RESEARCH ARTICLE

Production of NO, N_2O and N_2 by extracted soil bacteria, regulation by NO_2^- and O_2 concentrations

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Abstract

The oxygen control of denitrification and its emission of $NO/N_2O/N_2$ was investigated by incubation of Nycodenz-extracted soil bacteria in an incubation robot which monitors O_2, NO, N_2O and N_2 concentrations (in $He+O_2$ atmosphere). Two consecutive incubations were undertaken to determine (1) the regulation of denitrification by O_2 and NO_2^- during respiratory O_2 depletion and (2) the effects of re-exposure to O_2 of cultures with fully expressed denitrification proteome. Early denitrification was only detected (as NO and N_2O) at $\leq$ 80 μM O_2 in treatments with NO_2^-, and the rates were three orders of magnitude lower than the rates observed after oxygen depletion (with N_2 as the primary product). When re-exposed to O_2, the cultures continued to denitrify (8–55% of the rates during the foregoing anoxic phase), but its main product was N_2O. The N_2O reductase activity recovered as oxygen was being depleted. The results suggest that expression of the denitrifying proteome may result in significant subsequent aerobic denitrification, and this has profound implications for the understanding and modelling of denitrification and N_2O emission. Short anoxic spells caused by transient flooding during rainfall, could lead to subsequent unbalanced aerobic denitrification, in which N_2O is a major end product.

Introduction

Denitrification is the sequential dissimilatory reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2) catalysed by nitrate reductases (NaR or Nap), nitrite reductase (NiR), nitric oxide reductase (NoR) and nitrous oxide reductase (N_2OR), respectively (Zumft, 1997). Transient accumulation of intermediates (NO_2^-, NO and N_2O) is often observed at the onset of denitrification, which has been ascribed to enzyme kinetics, either alone (Betlach & Tiedje, 1981; Almeida *et al.*, 1997) or together with sequential gene expression (Philippot *et al.*, 2001; Bakken & Dörsch, 2007). Production of the intermediates NO and N_2O is of concern because of their role in the destruction of stratospheric ozone (Badr & Probert, 1993) and because N_2O is a potent greenhouse gas. Denitrification was traditionally thought to be inhibited in the presence of O_2 as O_2 is preferred over NO_3^- as a terminal electron acceptor during respiration (Knowles, 1982; Lloyd, 1993). However, there is now substantial evidence for the simultaneous respiration of O_2 and NO_3^- by different genera of cultured bacteria within the *Alpha-*, *Beta-* and *Gammaproteobacteria*, confirming the occurrence of aerobic denitrification under both alternating oxic/anoxic phases and fully aerated conditions (Robertson & Kuenen, 1984; Lloyd *et al.*, 1987; Robertson *et al.*, 1989, 1995; Bonin & Gilewicz, 1991; Baumann *et al.*, 1996).

Specific investigations of aerobic denitrification have mainly employed pure cultures of bacterial strains isolated from soils (Lloyd *et al.*, 1987; Patureau *et al.*, 2000), marine sediments (Lloyd *et al.*, 1987) or wastewater treatment plants (Robertson & Kuenen, 1984; Takaya *et al.*, 2003). N_2O and N_2 have been shown to be produced in bacterial cultures under O_2 concentrations ranging from near anoxic to *c.* 90% air saturation (Robertson & Kuenen, 1984; Körner & Zumft, 1989). The most studied denitrifying strain is *Paracoccus pantotrophus* (formerly *Thiosphaera pantotropha*) which can produce N_2O and N_2 during corespiration of O_2 and NO_3^- (Robertson & Kuenen, 1984; Bell & Ferguson, 1991). A periplasmic respiratory nitrate reductase (Nap) is

synthesized during aerobic growth of *P. pantotrophus* and is also expressed by this organism under anoxic conditions (Bell *et al.*, 1990), although 'anaerobic' denitrification generally proceeds via a membrane-bound (NaR) enzyme (Bell *et al.*, 1990; Sears *et al.*, 1993). All other enzymes involved in aerobic denitrification are believed to be the same as for anaerobic denitrification (Robertson & Kuenen, 1990).

Constitutive expression of NiR and NoR can be understood as a protective mechanism against cytotoxic concentrations of NO_2^- and NO (Knowles, 1982). In contrast there appears to be no physiological gain from N_2O reduction while O_2 is nonlimiting. In accordance with this there is evidence from culture experiments that N_2O is the predominant end product of denitrification by most denitrifiers in the presence of O_2 (Otte *et al.*, 1996; Takaya *et al.*, 2003), with several culture studies having demonstrated higher ratios of N_2O-to-N_2 with increased O_2 concentration. This has been attributed to a greater O_2 sensitivity of the N_2O reductase (Hochstein *et al.*, 1984; Otte *et al.*, 1996), suggesting, if all other enzymatic rates are similar to the anaerobic process, that aerobic denitrification could be of major environmental significance as a source of N_2O, especially if it is 'the rule rather than the exception' (Lloyd *et al.*, 1987).

Despite this mounting evidence, denitrification in soil is still considered as an anaerobic N-oxide reduction pathway, and modelled accordingly (Bakken & Dörsch, 2007). Here, we report on two experiments in which, for the first time, an indigenous soil bacterial community was used to investigate whether denitrification could proceed in the presence of O_2. Such experiments are not feasible with intact soil or soil slurries, because anoxic microsites may occur in such systems despite an overall oxic environment. In the present experiment, we used well-dispersed bacterial cells extracted from soil by density gradient centrifugation (Bakken & Lindahl, 1995), and incubated them in a robotized incubation system which allows a monitoring of the oxygen concentration as well as of the net production of the three gaseous products of denitrification, NO, N_2O and N_2 (Molstad *et al.*, 2007; Bergaust *et al.*, 2008).

The objectives were to determine, under controlled environmental conditions, the regulation of aerobic denitrification by changes in O_2 and NO_2^- concentrations (Experiment 1) and to determine the kinetics of NO, N_2O and N_2 production under transient changes in O_2 concentration (Experiment 2). NO_2^- was hypothesized to be an important trigger for aerobic denitrification (Experiment 1) as it has previously been shown to induce NiR and N_2OR (Körner & Zumft, 1989) and micro-aerobic denitrification in *Agrobacterium tumefaciens* (Bergaust *et al.*, 2008). Aerobic denitrification was hypothesized to be greater when switching from anoxic to oxic conditions (Experiment 2), driven by the nitrogen reductases synthesized during the anoxic phase (Körner & Zumft, 1989; Otte *et al.*, 1996). Because ammonia-oxidizing bacteria (AOB) are able to produce N_2O, both under oxic (Jiang & Bakken, 1999) and anoxic conditions (Shaw *et al.*, 2006), we included treatments with 10 Pa acetylene (C_2H_2) in the headspace to inhibit the metabolism of these bacteria. To our knowledge, there is no evidence that Crenarchaeal ammonia oxidation (Nicol *et al.*, 2006) differs from bacterial ammonia oxidation regarding sensitivity to C_2H_2 (Hayatsu *et al.*, 2008).

