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Insights into the Effect of Soil pH on N_2O and N_2 Emissions and Denitrifier Community Size and Activity▽

Jiří Čuhel,[1,2]* Miloslav Šimek,[1,2] Ronnie J. Laughlin,[3] David Bru,[4,5] Dominique Chèneby,[4,5] Catherine J. Watson,[3] and Laurent Philippot[4,5]

Biology Centre of the ASCR, v. v. i., Institute of Soil Biology, Na Sádkách 7, 370 05 České Budějovice, Czech Republic[1]; University of South Bohemia, Faculty of Science, Branišovská 31, 370 05 České Budějovice, Czech Republic[2]; Agri-Food and Biosciences Institute, Newforge Lane, Belfast, Northern Ireland, United Kingdom[3]; INRA, UMR 1229, F-21000 Dijon, France[4]; and Université de Bourgogne, UMR 1229, F-21000 Dijon, France[5]

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The objective of this study was to investigate how changes in soil pH affect the N_2O and N_2 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 H_2SO_4 solution (acidic soil), or water (natural pH soil) over 10 months. At the site, we determined field N_2O and N_2 emissions using the ^{15}N 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 $N_2O/(N_2O + N_2)$ ratio increased with decreasing pH due to changes in the total denitrification activity, while no changes in N_2O production were observed. Denitrification activity and N_2O 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 $N_2O/(N_2O + N_2)$ 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 NO_3^- via NO_2^- to gaseous NO, N_2O, and N_2, 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 N_2O emissions (7). N_2O is a potent greenhouse gas which has a global warming potential about 320 times greater than that of CO_2 and has a lifetime of approximately 120 years (32). In the stratosphere, N_2O can also react with O_2 to produce NO, which induces the destruction of stratospheric ozone (8). N_2O 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 N_2O is an intermediate in the denitrification pathway, both the amount of N_2O produced and the $N_2O/(N_2O + N_2)$ ratio are important in understanding and predicting N_2O fluxes from soils.

The main environmental factors known to influence the $N_2O/(N_2O + N_2)$ ratio are pH, organic carbon and NO_3^- availability, water content, and O_2 partial pressure (50). Soil pH is one of the most important factors influencing both denitrification and N_2O production (43). In general, the denitrification rate increases with increasing pH values (up to the optimum pH) while, in contrast, the $N_2O/(N_2O + N_2)$ 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 N_2 emissions (16). Nevertheless, ^{15}N tracing experiments based on the addition of a labeled denitrification substrate to soil offer a useful tool to quantify emissions of both N_2O and N_2 *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 N_2O emissions (5, 21). A recent study reported a negative relationship between the proportion of bacteria genetically capable of reducing N_2O within the total bacterial community and the $N_2O/(N_2O + N_2)$ 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* N_2O and N_2 emissions, denitrifying enzyme activity (DEA), and potential N_2O 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 ^{15}N tracer method was used to provide information on N emissions. In addition to measuring

* Corresponding author. Mailing address: Biology Centre of the ASCR, v. v. i., Institute of Soil Biology, and University of South Bohemia, Faculty of Science, Na Sádkách 7, 370 05 České Budějovice, Czech Republic. Phone: 420 387 775 767. Fax: 420 385 310 133. E-mail: jiri.cuhel@seznam.cz.
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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 H_2SO_4 solution to decrease the pH, and (iii) four random plots were amended with water (control plots with a natural pH). The KOH and H_2SO_4 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 H_2SO_4 (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 H_2SO_4 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 m^2; volume, 10 dm^3), 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 ^{15}N-labeled tracer, we measured the natural rates of N_2O and CO_2 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 N_2O 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 CO_2 analyses were collected using a 5-ml syringe and stored in pre-evacuated (<100 Pa), septum-capped, 3.5-ml vials (Venoject). N_2O and CO_2 were quantified using 6890N and 6850N gas chromatographs (Agilent Technologies) equipped with μECD and TCD detectors, respectively. Integration of measured peaks and N_2O and CO_2 quantification were conducted using GC ChemStation software (Rev. B.02.01.; Agilent Technologies).

