties, the effect of soil pH is often investigated by modifying the pH gradually (1, 33), typically by the application of lime. This method of pH manipulation can take years, with repeated lime applications being required. In the present study, we significantly modified the soil pH by short-term application (three times in 10 months) of H_2SO_4 and KOH so-

FIG. 4. Abundances of the 16S rRNA, *narG*, *napA*, *nirS*, *nirK*, and *nosZ* genes in soils with different pH treatment expressed as the number of gene copies ng^{-1} of DNA (A), the specific denitrification gene/16S rRNA gene ratio (B), and *nosZ*/*nirS*, *nosZ*/*nirK*, and *nosZ*/*(nirS + nirK)* ratios (C). Mean values and standard deviations are shown ($n = 12$). The different letters above the bars indicate significant differences between the specific pH treatments ($P < 0.05$).

lutions. Such additions of KOH or H_2SO_4 solutions to the soil also represent a source of exchangeable potassium cations and sulfate anions, respectively, which might also have direct or indirect side effects on the microbial community. The shift in

soil pH was accompanied by changes in soil functioning, leading to modifications of other soil properties (Table 1). Thus, the relatively high NH_4^+ concentration and low NO_3^- concentration in the acidic soil (H_2SO_4) suggest a decrease in nitrifying activity, as previously reported (12, 34). In contrast, the opposite was found in the alkaline soil, suggesting a higher nitrification rate, which could have stimulated denitrification. The application of the acid and hydroxide to the soil also affected the vegetation cover, which probably caused the observed differences in the CO_2 emissions and moisture content between the pH treatments (Table 1). Therefore, the correlations between pH and N fluxes or denitrifying community size observed in our work might not represent a direct causal relationship, as confounding effects cannot be ruled out.

Analysis of the denitrification process in the soils with different pH treatments revealed a significant decrease in N gas production with soil pH for both cumulative N fluxes *in situ* and DEA (Fig. 2A and 3A), which is in agreement with previous findings of lower denitrification rates in acidic soil (13, 45). Denitrification was the major process responsible for N_2O emissions in all three soils (Table 2); the fraction of the N_2O flux derived from the denitrifying NO_3^- pool (d_p) was 0.72 (72%) averaged over all treatments at 4, 8, and 26 h after the application of ^{15}N -labeled NO_3^- . In contrast to the total N fluxes in the field and DEA, we did not find any differences in N_2O emissions between the pH treatments for either the cumulative *in situ* N_2O emissions (Fig. 2A) or potential N_2O emissions (Fig. 3A). This result was surprising since higher N_2O emissions in acidic soils have been reported in several studies (30, 54). For example, Weslien et al. (54) found a strong negative correlation between N_2O emissions *in situ* and soil pH ($r = 0.93$, $P < 0.01$) from a Swedish organic soil with a pH range of 3.6 to 5.9. One of the possible reasons for the lack of difference in N_2O fluxes between the pH treatments in our study could be the relatively moderate change in pH obtained in the field, which might have been too limited to lead to changes in N_2O emissions estimated by the ^{15}N tracer experiment or the potential assay. We also found that the percentage of the applied nitrate (10 kg N ha^{-1}) emitted as N_2O *in situ* was between 1.8 and 2.8%, which is higher than the estimate of 1.25% according to the Intergovernmental Panel on Climate Change (IPCC) (22) but in agreement with recent studies reporting emission rates of up to 2.5% and suggesting that the IPCC methodology underestimates N_2O emissions (15). Interestingly, the potential N_2O production rates estimated per g N $\text{m}^{-2} 74 \text{ h}^{-1}$ (Fig. 3A) were ca. 3,500, 1,900, and 1,100 times higher than the *in situ* natural N_2O emissions from the acidic, natural pH, and alkaline soils, respectively (Table 1), and ca. 180, 210, and 40 times higher than the cumulative losses of N_2O over 74 h after the amendment with ^{15}N -labeled NO_3^- (Fig. 2A). The DEA assay optimizes the N production rate under laboratory conditions from the pool of denitrification enzymes present in the soil at the time of sampling and consists of incubating the soil at a temperature of 25°C without oxygen and with the addition of NO_3^- and a surplus of electron donor (glucose). Incubation of the soil under these non-limiting denitrifying conditions resulted in the stimulation of the rates observed in our study.