Materials and methods

Extraction of bacteria from soil

To be able to identify the occurrence of aerobic denitrification, it is vital to adequately control environmental conditions, particularly the O_2 concentration the bacterial cells are exposed to. To facilitate this, bacteria were separated from soil particles, and incubated as dispersed stirred suspensions in gas-tight flasks. Peat soil, classified as a Sapric Histosol (FAO, 1998) was sampled from Fureneset in Fjaler (Western Norway). This soil had 400 m^{-3} ha^{-1} of shell sand mixed into the top 30 cm in 1978 and a pH of 7.1 (Sognnes *et al.*, 2006). Separation was undertaken by dispersion and subsequent density gradient (Nycodenz) centrifugation (7500 rev min^{-1}, 2 h at 6 °C), according to the method of Bakken & Lindahl (1995). The method was slightly modified in that a density of 1.26 g mL^{-1} (Nyocenz cushion) was used, based on preliminary experiments with varying densities of the Nycodenz cushion (1.18–1.31 g mL^{-1}). We found that with the present organic soil, higher densities than 1.26 resulted in more impurities and the presence of a few cell aggregates (direct fluorescence microscopic counts of acridine orange stained cells), which were important to be avoided in this experiment. A density of 1.26 g mL^{-1} eliminated this problem and a reasonable yield of cells (1.7×10^9 cells g^{-1} soil, 7.5% of total numbers in the soil).

Culture conditions and general incubation procedure

Culturing was done in sterile serum flasks (120 mL) with 50-mL liquid medium and teflon magnetic stirrers. The medium contained 25 mM HEPES buffer, 1.55 mM KH_2PO_4, 3 mM $NH_4SO_4^-$, 5 mM glucose, 2 mM KNO_3^-, 1.76 mg L^{-1} EDTA, 10 mg L^{-1} $ZnSO_4$, 5 mg L^{-1} $FeSO_4$, 1.5 mg L^{-1} $MnSO_4$, 0.4 mg L^{-1} $CuSO_4$, 0.25 mg L^{-1} $Co(NO_3^-)_2$, 0.15 mg L^{-1} H_3BO_3, 1 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} thiamine and 1 mg L^{-1} biotin. The pH of the medium was adjusted to 7.1 with 1 M NaOH. The flasks were capped with butyl-rubber septa and gas-tight aluminium crimp seals with a small amount of laboratory grease placed around the rim of the flask to further reduce any potential leaks. Flasks were repeatedly evacuated and filled with helium (He) (five cycles) under constant stirring to remove most background gases

from the water phase and headspace. Headspace O_2 concentration was adjusted by injecting O_2 (100% v/v) with a gas-tight syringe to achieve the desired initial incubation concentrations. The final overpressure in the flasks was released through a 16-mm needle attached to a syringe without a plunger. To prevent any external air entering the flask the syringe was filled with a few milliliters of water. The flasks were then inoculated by injecting 1.6 mL of the suspensions of extracted soil bacteria containing 10^{10} cells (total microscopic counts), resulting in a final cell density of 2×10^8 cells mL^{-1}, and incubated in the robotized incubation system described in detail by Molstad *et al.* (2007). In short, the system consists of a thermostated water bath (15 °C) with a submersible magnetic stirrer (Variomag HP15, H+P Labortechnique GmbH, Munich, Germany) for continuous stirring of 15 serum flasks. Headspace gas is sampled and analysed automatically by an autosampler (Gilson Model 222, Gilson, leBel France) and a Gilson Minipuls3 peristaltic pump attached to a MicroGC (Varian CP4900). The gas chromatograph (GC) has two columns; a 10 m poraPLOT U for N_2O and CO_2 analysis (oven temperature 36 °C) and a 20 m 5 Å Molsieve (oven temperature 50 °C) for N_2 and O_2 analysis. The outlet of the GC injection loop is coupled to a T-piece junction with a continuous He flow, which carries sample gas further into the inlet of a chemiluminescence NO_x-analyser (Model 200A, Advanced Pollution Instrumentation, San Diego, CA), for the determination of NO concentrations. After gas sampling, the peristaltic pump is reversed thus replacing the sampled gas with an equal volume of He. Thus, the sampling does not alter the headspace gas pressure, but leads to a significant dilution (3%) of the headspace gas. This dilution was taken into account when calculating rates of gas production. O_2 concentrations in the liquid are calculated as a function of measured O_2 transport rate from headspace to the liquid, and an empirically determined transport coefficient, as described below.

Experimental conditions

Two experiments were undertaken. In the first, cultures were incubated with different initial headspace O_2 concentrations (0.1, 5.2 and 20 vol% O_2 in headspace; 1–2, 80 and 300 μM O_2 in liquid) and different concentrations of NO_2^- (0, 0.1, 0.5 and 2 mM). Duplicates of the 5.2 vol% O_2 treatment were amended with C_2H_2 (10 Pa), generated from calcium carbide, to inhibit any NO and N_2O production from NH_3 oxidation. Headspace samples were taken automatically every 2 h over a 118-h period. In the second experiment the treatments with an initial O_2 level of 5.2 vol% were re-exposed to their initial O_2 (5.2 vol%, 80 μM) and NO_2^- concentrations (0, 0.1, 0.5 and 2 mM NO_2^-) once anoxic conditions had been achieved and all NO_3^- and NO_2^- had been reduced. To ensure ample amounts of carbon substrates during this second incubation, 20 mL of the culture was removed and replenished with new medium (same component concentration as above). The flask was resealed with a new septum, flushed with He (repeated evacuation and filling as described above), adjusted to 5.2 vol% headspace O_2 concentration, refilled with 10 Pa C_2H_2 (those which had C_2H_2 during the first incubation), and incubated as previously in Experiment 1.

Initial traces of NO were detected in response of NO_2^- additions, and we tested whether this could derive from chemical decomposition. Flasks were inoculated with autoclaved suspensions of soil bacteria (same concentrations as in the culture experiment), and incubated with and without 2 mM NO_2^- and at two oxygen concentrations (near-zero and 5.2 vol% O_2).

Data analysis

Measured gas concentrations were corrected for dilution by He injection, and influx per injection and leaks through tubing and septa determined from parallel flasks with He atmosphere and sterile medium (see Molstad *et al.*, 2007 for details). This correction was necessary to obtain correct estimates of oxygen consumption and cumulative nitrogen-gas production. Published gas-solubility coefficients (Wilhelm *et al.*, 1977) were used to calculate the total amount of gas per flask (gas+liquid phase), assuming equilibrium between headspace and liquid, which is acceptable for all gases except oxygen (Molstad *et al.*, 2007). To obtain correct estimates of O_2 concentrations in the bulk liquid during oxygen depletion, the transport rate was taken into account. In theory, the O_2 transport between the liquid phase and the headspace is a function of the concentrations in the two phases, O_2 solubility, and a coefficient for transport of O_2 between the liquid and the headspace:

Transport between liquid and headspace

$$V = k_t \times (k_H \times P_{O_2} - [O_2]) \quad (1)$$

where V is the transport rate (mol O_2 s^{-1}); k_t is the coefficient for transport from gas to liquid (L s^{-1}); P_{O_2} is the O_2 partial pressure in headspace (atm); k_H is the solubility of O_2 in water (mol L^{-1} atm^{-1}); $[O_2]$ is O_2 concentration in the liquid (mol L^{-1}). k_t was determined previously to be 2.75×10^{-4} L s^{-1} for the conditions (temperature, liquid volume and stirring) used in the present experiment (Molstad *et al.*, 2007). Solving (1) for $[O_2]$ gives $[O_2] = k_H P_{O_2} - V/k_t$, which was used to calculate the average O_2 concentration in the liquid for each time increment between two samplings (estimating V by the measured decline in P_{O_2}).

