

Production of NO, N₂O and N₂ by extracted soil bacteria, regulation by NO₂⁻ and O₂ concentrations

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Abstract

The oxygen control of denitrification and its emission of NO/N₂O/N₂ was investigated by incubation of Nycodenz-extracted soil bacteria in an incubation robot which monitors O₂, NO, N₂O and N₂ concentrations (in He+O₂ atmosphere). Two consecutive incubations were undertaken to determine (1) the regulation of denitrification by O₂ and NO₂⁻ during respiratory O₂ depletion and (2) the effects of re-exposure to O₂ of cultures with fully expressed denitrification proteome. Early denitrification was only detected (as NO and N₂O) at ≤ 80 μM O₂ in treatments with NO₂⁻, and the rates were three orders of magnitude lower than the rates observed after oxygen depletion (with N₂ as the primary product). When re-exposed to O₂, the cultures continued to denitrify (8–55% of the rates during the foregoing anoxic phase), but its main product was N₂O. The N₂O 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 N₂O emission. Short anoxic spells caused by transient flooding during rainfall, could lead to subsequent unbalanced aerobic denitrification, in which N₂O is a major end product.

Introduction

Denitrification is the sequential dissimilatory reduction of nitrate (NO₃⁻) to nitrite (NO₂⁻), nitric oxide (NO), nitrous oxide (N₂O) and dinitrogen (N₂) catalysed by nitrate reductases (NaR or Nap), nitrite reductase (NiR), nitric oxide reductase (NoR) and nitrous oxide reductase (N₂OR), respectively (Zumft, 1997). Transient accumulation of intermediates (NO₂⁻, NO and N₂O) 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 N₂O is of concern because of their role in the destruction of stratospheric ozone (Badr & Probert, 1993) and because N₂O is a potent greenhouse gas. Denitrification was traditionally thought to be inhibited in the presence of O₂ as O₂ is preferred over NO₃⁻ as a terminal electron acceptor during respiration (Knowles, 1982; Lloyd, 1993). However, there is now

substantial evidence for the simultaneous respiration of O₂ and NO₃⁻ 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). N₂O and N₂ have been shown to be produced in bacterial cultures under O₂ 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 N₂O and N₂ during corespiration of O₂ and NO₃⁻ (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 NO₂⁻ and NO (Knowles, 1982). In contrast there appears to be no physiological gain from N₂O reduction while O₂ is nonlimiting. In accordance with this there is evidence from culture experiments that N₂O is the predominant end product of denitrification by most denitrifiers in the presence of O₂ (Otte *et al.*, 1996; Takaya *et al.*, 2003), with several culture studies having demonstrated higher ratios of N₂O-to-N₂ with increased O₂ concentration. This has been attributed to a greater O₂ sensitivity of the N₂O 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 N₂O, 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 O₂. 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, N₂O and N₂ (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 O₂ and NO₂⁻ concentrations (Experiment 1) and to determine the kinetics of NO, N₂O and N₂ production under transient changes in O₂ concentration (Experiment 2). NO₂⁻ was hypothesized to be an important trigger for aerobic denitrification (Experiment 1) as it has previously been shown to induce NiR and N₂OR (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 ammo-

nia-oxidizing bacteria (AOB) are able to produce N₂O, both under oxic (Jiang & Bakken, 1999) and anoxic conditions (Shaw *et al.*, 2006), we included treatments with 10 Pa acetylene (C₂H₂) 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 C₂H₂ (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 O₂ 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 Furenset 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 (Nycodenz 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 × 10⁹ 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 KH₂PO₄, 3 mM NH₄SO₄⁻, 5 mM glucose, 2 mM KNO₃⁻, 1.76 mg L⁻¹ EDTA, 10 mg L⁻¹ ZnSO₄, 5 mg L⁻¹ FeSO₄, 1.5 mg L⁻¹ MnSO₄, 0.4 mg L⁻¹ CuSO₄, 0.25 mg L⁻¹ Co(NO₃)₂, 0.15 mg L⁻¹ H₃BO₃, 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 O₂ concentration was adjusted by injecting O₂ (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 10¹⁰ cells (total microscopic counts), resulting in a final cell density of 2 × 10⁸ 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 Labortechnik 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 N₂O and CO₂ analysis (oven temperature 36 °C) and a 20 m 5 Å Molsieve (oven temperature 50 °C) for N₂ and O₂ 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. O₂ concentrations in the liquid are calculated as a function of measured O₂ 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 O₂ concentrations (0.1, 5.2 and 20 vol% O₂ in headspace; 1–2, 80 and 300 μM O₂ in liquid) and different concentrations of NO₂⁻ (0, 0.1, 0.5 and 2 mM). Duplicates of the 5.2 vol% O₂ treatment were amended with C₂H₂ (10 Pa), generated from calcium carbide, to inhibit any NO and N₂O production from NH₃ oxidation. Headspace samples were taken automatically every 2 h over a 118-h period. In the second experiment the treatments with an initial O₂ level of 5.2 vol% were re-exposed to their initial O₂ (5.2 vol%, 80 μM) and NO₂⁻ concentrations (0, 0.1, 0.5 and 2 mM NO₂⁻) once anoxic conditions had been achieved and all NO₃⁻ and NO₂⁻ had been reduced. To ensure ample amounts of carbon sub-