The ^{15}N gas flux method was used to measure the N_2O and N_2 emissions, and nitrogen was added as ^{15}N-labeled KNO_3 at a 60 atom% excess at a rate of 10 kg N ha^{-1}. The required amount of N was dissolved in 250 ml H_2O 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 N_2O and CO_2 emissions. Fluxes of N gases were determined five times following KNO_3 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 ^{15}N content of N_2O and the ^{15}N content of the N_2 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 ^{45}R ($^{45}I/^{44}I$) and ^{46}R ($^{46}I/^{44}I$) to be calculated for N_2O. The sources of N_2O 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 N_2, the ion currents at m/z 28, 29, and 30 enabled the ^{29}R ($^{29}I/^{28}I$) and ^{30}R ($^{30}I/^{28}I$) molecular ratios to be determined. Differences between the molecular ratios in enriched and normal atmospheres were calculated as $\Delta^{29}R$ and $\Delta^{30}R$. The flux of N_2 was calculated by using data for $\Delta^{29}R$ and $\Delta^{30}R$ to calculate the enrichment of the denitrifying pool ($^{15}X_N$) and then the N_2 flux according to Mulvaney and Boast (31). The total N fluxes during the 74-h period after the application of ^{15}N-labeled KNO_3 were calculated by integration, assuming a linear change in flux rates between observation times.

Soil sampling and analyses. On the day of labeled KNO_3 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-H_2O 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 (NH_4^+, NO_3^-) 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 KNO_3. 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 N_2O emission, respectively. After 30 and 60 min, 0.5-ml headspace samples were taken with a gas-tight syringe and N_2O 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 polyvinylpolypyrrolidone 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 cd_1 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-μl PCR mixture contained 12.5 μl of SYBR green PCR Master Mix (Absolute QPCR SYBR green Rox ABgen), 1 μM 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

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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_2O 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_2O 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 KNO_3 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 NH_4^+ concentration was higher in the acidic soil than in the natural pH and alkaline soils, while the NO_3^- 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 CO_2 and N_2O determined before the application of ^{15}N-labeled KNO_3 were also affected by the treatments. Thus, significantly higher CO_2 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, N_2O 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* N_2O and N_2 emissions after the application of ^{15}N-labeled NO_3^-.** Figure 1 shows the time course of the total N fluxes ($N_2O + N_2$) and of the N_2O fluxes from the soils with different pH treatment during the 74 h after the ^{15}N-labeled KNO_3 amendment. Higher N gas emissions were observed in

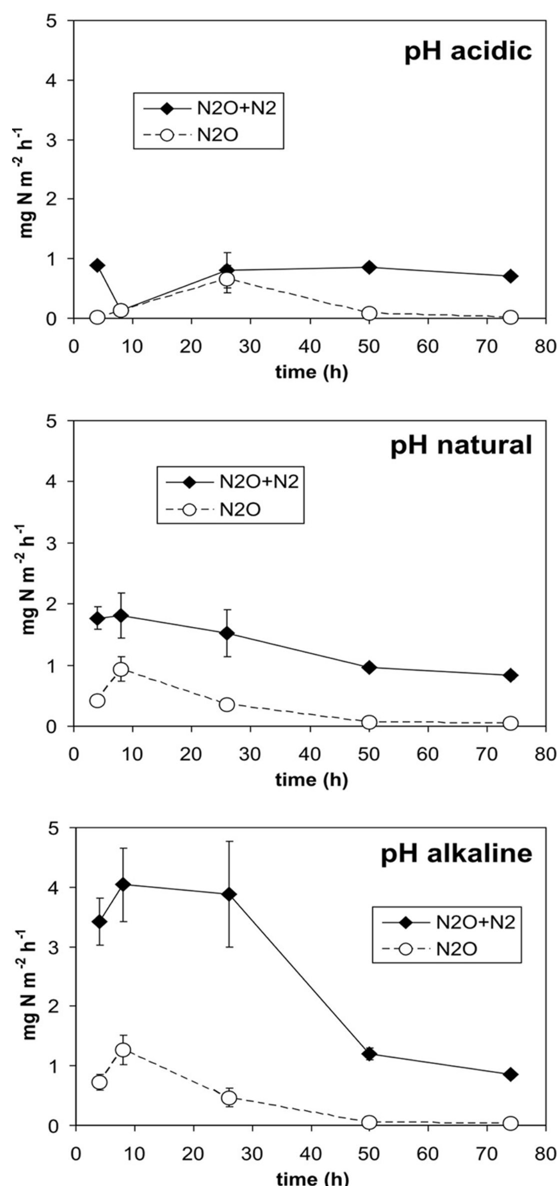

FIG. 1. Dynamics of total N ($N_2O + N_2$) and N_2O *in situ* fluxes after the addition of ^{15}N-labeled NO_3^- to acidic, natural pH, and alkaline soils. Mean values ± standard errors of the means are shown (n = 12).