For well-dispersed suspensions of single cells, the O_2 concentration at the cell surface is 90–98% of that in the bulk liquid (see Bergaust *et al.*, 2008 for details). This

condition was met at the onset of the first incubation of cells extracted from soil. In fact, the presence of flocculated cells was confirmed by visual inspection of the flasks at the end of the incubation experiment, although the majority of cells appeared to be well dispersed. Cells contained in aggregates could in theory experience much lower O_2 concentrations than that calculated for the bulk liquid.

Relative enzyme rates ($\mu mol\ N\ L^{-1}\ h^{-1}$) for NiR, NoR and N_2OR were calculated as follows:

$$N_2OR = \frac{\Delta N_2}{t}$$

$$NoR = N_2OR + \left(\frac{\Delta N_2O}{t}\right)$$

$$NiR = NoR + \left(\frac{\Delta NO}{t}\right)$$

where Δ signifies the change in gas species ($\mu mol\ flask^{-1}$) over time (t).

Results

Experiment 1: NO, N_2O and N_2 production with varying initial O_2 and NO_2^- concentrations

In the treatments without NO_2^- (Fig. 1), detectable accumulation of NO, N_2O and N_2 occurred as O_2 was near to depletion in all treatments. In the treatments with initial O_2 at 80 and 300 μM, detectable NO accumulation started as the O_2 concentration reached 7 and 10 μM, respectively. In the treatment with initial O_2 at 1–2 μM, detectable NO accumulation started as O_2 reached 0.4–0.5 μM. N_2O accumulation coincided with that of NO, whereas detectable N_2 accumulation started *c.* 10 h later in all treatments.

The addition of 2 mM NO_2^- (Fig. 2 vs. Fig. 1) clearly retarded O_2 consumption at low O_2 concentrations (Fig. 2a vs. 1a and Fig. 3), induced significant NO accumulation from time zero at the two lowest initial O_2 treatments and induced accumulation of N_2O from time zero in the 1–2 μM O_2 treatment (Fig. 2c). Thus, at the lowest initial O_2, NO_2^- induced a transient accumulation of NO (initial rates 1.5 nmol NO per flask h^{-1}) and a sustained accumulation of N_2O (*c.* 1 nmol N_2O per flask h^{-1}) throughout the first 80 h of incubation. In contrast, at 80 μM initial O_2, NO_2^- induced an early but transient NO accumulation (initial rate *c.* 0.5 nmol NO per flask h^{-1}). Thereafter neither NO nor N_2O accumulation could be detected until O_2 reached the same low concentrations as for the treatments without NO_2^- (5–10 μM, Fig. 2).

Figure 3 allows a closer inspection of the effect of NO_2^- at 80 μM initial O_2. First, it caused an initial transient aerobic NO accumulation, proportional with the NO_2^- concentration (Fig. 3b). In contrast, initial NO accumulation (0–20 h) in flasks with sterilized suspensions of soil bacteria and 2 mM NO_2^- was $< 10\%$ of that in the nonsterile cultures (results not shown). As found for the other oxygen treatments (Fig. 2 vs. Fig. 1), 2 mM NO_2^- caused a retardation of oxygen consumption, whereas lower NO_2^- concentrations did not (Fig. 3a). It also caused a similar retardation of the

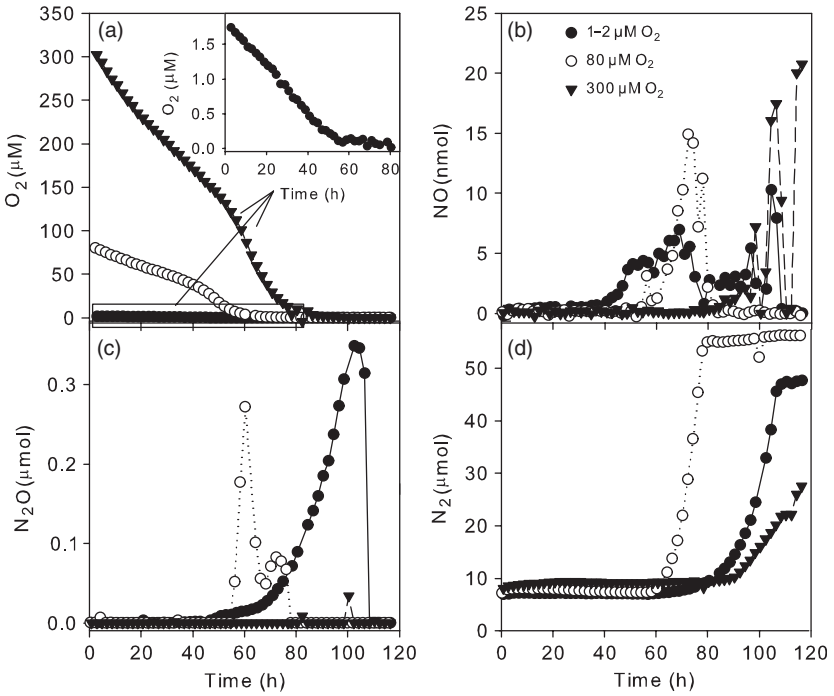

Fig. 1. First incubation of cells extracted from soil, medium without NO_2^-. The graphs show oxygen concentrations in the liquid (a) and total amounts of nitrogen gases per flasks (b–d). The different initial oxygen treatments are indicated in the graph. All media contained 2 mM NO_3^-.

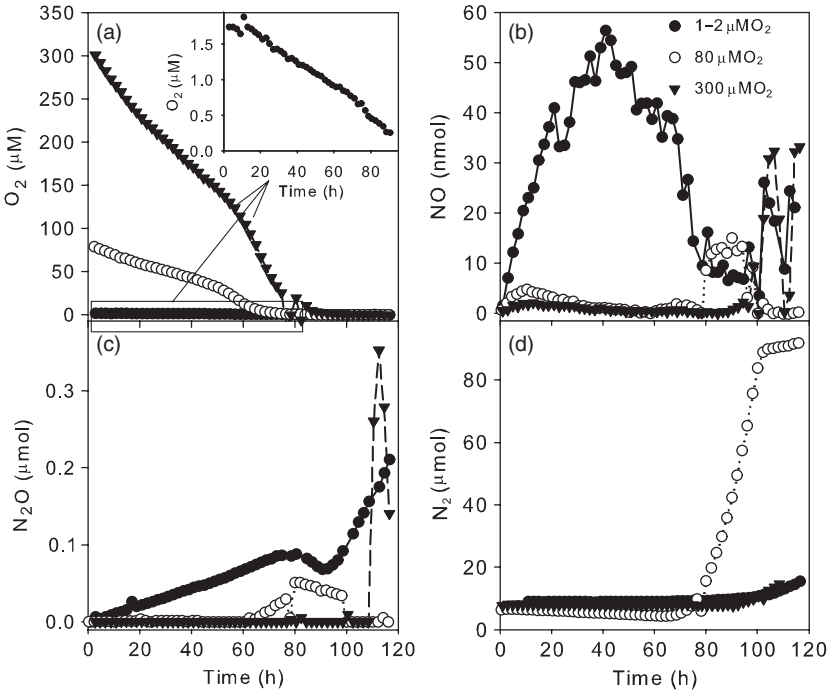

Fig. 2. First incubation of cells extracted from soil, medium with 2 mM NO_2^-. The graphs show oxygen concentrations in the liquid (a) and total amounts of nitrogen gases per flasks (b–d). The different initial oxygen treatments are indicated in the graph. All media contained 2 mM NO_3^- and 2 mM NO_2^-.