strates 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 O₂ concentration, refilled with 10 Pa C₂H₂ (those which had C₂H₂ during the first incubation), and incubated as previously in Experiment 1.

Initial traces of NO were detected in response of NO₂⁻ 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 NO₂⁻ and at two oxygen concentrations (near-zero and 5.2 vol% O₂).

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 O₂ concentrations in the bulk liquid during oxygen depletion, the transport rate was taken into account. In theory, the O₂ transport between the liquid phase and the headspace is a function of the concentrations in the two phases, O₂ solubility, and a coefficient for transport of O₂ 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 O₂ s⁻¹); k_t is the coefficient for transport from gas to liquid (L s⁻¹); P_{O_2} is the O₂ partial pressure in headspace (atm); k_H is the solubility of O₂ in water (mol L⁻¹ atm⁻¹); $[O_2]$ is O₂ 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 O₂ 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 O₂ 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 O₂ concentrations than that calculated for the bulk liquid.

Relative enzyme rates ($\mu\text{mol N L}^{-1} \text{h}^{-1}$) for NiR, NoR and N₂OR were calculated as follows:

$$\begin{aligned} \text{N}_2\text{OR} &= \frac{\Delta\text{N}_2}{t} \\ \text{NoR} &= \text{N}_2\text{OR} + \left(\frac{\Delta\text{N}_2\text{O}}{t}\right) \\ \text{NiR} &= \text{NoR} + \left(\frac{\Delta\text{NO}}{t}\right) \end{aligned}$$

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

Results

Experiment 1: NO, N₂O and N₂ production with varying initial O₂ and NO₂⁻ concentrations

In the treatments without NO₂⁻ (Fig. 1), detectable accumulation of NO, N₂O and N₂ occurred as O₂ was near to depletion in all treatments. In the treatments with initial O₂ at 80 and 300 μM , detectable NO accumulation started as the O₂ concentration reached 7 and 10 μM , respectively. In

the treatment with initial O₂ at 1–2 μM , detectable NO accumulation started as O₂ reached 0.4–0.5 μM . N₂O accumulation coincided with that of NO, whereas detectable N₂ accumulation started *c.* 10 h later in all treatments.

The addition of 2 mM NO₂⁻ (Fig. 2 vs. Fig. 1) clearly retarded O₂ consumption at low O₂ concentrations (Fig. 2a vs. 1a and Fig. 3), induced significant NO accumulation from time zero at the two lowest initial O₂ treatments and induced accumulation of N₂O from time zero in the 1–2 μM O₂ treatment (Fig. 2c). Thus, at the lowest initial O₂, NO₂⁻ induced a transient accumulation of NO (initial rates 1.5 nmol NO per flask h⁻¹) and a sustained accumulation of N₂O (*c.* 1 nmol N₂O per flask h⁻¹) throughout the first 80 h of incubation. In contrast, at 80 μM initial O₂, NO₂⁻ induced an early but transient NO accumulation (initial rate *c.* 0.5 nmol NO per flask h⁻¹). Thereafter neither NO nor N₂O accumulation could be detected until O₂ reached the same low concentrations as for the treatments without NO₂⁻ (5–10 μM , Fig. 2).