Calculation of the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio showed a decreasing molar ratio with increasing soil pH and denitrification

rates, in agreement with previous studies (43). However, the range of the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio was different according to the approach used. Thus, it varied between 15.1 and 28.5% and between 10.5 and 79.2% when calculated using the *in situ* measurement and potential denitrification, respectively (Fig. 2B and 3B). Since NO_3^- is preferred over N_2O as an electron acceptor, the high nitrate concentration in the DEA assay may explain the high $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio. Nevertheless, notwithstanding the differences between the two approaches, the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio in the field was significantly correlated with the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio calculated from the DEA assay under laboratory conditions ($r = 0.379$, $P = 0.023$). Moreover, the total N fluxes *in situ* were significantly correlated with potential denitrification (DEA). This is an important finding indicating that although the magnitudes of the fluxes were different, determination of the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio under laboratory conditions can reflect treatment differences in the field. We found soil pH to be the best predictor of the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio determined by both DEA and *in situ* N flux measurements (Table 3). Similarly, simultaneous determination of N_2O and N_2 emissions from intact soil cores taken from a beech forest ecosystem with differences in pH values showed that pH was the only significant determinant of the $\text{N}_2/\text{N}_2\text{O}$ ratio (11). In our study, soil pH was also the best predictor of total N fluxes *in situ* but not of DEA, which was correlated more with C_{mic} , NO_3^- , and soil moisture. Overall, our results confirm the role of soil pH *in situ* in determining the nature of the denitrification end products and process rates. It is, however, important to underline the fact that in our experimental field study many variables, such as pH and NO_3^- , correlated with each other.

Most recent studies investigating linkages between denitrification activity and denitrifying community ecology have focused on denitrifier diversity and process rates without considering the nature of the denitrification end products (13, 17, 26, 40). However, recent studies have shown that some denitrifiers can have a truncated denitrification pathway and lack the *nosZ* gene encoding nitrous oxide reductase (24, 37), which results in the emission of N_2O as the end product, whatever the environmental conditions. In the present study, we therefore quantified the *narG*, *napA*, *nirK*, *nirS*, and *nosZ* denitrification genes to verify whether differences in the denitrification rates and in the $\text{N}_2\text{O}/(\text{N}_2\text{O} + \text{N}_2)$ ratio could be related to changes in the size of the microbial community possessing the different denitrification genes. In our study, the abundance of the *nosZ* gene was less than 6% of the total abundance of the *nirS* and *nirK* genes. A higher abundance of the *nirS* and *nirK* genes than of the *nosZ* gene has already been observed in several studies (3, 19, 38). However, in a recent study by Hallin et al. (17) using the same primers that we used in our study, similar densities of the *nirK* gene or *nirS* and *nosZ* genes were found, which rules out the possibility that the imbalanced densities of the *nirK*, *nirS*, and *nosZ* genes are due to the fact that the *nosZ* primers may not be as universal as those for the *nirK* and *nirS* genes. Together, these results indicate that, in some environments, a significant fraction of the denitrifiers lacks the *nosZ* gene and therefore these denitrifiers are not genetically able to reduce N_2O , as suggested by bacterial genome analysis (24). Higher copy numbers of all of the denitrification genes and the 16S rRNA gene were observed in the natural pH soil (Fig. 4A), in