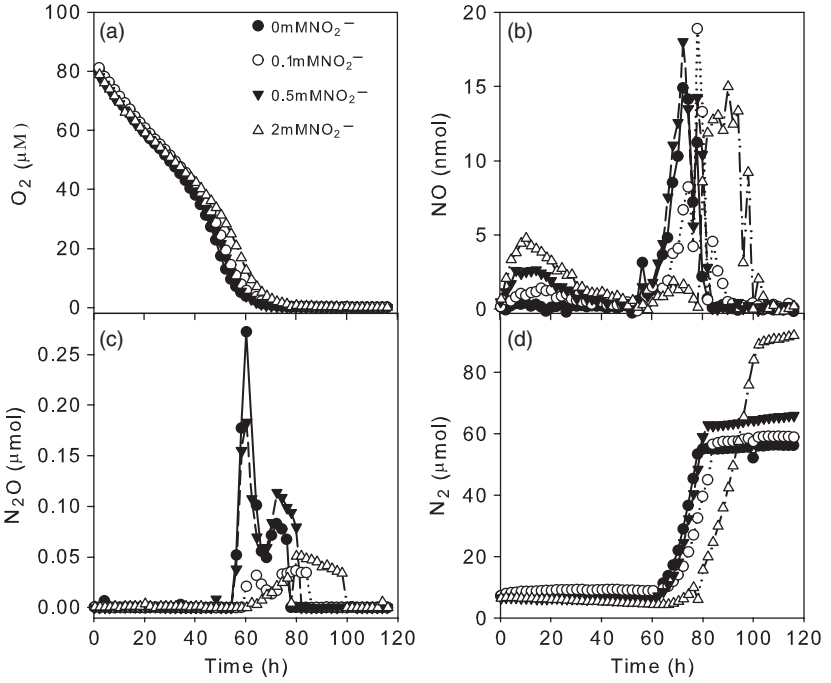

Fig. 3. First incubation, effects of varying concentrations of NO_2^-. Treatment with initial 80 μM O_2 and no C_2H_2. The graphs show oxygen concentrations in the liquid (a) and total amounts of nitrogen gases per flasks (b–d). The different NO_2^- treatments are indicated in the graph.

appearance of both NO, N_2O and N_2. Detectable N_2 production for all four treatments commenced 5–10 h later than NO and N_2O accumulation (Fig. 3d) and by the end of the incubation 90–97% of added N (NO_3^-+NO_2^-) was recovered as N_2.

Once rapid denitrification was initiated, driven by O_2 depletion, the products were completely dominated by N_2, and the rates were similar for all treatments: 2–3 μmol N_2 per flask h^{-1}. These rates are three orders of magnitude higher than the detected net rates of NO and/or

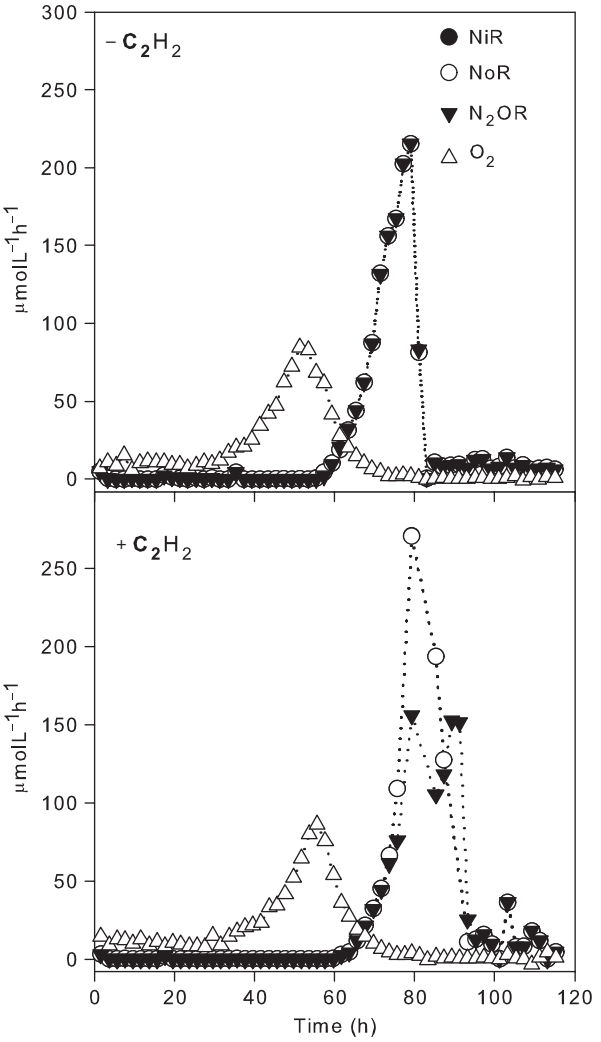

Fig. 4. Effects of 10 Pa C_2H_2 during the first incubation of extracted cells. The graphs show the estimated rates of reduction of the different electron acceptors (μmol O_2 L^{-1} h^{-1} and μmol N L^{-1} h^{-1} for the nitrogen species) for incubation with and without 10 Pa C_2H_2 in headspace (indicated in graphs). Initial O_2 was 5.2 vol% in headspace, the medium contained 2 mM NO_3^- and 0.5 mM NO_2^-. NiR, rate of NO_2^- reduction to NO; NoR, rate of NO reduction to N_2O; N_2OR, rate of N_2O reduction to N_2; O_2, rate of O_2-reduction.

N_2O accumulation in the various treatments during the first aerobic part of the incubation.

For all the treatments in which N_2 accumulation reached a plateau (Figs 1d, 2d and 3d), the final N_2 accumulation accounted for 90–97% of the added NO_3^- or NO_2^-. In general, the transient accumulation of NO and N_2O was marginal compared with the production of N_2. This implies that throughout the entire period with active denitrification, most of the NO_3^- or NO_2^- was efficiently reduced to N_2, i.e. the rates of NO and N_2O reduction were almost equal to the rate of NO_2^- reduction to NO. This is illustrated in Fig. 4, which shows the estimated rates of O_2 and N-oxide reduction in the 80 μM O_2 treatment with 2 mM NO_3^- and 0.5 mM NO_2^-, with and without 10 Pa C_2H_2 in the headspace. The O_2 respiration peaked around 50 h for both treatments, then declined with corresponding increases in rates of the N-oxide reductases. In the absence of C_2H_2, the rates of NO and N_2O reduction (NoR and N_2OR) were practically equal to the rates of NO production (NiR) during active denitrification, thus the three curves shown in Fig. 4 are identical (hence seen as a single graph). In the presence of 10 Pa C_2H_2, however, N_2O reduction appeared to be partly inhibited during the mid-phase of the most active denitrification, i.e. NO reduction rates significantly exceeded the rate of N_2O reduction. This reflected a higher transient N_2O accumulation in the presence of 10 Pa C_2H_2 (16–31 μmol N_2O per flask, data not shown).

The NO concentrations in the liquid can be calculated from the data presented in Figs 1–3; 1 nmol NO per flask is equivalent to 0.75 nM NO in the liquid. Thus, NO concentrations were typically in the 5–30 nM range during active denitrification.

