

# Denitrification gene pools, transcription and kinetics of NO, N<sub>2</sub>O and N<sub>2</sub> production as affected by soil pH

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## Keywords

denitrification; gene transcription; gas kinetics; liming; soil pH.

## Introduction

Denitrification is the most important source of N<sub>2</sub>O emission, and soils account for 70% of the total load of N<sub>2</sub>O in the atmosphere (Mosier, 1998). The strong specific global warming effect of N<sub>2</sub>O compared with that of CO<sub>2</sub> implies that the performance of ecosystems as reactors in the climate system is strongly influenced by their N<sub>2</sub>O emission, despite the fact that N<sub>2</sub>O fluxes are low compared with those of CO<sub>2</sub>. This was clearly demonstrated in a recent study by Crutzen *et al.* (2008), concluding that the potential global cooling effect of biofuel production (by substituting fossil C) was negated by N<sub>2</sub>O emissions if including the entire life cycle of the fertilizer N. Such life cycle calculations are ultimately based on assumed product stoichiometry (N<sub>2</sub>O:N<sub>2</sub>) of the ecosystems involved. The empirical basis for the product stoichiometry of denitrification is weak, however, resulting in very uncertain life cycle estimates of N<sub>2</sub>O emissions (Schlesinger, 2009). This underscores the need to improve our knowledge of the N<sub>2</sub>O:N<sub>2</sub> product

## Abstract

The N<sub>2</sub>O:N<sub>2</sub> product ratio of denitrification is negatively correlated with soil pH, but the mechanisms involved are not clear. We compared soils from field experiments where the pH had been maintained at different levels (pH 4.0–8.0) by liming (≥ 20 years), and quantified functional gene pools (*nirS*, *nirK* and *nosZ*), their transcription and gas kinetics (NO, N<sub>2</sub>O and N<sub>2</sub>) of denitrification as induced by anoxic incubation with and without a carbon substrate (glutamate). Denitrification in unamended soil appeared to be based largely on the activation of a pre-existing denitrification proteome, because constant rates of N<sub>2</sub> and N<sub>2</sub>O production were observed, and the transcription of functional genes was below the detection level. In contrast, glutamate-amended soils showed sharp peaks in the transcripts of *nirS* and *nosZ*, increasing the rates of denitrification and pH-dependent transient accumulation of N<sub>2</sub>O. The results indicate that the high N<sub>2</sub>O:N<sub>2</sub> product ratio at low pH is a post-transcriptional phenomenon, because the transcription rate of *nosZ* relative to that of *nirS* was higher at pH 6.1 than at pH 8.0. The most plausible explanation is that the translation/assembly of N<sub>2</sub>O reductase is more sensitive to low pH than that of the other reductases involved in denitrification.

ratio of denitrification in various soils, and how it is regulated.

Denitrification in soil is affected by a number of factors. One important controller seems to be soil pH, which, although the mechanisms are not well understood, seems to have a direct effect on denitrifying prokaryotes. In addition, pH exerts a plethora of indirect effects through its influence on physical, chemical and biological properties and processes (Bremner, 1997). In a review covering 50 years of research on soil pH effects on denitrification, Simek & Cooper (2002) found evidence for a positive, but somewhat variable relationship between soil pH and overall denitrification rates, and a consistent negative relationship between soil pH and the N<sub>2</sub>O:N<sub>2</sub> product ratio of denitrification. This suggests that liming of slightly acidic agricultural soils could efficiently reduce the overall N<sub>2</sub>O emission induced by fertilizers, be it by reducing direct N<sub>2</sub>O emissions from the fertilized soils, reducing the nitrate leaching by increased denitrification in agricultural fields (thus reducing the N<sub>2</sub>O emission further down in catchments) or both. Surprisingly

few attempts have been made deliberately to explore such pH manipulation as a method to reduce  $\text{N}_2\text{O}$  emissions. A notable exception is the recent field studies by Zaman *et al.* (2007, 2008), which indicated that moderate liming may enhance denitrification while reducing the  $\text{N}_2\text{O}:\text{N}_2$  product ratio. One reason for the general reluctance to manipulate pH to mitigate  $\text{N}_2\text{O}$  emissions may be the lack of understanding of the mechanisms involved.

Soil pH exerts direct control on the metabolism of the organisms present, but will also represent a long-term selective pressure, because pH is a stable characteristic determined primarily by the quality of the parent material and climate (and liming for agricultural soils). Soil pH appears to be a master variable determining the entire bacterial community composition of soils (Frostegård *et al.*, 1993; Fierer & Jackson, 2006). The selective pressure on the functional characteristics of denitrifying communities of soils was investigated by Simek *et al.* (2002), who exposed microbial communities from different soils to a range of pH levels. The results showed that the communities had different pH optima for potential denitrification, and these were correlated with the soils' native pH.

Investigations of the direct effect of pH on the denitrification process have demonstrated that the  $\text{N}_2\text{O}:\text{N}_2$  product ratio increases with decreasing pH, and this appears to be independent of the pH optimum for the organisms, as judged by the comparison of soil communities with different pH optima (Simek & Cooper, 2002). This could be taken to suggest that acidity represents a universal constraint on the reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$ . The explanation for the observed effects of pH on the  $\text{N}_2\text{O}:\text{N}_2$  product ratio has for a long time been that the enzyme  $\text{N}_2\text{O}$  reductase, which is a periplasmic enzyme in gram-negative bacteria, is more sensitive to low pH than the other reductases involved in denitrification (Thomsen *et al.*, 1994; Simek *et al.*, 2002). This explanation was challenged by L. Bergaust, M. Yuejian, L. Bakken & Å. Frostegård (unpublished data), who measured denitrification gene expression in *Paracoccus denitrificans*. The results provided compelling evidence that the primary reason for the low relative  $\text{N}_2\text{O}$ -reductase activity at suboptimal pH (6.0) was that low pH impaired or delayed the assembly of this protein in the periplasm. The phenomenon may have profound implications for interpretation of the effect of pH on observed  $\text{N}_2\text{O}$  and  $\text{N}_2$  production kinetics in soil, but it is currently to be considered a hypothesis, because we do not know whether the indigenous denitrifying organisms resemble *P. denitrificans* in this respect.

