



Comparison of denitrifying communities in organic soils: kinetics of NO_3^- and N_2O reduction

Liv Holtan-Hartwig^{a,*}, Peter Dörsch^b, Lars Reier Bakken^b

^aCentre for Soil and Environmental Research, N-1432, Ås, Norway

^bDepartment of Soil and Water Sciences, Agricultural University of Norway, PO Box 5028, N-1432, Ås, Norway

Accepted 14 November 1999

Abstract

Our aim was to determine whether intrinsic differences in the denitrifier communities existed in farmed organic soils from three different sites (Germany, Sweden and Finland) on which field fluxes of N_2O had been measured continuously over 2 y. To estimate enzyme kinetic parameters (i.e. V_{\max} and K_m) for N_2O reductase, NO_3^- was first removed by a combination of soil washing and anaerobic incubation. Then the samples were incubated anaerobically (as slurries) with or without added NO_3^- , N_2O and C_2H_2 . The estimated half saturation constants for N_2O reductase were similar for all soils, and very low ($K_m = 0.1\text{--}0.4 \mu\text{M}$) compared to other investigations. In response to a prolonged anaerobic conditioning incubation (48 h), the K_m values increased significantly and similarly for all soils, suggesting a shift in the dominant members of the denitrifier communities. The ratio between the estimated V_{\max} values for NO_3^- reduction-to- N_2O and N_2O reduction-to- N_2 was much lower for the Swedish than for the other two, indicating that the community of the Swedish soil would be more efficient in reducing NO_3^- all the way to N_2 . This difference is congruent with differences in annual field fluxes. The results thus suggest that intrinsic differences in community composition of soils exist, with consequences for the emission of N_2O . Prolonged anaerobic incubation (48 h at 20°C) resulted in a convergence of the communities towards similar ratios between the two V_{\max} values, suggesting that the apparent intrinsic differences may disappear in response to such severe treatment. Thus, the persistence of such patterns may depend on the drainage capacity of the soils. The results indicate that qualities of the denitrifying community must be taken into account when trying to understand and to model field fluxes of N_2O from soils. They also illustrate how global trace gas emissions can be affected by changes in the community compositions of soils. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Denitrification; N_2O emission; Organic soils; Kinetic parameters

1. Introduction

The functioning of the denitrifying community may be a crucial factor in regulating the emission of N_2O to the atmosphere, since the emission is a net result of N_2O production and reduction. This balance between production and reduction of N_2O is controlled by diffusion conditions and the enzymatic activities of the denitrifying community. The latter is subject to short-term variations since each denitrifying organism is able

to regulate the enzyme concentration depending on the conditions (primarily by the supply of oxygen, C, NO_3^- , NO_2^- and possibly N_2O). Exposure to anaerobic conditions results in the induction of the different denitrifying enzymes, and under most circumstances, the induction of N_2O reductase appears to lag behind the others, resulting in transient accumulation (or emission) of N_2O (Firestone and Tiedje, 1979; Dendooven and Anderson, 1995).

In modelling denitrification and N_2O emission, it is often assumed that the denitrifying communities of soils are identical in terms of affinities for electron acceptors, relative reaction rates of the different steps, and the time it takes for inducing the different enzymes (under equal conditions). Most experimental papers on

* Corresponding author. Fax: +47-64-94-81-10.

E-mail address: liv.holtan-hartwig@jordforsk.nlh.no (L. Holtan-Hartwig).

denitrification and N_2O emission reflect the same implicit assumption in emphasising the processes' dependency on soil physics, chemistry and substrate supplies, rather than the composition and physiological properties of the active organisms. This bias has been challenged more or less explicitly in some papers (e.g. Firestone et al., 1980; Munch, 1989; Dendooven et al., 1996; Chèneby et al., 1998) and is discussed in a broader context by Schimel and Gullledge (1998), who suggest that the microbial community composition exerts a direct control on the fluxes of both CH_4 and N_2O , in the sense that differences in the gas fluxes between soils are at least partly attributable to differences in physiological properties of the communities. We have attempted to explore such possible differences between the denitrifying communities of soils.

The experiments were a part of a larger research programme entitled "Greenhouse Gas Emission from Farmed Organic Soils" (GEFOS), in which field fluxes of N_2O were measured intensively over 2 y on farmed organic soils in Germany, Finland and Sweden. We took soil samples from these three sites and conducted a series of laboratory experiments designed to estimate kinetic constants (V_{max} and K_m) for NO_3^- reduction (to N_2O) and N_2O reductase.

Comparisons of kinetic differences between denitrifying communities are not trivial, since the activity status of the potentially active bacteria is temporally fluctuating according to the availability of substrates and electron donors. As a result, attempts to characterise the denitrifying communities in situ might reveal profound differences. Such observations might, however, merely reflect differences in the recent exposures to drought or flooding (prior to measurements), rather than an intrinsic difference due to community composition. In order to explore possible intrinsic differences in kinetic profiles between the soils, we therefore attempted to characterise the actively denitrifying communities after a standardised set of prior treatments. Before the experiments, the soils were sieved, and stored moist at 4°C for at least 2 months. The soils were then incubated anaerobically in a slurry with glutamic acid so as to ensure ample supply of carbon and energy substrate to a significant (but unknown) fraction of the denitrifying bacteria. The kinetic measurements were conducted after complete removal of NO_3^- from the anaerobic slurries. Kinetic parameters after a brief (2-h) and a long anaerobic conditioning incubation (48 h) were compared.

2. Materials and methods

2.1. Soil

Topsoils (0–30 cm) were collected (10–12 soil cores of 7 cm dia were bulked) from the arable field research

sites of the EU project GEFOS, in Germany (48°40' N, 11°04' E), Sweden (58°20' N, 13°30' E) and Finland (62°55' N, 29°30' E). The Finnish soil was collected in May 1997 and the German and Swedish soils in October 1997. Soil and climatic properties are provided in Table 1. The soils were sieved (4 mm) and stored at field moisture in plastic bags at 4°C until used. The experiments were conducted over a period lasting from 2 to 12 months after sampling. Each type of experiment was conducted for all the three soils within 1 month.