Figure 3 allows a closer inspection of the effect of NO₂⁻ at 80 μM initial O₂. First, it caused an initial transient aerobic NO accumulation, proportional with the NO₂⁻ concentration (Fig. 3b). In contrast, initial NO accumulation (0–20 h) in flasks with sterilized suspensions of soil bacteria and 2 mM NO₂⁻ 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 NO₂⁻ caused a retardation of oxygen consumption, whereas lower NO₂⁻ concentrations did not (Fig. 3a). It also caused a similar retardation of the

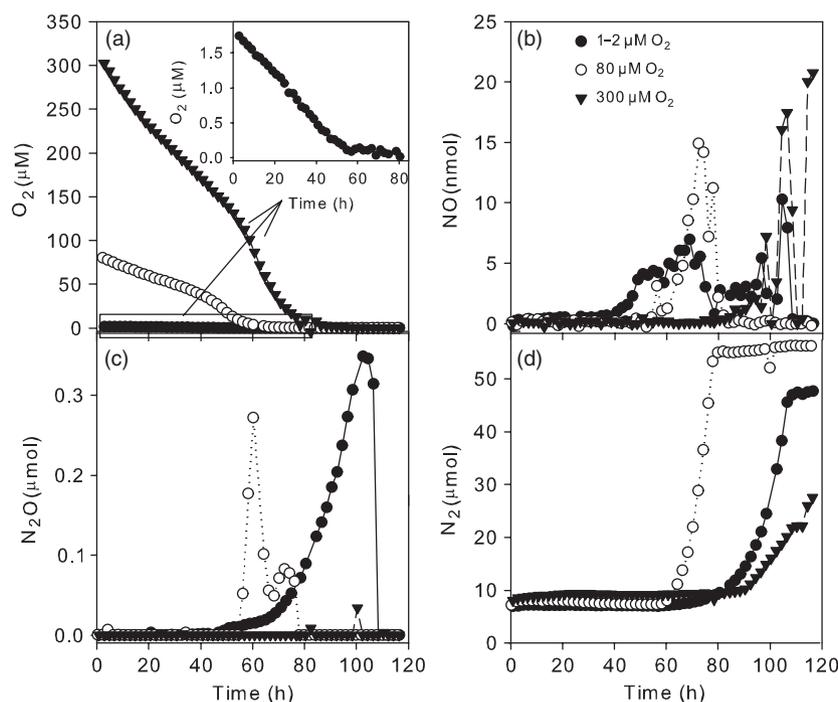


Fig. 1. First incubation of cells extracted from soil, medium without NO₂⁻. The graphs show oxygen concentrations in the liquid (a) and total amounts of nitrogen gases per flasks (b–d). The different initial oxygen treatments are indicated in the graph. All media contained 2 mM NO₂⁻.

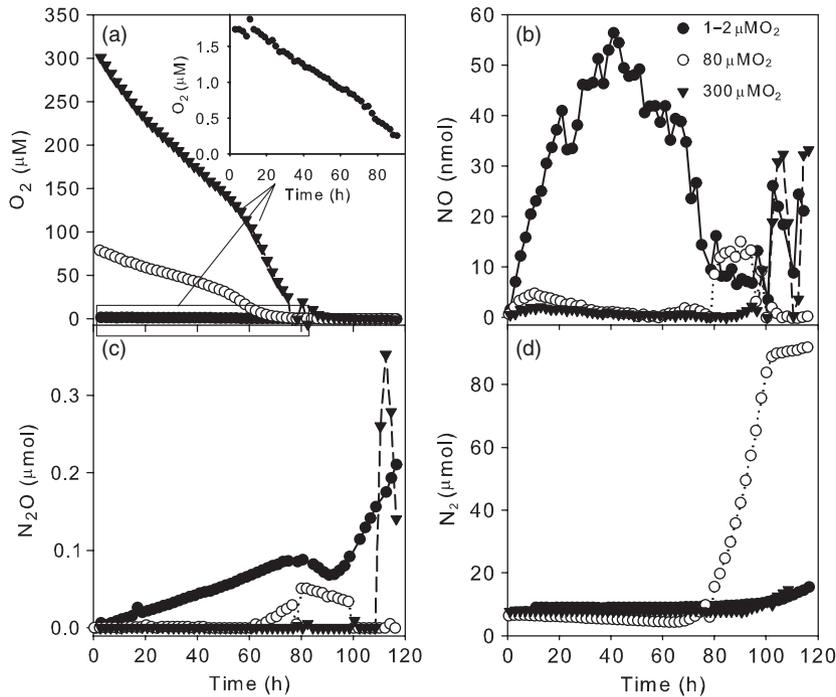


Fig. 2. First incubation of cells extracted from soil, medium with 2 mM NO₂⁻. The graphs show oxygen concentrations in the liquid (a) and total amounts of nitrogen gases per flask (b-d). The different initial oxygen treatments are indicated in the graph. All media contained 2 mM NO₃⁻ and 2 mM NO₂⁻.