agreement with the soil respiration data (determined as CO₂ emissions, Table 1), indicating that both denitrifiers and total bacteria were affected by the pH treatment. However, the abundance of the denitrification genes was not correlated with total N fluxes *in situ* and only the abundance of the *nirS* gene was correlated with DEA (Table 3). A positive correlation between *nirS* gene abundance and DEA was also reported by Philippot et al. (38) and Chroňáková et al. (6) at sampling sites in the same grassland area while, similar to our results, the abundance of the *nosZ* gene was uncoupled from the denitrification activity in the studies by Miller et al. (28, 29) and Dandie et al. (10). In contrast, other studies reported significant correlations between the *nirK* or *nosZ* gene copy numbers and potential denitrification rates (17, 52). Even though denitrification is a facultative respiratory process and is therefore primarily regulated by nitrate, carbon, and oxygen availability, it is possible that the size of the denitrifying community contributes to the regulation of the process rates when conditions are favorable for denitrification. Such a correlation between the size and activity of the denitrifying community is even more likely when the measured rates result from the activity of all of the denitrification enzymes present in the sample, such as in the DEA assay. However, the discrepancies between the studies cited above underline the complexity of the hierarchical regulation of biochemical processes (42) and indicate that counting of denitrifiers is not enough for a comprehensive understanding of N fluxes by denitrification. In contrast to total N fluxes and DEA, the N₂O/(N₂O + N₂) ratio estimated both *in situ* and in the laboratory was negatively correlated with the abundance of *nirS* and to a lesser extent with that of *napA* (only for the N₂O/[N₂O + N₂] ratio estimated by DEA) and *narG* (Table 3). Further experiments are required over a broader range of pHs to verify whether there is a relationship between the size of the denitrifying community possessing these genes and the nature of the denitrification end products.

To better understand the relationships among soil pH, denitrification activity, and the denitrifying community, we calculated the proportion of denitrifiers performing the different steps in the denitrification cascade within the total bacterial community (Fig. 4B) and found a positive correlation between the *nirS*/16S rRNA gene ratio and the soil pH. This correlation, which suggests that NirS denitrifiers are more sensitive to soil pH than the rest of the total bacterial community, has not, however, been observed in previous studies and therefore could represent a spurious relationship (17, 38). Nitrite reductases, which reduce nitrite to gaseous NO, are key enzymes of the denitrification pathway, and bacteria must have this reductase to be recognized as denitrifiers (50). Denitrifying bacteria possess either a cytochrome *cd*₁ nitrite reductase (NirS) or a copper nitrite reductase (NirK) (56), but both enzymes perform the same reaction and are interchangeable. The increase in the *nirS*/16S rRNA gene ratio with soil pH but not of the *nirK*/16S rRNA gene ratio supports the findings from recent studies suggesting niche differentiation between denitrifiers possessing the two types of nitrite reductases (17, 38). We found that the ratio of all of the denitrification genes, but not the *nirK*/16S rRNA gene ratio, was correlated significantly with DEA (Table 3), suggesting that the proportion of denitrifiers within the total bacterial community could be more important for denitrification rates than the size of the denitrifier community itself. Probably due to the significant correlation between the *nirS*,

narG, and *napA* gene copy numbers and the N₂O/(N₂O + N₂), the *nirS*/16S rRNA gene, *narG*/16S rRNA gene, *napA*/16S rRNA gene, *nosZ*/*nirS*, and *nosZ*/(*nirS* + *nirK*) ratios were also correlated with the N₂O/(N₂O + N₂) ratio determined in the laboratory. This putative influence of the proportion of denitrifiers on N₂O production might also be interlaced with the influence of the denitrifier community composition (5), which has been shown to be affected by shifts in soil pH (13, 17). In contrast to the results of Philippot et al. (38) suggesting that the proportion of bacteria able to reduce N₂O could be of importance in determining the nature of the denitrification end products, we did not find any negative correlation between the proportion of denitrifiers possessing the *nosZ* gene and the N₂O/(N₂O + N₂) ratio. This indicates that, not surprisingly, the routes for N₂O production are numerous and the relative importance of the denitrifier community composition, denitrification enzyme regulation (41), and the proportion of denitrifiers lacking the gene for an N₂O reductase remains to be experimentally demonstrated.

To conclude, our results indicate that manipulation of soil pH affected the N₂O/(N₂O + N₂) ratio, which increased with decreasing pH due to changes in the total denitrification activity but not in N₂O production. We also showed that denitrification activity and N₂O emissions measured under laboratory conditions were correlated with N fluxes *in situ* and therefore could reflect treatment differences in the field. The size of the denitrifying community was uncoupled from *in situ* N fluxes, but the potential denitrification was significantly correlated with the number of NirS denitrifiers. We also found a relationship between the *narG*, *napA*, and *nirS* gene copy numbers and the N₂O/(N₂O + N₂) molar ratio which requires further understanding. However, in this study, the proportion of denitrifiers capable of reducing N₂O did not seem to have a role in determining the N₂O/(N₂O + N₂) ratio. It is crucial in future studies to continue to bridge the gap between studies of denitrifier ecology and of N fluxes for a comprehensive understanding of the role of denitrifier community ecology in determining not only total denitrification rates but also the nature of the denitrification end products.