2.2. Anaerobic incubation routines and gas sampling

All incubations and gas samplings were carried out as follows: bottles (120 ml) with soil (corresponding to 4–8 g dry weight) and 16–32 ml 1 mM glutamic acid solution were capped with butyl rubber stoppers (type 20-B3P, Chromacol Ltd, London) and metal holders. The slurries were made anaerobic by eight cycles of evacuation and flushing with 1.5 atm He (lasting a total of about 30 min); the slurries were stirred by magnetic stirring (600 rev min⁻¹) during this treatment. A final over-pressure in the bottles was released through a 0.5 mm (id) cannula which was pierced through the rubber septum. To prevent O_2 from entering during venting, the cannula was mounted on a 5 ml plastic syringe (without piston) filled with 0.5 ml distilled water. All bottles were incubated in a waterbath shaker (stroke 40 mm, 200 rev min⁻¹) at 20 °C, except for the 2 h preincubation which was conducted at 30°C. Gas samples (0.2–1.0 ml, depending on expected N_2O concentrations) were periodically withdrawn from the bottles by precision gas syringes (VICI Precision Sampling, Inc. Baton Rouge, Louisiana, USA). To prevent any O_2 from entering during this sampling, the syringes were first filled with autoclaved water and emptied so that no air was present in the cannula when piercing the septa. The gas samples were injected into He-filled (1 atm) 11.6 ml vials (10-CV, Chromacol Ltd, London) capped with butyl rubber septa. These diluted gas samples were then analysed for N_2O and CO_2 (for further details see below). Experiments for each soil and treatment were run in triplicate.

2.3. Removal of NO_3^-

We tested two methods of removing NO_3^- from the soil thereby facilitating a direct determination of V_{max} and K_m of N_2O reductase: (i) washing of soil followed by a short-term anaerobic incubation and (ii) long-term anaerobic incubation of fresh soil.

2.3.1. Washing and short-term anaerobic incubation

To enable a quick and efficient drainage of soils, we used 250 ml centrifugation buckets equipped with a

Table 1
Soil properties

	C (%)	pH (H ₂ O)	Average ground water depth (cm)	Bulk density (kg m ⁻³)	Mean temp (°C)	Annual precip. (mm)
Finnish	23	6.0	1.06	320	2.2	612
German	35	5.4	0.67	360	7.1	579
Swedish	24	7.1	0.60	270	4.9	645

screen 35 cm above the bottom of the tube and a removable bottom (Fig. 1). Glass microfibre filters (Whatman GF/C no. 1822055. Diam. 55 mm. Particle retention in liquid; 1.2 µm) were placed on the screen, and 30 g fresh soil were added to each bucket (two buckets per soil type). Then 45 ml of 1 mM glutamic acid solution was added gently onto the soil surface, to avoid dispersing the soils (which would clog the filters). The buckets were centrifuged at 1500 × g for 3–9 min, the bottom part of the tube was unscrewed, and the liquid solution was discharged. The flooding of glutamic acid solution, centrifuging and discharging of solution was repeated three times to minimise the residual NO₃⁻. The necessary centrifugation time to efficiently drain the soil increased through the washing, from 3 min (first run) to 9 min (last run). Immediately after the last centrifuge run, the soil from both buckets was pooled in a beaker and thoroughly mixed. In order to prevent new formation of NO₃⁻, the soil was kept cold during the washing (centrifuge temperature was 2°C) and the subsequent weighing procedure by using cooled (4°C) equipment and solutions, and by placing buckets and bottles on ice. The washed soil was then incubated as a slurry as described. Half of the bottles were provided with 12 ml 100% C₂H₂ in order to block the N₂O reduction step of denitrification (Balderstone et al., 1976, Yoshinari et al., 1977). The moment of addition of C₂H₂ was regarded as the start of incubation (*t* = 0). The bottles were incubated for 4.5 h at 30 ± 1°C. Washing the soil had only a

minor effect on the production and reduction of N₂O as judged from the accumulation of N₂O in the presence and absence of C₂H₂. In bottles with washed soil, the concentrations during a 350 min incubation were 4–13% lower than in bottles with unwashed soil.

2.3.2. Long-term anaerobic incubation of fresh (not washed) soil

Anaerobic soil slurries were prepared as described previously. Half of the bottles were provided with C₂H₂, and all bottles were incubated for 72 h at 20 ± 1°C. To ensure substrate for activity and growth during the whole incubation, 0.5 ml of deoxygenated 40 mM glutamic acid solution was provided through the septum with a syringe each 24 h.

The liquid phase was sampled for NO₃⁻ determination several times during the incubation. The samples were withdrawn with a syringe after a brief sedimentation of coarse soil particles by placing the bottles in a swing out rotor at 1500 rev min⁻¹ for 7 min. The NO₃⁻ content in samples of the soil slurries was determined according to Christensen and Tiedje (1988), using conversion of NO₃⁻ to N₂O by the bacterium *Pseudomonas chlororaphis*. The measurements revealed a correspondence between the stabilisation of the headspace N₂O concentrations in C₂H₂ treated bottles and the disappearance of NO₃⁻ from the slurries. We concluded that a 2-h anaerobic incubation was sufficient to remove all NO₃⁻ from the washed soil slurries, whereas 48-h anaerobic incubation was necessary to remove all NO₃⁻ from slurries of fresh soil. We decided to employ both procedures of removal of NO₃⁻, thus comparing endogenous and “induced” denitrifier communities.

The short-term NO₃⁻ removal procedure is denoted *wash-2h*. The soil slurries were allowed to equilibrate to 20°C for 30 min (waterbath shaker with thermostat) before the kinetic measurements were initiated.

The long-term NO₃⁻ removal procedure is denoted *fresh-48h*. The flushing of bottles by alternate evacuation and addition of He was repeated after this incubation to remove any N₂O before the kinetic measurements started.

2.4. *V*_{max} of NO₃⁻ reduction to N₂O

After removing NO₃⁻ from the three soils (by wash-

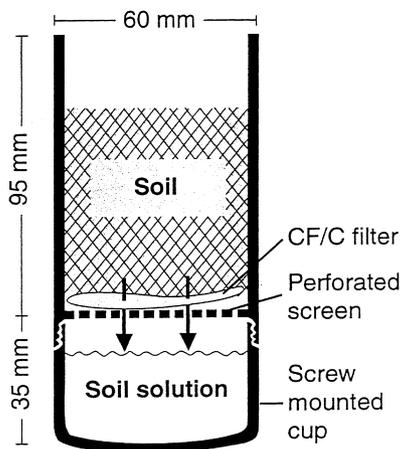


Fig. 1. Cross section of centrifugation bucket for soil washing.

2h or fresh-48h), we added 12 ml 100% C₂H₂ and deoxygenated KNO₃ solution to a final concentration of 1 mM NO₃⁻ in the slurry. The fresh-48h samples were provided with additional glutamic acid solution to a final concentration of 1 mM in the slurry (assuming that the previous aliquot had been completely consumed). Gas samples were taken every 30 min during a 3-h period. The V_{\max} of NO₃⁻ reduction was calculated from linear regressions of measured N₂O accumulation versus time for each bottle, assuming that the NO₃⁻ concentration was above enzyme saturation (Knowles, 1982).