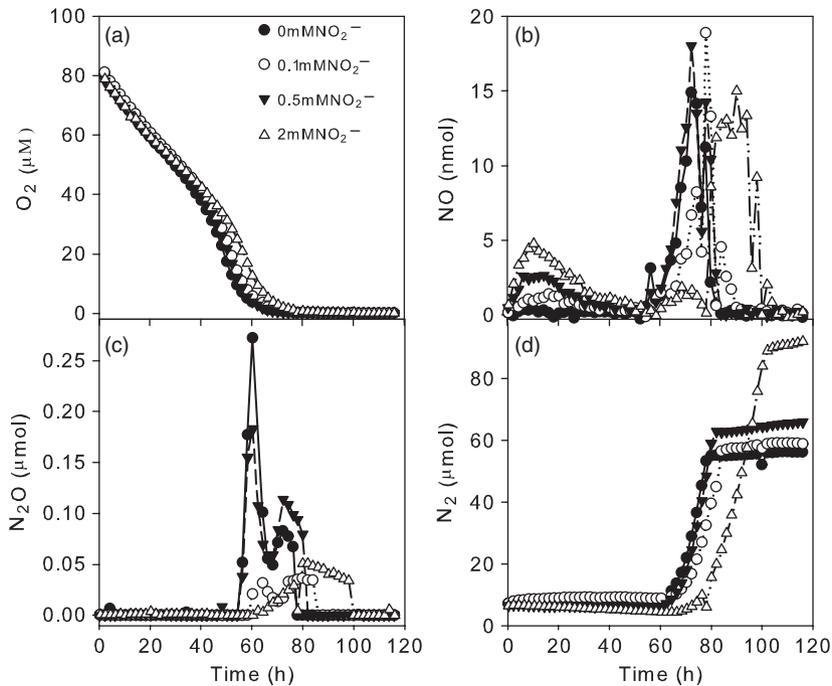


Fig. 3. First incubation, effects of varying concentrations of NO₂⁻. Treatment with initial 80 μM O₂ and no C₂H₂. The graphs show oxygen concentrations in the liquid (a) and total amounts of nitrogen gases per flask (b-d). The different NO₂⁻ treatments are indicated in the graph.

appearance of both NO, N₂O and N₂. Detectable N₂ production for all four treatments commenced 5–10 h later than NO and N₂O accumulation (Fig. 3d) and by the end of the incubation 90–97% of added N (NO₃⁻+NO₂⁻) was recovered as N₂.

Once rapid denitrification was initiated, driven by O₂ depletion, the products were completely dominated by N₂, and the rates were similar for all treatments: 2–3 μmol N₂ per flask h⁻¹. These rates are three orders of magnitude higher than the detected net rates of NO and/or