Molecular methods have provided tools to unravel the composition of indigenous prokaryote communities by DNA analysis (Amann *et al.*, 1995; Head *et al.*, 1998; Greer *et al.*, 2001), improved further by analyzing RNA, to capture the metabolically active organisms (Griffiths *et al.*, 2000;

Mahmood & Prosser, 2006; Chen *et al.*, 2007; Tourna *et al.*, 2008). Quantification of mRNA in environmental samples has long been restricted by methodological difficulties, but advances in extraction technologies have now made this feasible (Leininger *et al.*, 2006; Smith *et al.*, 2007; Baelum *et al.*, 2008; Nicolaisen *et al.*, 2008; Freitag & Prosser, 2009). In the present study, we have adapted such methods to quantify denitrification genes and their transcripts in soils during the transition from oxic to anoxic conditions, while monitoring gas kinetics using a robotized incubation system (Molstad *et al.*, 2007).

We have investigated the mechanisms involved in pH control of denitrification by quantifying pools of functional denitrification genes, their transcription and the kinetics of  $\text{NO}$ ,  $\text{N}_2\text{O}$  and  $\text{N}_2$  production in soils from long-term liming experiments. The gas kinetics during anoxic incubation corroborated previous findings regarding the effect of pH on  $\text{N}_2\text{O}:\text{N}_2$  product stoichiometry (Simek & Cooper, 2002): all three liming experiments demonstrated a practically identical gradual increase in the  $\text{N}_2\text{O}:\text{N}_2$  product ratio with decreasing soil pH. The investigations of gene pools and their transcription in combination with the kinetics of gas production unraveled several novel phenomena with profound implications for our understanding of denitrification in soil as controlled by pH.

## Materials and methods

### Soils

Soils were sampled from three long-term field experiments with randomized block designs in which soil pH had been increased to different levels by addition of lime or shell sand. All soil sampling was performed in early spring. The three soils were F: peat soil (Sapric Histosol, FAO/ISRIC/ISS, on average containing 45% soil organic C, 2% soil organic N) from Fjaler in western Norway (grown with meadow), in which pH had been increased to different levels by different amounts of shell sand (0, 200, 400 and 800  $\text{m}^3 \text{ha}^{-1}$ ) at establishment of the field experiment in 1978 (Sognnes *et al.*, 2006; Mørkved *et al.*, 2007); Å: clay loam (Stagnic Albeluvisol, FAO/ISRIC/ISS, 39% sand, 40% silt, 21% clay, 3% soil organic C, 0.22% organic N) from Ås in south east Norway (grown with wheat for several years) in which the pH had been increased by applying different levels of  $\text{CaCO}_3$ ; and Ö: silt soil (5% clay, 86% silt, 9% sand, 6% soil organic C, 0.55% soil organic N) from Öjebyn in Northern Sweden, in which the pH was maintained at different levels by frequent addition of  $\text{CaCO}_3$  for a period of 22 years before sampling. The sampled soils were sieved (2 mm for the mineral soils and 6 mm for the peat soil) while moist, and stored moist at 4 °C until used in the various incubations (6–12 months after sampling). The pH reported was

measured in a soil slurry with distilled water (2.5 mL g<sup>-1</sup> mineral soil, 10 mL g<sup>-1</sup> organic soil).

In the F-soil, addition of shell sand significantly reduced weight % soil organic C (measured by weight loss on ignition, results not shown) from 49% in the unlimed soil to ~40% in the most heavily limed soil (primarily by 'dilution' due to the large amounts of shell sand added). The % dry weight of the F-soils (both as stored soil, and after drainage before incubations) varied inversely with the % C in dry weight (40–50%) because the shell sand holds less water than the organic material. As a result, all the soil F samples used for quantification of genes and transcripts and for gas kinetic studies contained almost identical amounts of soil organic C per gram of fresh weight (~0.2 g C g<sup>-1</sup>). The number of gene copies and transcripts are all reported per gram of fresh weight, but can be transformed to a per gram of soil organic C by multiplying by 5.

### Gas kinetics under anoxic incubations

The first incubation experiment was performed to determine potential denitrification and its N<sub>2</sub>O:N<sub>2</sub> product ratio in all soils (nine from site F, six from site Å and 11 from site Ö) when incubated under anoxic conditions. Field moist soil (20 g fresh weight), with NO<sub>3</sub><sup>-</sup> concentrations measured directly from storage of 500, 40 and 200 µg NO<sub>3</sub><sup>-</sup>-N g<sup>-1</sup> soil dry weight for F, Å and Ö, respectively, was transferred to 120-mL serum flasks. Flasks were then sealed with air-tight, butyl-rubber septa and aluminum crimp caps and made anoxic by repeated evacuation and filling with He. Triplicate samples were prepared from each treatment plot. To one of the replicates, acetylene (C<sub>2</sub>H<sub>2</sub>) was added to the headspace (injection and subsequent release of overpressure, final concentration 10% v/v) to inhibit N<sub>2</sub>O reduction. The flasks were then incubated in a water bath at 15 °C and monitored for N<sub>2</sub>O accumulation by repeated sampling for GC analysis (Sitaula *et al.*, 1992) for a period of 21 h (Run 1). Sampled gas (2 mL per sampling) was replaced by injection of He, and this dilution was taken into account when calculating the emission of N<sub>2</sub>O (in flasks without C<sub>2</sub>H<sub>2</sub>) and denitrification (N<sub>2</sub>O in flasks with C<sub>2</sub>H<sub>2</sub>). The flasks with C<sub>2</sub>H<sub>2</sub> were then discarded, and the others (2 per soil) were incubated for 13 days (under anoxic condition). Nitrate was then added (all original nitrate was assumed to be denitrified) by injecting 1 mL of 36 mM NO<sub>3</sub><sup>-</sup> solution with a syringe (spread evenly over the soil surface), after which the headspace was replaced with He, with evacuation and filling as described above, to remove any remaining N<sub>2</sub>O. C<sub>2</sub>H<sub>2</sub> was added to one of the remaining flasks for each soil (10% v/v). The headspace was monitored again for N<sub>2</sub>O accumulation by frequent sampling over a period of 21 h (Run 2). The purpose of Run 2 was to investigate the effect of a prolonged anoxic incubation (13 days)

on potential denitrification and N<sub>2</sub>O:N<sub>2</sub> product stoichiometry.