2.5. K_m and V_{\max} of N₂O reduction

After removal of NO₃⁻, the K_m and V_{\max} for N₂O reductase was determined by two different assays, denoted single-high and five-dif, respectively.

2.5.1. Single-high assay

A single high dose injection of N₂O (1–6 μM N₂O in the soil slurry of wash-2h treated soils and 20–80 μM in fresh-48h) was introduced to triplicate bottles. Gas samples were periodically withdrawn from the headspace until near depletion of N₂O. The appropriate initial concentration of N₂O for each soil and each pretreatment had to be determined by preliminary experiments using various concentrations to determine approximate V_{\max} . Based on this V_{\max} , the initial concentrations were chosen so as to achieve a near depletion within 300 min. For each time increment between two samplings the N₂O consumption rate was calculated as $v_t = (S_n - S_{(n+1)}) / (t_{(n+1)} - t_n)$, with a corresponding substrate concentration, $S_t = (S_{(n+1)} + S_n) / 2$, where v is the apparent reduction rate of N₂O, t is the time, and S denotes the molar concentration of N₂O in liquid phase assuming equilibrium between gas and liquid phase. The subscript n and $(n + 1)$ indicate subsequent samplings.

2.5.2. Five-dif assay

A set of 15 bottles were loaded with five different N₂O concentrations (each concentration in triplicate). Five gas samples were then periodically withdrawn from each bottle during a 30-min incubation. The N₂O reduction rates for each initial concentration (0.3–40 μM) were calculated from linear regressions of the N₂O consumption in the bottles. The five-dif assay was only applied with soil slurries subjected to the fresh-48h NO₃⁻ removal procedure. This was due to the low activity and the low K_m of the fresh-2h soil (see results) which precluded this type of determination.

K_m and V_{\max} for N₂O reductase were estimated from the results of both assays by non linear regression (JMP, SAS Institute Inc.), using the function

$v = (V_{\max} * S) / (S + K_m)$, where v is the measured rate of N₂O reduction and S the calculated molar concentration of N₂O in liquid phase assuming equilibrium between gas and liquid phase. Initial equilibrium following N₂O injection was obtained by vigorously handshaking bottles for 15 s immediately after injection. This was confirmed in preliminary experiments with autoclaved soil slurries (data not shown).

2.6. Analysis and calculations of gas content

Concentrations (μl l⁻¹) of N₂O in the 11.6-ml dilution vials were determined by gas chromatography as described in detail by Sitaula et al. (1992). The amount of N₂O in the bottles was calculated according to:

$$N_2O-N (\mu g \text{ bottle}^{-1}) = C_v * ((V_h + V_g) / V_h) * ((V_v + V_g) / V_g) * (M_{w(N_2)} / V_{N_2O}(T)) * (V_h + E(T) * V_1)$$

where C_v is concentration of N₂O determined by GC (μl l⁻¹); V_h is volume of headspace in bottle (l); V_v is volume of dilution vial (11.6 ml); V_g is volume of gas sample taken from bottle to the dilution vial (l); $M_{w(N_2O-N)}$ is molecular weight of N₂ (28.0 g mole⁻¹); $V_{N_2O}(T)$ is mole volume at the experimental temperature T (l mole⁻¹); T is temperature (K); $E(T)$ is solubility constant for N₂O (ml N₂O ml⁻¹ H₂O) in water at T (Wilhelm et al., 1977; 680 μl N₂O ml⁻¹ H₂O at 293.15 K and 526 μl N₂O ml⁻¹ H₂O at 303.15 K); V_1 is volume of water in the incubation bottle (medium + soil moisture) (l).

The solubility constants for N₂O in water were tested in our laboratory and found to be in perfect agreement (<1% deviation) with the values given by Wilhelm et al. (1977).

2.7. Simulation of N₂O reduction

Simulation of N₂O reduction based on estimated kinetic parameters was performed to assess the likelihood of growth or enzyme induction, and to inspect the consequences of altered K_m in the experimental systems. The depletion of N₂O in the gas phase during incubation (no NO₃⁻ present) was simulated assuming instantaneous equilibrium between gas phase and liquid phase, and Michaelis–Menten kinetics of N₂O reduction in the liquid phase:

$$d(N_2O)/dt = DW * V_{\max} * [N_2O]_L / (K_m + [N_2O]_L)$$

where $d(N_2O)/dt$ is the rate of N₂O reduction in the whole bottle (nmoles h⁻¹). DW is the amount of soil (dry weight). V_{\max} is maximum N₂O-reduction rate (nmoles g soil dw⁻¹ h⁻¹). $[N_2O]_L$ is the molar concentration of N₂O in the liquid phase (assuming equilibrium between gas phase and liquid phase), and K_m is the half saturation constant for N₂O reductase. For

comparison of measured and simulated N₂O depletion, the model estimates of N₂O per bottle were transformed to $\mu\text{l l}^{-1}$ in the the gas phase.

3. Results

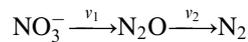
3.1. Removal of NO₃⁻

The time courses of N₂O production and consumption in short and long term incubations of washed and fresh soil, respectively, are shown in Figs. 2 and 3. As can be seen from the stabilisation of N₂O concentrations in C₂H₂ treated bottles, NO₃⁻ was depleted from washed soils after 2 h (Fig. 2), whereas 20–40 h of incubation were necessary to deplete NO₃⁻ in unwashed soil (Fig. 3). As mentioned previously, the absence of NO₃⁻ after stabilisation of the N₂O concentration was confirmed by measurements in preliminary experiments (data not shown). Assuming complete conversion of NO₃⁻ to N₂O in the presence of C₂H₂, the final N₂O–N concentrations reflect initial NO₃⁻–N concentration in the soils prior to the incubation (approximately 20, 50 and 75 $\mu\text{g NO}_3^- \text{N g}^{-1}$ in the Ger-

man, Swedish and Finnish soil, respectively; Fig. 3). The washing reduced the amounts to about 1 $\mu\text{g NO}_3^- \text{N g dw}^{-1}$ in Swedish and German and to about 2–2.5 $\mu\text{g NO}_3^- \text{N g dw}^{-1}$ in Finnish soil (Fig. 2).

The NO₃⁻ reduction in the unwashed soil slurries resulted in a slight increase in pH. We measured the pH in the slurries (consisting of soil and 1 mM glutamic acid) twice: (i) before they were made anaerobic and (ii) immediately after the 48-h anaerobic incubation. The pH in the German, Finnish and Swedish soil was found to change from 5.4 to 5.5, 6.0 to 6.5 and from 7.1 to 7.5, respectively.