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TABLE 2. Causes of N_2O flux in acidic, natural pH, and alkaline soils[a]

Parameter	Soil	Mean value ± 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 ± 0.22	0.73 ± 0.22	ND	ND
	Natural pH	0.75 ± 0.15	0.83 ± 0.12	0.61 ± 0.24	ND	ND
	Alkaline	0.83 ± 0.14	0.87 ± 0.13	0.63 ± 0.26	ND	ND
$d_N = (1 - d_D)$ (fraction)	Acidic	ND	0.52 ± 0.22	0.27 ± 0.22	ND	ND
	Natural pH	0.25 ± 0.15	0.17 ± 0.12	0.39 ± 0.24	ND	ND
	Alkaline	0.17 ± 0.14	0.13 ± 0.13	0.37 ± 0.26	ND	ND
a_D (enrichment; atom%)	Acidic	ND	0.56 ± 0.02	0.56 ± 0.03	ND	ND
	Natural pH	0.54 ± 0.03	0.55 ± 0.03	0.50 ± 0.06	ND	ND
	Alkaline	0.54 ± 0.05	0.55 ± 0.04	0.51 ± 0.07	ND	ND

[a] The fractions of the N_2O flux which were derived from the NO_3^- (denitrification) pool (d_D), from the NH_4^+ (nitrification) pool (d_N), and the enrichment (a_D) of the NO_3^- (denitrifying) pool (atom%) in acidic, natural pH, and alkaline soils are shown ($n = 12$).
[b] ND, not determined due to low N_2O fluxes.

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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 KNO_3. However, 50 to 74 h after the KNO_3 amendment, the total N fluxes were around 1 mg N m^{-2} h^{-1} in all treatments. In the control and alkaline plots, the N_2O emissions reached a maximum of ca. 1.1 mg N m^{-2} h^{-1} within 8 h after KNO_3 application and then decreased to ca. 0.4 mg N m^{-2} h^{-1}. In the acidic soil, the N_2O emissions increased up to 26 h before decreasing. In all three pH treatments, N_2 was the only gas product at the end of the experimental period.

Table 2 shows the fraction of the N_2O flux which was derived from the NO_3^- (denitrification) pool (d_D) together with the enrichment (a_D) of this pool and also the fraction from the NH_4^+ (nitrification) pool (d_N). Valid calculations could only be done, however, whenever there was a significant N_2O flux. Therefore, the values were not determined at 50 and 74 h after the KNO_3 addition for all three soils and 4 h after the KNO_3 addition for the acidic soil. In general, denitrification was the major process responsible for N_2O emissions in all three soils, accounting for ca. 61 to 87%. The exception was the acidic soil 8 h after KNO_3 addition, where the N_2O emissions were split equally between nitrification and denitrification. The enrichment of the denitrifying pool (a_D; on average, 54 atom%) was close to the enrichment of the added KNO_3 (60 atom%).

Cumulative fluxes of $N_2O + N_2$ 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 N_2 emissions were mostly responsible for the differences in the total N fluxes between the pH treatments, as the N_2O emissions were not significantly different (Tukey HSD test, $P >$ 0.24). The large differences in N_2 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 N_2O to the total cumulative N_2O + N_2 fluxes expressed as the $N_2O/(N_2O + N_2)$ molar ratio. The $N_2O/(N_2O + N_2)$ ratio increased with decreasing soil pH. The total losses of added NO_3-N (10 kg N ha^{-1}) as N_2O and N_2 (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 N_2O 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-

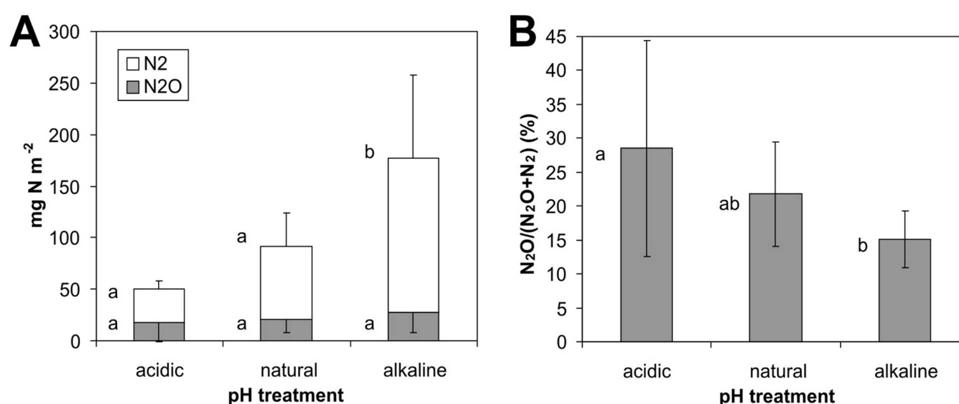

FIG. 2. *In situ* cumulative losses of N (separately as N_2O and N_2) (A) and relative N_2O production expressed as the $N_2O/(N_2O + N_2)$ molar ratio (B) over the 74 h after the addition of ^{15}N-labeled KNO_3 to acidic, natural pH, and alkaline soils. Mean values and ± standard deviations are shown ($n = 12$). 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 $N_2O/(N_2O + N_2)$ molar ratio