The second experiment was a repetition of the first, but using a robotized incubation system (Molstad *et al.*, 2007), which allows direct quantification of N<sub>2</sub> production (without C<sub>2</sub>H<sub>2</sub> inhibition). This experiment was performed with five soil samples from site F (pH 4.0–8.0). Essentially, the experimental procedure was equivalent to the foregoing, but somewhat refined. Soils were flooded with 2 mM NO<sub>3</sub><sup>-</sup> solution in Büchner funnels with paper filters and then drained by applying vacuum for 5 min to obtain a homogeneous distribution and an equal concentration of nitrate in all samples. This flooding/drainage procedure was repeated three times, followed by final evacuation–drainage for 15 min. Then 20 g wet weight of soil (equivalent to 4 g soil organic C per flask) was transferred to serum flasks (120 mL), which were sealed, made anoxic (He) as described above and transferred to the robotized incubation system (Molstad *et al.*, 2007) to monitor O<sub>2</sub>, N<sub>2</sub>, N<sub>2</sub>O and NO for 40 h (Run 1). One set of flasks (duplicates per sample) was monitored immediately after making the conditions anaerobic. The other set was left anoxic for 13 days (15 °C) and then supplied with additional nitrate (1 mL, 36 mM NO<sub>3</sub><sup>-</sup> solution) and reflashed with He (as described above) before monitoring gas production (Run 2). A parallel set of samples with 10% C<sub>2</sub>H<sub>2</sub> was included in each measurement, for comparison with the directly measured N<sub>2</sub> production.

Incubation of soil without addition of an extra carbon substrate resulted in near constant rates of denitrification and no detectable transcripts of functional denitrification genes, suggesting severe substrate limitation. A third series of anoxic incubations with glutamate added to the soils was conducted to determine the importance of the energy status of the denitrifying organisms. This used the same procedure as the second experiment, but the flooding/drainage treatment before incubation was performed with a solution containing both 2 mM nitrate and 10 mM glutamic acid, with pH adjusted to that of each soil.

### Extraction of nucleic acids

Total nucleic acids were isolated from intact soil using a beat-beating method combined with phenol–chloroform treatment as described by Griffiths *et al.* (2000) with minor modifications (Nicolaisen *et al.*, 2008). Only certified nuclease-free tubes and diethyl pyrocarbonate-treated solutions (Blumberg, 1987) were used for extraction. For DNA analysis, the DNA/RNA mixture was digested by RNase (Promega, Madison, WI) at 37 °C, purified using the Wizard DNA Clean-Up kit (Promega) and used for PCR. For RNA analysis, the DNA/RNA mixture was digested using the RNase-Free DNase Set (Qiagen Nordic, Norway) and then purified using the RNeasy mini kit (Qiagen Nordic). The

concentration and purity of total DNA and RNA were established using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE).

Copy numbers of *nirK*, *nirS*, *nosZ* and 16S rRNA genes were quantified by real-time PCR in F soil samples (pH 4.0, 6.1 and 8.0) taken at the start of the incubation experiment. Transcription of functional genes was analyzed in pH 4.0, 6.1 and 8.0 soil samples with and without glutamic acid before and during anoxic incubation. Gases were monitored continuously in a large number of flasks to capture periods of high activity. Guided by these measurements, destructive sampling was performed (using all the soil in a flask), at sampling times shown in Fig. 1, by snap freezing in liquid nitrogen. The samples were stored at  $-80^{\circ}\text{C}$  for nucleic acid extraction.

### Reverse transcription

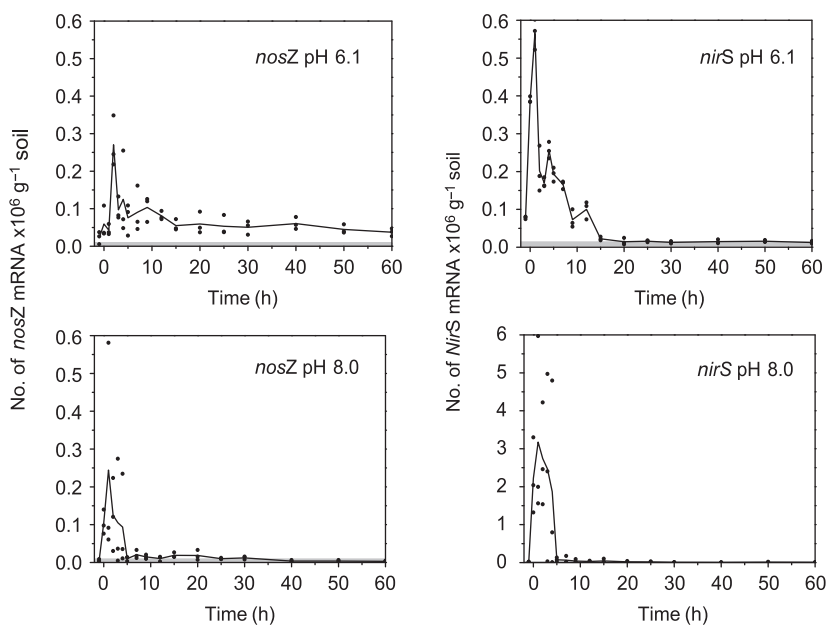
Reverse transcription of the purified RNA extract was performed with random hexamer primers ( $50\text{ ng}\mu\text{L}^{-1}$ ) using the Masterscript RT-PCR System (5 Prime GmbH, Hamburg, Germany) following the manufacturer's instructions. Control samples were prepared for each gene and analyzed by real-time PCR to ensure that all genomic DNA was removed by DNase. These controls contained the same concentration of soil RNA extract, but reverse transcription was not performed.