The community potentials for NO₃⁻ and N₂O reduction were assessed by calculating reaction rates from the initial linear part of the progress curves in Figs. 2 and 3. The reaction rates to be estimated were



The gross production of N₂O (v_1) was estimated by linear regression of data for bottles with C₂H₂. The reduction of N₂O (v_2) was estimated by subtracting measured net production (bottles without C₂H₂) from gross production. The relationship between the esti-

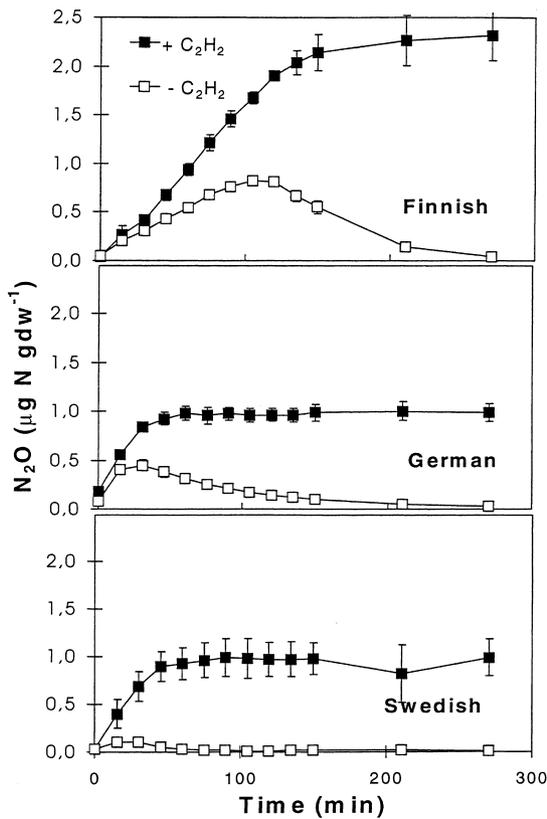


Fig. 2. Time course of N₂O concentration during anaerobic incubation of slurries of washed soil. Triplicate samples were incubated anaerobically (30°C) with (■) or without (□) addition of 10% C₂H₂. Bars indicate SD ($n = 3$).

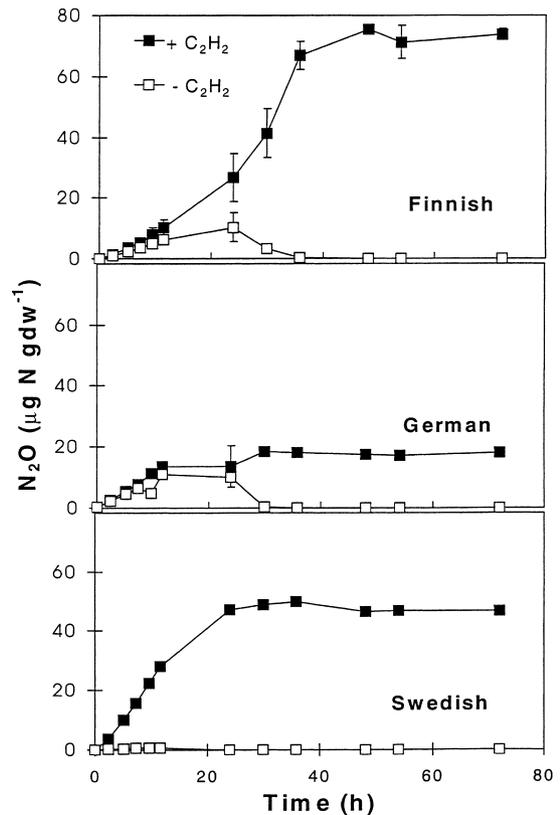


Fig. 3. Time course of N₂O concentration during anaerobic incubation of slurries of unwashed soil. Triplicate samples were incubated anaerobically (20°C) with or without C₂H₂. Bars indicate SD ($n = 3$).

mated initial N_2O production and reduction (v_1/v_2) for the short-term and the long-term incubation is shown in Fig. 4. In some of the treatments the data points were too sparse to make reliable regressions for the initial increase of N_2O concentration. Nevertheless, the two experiments reveal consistent differences between the three soils, in the sense that N_2O reduction was more efficient in the Swedish than in the two other soils.

3.2. Kinetic constants

Fig. 5 shows calculated N_2O reduction rates versus molar N_2O concentrations for the Finnish soil. Data were obtained from wash-2h and fresh-48h treated soils. For the latter treatment, short-term incubation of bottles with variable initial N_2O concentrations (five-dif assay) is compared to the N_2O depletion of a single high N_2O dose (single-high assay). Similar results were obtained with the other soils. Based on such measurements, K_m and V_{max} were estimated by non-linear regression as outlined in Section 2.5.

Estimated kinetic parameters from the different experiments are summarised in Table 2. The substrate affinity of N_2O reduction showed small differences between the soils, and a consistent increase in response to the 48-h conditioning incubation. The K_m values were much below $1 \mu M$ (0.1 – $0.4 \mu M$) after the wash-2h treatment, and 1.1 – $5.8 \mu M$ after fresh-48h. The V_{max} values of NO_3^- reduction were clearly higher after the 48-h anaerobic conditioning incubation

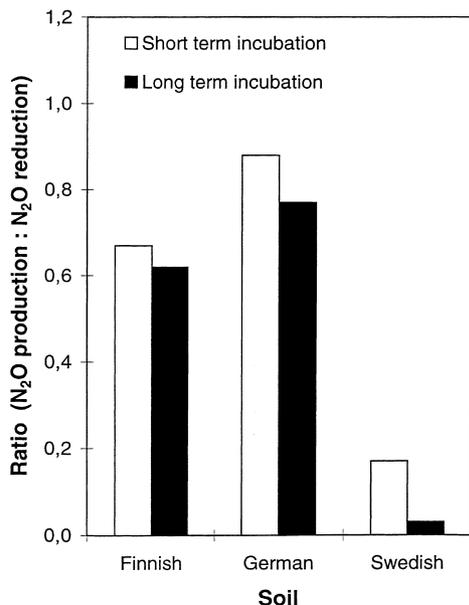


Fig. 4. Ratio between the estimated initial gross production and reduction of N_2O . The estimated rates are based on linear regression of N_2O accumulation in bottles with or without C_2H_2 (Figs. 2 and 3). See text for further explanation.