Experiment 2: Re-exposure to 80 μM O_2

Here, the cultures which had been incubated with initial 5.2 vol% O_2 in headspace (80 μM O_2) in Experiment 1 were re-exposed to 5.2 vol% in headspace, 2 mM NO_3^- and 0, 0.1, 0.5 or 2 mM NO_2^- (20 mL of the culture was removed and replaced with fresh medium, thus the initial cell density was 60% of that at the end of Experiment 1). Respiration depleted O_2 at nearly constant rate until reaching 20 μM (Figs 5a and 6a). During this oxic phase, NO concentrations fluctuated between 4 and 10 nmol per flask (i.e. 1–7 nM in the liquid) which was significantly lower than the initial NO accumulation in Experiment 1 (Fig. 2b). As the O_2 was depleted, the NO concentrations increased rapidly. Higher levels were reached in the treatments without C_2H_2 (40–140 nmol per flask), compared with that in the flasks with 10 Pa C_2H_2 (20–50 nmol per flask), and for both treatments the levels increased with increasing concentrations of NO_2^- (Figs 5b and 6b). The N_2O accumulation started from the very beginning of the incubation, and peaked at high levels (4–12 μmol N_2O per flask) before O_2 was depleted. The peak concentrations varied between treatments, and were not systematically related to the NO_2^- concentrations nor to the presence/absence of C_2H_2 in the headspace. At the onset of anoxic conditions, N_2O was efficiently reduced to zero in the treatments with C_2H_2 (Fig. 6), whereas it increased in the absence of C_2H_2 (with one exception). The N_2 accumulation rate was moderate through the first 20 h of the oxic phase (0.15–0.47 μmol N_2 h^{-1} per flask), and then increased gradually as O_2 was being depleted. The response pattern is more clearly visible

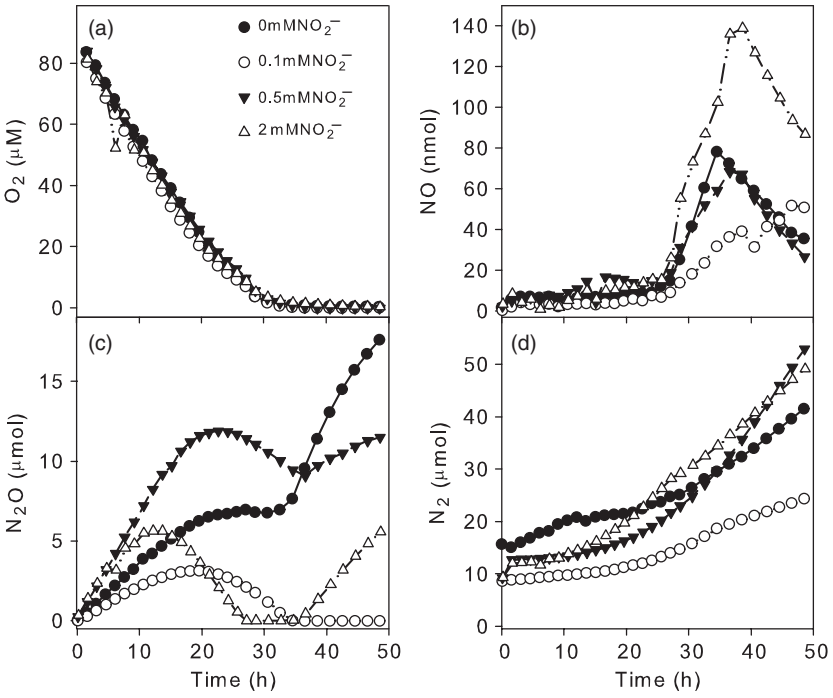

Fig. 5. Second incubation. Cell suspensions from the first incubation, during which oxygen was depleted, were amended with fresh medium and a new dose of O_2, and the original concentrations of NO_3^- and NO_2^- were restored. Results for treatments without C_2H_2. Graphs are equivalents to those in Figs 1–3. Treatments are indicated in the graph. Each culture was exposed to the same initial concentration of O_2 and NO_2^- as it experienced in the first incubation.

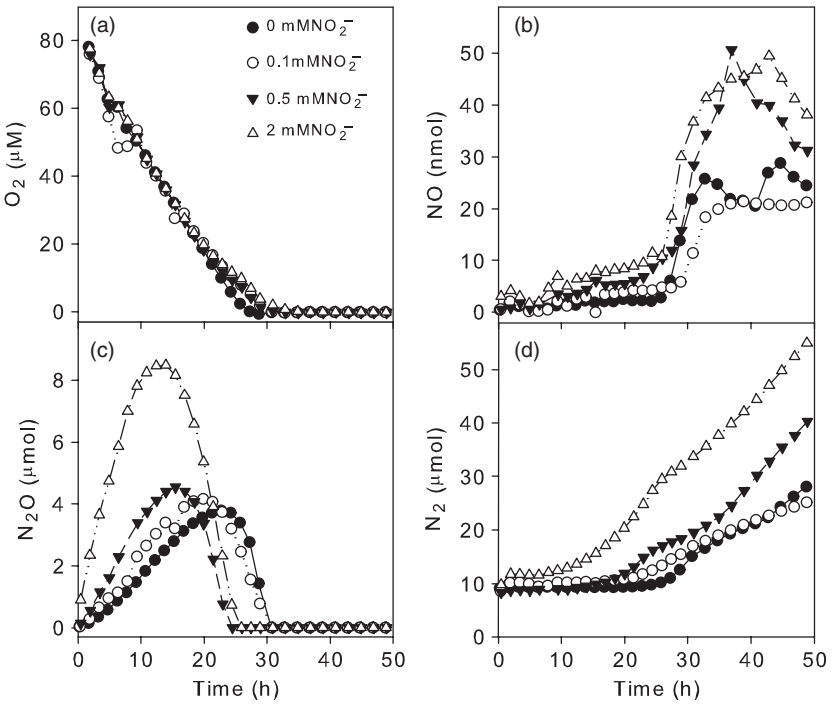

Fig. 6. Second incubation. Treatments equivalent to Fig. 5, but with 10 Pa C_2H_2 in headspace.

in Fig. 7, where the rates of O_2 and N-oxide reduction are shown. The rates of NO_2^- reduction to NO (NiR) are not visible because it practically equals that of NO reduction to N_2O. The rates of N_2O reduction to N_2 (N_2OR), however, were lower than that of NiR and NoR during the first 20 h of the oxic phase in both treatments (i.e. with and without 10 Pa C_2H_2) (Fig. 7). The estimated N_2OR activity was strongly negatively correlated with the bulk concentration of oxygen ($r = -0.94$, $P < 0.01$). The O_2 respiration rates peaked at around 30 h, for both treatments, then declined rapidly.