### Real-time PCR

The standard curves for the real-time PCR were constructed using *nirS*, *nirK*, *nosZ* and 16S rRNA gene fragments cloned

into plasmid pCR4-TOPO (3956 bp, Invitrogen, Paisley, UK). Genomic DNA was extracted from the pH 8.0 soil, which has a high denitrification activity. *NirS*, *nirK*, *nosZ* and 16S rRNA gene fragments were amplified with the primer pairs cd3af and r3cd (Michotey *et al.*, 2000), 1F and 5R (Braker *et al.*, 1998), Z-F and Z-R (Kloos *et al.*, 2001) and 27F and 518R (Lane, 1991; Muyzer *et al.*, 1993), respectively. The amplicons were gel-purified using the E.Z.N.A. Gel Extraction Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) and cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was extracted using a Qiagen Plasmid Mini Kit (Qiagen Nordic) and diluted in series to establish the standard curves. Plasmid concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Quantification of gene copies and their transcripts was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK), and data were analyzed using the SEQUENCE DETECTION SYSTEM (SDS 2.3 software; Applied Biosystems). The 20- $\mu\text{L}$  reaction mixture contained 0.2  $\mu\text{M}$  of each primer, 10  $\mu\text{L}$  of SYBR green PCR master mix (SYBR Premix Ex Taq with ROX, Takara Bio Co., Japan) and 0.4  $\mu\text{L}$   $50\times$  ROX and 2  $\mu\text{L}$  of template DNA. Quantitative PCR assays were carried out using the following temperature program: preincubation at  $94^{\circ}\text{C}$  for 20 s, 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 15 s, annealing and extension at  $60^{\circ}\text{C}$  for 60 s for all three genes, followed by a 20-s period at  $82^{\circ}\text{C}$ . Quantification of fluorescence took place during the 20-s period at  $82^{\circ}\text{C}$  to allow denaturation of primer dimers and unspecific PCR products. A melting



**Fig. 1.** Transcription of *nirS*, *nosZ*, expressed as copy numbers per gram of soil during anoxic incubation of soil F for 60 h after addition of glutamate. Gas kinetics are shown in Fig. 4. Results for each extract are shown (●), and the lines show the average for each time point ( $n=3$ ). The detection limit is indicated by the shaded area; see text for further explanation.

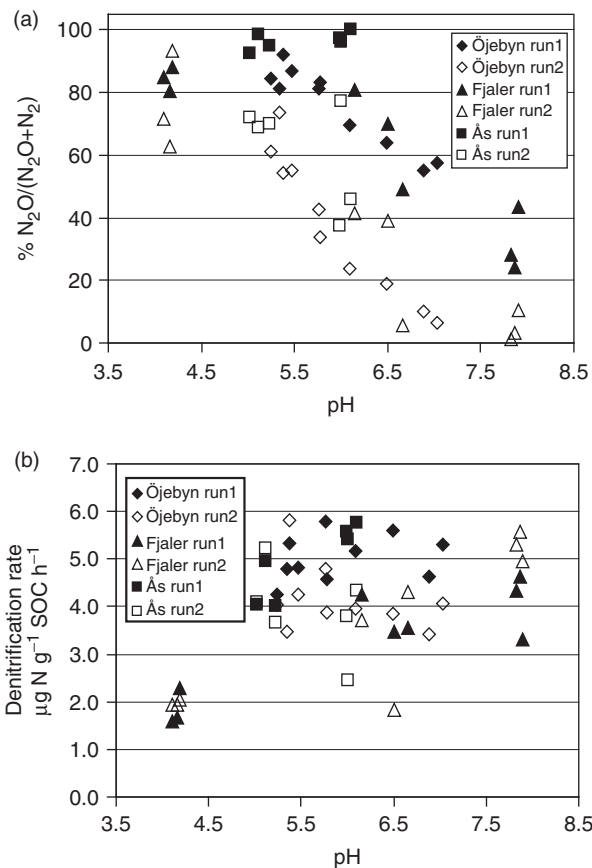
curve analysis (55–95 °C) was performed at the end of each PCR run to determine the specificity of the PCR reaction. PCR efficiency was calculated for the standard curve of each gene using the formula  $E = 10^{(-1/\text{slope})} - 1$ , where the slope was from the linear regression of  $C_t$  values against the logarithm of the copy numbers. PCR efficiency for soil samples was determined by amplifying the 16S rRNA gene from serially diluted soil DNA samples derived from the different pH soils. The real-time PCR runs were repeated several times for standard curves of each targeted gene, and  $C_t$  values for different dilutions were very stable on comparing different PCR runs. On calculating the average  $C_t$  values of four measurements for six dilutions ( $10^2$ – $10^7$ ;  $C_t = 13$ – $31$ ), the coefficient of variation was < 4.0% both for *nosZ* and for *nirS*, except in one case, when it was 5.7%.

The fraction of cDNA applied to each PCR tube was equivalent to that extracted from 1.7 mg soil fresh weight. Special attention was paid to the detection limit of the method because many samples yielded very weak signals. Control samples [no template controls (NTC)] were included in all runs, and the NTC average copy numbers were 3.6 copies per tube for *nosZ* (SD = 1.4,  $n = 4$ ); 6.0 copies per tube for *nirS* (SD = 2.1,  $n = 4$ ); and 4.0 copies per tube for *nirK* (SD = 2.4,  $n = 3$ ). These NTC average numbers were subtracted when calculating the copy numbers in the samples. The detection limit was set to 10 times the SD of the NTC values, which is substantially more conservative than that used routinely in analytical chemistry, which is ~3 times the SD of NTC (Skoog *et al.*, 1992).