(fresh-48h) compared to the 2-h conditioning incubation (wash-2h). The increase in V_{max} for NO_3^- reduction to N_2O was much higher for the Finnish and the Swedish soil than for the German. Similarly, the V_{max} values for N_2O reduction were higher in the 48-h anaerobic conditioning incubation, and again there were major differences between the soils.

The depletion curves for N_2O (single-high) of fresh-48h soil samples suggested a substantial upshift in activity of N_2O reductase 1–3 h after N_2O injection in all soils (illustrated in Fig. 8, to be commented on further). The estimates of V_{max} prior to the upshift (“before upshift”, Table 2) are based on linear regression, whereas the V_{max} (and K_m) estimates after the upshift (“after upshift”, Table 2) are based on non-linear regression as described in Section 2.5. Enzymatic parameters estimated by the single-high assay are therefore further separated into “before upshift” and “after upshift” categories in Table 2.

To assess physiological differences between the soils, the ratios between V_{max} for the two processes ($V_{max} NO_3^-$ reduction/ $V_{max} N_2O$ reduction) were examined. Differences in the ratios suggest that a major shift in the balance between the two processes occurred in re-

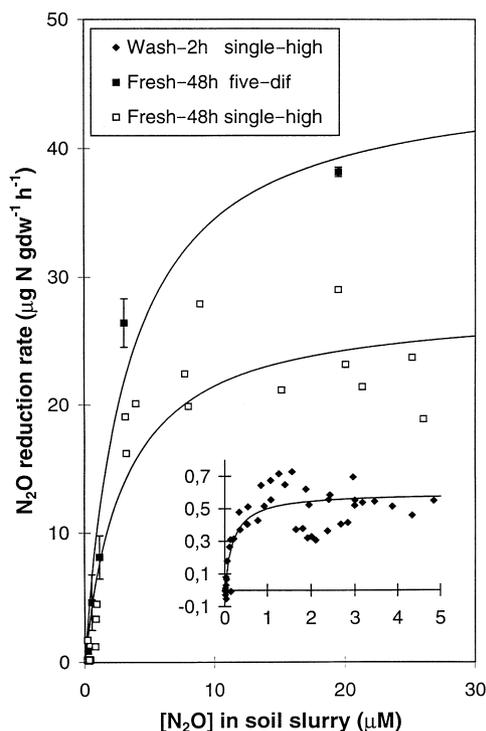


Fig. 5. Michaelis–Menten plot for the Finnish soil. Estimated N_2O reduction rates are plotted against molar N_2O concentration in the liquid phase. Results for two different conditioning incubations (wash-2h and fresh-48h) and two types of assays (single-high and five-dif) are shown. Error bars denote SD ($n = 3$). The curves are the fitted Michaelis–Menten’s function (V_{max} and K_m values are shown in Table 2). Data from the period before the upshift of N_2O reduction are not included. See text for further explanation.

Table 2

Kinetic constants for NO_3^- and N_2O reduction for slurries of Finnish, German and Swedish soils after being under anaerobic conditions for different durations (standard error (SE) of means in parantheses)

Soil	NO_3^- removal treatment ^a	NO_3^- reduction ^b V_{\max} ($\mu\text{g N g dw}^{-1} \text{h}^{-1}$)	N_2O reduction assay ^c	N_2O reduction		Calculated ratio $V_{\max} \text{NO}_3\text{red}/V_{\max}\text{N}_2\text{Ored}$
				V_{\max}^{bd} ($\mu\text{g N g dw}^{-1} \text{h}^{-1}$)	K_m^{d} (μM)	
Finnish	Wash-2 h	0.6 (0.0)	Single-high	0.6 (0.0)	0.2 (0.1)	1.0
	Fresh-48 h	8.3 (0.3)	Five-dif	45.9 (6.0)	3.3 (1.2)	0.2
			Single-high before upshift	17.7 (3.2)		0.5
			Single-high after upshift	27.9 (2.1)	3.0 (0.8)	0.3
German	Wash-2 h	0.8 (0.1)	Single-high	0.4 (0.1)	0.1 (0.1)	2.0
	Fresh-48 h	1.4 (0.1)	Five-dif	6.1 (0.3)	1.1 (0.2)	0.2
			Single-high before upshift	5.0 (0.6)		0.3
			Single-high after upshift	9.1 (0.5)	4.4 (0.9)	0.2
Swedish	Wash-2 h	1.3 (0.2)	Single-high	4.8 (0.1)	0.4 (0.05)	0.3
	Fresh-48 h	5.4 (0.5)	Five-dif	14.2 (1.2)	0.8 (0.2)	0.4
			Single-high before upshift	8.2 (1.1)		0.7
			Single-high after upshift	15.1 (2.5)	4.3 (2.3)	0.3

^a Wash-2h is 2-h anaerobic incubation of washed soil; fresh-48h is 48-h anaerobic incubation of native soil.

^b V_{\max} of NO_3^- reduction and N_2O reduction before shiftup are average values of three bottles (separate linear regressions for each bottle) with standard error.

^c See text for explanation.

^d V_{\max} and K_m of N_2O reduction after upshift were estimated by one non linear regression analysis (JMP, SAS Institute Inc.) of data from three replicate bottles.

sponse to the long (48-h) anaerobic incubation, but only for the Finnish and the German soils. In contrast, the Swedish soil already had a low ratio after the 2-h anaerobic incubation, and no further decrease took place during the 48-h incubation.

Fig. 6 shows the relationship between CO_2 production and $\text{NO}_3^-/\text{N}_2\text{O}$ reduction rates. Reasonable agreement between the measured and theoretical (dashed lines Fig. 6) relationship (based on electron balance of respiration of glutamic acid) suggests that most of the CO_2 produced derives from organisms using NO_3^- and N_2O as electron acceptors, and that fermentation was not important.

3.3. Simulation of N_2O reduction

Fig. 7 shows a N_2O depletion curve for the Swedish soil (wash-2h, single-high) together with three alternative simulations using three different K_m values (0.1, 0.4 and 0.8 $\mu\text{M N}_2\text{O}$) and the experimentally estimated V_{\max} ($=4.8 \mu\text{g N g dw}^{-1} \text{h}^{-1}$). The simulation using the experimentally determined K_m (0.4 μM) fits the data well. The other simulations ($K_m=0.1$ and 0.8 μM) were included to illustrate the sensitivity of the simulation. The residuals were included in the figure in order to inspect whether a time trend existed, which would have been created if significant induction or net growth took place (thus increasing V_{\max}). No such trend can be discerned, suggesting that the depletion curves were not influenced by growth/induction. Similar model exercises were executed for the wash-2h

single-high treatments in Finnish and German soil, and indicated a good fit between modelled and experimental V_{\max} and K_m values.