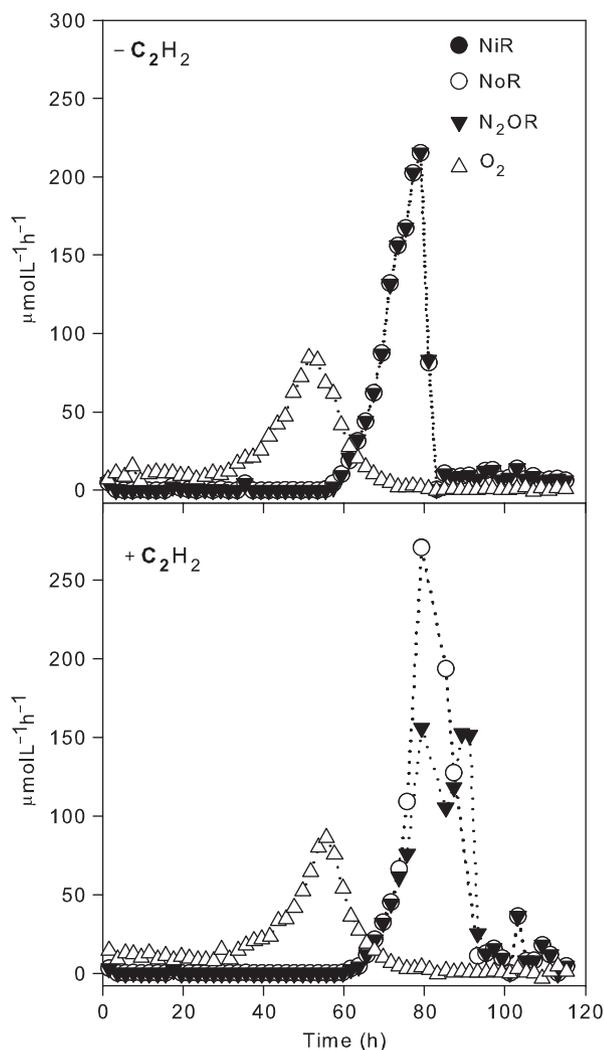


Fig. 4. Effects of 10 Pa C₂H₂ during the first incubation of extracted cells. The graphs show the estimated rates of reduction of the different electron acceptors ($\mu\text{mol O}_2 \text{L}^{-1} \text{h}^{-1}$ and $\mu\text{mol NL}^{-1} \text{h}^{-1}$ for the nitrogen species) for incubation with and without 10 Pa C₂H₂ in headspace (indicated in graphs). Initial O₂ was 5.2 vol% in headspace, the medium contained 2 mM NO₃⁻ and 0.5 mM NO₂⁻. NiR, rate of NO₂⁻ reduction to NO; NoR, rate of NO reduction to N₂O; N₂OR, rate of N₂O reduction to N₂; O₂, rate of O₂-reduction.

N₂O accumulation in the various treatments during the first aerobic part of the incubation.

For all the treatments in which N₂ accumulation reached a plateau (Figs 1d, 2d and 3d), the final N₂ accumulation accounted for 90–97% of the added NO₃⁻ or NO₂⁻. In general, the transient accumulation of NO and N₂O was marginal compared with the production of N₂. This implies that throughout the entire period with active denitrification, most of the NO₃⁻ or NO₂⁻ was efficiently reduced to N₂, i.e. the rates of NO and N₂O reduction were almost equal to the rate of NO₂⁻ reduction to NO. This is illustrated in Fig. 4,

which shows the estimated rates of O₂ and N-oxide reduction in the 80 $\mu\text{M O}_2$ treatment with 2 mM NO₃⁻ and 0.5 mM NO₂⁻, with and without 10 Pa C₂H₂ in the headspace. The O₂ respiration peaked around 50 h for both treatments, then declined with corresponding increases in rates of the N-oxide reductases. In the absence of C₂H₂, the rates of NO and N₂O reduction (NoR and N₂OR) 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 C₂H₂, however, N₂O 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 N₂O reduction. This reflected a higher transient N₂O accumulation in the presence of 10 Pa C₂H₂ (16–31 $\mu\text{mol N}_2\text{O}$ 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 $\mu\text{M O}_2$

Here, the cultures which had been incubated with initial 5.2 vol% O₂ in headspace (80 $\mu\text{M O}_2$) in Experiment 1 were re-exposed to 5.2 vol% in headspace, 2 mM NO₃⁻ and 0, 0.1, 0.5 or 2 mM NO₂⁻ (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 O₂ 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 O₂ was depleted, the NO concentrations increased rapidly. Higher levels were reached in the treatments without C₂H₂ (40–140 nmol per flask), compared with that in the flasks with 10 Pa C₂H₂ (20–50 nmol per flask), and for both treatments the levels increased with increasing concentrations of NO₂⁻ (Figs 5b and 6b). The N₂O accumulation started from the very beginning of the incubation, and peaked at high levels (4–12 $\mu\text{mol N}_2\text{O}$ per flask) before O₂ was depleted. The peak concentrations varied between treatments, and were not systematically related to the NO₂⁻ concentrations nor to the presence/absence of C₂H₂ in the headspace. At the onset of anoxic conditions, N₂O was efficiently reduced to zero in the treatments with C₂H₂ (Fig. 6), whereas it increased in the absence of C₂H₂ (with one exception). The N₂ accumulation rate was moderate through the first 20 h of the oxic phase (0.15–0.47 $\mu\text{mol N}_2 \text{h}^{-1}$ per flask), and then increased gradually as O₂ was being depleted. The response pattern is more clearly visible