## Results

### N<sub>2</sub>O and N<sub>2</sub> production

The results from the first incubation of all soils are shown in Fig. 2. The rate of N<sub>2</sub>O production (with and without C<sub>2</sub>H<sub>2</sub>) was almost constant for all samples throughout the 21-h incubation period (not shown), both for Run 1 (immediately after making the conditions anaerobic) and for Run 2, in which additional nitrate was supplied after 13 days of anoxic incubation. The estimated denitrification rates per gram of soil were much lower in mineral soils (Å, Ö) than in the peat soil (F) (0.2–0.5 and 1–2  $\mu\text{g N g}^{-1} \text{h}^{-1}$ , respectively; not shown), but when expressed per gram of soil organic C, there was essentially no difference between the three soils (Fig. 2b), and no effect of soil pH apart from the low denitrification rates in the very acid (unlimed) peat soil (pH 4.0). Furthermore, the denitrification rates were essentially the same during Runs 1 and 2; the 13 days of anoxic conditions did not result in significantly higher rates of denitrification. In contrast, the product ratios, expressed as % N<sub>2</sub>O:(N<sub>2</sub>O+N<sub>2</sub>), showed a consistent decline with



**Fig. 2.** Denitrification rates and gas product ratios [N<sub>2</sub>O:(N<sub>2</sub>+N<sub>2</sub>O)] during anoxic incubation of soils from three liming experiments: Fjaler, Öjebyn and Ås. Product ratios (a) are expressed as molar % N<sub>2</sub>O [N<sub>2</sub>O:(N<sub>2</sub>+N<sub>2</sub>O)] and denitrification rates (b) are expressed as  $\mu\text{g (N}_2\text{+N}_2\text{)-N g}^{-1} \text{SOC h}^{-1}$ . Rates were measured during the first 20 h of anoxic incubation (Run 1, closed symbols). More nitrate was added after incubation for 13 days and gas production was measured again for 20 h (Run 2, open symbols). N<sub>2</sub>+N<sub>2</sub>O production rates were estimated using the C<sub>2</sub>H<sub>2</sub> inhibition method.

increasing pH, and clearly lower values for Run 2 than Run 1 (Fig. 2a).

A similar trend was found on analyzing the five selected pH treatments from site F using the robotized incubation system (Fig. 3), in which the production of N<sub>2</sub> was quantified directly (not by C<sub>2</sub>H<sub>2</sub> inhibition). As in the previous experiment, N<sub>2</sub>O and N<sub>2</sub> increased linearly with time ( $r^2 > 0.9$ ) during incubation for 40 h (result not shown). Denitrification product ratios showed essentially the same pH response as in the previous experiment (Fig. 3 vs. Fig. 2a). The results for the flasks with 10% C<sub>2</sub>H<sub>2</sub> showed negligible N<sub>2</sub> accumulation, and N<sub>2</sub>O accumulations were within  $\pm 10\%$  of the sum of N<sub>2</sub>+N<sub>2</sub>O accumulation in the other flasks (results not shown).

Incubation of soils amended with glutamate resulted in nonlinear accumulation of denitrification products and

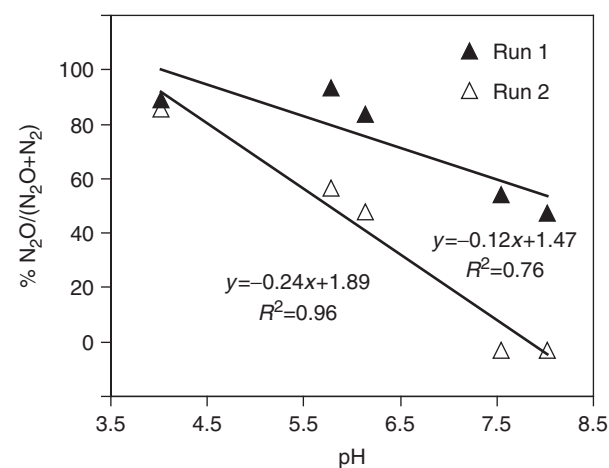
transient accumulation of  $N_2O$  that was clearly dependent on pH. The detailed gas kinetics throughout anoxic incubation is illustrated for three single flasks in Fig. 4a–c. The rate of  $N_2O$  accumulation for pH 4.0 soil was almost constant throughout the entire incubation, and  $N_2$  production was negligible (not visible in the graph, Fig. 4a). Transient  $N_2O$  accumulation was observed in soils with pH 6.1 and 8.0, reaching higher and lasting longer at pH 6.1 than at pH 8.0.  $N_2$  production rates increased drastically throughout the incubation, declining to zero ( $N_2$  reaching a plateau) when all nitrate had been reduced to  $N_2$ . The replicates for the

three pH levels shown in Fig. 4a–c were practically identical to those shown. The results for the other pH levels show a gradual decline in the transient  $N_2O$  peak with increasing pH. An  $N_2O$  index ( $I$ ) was calculated for each single flask to summarize the results across all replicates and pH levels and is an estimate of the average relative amount of  $N_2O$ -N as a fraction of  $(N_2O+N_2)$  during the entire period of active denitrification. Technically, the calculation of the index is shown for  $I_{N_2O}$ :

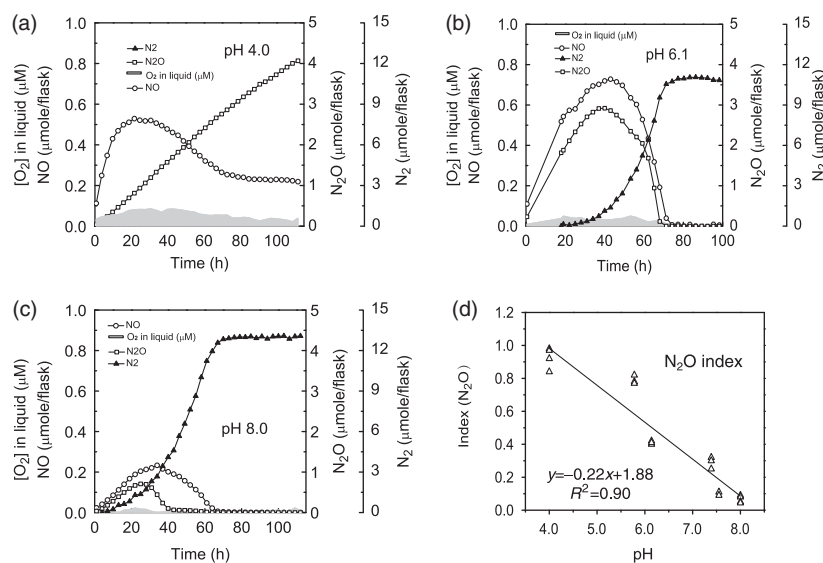
$$I_{N_2O} = \int_0^T N_2O / \left[ \int N_2O + \int N_2 \right]$$