The depletion of N_2O in a single fresh-48h sample

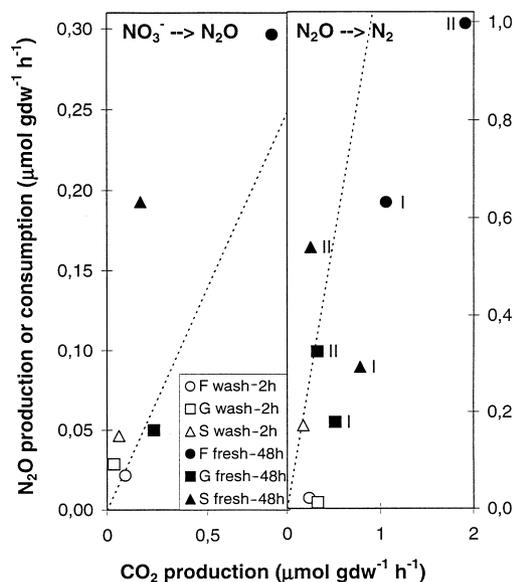


Fig. 6. CO_2 production rates versus NO_3^- and N_2O reduction rates in soil from the Finnish (F), German (G) and Swedish (S) sites. Soil slurries were exposed to a 2-h (wash-2h) and a 48-h (fresh-48h) anaerobic preincubation before the measurement. The dotted line represents the theoretical relation between N_2O -production and consumption and carbon oxidation assuming that glutamic acid was the sole C source and no fermentation occurred. 'I' denotes the period before and 'II' after the upshift in N_2O consumption.

of Swedish soil (Fig. 8) illustrates the upshift in N_2O reduction rate that was found in all soils after exposure to the 48-h conditioning incubation (fresh-48h). The deviations between measured and modelled depletion rates (residuals) were more considerable than for the wash-2 h samples shown in Fig. 7.

4. Discussion

4.1. Soil storage and removal of NO_3^-

The diverse geographical origin of the soils precluded any attempt to measure the “in situ” status of the three soil communities. But this was not a primary purpose of the study, since we wanted to investigate whether there existed more intrinsic differences between the communities. In situ enzyme activity values in soils are likely to vary over a large scale, depending on recent exposures of the communities to aerobic or anaerobic conditions. Such presumably ephemeral differences were not the focus of our study, however. A storage of the soils under equal conditions, as in our study, is then a better prior treatment than

any attempt to make undisturbed measurements of in situ activities.

For two reasons, we wanted to find a method by which all NO_3^- could be removed prior to the determination of V_{max} and K_m for N_2O reductase. One reason was to eliminate the confounding of N_2O production and reduction, hence allowing the kinetic constants to be determined directly from observed changes in N_2O concentrations. Secondly, the presence of NO_3^- could reduce N_2O reduction rate due to competition for electrons (Blackmer and Bremner, 1978).

The soil washing procedure did not remove all NO_3^- , but the amounts were sufficiently lowered to allow complete removal by a short anaerobic incubation. The necessity of an anaerobic incubation prior to measurements of kinetic constants would be unfortunate if the in situ enzyme status is to be determined, since even short incubations could allow some induction and de novo synthesis of denitrifying enzymes to take place (Smith and Tiedje, 1979). However, for our purpose, which was to compare kinetic properties of denitrifying enzymes in the three soils, enzyme induc-

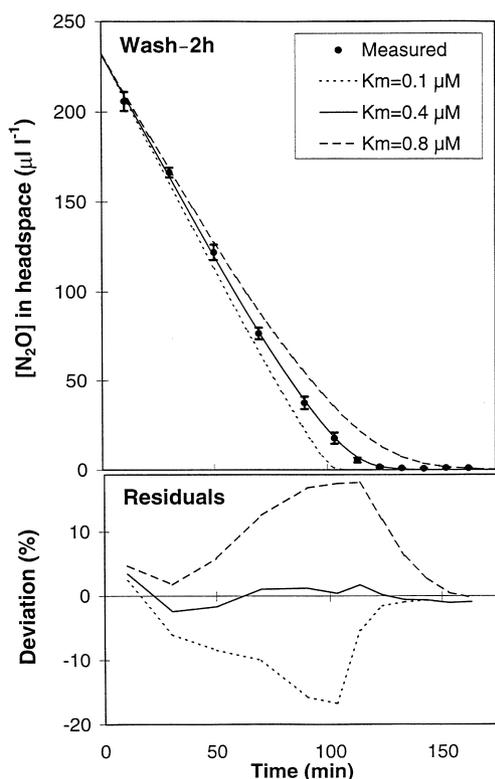


Fig. 7. Experimental and modelled N_2O depletion in wash-2h treated Swedish soil. The three model lines are based on a common V_{max} ($4.8 \mu\text{g N (g dw)}^{-1} \text{h}^{-1}$, taken from an independent determination) and three alternative K_m values. Residuals (modelled minus measured values) are shown below. Error bars denote SD ($n = 3$).

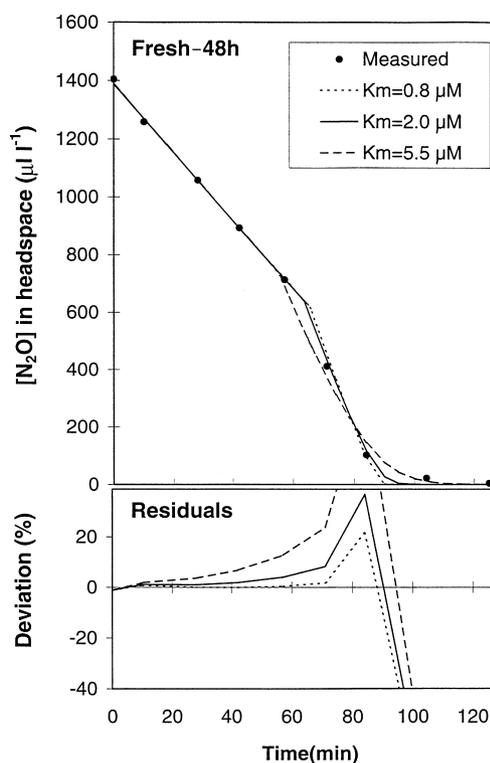


Fig. 8. Experimental (single-high, values from one bottle) and modelled N_2O depletion in fresh-48 h treated Swedish soil showing the upshift of N_2O reduction after about 1 h. The simulation prior to upshift is based on $V_{max} = 8.6 \mu\text{g N g dw}^{-1} \text{h}^{-1}$ (independently measured) and $K_m = 0.8 \mu\text{M}$. Three alternative depletion curves after the upshift are shown, based on three different K_m values and identical $V_{max} = 22.1 \mu\text{g N g dw}^{-1} \text{h}^{-1}$ (independently measured).

tion during the wash-2h prior treatment is not a serious problem.