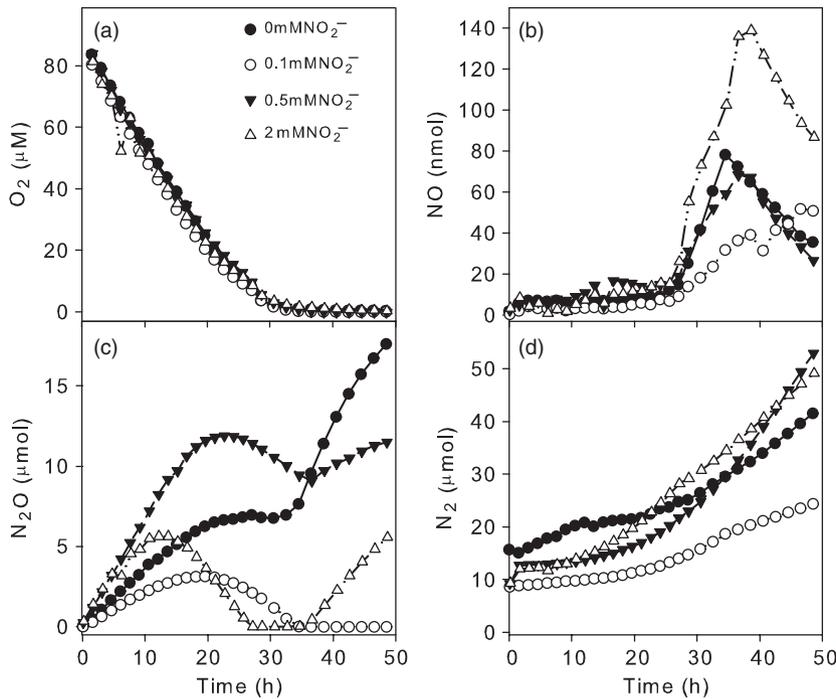


Fig. 5. Second incubation. Cell suspensions from the first incubation, during which oxygen was depleted, were amended with fresh medium and a new dose of O₂, and the original concentrations of NO₃⁻ and NO₂⁻ were restored. Results for treatments without C₂H₂. Graphs are equivalents to those in Figs 1–3. Treatments are indicated in the graph. Each culture was exposed to the same initial concentration of O₂ and NO₂⁻ as it experienced in the first incubation.

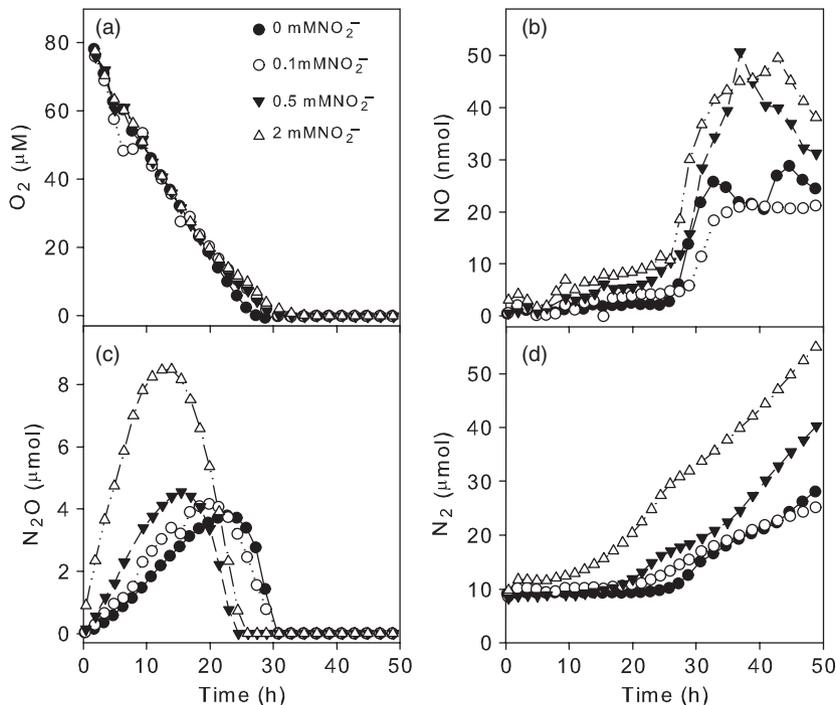


Fig. 6. Second incubation. Treatments equivalent to Fig. 5, but with 10 Pa C₂H₂ in headspace.

in Fig. 7, where the rates of O₂ and N-oxide reduction are shown. The rates of NO₂⁻ reduction to NO (NiR) are not visible because it practically equals that of NO reduction to N₂O. The rates of N₂O reduction to N₂ (N₂OR), 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 C₂H₂) (Fig. 7). The estimated N₂OR activity was strongly negatively correlated with the bulk concentration of oxygen ($r = -0.94$, $P < 0.01$). The O₂ respiration rates peaked at around 30 h, for both treatments, then declined rapidly.