Parameter	*P* value			
	In situ		In laboratory	
	$N_2O + N_2$	$N_2O/(N_2O + N_2)$ ratio	$N_2O + N_2$	$N_2O/(N_2O + N_2)$ 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
narG gene copy no.	−0.156	−0.339[c]	0.243	−0.452[b]
napA gene copy no.	0.122	−0.130	0.175	−0.470[b]
nirS gene copy no.	−0.012	−0.415[c]	0.441[b]	−0.602[a]
nirK gene copy no.	0.080	−0.005	−0.121	−0.186
nosZ gene copy no.	−0.062	−0.095	0.191	−0.261
narG/16S rRNA ratio	−0.139	−0.481[b]	0.544[a]	−0.386[c]
napA/16S rRNA ratio	0.387[c]	−0.304	0.657[a]	−0.686[a]
nirS/16S rRNA ratio	0.063	−0.493[b]	0.666[a]	−0.612[a]
nirK/16S rRNA ratio	0.101	−0.022	−0.120	−0.043
nosZ/16S rRNA ratio	−0.062	−0.167	0.409[c]	0.015
nosZ/*nirS* ratio	−0.275	0.433[b]	−0.491[b]	0.706[a]
nosZ/*nirK* ratio	−0.066	−0.124	0.349	0.005
nosZ/(*nirS* + *nirK*) 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 $N_2O/(N_2O + N_2)$ 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 ($N_2O + N_2$) 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 ng N g^{-1} 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 ($N_2O/[N_2O + N_2]$) was also highly dependent on the pH treatment,

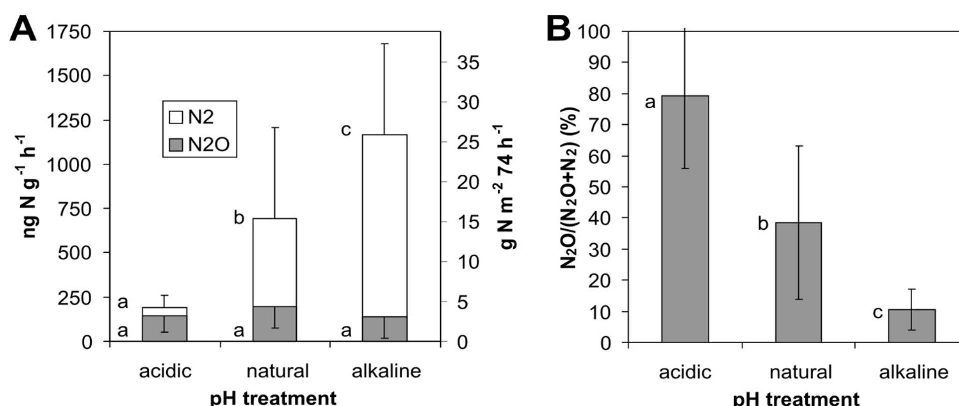

FIG. 3. DEA (separately as N_2O and N_2 production) of soils with different pH treatments (A) and relative N_2O production during DEA determination expressed as the $N_2O/(N_2O + N_2)$ molar ratio (B). DEA (A) was also estimated and is expressed as g N m^{-2} 74 h^{-1} (right vertical axis). Mean values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

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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 $N_2O/(N_2O + N_2)$ 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 $N_2O/(N_2O + N_2)$ 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 $N_2O/[N_2O + N_2]$ 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 $N_2O/(N_2O + N_2)$ molar ratio from the field measurements and from the DEA assay. Significant correlations between the $N_2O/(N_2O + N_2)$ 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).