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REFERENCES

- Adams, T. M., and S. N. Adams. 1983. The effects of liming and soil pH on carbon and nitrogen contained in the soil biomass. *J. Agric. Sci.* **101**:553–558.
- Arah, J. R. M. 1997. Apportioning nitrous oxide fluxes between nitrification and denitrification using gas-phase mass spectrometry. *Soil Biol. Biochem.* **29**:1295–1299.
- Baudoin, E., L. Philippot, D. Chèneby, L. Chapuis-Lardy, N. Fromin, D. Bru, B. Rabary, and A. Brauman. 2009. Direct seeding mulch-based cropping increases both the activity and abundance of denitrifier communities in a tropical soil. *Soil Biol. Biochem.* **41**:1703–1709.
- Bru, D., A. Sarr, and L. Philippot. 2007. Relative abundance of the membrane-bound and periplasmic nitrate reductase. *Appl. Environ. Microbiol.* **73**:5971–5974.

5. Cavigelli, M. A., and G. P. Robertson. 2000. The functional significance of denitrifier community composition in a terrestrial ecosystem. *Ecology* **81**: 1402–1414.
6. Chroňáková, A., V. Radl, J. Čuhel, M. Šimek, D. Elhottová, M. Engel, and M. Schlöter. 2009. Overwintering management on upland pasture causes shifts in an abundance of denitrifying microbial communities, their activity and N₂O-reducing ability. *Soil Biol. Biochem.* **41**:1132–1138.
7. Conrad, R. 1996. Soil microorganisms as controllers of atmospheric trace gases (H₂, CO, CH₄, OCS, N₂O, and NO). *Microbiol. Rev.* **60**:609–640.
8. Crutzen, P. J., and D. H. Ehhalt. 1977. Effects of nitrogen fertilizers and combustion on the stratospheric ozone layer. *Ambio* **6**:112–117.
9. Čuhel, J. 2004. B.S. thesis. Faculty of Biological Sciences, University of South Bohemia, České Budějovice, Czech Republic.
10. Dandie, C. E., D. L. Burton, B. J. Zebbarth, S. L. Henderson, J. T. Trevors, and C. Goyer. 2008. Changes in bacterial denitrifier community abundance over time in an agricultural field and their relationship with denitrification activity. *Appl. Environ. Microbiol.* **74**:5997–6005.
11. Dannenmann, M., K. Butterbach-Bahl, R. Gasche, G. Willibald, and H. Papen. 2008. Dinitrogen emissions and the N₂:N₂O ratio of a Rendzic Leptosol as influenced by pH and forest thinning. *Soil Biol. Biochem.* **40**: 2317–2323.
12. de Boer, W., and G. A. Kowalchuk. 2001. Nitrification in acid soils: microorganisms and mechanisms. *Soil Biol. Biochem.* **33**:853–866.
13. Enwall, K., L. Philippot, and S. Hallin. 2005. Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. *Appl. Environ. Microbiol.* **71**:8335–8343.
14. Firestone, M. K., R. B. Firestone, and J. M. Tiedje. 1980. Nitrous oxide from soil denitrification: factors controlling its biological production. *Science* **208**: 749–751.
15. Giles, J. 2005. Fallout of fertilizers set too low, studies warn. *Nature* **434**:262.
16. Groffman, P. M., M. A. Altabet, J. K. Bohlke, K. Butterbach-Bahl, M. B. David, M. K. Firestone, A. E. Giblin, T. M. Kana, L. P. Nielsen, and M. A. Voytek. 2006. Methods for measuring denitrification: diverse approaches to a difficult problem. *Ecol. Appl.* **16**:2091–2122.
17. Hallin, S., C. M. Jones, M. Schlöter, and L. Philippot. 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. *ISME J.* **3**:597–605.
18. Henry, S., E. Baudouin, J. C. López-Gutiérrez, F. Martin-Laurent, A. Brauman, and L. Philippot. 2004. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *J. Microbiol. Methods* **59**:327–335. (Erratum, **61**:289–290.)