where the integrals are the area under the curve for each gas product (the straight line between each point of measurement), limited to the period with active denitrification. Thus,  $T$  is the time when practically all added  $NO_3^-$  is recovered as  $N_2$ , except for the pH 4.0, for which the experiment was terminated when only ~30% of the added  $NO_3^-$  had been recovered as  $N_2O$ . In this case,  $T$  indicates the end of the experiment. Figure 4d shows these indices for single flasks.  $I_{N_2O}$  shows a consistent response to pH over the entire pH range, very similar to that observed in unamended soil (Figs 2a and 3). An alternative way to illustrate the gradual response to pH is shown in Supporting Information (Fig. S1), where the  $N_2O : (N_2O+N_2)$  ratio is plotted against the cumulated recovery of  $NO_3^-$  as  $N_2+N_2O+NO$ .

The constant low oxygen concentration (expressed as  $\mu M$  in liquid) throughout incubation (Fig. 4a–c, shaded areas) reflects continuous oxygen consumption, because there is inevitable flux of oxygen into the flasks by leaks through the septa and the injection system (Molstad *et al.*, 2007). The oxygen consumption rates were 6–7 nmol per flask  $h^{-1}$  (not



**Fig. 3.** Denitrification product [ $N_2O : (N_2 + N_2O)$ ] ratio (%) vs. pH for five soil samples from the liming experiment at Fjaler. Experimental details as for Fig. 2, but the  $N_2$  production rates were measured directly (not with the  $C_2H_2$  inhibition method). Rates were measured during the first 40 h of anoxic incubation (Run 1, closed symbols). More nitrate was added after incubation for 13 days and gas production was measured again for 40 h (Run 2, open symbols).



**Fig. 4.** Kinetics of NO,  $N_2O$  and  $N_2$  during incubation of intact soil samples amended with 2 mM nitrate and 10 mM glutamic acid (flooding and drainage immediately before incubation). The experiment was run in triplicate (with soil samples from three replicate field plots for each pH level), but the figure (a, b, c) shows the results for single flasks for only three pH values. (Replicates were essentially identical, as indicated in the text.) The measured oxygen concentration is illustrated (shaded area) as  $\mu M$  in liquid, estimated from the measured headspace concentration (assuming equilibrium). (d) The  $N_2O$  index of single flasks for all treatments ( $n = 3$  for each). The  $N_2O$  index is a measure of the relative amount of  $N_2O$  transiently accumulating during active denitrification (see text for further explanation).

shown). The pH 8.0 soil sustained a significantly lower oxygen concentration than pH 4.0 soil (comparison of three replicates of each, results not shown). Oxygen concentrations for intermediate pH levels were somewhat variable, but inspection of all treatments by linear regression of average oxygen concentration vs. pH suggests a decreasing level with increasing pH ( $r^2 = 0.62$ ).

### Molecular analysis

Nucleic acids of good quality and quantity were obtained after crude extraction and purification. The total DNA and RNA yields were 16.1–26.4 and 2.3–7.2  $\mu\text{g g}^{-1}$  wet weight soil, respectively. The  $A_{260\text{ nm}}:A_{280\text{ nm}}$  ratios, measured using a Nanodrop spectrophotometer, were  $> 1.7$  for all samples. No bands were obtained in agarose gels of the control samples (treated with DNase, but no reverse transcriptase), demonstrating the successful removal of DNA by DNase. Standard curves for calibration of real-time PCR were linear for all the genes studied ( $10^2$ – $10^7$  copies;  $r^2 = 0.994$ – $0.999$ ), and PCR efficiency was 94.5%, 90.5% and 90.3% for *nosZ*, *nirS* and 16S rRNA genes, respectively. PCR efficiency for soil-derived nucleic acids, estimated from amplification of the 16S rRNA gene in dilution series of DNA extracted from soil, was 90.3%, 98.7% and 96.0% for pH 4.0, 6.1 and 8.0 soil samples, respectively. Melting curve analysis showed one distinct peak for each expected PCR product and no nonspecific peaks. No primer dimer peaks were formed for *nirS* and 16S rRNA genes. One primer dimer peak was formed when *nosZ* was targeted, but this was completely denatured at 82 °C and did not affect the accuracy (see Fig. S3).

### Abundance of *nirS*, *nirK* and *nosZ* genes

The copy numbers of the functional genes *nirS*, *nirK* and *nosZ* relative to those of 16S rRNA genes in the same sample are presented in Fig. 5. The copy numbers of all three functional genes showed a positive relationship with pH. *NirS* copy number increased approximately 170-fold, from  $1.8 \times 10^{-4}$  copies per 16S rRNA gene at pH 4.0 to  $3.0 \times 10^{-2}$  at pH values  $> 7.5$ . *NirK* copy number increased 110-fold over the same pH interval, from  $1.5 \times 10^{-4}$  to  $1.7 \times 10^{-2}$

copies per 16S rRNA gene. The copy number of *nosZ* also increased with pH, but only 20-fold, from an average of  $1.0 \times 10^{-3}$  copies per 16S rRNA gene copy at pH 4.0 to  $1.9 \times 10^{-2}$  copies per 16S rRNA gene copy at pH values  $> 7.5$ . The largest increase in all three functional genes took place between pH 4.0 and 6.1.