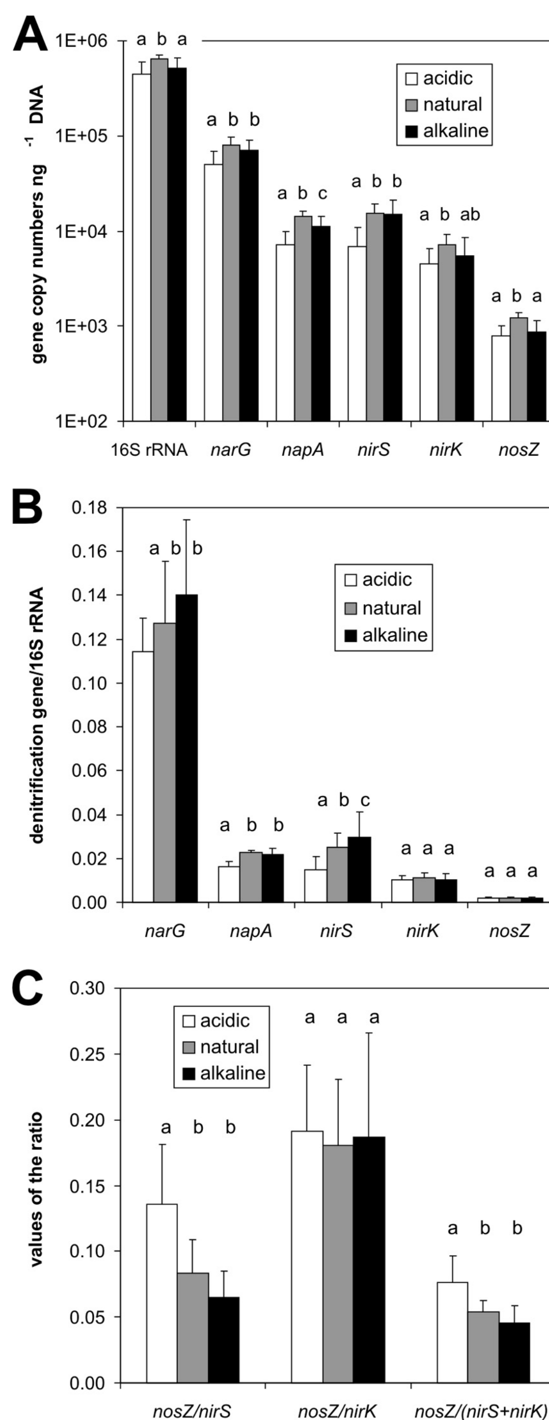

FIG. 4. Abundances of the 16S rRNA, *narG*, *napA*, *nirS*, *nirK*, and *nosZ* genes in soils with different pH treatment expressed as the number of gene copies ng^{-1} of DNA (A), the specific denitrification gene/16S rRNA gene ratio (B), and *nosZ*/*nirS*, *nosZ*/*nirK*, and *nosZ*/(*nirS* + *nirK*) 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 ($P < 0.05$).

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 solutions. 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

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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_D) 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 m^{-2} 74 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 nonlimiting denitrifying conditions resulted in the stimulation of the rates observed in our study.

Calculation of the $N_2O/(N_2O + N_2)$ ratio showed a decreasing molar ratio with increasing soil pH and denitrification rates, in agreement with previous studies (43). However, the range of the $N_2O/(N_2O + N_2)$ 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 $N_2O/(N_2O + N_2)$ ratio. Nevertheless, notwithstanding the differences between the two approaches, the $N_2O/(N_2O + N_2)$ ratio in the field was significantly correlated with the $N_2O/(N_2O + N_2)$ 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 $N_2O/(N_2O + N_2)$ ratio under laboratory conditions can reflect treatment differences in the field. We found soil pH to be the best predictor of the $N_2O/(N_2O + N_2)$ 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 N_2/N_2O 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 $N_2O/(N_2O + N_2)$ 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 then 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

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agreement with the soil respiration data (determined as CO_2 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 $N_2O/(N_2O + N_2)$ 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 $N_2O/[N_2O + N_2]$ 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 $N_2O/(N_2O + N_2)$, 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 $N_2O/(N_2O + N_2)$ ratio determined in the laboratory. This putative influence of the proportion of denitrifiers on N_2O 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 N_2O 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 $N_2O/(N_2O + N_2)$ ratio. This indicates that, not surprisingly, the routes for N_2O 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 N_2O reductase remains to be experimentally demonstrated.

To conclude, our results indicate that manipulation of soil pH affected the $N_2O/(N_2O + N_2)$ ratio, which increased with decreasing pH due to changes in the total denitrification activity but not in N_2O production. We also showed that denitrification activity and N_2O 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 $N_2O/(N_2O + N_2)$ molar ratio which requires further understanding. However, in this study, the proportion of denitrifiers capable of reducing N_2O did not seem to have a role in determining the $N_2O/(N_2O + N_2)$ 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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