19. Henry, S., D. Bru, B. Stres, S. Hallet, and L. Philippot. 2006. Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. *Appl. Environ. Microbiol.* **72**:5181–5189.
20. Hofstra, N., and A. F. Bouwman. 2005. Denitrification in agricultural soils: summarizing published data and estimating global annual rates. *Nutrient Cycling Agroecosyst.* **72**:267–278.
21. Holtan-Hartwig, L., P. Dörsch, and L. R. Bakken. 2000. Comparison of denitrifying communities in organic soils: kinetics of NO₃[−] and N₂O reduction. *Soil Biol. Biochem.* **32**:833–843.
22. Houghton, J. T., L. G. Meira Filho, B. A. Callander, N. Harris, N. Kattenberg, and K. Maskell (ed.). 1996. *Climate change 1995: the science of climate change*. Cambridge University Press, Cambridge, United Kingdom.
23. Hynš, J., P. Brůček, and M. Šimek. 2007. Nitrous oxide emissions from cattle-impacted pasture soil amended with nitrate and glucose. *Biol. Fert. Soils* **43**:853–859.
24. Jones, C. M., B. Stres, M. Rosenquist, and S. Hallin. 2008. Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. *Mol. Biol. Evol.* **25**:1955–1966.
25. López-Gutiérrez, J. C., S. Henry, S. Hallet, F. Martin-Laurent, G. Catroux, and L. Philippot. 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. *J. Microbiol. Methods* **57**: 399–407.
26. Ma, W. K., A. Bedard-Haughn, S. D. Siciliano, and R. E. Farrel. 2008. Relationship between nitrifier and denitrifier community composition and abundance in predicting nitrous oxide emissions from ephemeral wetland soils. *Soil Biol. Biochem.* **40**:1114–1123.
27. Martin-Laurent, F., L. Philippot, S. Hallet, R. Chaussod, J. C. Germon, G. Soulas, and G. Catroux. 2001. DNA extraction from soils: old bias for new microbial diversity analysis methods. *Appl. Environ. Microbiol.* **67**:2354–2359.
28. Miller, M. N., B. J. Zebbarth, C. E. Dandie, D. L. Burton, C. Goyer, and J. T. Trevors. 2008. Crop residue influence on denitrification, N₂O emissions and denitrifier community abundance in soil. *Soil Biol. Biochem.* **40**:2553–2562.
29. Miller, M. N., B. J. Zebbarth, C. E. Dandie, D. L. Burton, C. Goyer, and J. T. Trevors. 2009. Influence of liquid manure on soil denitrifier abundance, denitrification, and nitrous oxide emissions. *Soil Sci. Soc. Am. J.* **73**:760–768.
30. Mkhabela, M. S., R. Gordon, D. Burton, A. Madani, and W. Hart. 2006. Effect of lime, dicyandiamide and soil water content on ammonia and nitrous oxide emissions following application of liquid hog manure to a marshland soil. *Plant Soil* **284**:351–361.
31. Mulvaney, R. L., and C. W. Boast. 1986. Equations for determination of nitrogen-15 labeled dinitrogen and nitrous oxide by mass spectrometry. *Soil Sci. Soc. Am. J.* **50**:360–363.
32. Nakicenovic, N., and R. Swart (ed.). 2000. *Special report on emissions scenarios: a special report of Working Group III of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge, United Kingdom.
33. Nicol, G. W., S. Leininger, C. Schleper, and J. I. Prosser. 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. *Environ. Microbiol.* **10**:2966–2978.
34. Nugroho, R. A., W. F. M. Röling, A. M. Laverman, and H. A. Verhoef. 2007. Low nitrification rates in acid Scots pine forest soils are due to pH-related factors. *Microb. Ecol.* **53**:89–97.
35. Parkin, T., A. J. Sextstone, and J. M. Tiedje. 1985. Adaption of denitrifying populations to low soil pH. *Appl. Environ. Microbiol.* **49**:1053–1056.