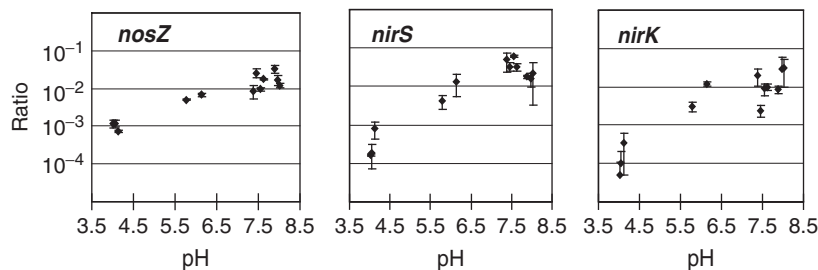
The *nos:nir* ratio, i.e. the copy numbers of genes coding for  $\text{N}_2\text{OR}$  relative to those coding for nitrite reductases [copy number of *nosZ*:copy number of (*nirS*+*nirK*)] was sixfold higher for the unlimed (pH~4) soils (average *nos:nir* = 3.1; SD = 1.1,  $n = 8$ ) than in limed soils (average *nos:nir* = 0.5, SD = 0.07,  $n = 27$ ). No clear effect of pH on the *nos:nir* ratio could be discerned within the limed soils (pH 5.5–8.0).

### Gene expression at different pH values

No significant expression of denitrification genes could be measured in soil to which no carbon had been added and *nirK*-, *nirS*- and *nosZ*-mRNA copy numbers were all below or close to the detection limit (calculated as 10 times the NTC SD, which was  $8.4 \times 10^3$ ,  $1.3 \times 10^4$  and  $4.3 \times 10^3$  copies  $\text{g}^{-1}$  soil for *nosZ*, *nirS* and *nirK*, respectively), except for a reading for *nosZ* of 100 copies in a single tube after 3 h (pH 8.0 soil).

Soils were amended with 10 mM glutamic acid by flooding and draining to stimulate a higher activity, and thereby increase the gene transcription. This resulted in complete denitrification after about 70 and 50 h for pH 6.1 and 8.0 soil, respectively (Fig. 4). Sharp peaks of gene transcription were observed for both *nirS* and *nosZ* in response to anaerobiosis at pH 6.1 and 8.0 (Fig. 1), but the transcripts of *nirK* were below the detection limits throughout (results not shown). For the pH 4.0 soil, gene transcription was invariably below the detection limit (results not shown).

The ratio of *nosZ*:*nirS* transcript copy number per gram of soil (*nosZ*:*nirS*) is of particular interest. Careful inspection of the data presented in Fig. 1 (note that the axis showing *nirS* in pH 8.0 soil has a different scaling) shows that the *nosZ*:*nirS* ratio was generally higher in pH 6.1 than in pH 8.0 soil during the peak transcription (0–15 h). Another conspicuous contrast is that the number of *nosZ* transcripts was maintained at around  $0.1 \times 10^6$  copies  $\text{g}^{-1}$  in



**Fig. 5.** The copy number of the functional genes (*nirS*, *nirK* and *nosZ*) in soil samples from the liming experiment at Fjaler (F). Copy numbers are expressed relative to those of the 16S rRNA gene. The real-time PCR were run in triplicate for each soil sample, and average values and SDs are shown.

pH 6.1 soil throughout the period of active denitrification (compare Figs 4 and 1), whereas in pH 8.0 soil, it declined to much lower values after only 5 h. Copy numbers of gene transcripts expressed as the ratio of mRNA:DNA for each gene were very low, never exceeding one gene transcript per gene copy (Fig. S2).

## Discussion

Anoxic incubation of soil to which no substrate had been added resulted in near constant rates of  $N_2$  and  $N_2O$  production and a clear negative relationship between the  $N_2O:N_2$  product ratio and soil pH. This relationship was remarkably similar for all soils from the three long-term field liming experiments. The product ratio was reduced somewhat when measured after 13 days of anoxic incubation (Fig. 2a), but the relationship with pH was retained. In contrast, the total denitrification rate ( $N_2+N_2O$  production) did not appear to be controlled by pH; the denitrification rate, when expressed as per gram of soil organic C (which is a good predictor of the potential respiratory activity of soils), was virtually unaffected by pH, except for the low values recorded for the most acid organic soil (Fig. 2b).

The near constant rates of denitrification in unamended soil suggested a lack of *de novo* synthesis of denitrification enzymes, which would imply that the observed denitrification is using an existing denitrification proteome. We hypothesized that this could be due to severe starvation prevailing in unamended bulk soil, and the effect of adding an easily available carbon substrate strengthened this view. The addition of glutamate resulted in exponential increases in the denitrification rate and a final recovery of all added nitrate as  $N_2$  for all pH values, except for the most acidic soil. There was a transient  $N_2O$  accumulation that was strongly controlled by pH and the response of the average  $N_2O:(N_2+N_2O)$  product ratio to pH during the entire phase of denitrification (as estimated by the  $N_2O$  index) was very similar to that for unamended soil (compare Fig. 4d with Figs 2a and 3). This demonstrates that enhancing the substrate input can indeed induce a high relative  $N_2O$  reductase activity ( $N_2OR$ ) at almost any soil pH. However, pH exerts a strong effect on the timing of  $N_2OR$  activity, which lagged behind that of nitrate reductase, nitrite reductase and nitric oxide reductase in acidic soils more than in alkaline soils.

These observations show the pervasive control of pH on the relative  $N_2OR$  activity under denitrifying conditions, and hence the propensity of soils to emit  $N_2O$ . They raise, however, more questions than answers regarding the mechanisms involved. In theory, the weak relative  $N_2OR$  activity at low pH could be due to the selection of organisms with truncated denitrification, i.e. lacking the gene coding

for  $N_2OR$  (*nosZ*). The results lend no support to this hypothesis, however, because the ratio between the gene copy numbers of *nosZ* and *nirS+nirK* was actually at a maximum in the unlimed soil (pH 4) and unaffected by pH in the limed soils (pH 5.5–8.0). It could also be due to pH control of the relative transcription of *nosZ* compared with that of *nirS* or *nirK*. Again, there is no support in the data; the relative number of *nosZ* transcripts was higher in the pH 6.1 soil than in the pH 8 soil. A more plausible explanation is that pH affects the translation or the activity of  $N_2OR$ , as demonstrated for *P. denitrificans* (L. Bergaust, M. Yuejian, L. Bakken & Å. Frostegård, unpublished data). They found that *P. denitrificans* produced an increasing relative amount of  $N_2O$  with decreasing pH, not because the relative transcription of *nosZ* was affected by low pH, but because translation or protein assembly of  $N_2OR$  was negatively affected as well as its activity.