4.2. Kinetic constants

The term denitrifying community of a soil comprises all bacteria that are capable of reducing nitrate or nitrite to NO, N₂O or N₂. In the present text, we use the term actively denitrifying community to denote the fraction that is active under the given circumstances. The use of anaerobic slurry incubation with glutamic acid as the sole added C-source is unlikely to ensure high activity of all potentially active denitrifying bacteria in the soil. This is not a problem, however, since we are presumably studying the same “functional” fraction in all soils.

It should be kept in mind that the increase in the activity values that took place during the anaerobic incubation (Table 2) is likely to be a result of three phenomena; an increased metabolic activity due to addition of glutamic acid, induction of denitrifying enzymes as a result of anaerobic conditions, and net growth. Since these phenomena are likely to occur in (partly overlapping) fractions of the community, it may cause changes in the apparent affinity for the substrate, provided that the community contains organisms with different substrate affinities.

For the sake of simplicity, we have used the term “upshift” to denote the sudden change in activity value appearing 1–3 h after N₂O injection in the fresh-48h single-high samples, acknowledging that it may include both enzyme induction and increased metabolic activity due to substrate input (glutamic acid added after 48 h).

The estimated K_m values (Table 2) were low compared to those observed for various denitrifying bacteria, which range from 0.5 to 100 μM (Conrad, 1996). High K_m values may be biased, however, due to substrate diffusion limitation which will cause erroneously high K_m estimates (Firestone, 1982). Diffusion limitation was minimised in the present experiment by the vigorous shaking of the slurries. The method of K_m calculation (discrete intervals of a differential equation) from the progress curve in single-high experiments is an approximation which will give slightly too high K_m estimates. We do not believe, however, that this bias is large enough to confound the major trends. The simulation exercises (Figs. 7 and 8) also support that the estimated K_m values are reasonable estimates for the dominant denitrifying bacteria in the soils. The fact that these K_m values shifted consistently towards higher values after the fresh-48h treatment suggests that such long anaerobic incubation caused a shift in the composition of the community of active denitrifying bacteria. This could be due to induction of new and different enzymes or rapid growth by a fraction of

the community (Schimel, 1995; Schimel and Gullede, 1998). A similar shift occurred in all soils, suggesting that this is a general phenomenon.

The V_{\max} values, and especially that for N₂O reduction, were more variable among soils (Table 2). The V_{\max} for N₂O reduction of the Finnish and German soil was fairly low (0.6 and 0.4 $\mu\text{g N g dw}^{-1} \text{h}^{-1}$) after the 2-h conditioning incubation (wash-2h), while it was about 10-fold higher (4.8 $\mu\text{g N g dw}^{-1} \text{h}^{-1}$) in the Swedish soil. The anaerobic conditioning incubation lasting for 48 h (fresh-48h) resulted in a V_{\max} that was roughly 50, 17, and 3 times higher than after the 2-h conditioning incubation for the Finnish, German, and Swedish soil, respectively.

The ratios between the V_{\max} values for NO₃⁻ reduction and N₂O reduction (Table 2) appear to be of greatest interest regarding the role of the communities in regulating the emissions of N₂O to the atmosphere. This ratio is a relative measure of each community's ability to reduce NO₃⁻ all the way to N₂ (i.e. with minimal losses of N₂O). Thus, the low ratio (0.3) for the Swedish soil after the wash-2h treatment suggests that the actively denitrifying community in this soil possesses a higher capacity for avoiding losses of N₂O compared to the Finnish and German soils. This difference between the soils matches the preliminary experiments (Figs. 2 and 3), in which practically no N₂O accumulation in the Swedish soil compared to the others was found. There is also a striking congruence between our laboratory observations and the 2 y field flux measurements for these sites, which showed much lower annual emissions for the Swedish (4.0 kg N₂O–N ha⁻¹ y⁻¹) than from the Finnish (8.3 kg N₂O–N ha⁻¹ y⁻¹) and German (14.6 kg N₂O–N ha⁻¹ y⁻¹) sites (Leif Klemetsson et al., 1999). This is of course, however, not proof of an independent control over N₂O emission by the composition of the community.

The fact that the communities converged towards similar characteristics after 48 h could be taken to suggest that the German and Finnish soils have the capacity to support an equally “efficient” denitrifying community (i.e. with low N₂O emission) as the Swedish soil, provided that they were exposed to prolonged periods of flooding. The relevance of this is uncertain, however, since most of the denitrification-derived N₂O emission from drained soils is likely to stem from organisms exposed to intermittent aerobic/microaerobic/anaerobic conditions.

Dendooven and Anderson (1995) used a mathematical procedure to estimate enzyme characteristics in their experiments. They estimated the de novo synthesis of denitrification enzymes in a clayey soil under pasture in response to anaerobiosis, and found that it appeared to occur in a sequential order: NO₃⁻ reductase was formed within 2–3 h, NO₂⁻ reductase between 2 and 12 h and nitrous oxide reductase

between 24 and 42 h. The same sequential synthesis pattern of nitrogenous oxide reductases was found by Firestone and Tiedje (1979) and Firestone et al. (1980), and is in some agreement with our observations, in the sense that the fresh-48h treatment decreased the ratio ($V_{\max} \text{NO}_3^- \text{red.}/V_{\max} \text{N}_2\text{O red.}$) compared to the wash-2h treatment (Table 2). This was, however, only observed for the Finnish and German soils. In contrast, the Swedish soil had a low ratio after the wash-2h treatment, and did not decrease in response to a long lasting anaerobic incubation prior to determination of kinetic constants.

The considerable upshift of V_{\max} for N_2O reduction found some hours after N_2O injection in fresh-48h single-high samples (Table 2 and Fig. 8) might be due to induction of additional N_2O reductase as a response to the high N_2O concentration. Some kind of recovery or resuscitation might also have taken place. A possible stress during the conditioning incubation could be starvation due to depletion of electron acceptors (N_2O and NO_3^-) towards the end of the 48-h conditioning incubation (see Fig. 3). A lack of electron acceptors during anaerobic conditions represents a severe starvation for denitrifying bacteria (Højberg et al., 1997).