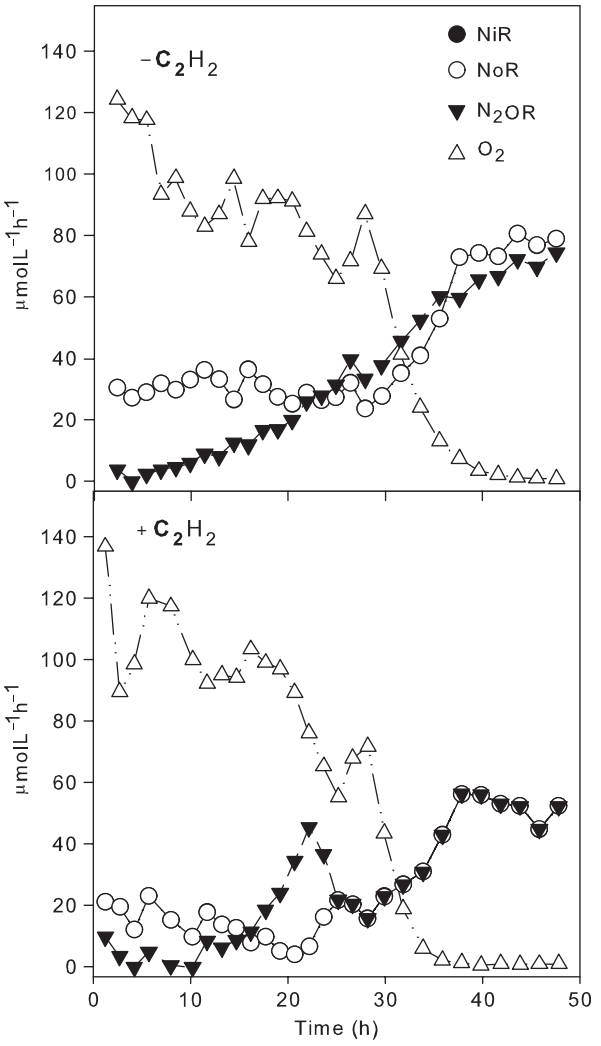

Fig. 7. Effects of 10 Pa C_2H_2 during the second incubation of extracted cells. The graphs show the estimated rates of reduction of the different electron acceptors (μmol O_2 L^{-1} h^{-1} and μmol N L^{-1} h^{-1} for the nitrogen species) for incubation with and without 10 Pa C_2H_2 in headspace (indicated in graphs). Initial O_2 was 5.2 vol% in headspace, the medium contained 2 mM NO_3^- and 0.5 mM NO_2^-. NiR, rate of NO_2^- reduction to NO; NoR, rate of NO reduction to N_2O; N_2OR, rate of N_2O reduction to N_2; O_2, rate of O_2 reduction.

The denitrification rates under oxic conditions (Figs 5 and 6), estimated as the sum of NoR and N_2OR activity (i.e. the net accumulation rate of N_2 plus that of N_2O), ranged from 0.2–1 μmol N_2O+N_2 per flask h^{-1}. In comparison, the maximum denitrification rates during the preceding anoxic phase (Figs 1 and 2) were 3–4 μmol N_2 per flask h^{-1} (N_2O was insignificant in this case). For a true comparison of these rates, however, we must take into account that the cell density was reduced with 40% before the secondary oxic phase (20 mL culture volume was removed and replaced with fresh medium). Thus, we should compare rates of 1.8–2.4 μmol N_2O+N_2 per flask h^{-1} in the first anoxic phase with 0.2–1 μmol N_2O+N_2 per flask h^{-1} during the following oxic phase. Thus, the measured denitrification rates during the secondary oxic phase were 8–55% of the maximum rates during the preceding anoxic phase.

Discussion

Denitrification in cells not previously exposed to anoxia

A prerequisite for identifying aerobic denitrification, *sensu stricto*, is to precisely know the O_2 concentration at the bacterial cell surface. However, if cells aggregate, there is uncertainty whether strictly uniform O_2 tensions have been maintained throughout the culture. In our experiments we can only be certain that all cells were fully exposed to O_2 during initial incubation (0–20 h), as later on the possibility of cell aggregation could not be excluded. Hence, some cells could be experiencing lower O_2 concentrations than in the bulk liquid. Therefore, caution is required in the interpretation of N-oxide production later on during the incubation while O_2 was still present in the liquid.

The early (0–20 h) NO accumulation in proportion to the NO_2^- concentration at 80 μM O_2 (Figs 2b and 3b) could in theory be due to chemical decomposition of NO_2^- rather than biological (Conrad, 1996). However, the early NO accumulations in flasks with sterilized suspensions of soil bacteria was $< 10\%$ of that in nonsterile ones (at the same oxygen and NO_2^- concentration). NO emission in response to NO_2^- addition to autoclaved soil is normally found to be higher than that in response to NO_2^- addition to nonsterile soil (Conrad, 1996). Thus, there is strong evidence that the early NO accumulation observed is biological, and could be taken as an indication of aerobic denitrification *sensu stricto*. It appears, however, that only NiR was active, because only NO was detected. It cannot be excluded that both NoR and N_2OR became active as well (thus causing the observed depletion of NO), without being detected as an increase in N_2O and N_2 since the detection limit for N_2O and N_2 accumulation is much higher than that for NO (approximate detection limits are 150–200 nmol N_2 per flask, 2.5 nmol N_2O per flask and 1 nmol NO per flask, Molstad *et al.*, 2007). The rate of NO accumulation suggests a NiR activity of 0.7 nmol NO per flask h^{-1} in the 2 mM NO_2^- treatment (Fig. 3), and possibly somewhat higher if NoR and N_2OR were active from the very beginning of the incubation. The absence of NO accumulation in the treatment without NO_2^- could be taken as a proof for the absence of NaR, but is probably not. A low NaR activity (similar to the apparent NiR activity) would not produce high enough NO_2^- concentrations to induce the observed NO accumulation.

Thus, we tentatively conclude that the experiment demonstrates that millimolar concentrations of NO_2^- can induce a low denitrification rate at a moderately high O_2 concentration (5.2 vol%, 80 μM), but not at full aeration (300 μM). A similar observation was made with cultures of *A. tumefaciens*, where 2 mM NO_2^- induced an onset of NO accumulation as the O_2 concentration reached below 17 μM (Bergaust *et al.*, 2008). We hesitate to use the term aerobic denitrification, however. The original idea of aerobic denitrification is that it would occur even at full aeration (Robertson & Kuenen, 1984; Lloyd *et al.*, 1987). This was clearly not the case here (Fig. 2b).

At a later stage of the incubation, high denitrification rates were initiated as some O_2 was still present (7–10 μM, i.e. equivalent to 2–3% of full aeration), with or without NO_2^- present (Fig. 3). However, at this stage of the incubation cell aggregates were visibly present, hence the observed denitrification in the presence of oxygen could have been taking place in localized microsites with reduced O_2.

The treatment with initially near-zero oxygen concentrations (1–2 μM, Figs 1 and 2) is somewhat perplexing at first sight: in the absence of NO_2^-, no denitrification occurred during the first 40 h of incubation, through which the O_2 concentration dropped from 1.8 to 0.5 μM. This could be taken as evidence that < 1 μM O_2 is necessary for induction of denitrification, but is probably not. It seems more likely that the transcription of denitrification gene was initiated at the very beginning of the incubation at 1–2 μM O_2. If so, however, we should expect detectable denitrification within 1–2 h, based on observed expression kinetics in denitrifying bacteria (see for instance Härtig & Zumft, 1999). The fact that it took 40 h rather than 1–2 h could be explained by near absence of constitutive Nar, because this would result in severe energy limitation due to limited electron flow (as oxygen was abruptly lowered to 1–2 μM). This energy limitation would be relieved when a minimum of NaR is produced. In contrast, NO_2^- induced an immediate onset of denitrification at the lowest initial O_2 concentration treatment when concentrations were 1.7 μM. Taken together, these results suggest that NiR is constitutively expressed at a much higher level than NaR. This corroborates the interpretations above regarding NO_2^- effects on the early NO accumulation in the treatments with initially 5.2 vol%. Equivalent indirect evidence for low/zero constitutive expression of NaR in denitrifying bacteria has been found other studies as well (Højberg *et al.*, 1997; Bergaust *et al.*, 2008), warranting more focused studies of this phenomenon in the future.