36. Paul, E. A., and F. E. Clark. 1996. *Soil microbiology and biochemistry*. Academic Press, San Diego, CA.
37. Philippot, L. 2002. Denitrifying genes in bacterial and archaeal genomes. *Biochim. Biophys. Acta* **1577**:355–376.
38. Philippot, L., J. Čuhel, N. P. A. Saby, D. Chêneby, A. Chroňáková, D. Bru, D. Arrouays, F. Martin-Laurent, and M. Šimek. 2009. Mapping field-scale spatial distribution patterns of size and activity of the denitrifier community. *Environ. Microbiol.* **11**:1518–1526.
39. Philippot, L., S. Hallin, and M. Schlöter. 2007. Ecology of denitrifying prokaryotes in agricultural soil. *Adv. Agron.* **96**:249–305.
40. Rich, J. J., and D. D. Myrold. 2004. Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. *Soil Biol. Biochem.* **36**:1431–1441.
41. Richardson, D., H. Felgate, N. Watmough, A. Thomson, and E. Baggs. 2009. Mitigating release of the potent greenhouse gas N₂O from the nitrogen cycle—could enzymic regulation hold the key? *Trends Biotech.* **27**:388–397.
42. Röling, W. F. M. 2007. Do microbial numbers count? Quantifying the regulation of biogeochemical fluxes by population size and cellular activity. *FEMS Microbiol. Ecol.* **62**:202–210.
43. Šimek, M., and J. E. Cooper. 2002. The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. *Eur. J. Soil Sci.* **53**:345–354.
44. Šimek, M., and D. W. Hopkins. 1999. Regulation of potential denitrification by soil pH in long-term fertilized arable soil. *Biol. Fert. Soils* **30**:41–47.
45. Šimek, M., L. Jiřová, and D. W. Hopkins. 2002. What is the so-called optimum pH for denitrification in soil? *Soil Biol. Biochem.* **34**:1227–1234.
46. Smith, M. S., and J. M. Tiedje. 1979. Phases of denitrification following oxygen depletion in soil. *Soil Biol. Biochem.* **11**:261–267.
47. Stevens, R. J., and R. J. Laughlin. 1998. Measurement of nitrous oxide and di-nitrogen emissions from agricultural soils. *Nutrient Cycling Agroecosyst.* **52**:131–139.
48. Stevens, R. J., R. J. Laughlin, G. J. Atkins, and S. J. Prosser. 1993. Automated determination of nitrogen-15-labeled dinitrogen and nitrous oxide by mass-spectrometry. *Soil Sci. Soc. Am. J.* **57**:981–988.
49. Stevens, R. J., R. J. Laughlin, and J. P. Malone. 1998. Measuring the mole fraction and source of nitrous oxide in the field. *Soil Biol. Biochem.* **30**:541–543.
50. Tate, R. L. 1995. *Soil microbiology*. John Wiley, New York, NY.
51. Thomsen, J., T. Geest, and R. Cox. 1994. Mass spectrometric studies of the effect of pH on the accumulation of intermediates in denitrification in *Paracoccus denitrificans*. *Appl. Environ. Microbiol.* **60**:536–541.
52. Throbäck, I. N., M. Johansson, M. Rosenquist, M. Pell, M. Hansson, and S. Hallin. 2007. Silver (Ag⁺) reduces denitrification and induces enrichment of novel *nirK* genotypes in soil. *FEMS Microbiol. Lett.* **270**:189–194.
53. Vance, E. D., P. C. Brookes, and D. S. Jenkinson. 1987. An extraction method for measuring soil microbial biomass C. *Soil Biol. Biochem.* **19**:703–707.
54. Weslien, P., Å. K. Klemetsson, G. Björjesson, and L. Klemetsson. 2009. Strong pH influence on N₂O and CH₄ fluxes from forested organic soils. *Eur. J. Soil Sci.* **60**:311–320.
55. Zbíral, J., I. Honsa, and S. Malý. 1997. Soil analyses, part III. Czech Central Institute for Supervising and Testing in Agriculture, Brno, Czech Republic. (In Czech.)
56. Zumft, W. G. 1997. Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* **61**:533–616.