Although mRNA extraction from environmental samples and downstream quantification techniques have been improved drastically in recent years, they still have some inherent limitations and biases, such as the efficiency of the extraction and purification methods, the universality of the primers and enzyme inhibition by humic acids in RNA and DNA extracts. In the present study, however, the focus was not on the absolute quantification of mRNA copies, but on the relative amounts of *nir* and *nos* transcripts in acid vs. alkaline soils. Although soil pH may have affected the efficiency of mRNA extraction, it is likely that this bias would be equal for the transcripts (*nosZ* and *nirS*) and the relationship between pH and observed mRNA for *nosZ:nirS* would not be biased.

Surprisingly, we were unable to detect transcripts of the Cu-containing nitrite reductase *nirK*, although the gene pool of *nirK* was of a magnitude similar to that of the cytochrome cd1 nitrite reductase *nirS*. This is in contrast to some findings from other environments. In a study of marine sediments from different locations, *nirS* was successfully amplified from all samples, while *nirK* could only be detected in some (Braker *et al.*, 2000). In contrast, *nirK* copies were detected from both DNA and mRNA in the rhizospheres of the three legumes, while no *nirS* gene or transcript could be found (Sharma *et al.*, 2005). These studies may suggest that denitrifier communities are dominated by either *nirK* or *nirS*, but our study suggests another picture, in which *nirS* and *nirK* gene copies were found in similar amounts while only *nirS* transcripts were detected. This would indicate that the microorganisms carrying *nirS* and *nirK* were equally abundant, but that only those carrying *nirS* were activated under the current experimental conditions. This could mean that it is the gene expression, rather than the gene types, which is strongly selected under different environmental conditions. We cannot exclude, however, the possibility that the *nirK* primers used in this

study (Braker *et al.*, 1998, 2000) captured only a fraction of the population of denitrifiers carrying this gene, and that the active organisms carried somewhat different *nirK* genes, whose transcripts could not be detected by our primers.

The ratio of mRNA transcript:gene abundance may describe transcriptional activity more accurately than absolute numbers. In a pure culture study of *Pseudomonas* sp. strain P51, the highest expression level of the *tcbC* gene, measured by competitive reverse transcriptase (RT)-PCR, was about 20 mRNA copies per cell (Meckenstock *et al.*, 1998). In our study, the highest gene expression ratios were < 1.0 for *nirS* and 0.1 for *nosZ* (Fig. S2), much lower than those from pure cultures. Similar ratios were found in other studies. Quantification of *tfdA* gene transcripts per *tfdA* gene copy in phenoxyacetic acid-degrading soils showed the highest ratios between 0.02 and 0.05 (Baelum *et al.*, 2008). The highest ratio recorded for the *mcrA* gene encoding methyl coenzyme M reductase in methanogens in peat soil was about 2.0 (Freitag & Prosser, 2009). The ratios found from these *in situ* measurements are all in the lower range of those reported from pure cultures, which probably indicates that only a fraction of the structurally intact cells in soils are actually able to respond to substrate inputs. It should be kept in mind, though, that pure culture studies show large variations in the ratio of mRNA transcript:gene abundance, both when comparing different genes and when comparing the same gene in different strains of bacteria (Sabersheikh & Saunders, 2004).

Using gene-specific primers in the RT-PCR is an alternative way to quantify the mRNA, which might be considered to be more efficient by producing specific cDNA fragments. We also used gene-specific primers for all the functional genes using the pH 8.0 samples, but no clear bands were found in the agarose gel. Nicolaisen *et al.* (2008) found some nonspecific PCR products by amplifying cDNA produced by gene-specific primers from soil mRNA. Although the reason is still not clear, random primers seem to be more suitable for producing cDNA sequences from soil and these can also be used for analyzing the expression of other functional genes.

In conclusion, this study demonstrated a profound effect of pH on the denitrification activity (in response to substrate addition) and on the N<sub>2</sub>O yield in soil. The denitrification product ratios [N<sub>2</sub>O:(N<sub>2</sub>+N<sub>2</sub>O)] declined from low to high pH soils in the pH range 4.0–8.0. The underlying biological mechanism was explored by assessing the abundance and expression of functional genes. Neither the gene pools (*nirS* vs. *nosZ*) nor their transcription rates could explain the observed effects of low pH on N<sub>2</sub>O reductase (N<sub>2</sub>OR) activity. This implies that the detrimental effect of low pH on N<sub>2</sub>OR occurs at a post-transcriptional level, either by interfering with translation, protein assembly or by directly affecting the activity of the functional enzyme.

Further investigations with extracted soil prokaryotes have been initiated to unravel the mechanisms involved. The investigation demonstrates the potential of combining transcription analyses with in-depth functional analyses in soil, as underscored by Freitag & Prosser (2009).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Measured N<sub>2</sub>O/(N<sub>2</sub>O+N<sub>2</sub>) ratio in the incubation flask, plotted against the amount of NO<sub>3</sub>-N recovered as (N<sub>2</sub>O+NO+N<sub>2</sub>).

**Fig. S2.** Transcription of *nirS* and *nosZ*, expressed as ratio between number of transcripts and number of the corresponding genes during the 60 h anoxic incubation (gas kinetics shown in Fig. 3).

**Fig. S3.** Melting curve of the *nosZ* gene showing a primer dimer peak at around 77 °C, the fluorescent signals were measured at 82 °C, primer dimers were completely dena-

tured at this temperature and did not adversely affect the quantification accuracy.

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