In summary, the presence of NO_2^- in our extracted cells induced detectable denitrification rates under micro-oxic, whereas NO_3^- did not. This may reflect constitutive expression of NiR, which has been reported for denitrifying bacteria isolated from soil (Ka *et al.*, 1997), and previous findings that the cd_1-type nitrite reductase can remain active in the presence of molecular oxygen (Ferguson, 1994). Constitutive expression of NiR might be an advantage by protecting against toxic spikes of NO_2^- (detoxification). It may also help the organisms to survive during rapid transition from oxic to anoxic conditions, because it may provide the necessary minimum of energy (provided that traces of NO_2^- are present) to express the entire denitrification proteome.

Denitrification under oxic conditions following anoxic conditions

During anaerobic respiration (Experiment 1) the entire denitrification pathway was active and the reductases NiR, NoR and N_2OR were almost in a state of equilibrium. Upon switching back from anoxic to oxic conditions, active denitrification occurred, amounting to 8–55% of the activity observed during the preceding anoxic phase (see calculations in Results). All denitrification enzymes were active during this oxic phase, but N_2OR was clearly more severely inhibited by oxygen than the other enzymes (NaR, NiR and NoR).

This sustained denitrification at 80 μM O_2 is no proof of aerobic denitrification *sensu stricto*, because an unknown fraction of the activity may have taken place in cell aggregates with lower interior O_2 concentrations than that in the bulk liquid. Nevertheless, there must have been a substantial part of cells that were fully exposed to O_2 such as any free cells in the media and cells on the outer edge of bacterial aggregates. It seems likely, therefore, that at least a fraction of the community sustained denitrification despite being exposed to 80 μM O_2, but that their N_2OR activity was more inhibited by O_2 than the other reductases. As O_2 was being depleted the rates of N_2O reduction gradually recovered, either by reactivating existing enzymes or *de novo* enzyme synthesis of N_2OR.

Here we observed the composite response of bacteria extracted from a soil microbial community within which the different reductases can be expected to differ in terms of their regulatory mechanisms, sensitivity of N_2O reductases to O_2, or possession of mechanisms to protect the enzymes against O_2 (Bell & Ferguson, 1991). For example, while the N_2O reductase of *P. pantotrophus* has been shown to be active and stable in the presence of O_2 enabling aerobic denitrification to proceed effectively to N_2 (Berks *et al.*, 1993), the N_2OR of *Alcaligenes faecalis* and *Paracoccus denitrificans* appear to be more sensitive to O_2 (Baumann *et al.*, 1996; Otte *et al.*, 1996). Thus possession of an active N_2O reductase in oxic conditions appears to be organism dependent.

Microbial source of gaseous nitrogen

Ammonia oxidation was expected to be an insignificant source of NO or N_2O in the present experiments due to low extraction efficiency of ammonia oxidizers (AO, be it Bacteria or Archaea or both) in Nycodenz, which is up to 10-fold lower compared with heterotrophic bacteria (Aakra *et al.*, 2000). Nevertheless, we included treatments with 10 Pa C_2H_2, in order to check this contribution. The results prove that ammonia oxidation was an insignificant source of N_2O during the oxic phase, as judged by the absence of any C_2H_2 effect on gas production (Fig. 7). Ammonia oxidation cannot possibly have been significant during the first anoxic phase either, as judged by the higher N_2O accumulation rate with than without C_2H_2 (Fig. 4). The only result which could be taken as an evidence for a significant role of AO is the higher N_2O production rate in the absence than in the presence of 10 Pa C_2H_2 during the second anoxic phase (Fig. 5 vs. Fig. 6). If this difference was due to AO, however, this source of N_2O would require a massive presence of AO in the cultures, which seems unlikely as judged by their insignificance during the foregoing incubation. We are more inclined to believe that the positive effect of C_2H_2 on the relative N_2OR activity during the second incubation could be ascribed to an overexpression during the late phase of the first anoxic period in the presence of C_2H_2, due to its apparent partial inhibition of N_2OR. Partial inhibition of N_2OR by 10 Pa C_2H_2 is not unlikely, although the concentration is three orders of magnitude lower than that used to achieve complete inhibition in the C_2H_2 inhibition assay of denitrification. We acknowledge that this is only a speculative interpretation of the somewhat surprising effect of C_2H_2 during the late anoxic phase. However, whatever the mechanism is, it hardly alters the main conclusions regarding oxygen regulation of denitrification and the relative activity of N_2OR.

We acknowledge that cell extraction on Nycodenz may be biased; cells in deep cavities and cells embedded in firm organic materials are less likely to be extracted than loosely attached cells in macropores. Such loosely attached cells in macropores are probably experiencing shorter and more infrequent anoxic spells than the deeply embedded cell. Hence, bacteria from the most oxic microenvironments could be overrepresented in the bacterial fraction. This question could be addressed by studying sequential extraction of cells, as done by Almås *et al.* (2005).

Conclusions

The experiment shows that aerobic denitrification in this soil-derived community is insignificant without prior exposure to an anoxic phase. The role of NO_2^- as an inducer of aerobic denitrification is unclear. Cells which are exposed to short anoxic periods, for instance created by flooding and subsequent drainage will probably keep on denitrifying even once the O_2 supply is partly or fully restored. And this aerobic/microaerobic denitrification would be a much stronger N_2O emitter than that occurring during anoxia, due to the apparently high O_2 inactivation of N_2OR. More soils should be tested to confirm the community response we observed. Our results are of significance to soil N_2O emission models, which do not explicitly include the inhibition of N_2O reductase by O_2 and the unbalanced denitrification process as a result of O_2 fluctuations.

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References

Aakra Å, Hesselsoe M & Bakken LR (2000) Surface attachment of ammonia-oxidizing bacteria in soil. *Microb Ecol* **39**: 222–235.

Almås ÅR, Mulder J & Bakken LR (2005) The trace metal exposure of soil bacteria depends on their position in the soil matrix. *Environ Sci Technol* **39**: 5927–5932.

Almeida JS, Reis MAM & Carrondo MJT (1997) A unifying kinetic model of denitrification. *J Theoret Biol* **186**: 241–249.

Badr O & Probert SD (1993) Environmental impacts of atmospheric nitrous-oxide. *Appl Energy* **44**: 197–231.

Bakken LR & Dörsch P (2007) Nitrous oxide emission and global changes: modelling approaches. *Biology of the Nitrogen Cycle* (Bothe H, Ferguson SJ & Newton WE, eds), pp. 381–395. Elsevier, Amsterdam.

Bakken LR & Lindahl V (1995) Recovery of bacterial cells from soil. *Nucleic Acids in the Environment Methods and Applications* (Trevors JT & van Elsas JD, eds), pp. 9–27. Springer-Verlag, Berlin.

Baumann B, Snozzi M, Zehnder AJB & VanderMeer JR (1996) Dynamics of denitrification activity of *Paracoccus denitrificans* in continuous culture during aerobic–anaerobic changes. *J Bacteriol* **178**: 4367–4374.