In our laboratory experiments we provided unlimited amounts of carbon, constant temperature, and either no (after washing or anaerobic incubation) or a known amount of NO_3^- (1 mM KNO_3 given to samples receiving C_2H_2). The pH is the only important factor that was not kept controlled during the experiments. The reduction of N_2O is known to be more sensitive to acidic conditions than is that of NO_3^- , resulting in an increased $\text{N}_2\text{O}/\text{N}_2$ product ratio with decreasing pH (Nömmik, 1956; Koskinen and Keeney, 1982). This could be taken as an indication that the soil pH was the main reason for the efficient N_2O reduction in the Swedish soil (pH 7.1) compared to the Finnish (pH 6.0) and German (pH 5.4) soil. However, this congruence between pH and V_{\max} ratios (Table 2) disappeared after 48 h, although the measured changes in pH was negligible. This shows that a direct effect of pH on N_2O reductase does not necessarily override the effect of the composition of the active organisms/enzymes. Another point is that in the absence of nitrate, the pH has been found to have minimal effect on the $\text{N}_2\text{O}/\text{N}_2$ ratio (Blackmer and Bremner, 1978; Firestone et al., 1980). In our measurements of N_2O reductase, no NO_3^- was present, which makes it likely that the pH did not play a major role.

The results demonstrate that microbial factors may be of great importance for regulating the N_2O flux. Models of N_2O emission and other microbiological processes should take into account that different soils may harbour different communities, and the kinetics of microbial growth and enzyme induction should be modelled explicitly. We believe that the microbial com-

munity structure influences, to a greater extent than has been taken into consideration up to now, the global trace gases (Schimel, 1995; Schimel and Gullledge, 1998) and turnover of solutes in soil, and water.

Acknowledgements

The project was funded by the EU-project GEFOS and the Research Council of Norway. Technical support of Hege Oustad is gratefully acknowledged.

References

- Balderstone, W.L., Sherr, B., Payne, W.J., 1976. Blockage by acetylene of nitrous oxide reduction in *Pseudomonas perfectomarimus*. *Applied and Environmental Microbiology* 31, 504–508.
- Blackmer, A.M., Bremner, J.M., 1978. Inhibitory effect of nitrate on reduction of N_2O to N_2 by soil microorganisms. *Soil Biology & Biochemistry* 10, 187–191.
- Chèneby, D., Hartmann, A., Hénault, C., Topp, E., Germon, J.C., 1998. Diversity of denitrifying microflora and ability to reduce N_2O in two soils. *Biology and Fertility of Soils* 28, 19–26.
- Christensen, S., Tiedje, J.M., 1988. Sub-parts-per-billion nitrate method: use of an N_2O -producing denitrifier to convert NO_3^- or $^{15}\text{NO}_3^-$ to N_2O . *Applied and Environmental Microbiology* 54, 1409–1413.
- Conrad, R., 1996. Soil microorganisms as controllers of atmospheric trace gases (H_2 , CO_2 , CH_4 , OCS , N_2O , and NO). *Microbiological Reviews* 60, 609–640.
- Dendooven, L., Anderson, J., 1995. Use of a “least square” optimization procedure to estimate enzyme characteristics and substrate affinities in the denitrification reactions in soil. *Soil Biology & Biochemistry* 27, 1261–1270.
- Dendooven, L., Jackson, S.M., Anderson, J.M. 1996. Denitrification as affected by anaerobic and aerobic events. In: Van Cleemput, O., Hofman, G., Vermoesen, A. (Eds.), *Progress in Nitrogen Cycling Studies, Proceedings of the 8th Nitrogen Workshop held at the University of Ghent, 5–8 September, 1994*. Kluwer Academic Publishers, Dordrecht, pp. 537–542.
- Firestone, M.K., 1982. Biological denitrification. In: Stevenson, F.J. (Ed.), *Nitrogen in Agricultural Soils*. Agronomy Monographs, vol. 22. Soil Science Society of America, Madison, pp. 289–326.
- Firestone, M.K., Tiedje, J.M., 1979. Temporal change in nitrous oxide and dinitrogen from denitrification following onset of anaerobiosis. *Applied and Environmental Microbiology* 38, 673–679.
- Firestone, M.K., Firestone, R.B., Tiedje, J.M., 1980. Nitrous oxide from soil denitrification: factors controlling its biological production. *Science* 208, 749–751.
- Højberg, O., Binnerup, S.J., Sørensen, J., 1997. Growth of silicone-immobilized bacteria on polycarbonate membrane filters, a technique to study microcolony formation under anaerobic conditions. *Applied and Environmental Microbiology* 63, 2920–2924.
- Klemedtsson, L., Weslien, P., Kasimir Klemedtsson, Å., Silvola, J., Maljanen, M., Martikainen, P., Dörsch, P., van der Pol-van Dasselaar, A., Oenema, O., Corré, W., Holtan-Hartwig, L., Bakken, L., Christensen, S., Priemé, A., Jensen, N.O., Klein Gunnewiek, H.J.T., Leffelaar, P.A. 1999. Greenhouse gas emissions from farmed organic soils. In: Valentini, R., Brüning, C. (Eds.), *Greenhouse Gases and their Role in Climate Change: The Status of Research in Europe*. EUR 19085. Office for Official Publication of the European Community, Luxembourg, pp. 57–65.

- Knowles, R., 1982. Denitrification. *Microbiological Reviews* 46, 43–70.
- Koskinen, W.C., Keeney, D.R., 1982. Effect of pH on the rate of gaseous products of denitrification in a silt loam soil. *Soil Biology and Biochemistry* 14, 1165–1167.
- Munch, J.C., 1989. Organism specific denitrification in samples of an Udifluent with different nitrate concentrations. *Zeitschrift für Pflanzenernaehrung und Bodenkunde* 152, 395–400.
- Nõmmik, H., 1956. Investigation on denitrification in soil. *Acta Agriculturae Scandinavica* 6, 195–228.
- Schimel, J., 1995. Ecosystem consequences of microbial diversity and community structure. *Ecological Studies* 113, 239–254.
- Schimel, J.P., Gullledge, J., 1998. Microbial community structure and global trace gases. *Soil Biota and Global Change* 4, 745–758.
- Sitaula, B., Luo, J., Bakken, L.R., 1992. Rapid analysis of climate gases by wide bore capillary gas chromatography. *Journal of Environmental Quality* 21, 493–496.
- Smith, M.S., Tiedje, J.M., 1979. Phases of denitrification following oxygen depletion in soil. *Soil Biology & Biochemistry* 11, 261–267.
- Wilhelm, E., Battino, R., Wilcock, R.J., 1977. Low-pressure solubility of gases in liquid water. *Chemical Reviews* 77, 219–262.
- Yoshinari, T., Hynes, R., Knowles, R., 1977. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. *Soil Biology & Biochemistry* 9, 177–183.