Bell LC & Ferguson SJ (1991) Nitric and nitrous oxide reductases are active under aerobic conditions in cells of *Thiosphaera pantotropha*. *Biochem J* **273**: 423–427.

Bell LC, Richardson DJ & Ferguson SJ (1990) Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha* – the periplasmic enzyme catalyzes the 1st step in aerobic denitrification. *FEBS Lett* **265**: 85–87.

Bergaust L, Shapleigh J, Frostegård Å & Bakken L (2008) Transcription and activities of NO_x reductases in *Agrobacterium tumefaciens*: the influence of nitrate, nitrite and oxygen availability. *Environ Microbiol* doi: 10.1111/j.1462-2920.2007.01557.x.

Berks BC, Baratta D, Richardson DJ & Ferguson SJ (1993) Purification and characterization of a nitrous-oxide reductase from *Thiosphaera pantotropha* - implications for the mechanism of aerobic nitrous-oxide reduction. *FEBS J* **212**: 467–476.

Betlach MR & Tiedje JM (1981) Kinetic explanation for accumulation of nitrite, nitric oxide, and nitrous oxide during bacterial denitrification. *Appl Environ Microbiol* **42**: 1074–1084.

Bonin P & Gilewicz M (1991) A direct demonstration of "co-respiration" of oxygen and nitrogen oxides by *Pseudomonas nautica*: some spectral and kinetic properties of the respiratory components. *FEMS Microbiol Lett* **80**: 183–188.

Conrad R (1996) Soil microorganisms as controllers of atmospheric trace gases (H-2, CO, CH_4, OCS, N_2O, and NO). *Microbiol Rev* **60**: 609–640.

FAO (1988) FAO/Unesco soil map of the world revised legend, with corrections. World Soil, Resources, Report, 60, Rome (reprinted as technical paper 20, ISRIC, Wageningen, 1994).

Ferguson SJ (1994) Denitrification and its control. *Antonie Van Leeuwenhoek* **66**: 89–110.

Härtig E & Zumft W (1999) Kinetics of *nirS* expression (cytochrome cd_1 nitrite reductase) in *Pseudomonas stutzeri* during the transition from aerobic respiration to denitrification: evidence for a denitrification-specific nitrate- and nitrite-responsive regulatory system. *J Baceriol* **181**: 161–166.

Hayatsu M, Tago K & Saito M (2008) Various players in the nitrogen cycle: diversity and functions of the microorganisms involved in nitrification and denitrification. *Soil Sci Plant Nutr* **54**: 33–45.

Hochstein LI, Betlach M & Kritikos G (1984) The effect of oxygen on denitrification during steady-state growth of *Paracoccus halodenitrificans*. *Arch Microbiol* **137**: 74–78.

Højberg O, Binnerup SJ & Sørensen J (1997) Growth of silicone-immobilized bacteria on polycarbonate membrane filters, a technique to study microcolony formation under anaerobic conditions. *Appl Environ Microbiol* **63**: 2920–2924.

Jiang QQ & Bakken LR (1999) Nitrous oxide production and methane oxidation by different ammonia-oxidizing bacteria. *Appl Environ Microbiol* **65**: 2679–2684.

Ka JO, Urbance J, Ye RW, Ahn TY & Tiedje JM (1997) Diversity of oxygen and N-oxide regulation of nitrite reductases in denitrifying bacteria. *FEMS Microbiol Lett* **156**: 55–60.

Knowles R (1982) Denitrification. *Microbiol Rev* **46**: 43–70.

Körner H & Zumft WG (1989) Expression of denitrification enzymes in response to the dissolved-oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. *Appl Environ Microbiol* **55**: 1670–1676.

Lloyd D (1993) Aerobic denitrification in soils and sediments – from fallacies to facts. *Trends Ecol Evol* **8**: 352–356.

Lloyd D, Boddy L & Davies KJP (1987) Persistence of bacterial denitrification capacity under aerobic conditions – the rule rather than the exception. *FEMS Microbiol Ecol* **45**: 185–190.

Molstad L, Dörsch P & Bakken LR (2007) Robotized incubation system for monitoring gases (O_2, NO, N_2O N_2) in denitrifying cultures. *J Microbiol Methods* **71**: 202–211.

Nicol GW, Tscherko D, Chang L, Hammesfahr U & Prosser JI (2006) Crenarchaeal community assembly and microdiversity in developing soils at two sites associated with deglaciation. *Environ Microbiol* **8**: 1382–1393.

Otte S, Grobben NG, Robertson LA & Jetten MSM (1996) Nitrous oxide production by *Alcaligens faecalis* under transient and dynamic aerobic and anaerobic conditions. *Appl Environ Microbiol* **62**: 2421–2426.

Patureau D, Zumstein E, Delgenes JP & Moletta R (2000) Aerobic denitrifiers isolated from diverse natural and managed ecosystems. *Microb Ecol* **39**: 145–152.

Philippot L, Mirleau P, Mazurier S, Siblot S, Hartmann A, Lemanceau P & Germon JC (2001) Characterization and transcriptional analysis of *Pseudomonas fluorescens* denitrifying clusters containing the nar, nir, nor and nos genes. *Biochim Biophys Acta – Gene Struct Expression* **1517**: 436–440.

Robertson LA & Kuenen JG (1984) Aerobic denitrification – a controversy revived. *Arch Microbiol* **139**: 351–354.

Robertson LA & Kuenen JG (1990) Physiological and ecological aspects of aerobic denitrification, a link with heterotrophic nitrification. *Denitrification in Soil and Sediment* (Revsbech NP & Sørensen J, eds), pp. 91–104. Plenum Press, New York.

Robertson LA, Cornelisse R, Devos P, Hadioetomo R & Kuenen JG (1989) Aerobic denitrification in various heterotrophic nitrifiers. *Antonie Van Leeuwenhoek J Microbiol* **56**: 289–299.

Robertson LA, Dalsgaard T, Revsbech NP & Kuenen JG (1995) Confirmation of 'aerobic denitrification' in batch cultures, using gas chromatography and ^{15}N mass spectrometry. *FEMS Microbiol Ecol* **18**: 113–119.

Sears HJ, Ferguson SJ, Richardson DJ & Spiro S (1993) The identification of a periplasmic nitrate reductase in *Paracoccus denitrificans*. *FEMS Microbiol Lett* **113**: 107–111.

Shaw LJ, Nicol GW, Smith Z, Fear J, Prosser JI & Baggs EM (2006) *Nitrosospira* spp. can produce nitrous oxide via a nitrifier denitrification pathway. *Environ Microbiol* **8**: 214–222.

Sognnes LS, Fystro G, Opstad SL, Arstein A & Borresen T (2006) Effects of adding moraine soil or shell sand into peat soil on physical properties and grass yield in western Norway. *Acta Agriculturae Scandinavica Section B-Soil Plant Sci* **56**: 161–170.

Takaya N, Catalan-Sakairi MAB, Sakaguchi Y, Kato I, Zhou ZM & Shoun H (2003) Aerobic denitrifying bacteria that produce low levels of nitrous oxide. *Appl Environ Microbiol* **69**: 3152–3157.

Wilhelm E, Battino R & Wilcock RJ (1977) Low-pressure solubility of gases in liquid water. *Chem Rev* **77**: 219–262.

Zumft WG (1997) Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* **61**: 533–616.