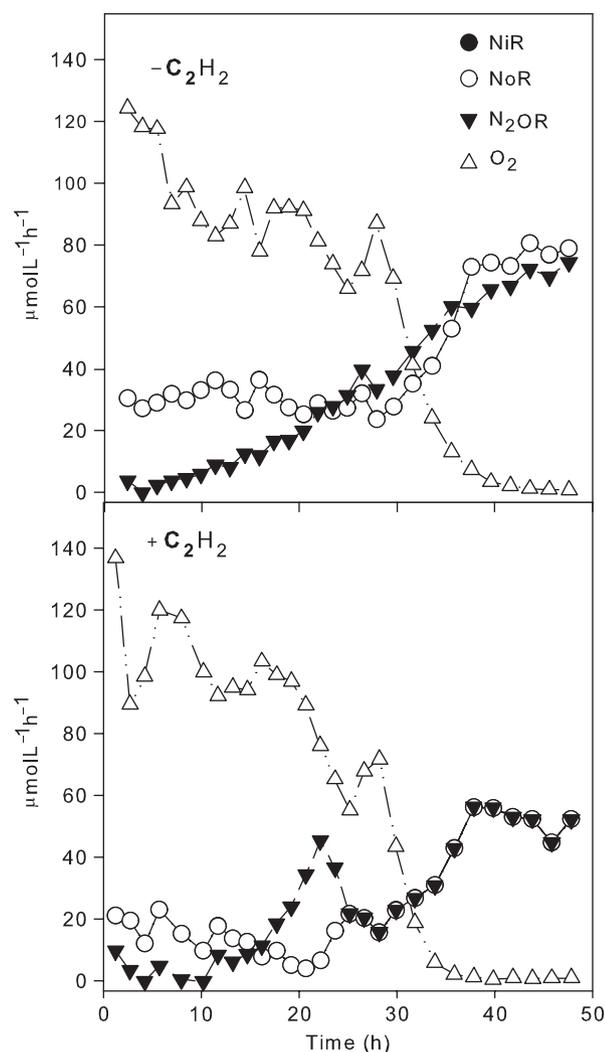


Fig. 7. Effects of 10 Pa C₂H₂ during the second incubation of extracted cells. The graphs show the estimated rates of reduction of the different electron acceptors ($\mu\text{mol O}_2 \text{ L}^{-1} \text{ h}^{-1}$ and $\mu\text{mol N L}^{-1} \text{ h}^{-1}$ for the nitrogen species) for incubation with and without 10 Pa C₂H₂ in headspace (indicated in graphs). Initial O₂ was 5.2 vol% in headspace, the medium contained 2 mM NO₃⁻ and 0.5 mM NO₂⁻. NiR, rate of NO₂⁻ reduction to NO; NoR, rate of NO reduction to N₂O; N₂OR, rate of N₂O reduction to N₂; O₂, rate of O₂ reduction.

The denitrification rates under oxic conditions (Figs 5 and 6), estimated as the sum of NoR and N₂OR activity (i.e. the net accumulation rate of N₂ plus that of N₂O), ranged from 0.2–1 $\mu\text{mol N}_2\text{O} + \text{N}_2$ per flask h⁻¹. In comparison, the maximum denitrification rates during the preceding anoxic phase (Figs 1 and 2) were 3–4 $\mu\text{mol N}_2$ per flask h⁻¹ (N₂O 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 $\mu\text{mol N}_2\text{O} + \text{N}_2$ per flask h⁻¹ in the first anoxic phase with 0.2–1 $\mu\text{mol N}_2\text{O} + \text{N}_2$ 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 O₂ concentration at the bacterial cell surface. However, if cells aggregate, there is uncertainty whether strictly uniform O₂ tensions have been maintained throughout the culture. In our experiments we can only be certain that all cells were fully exposed to O₂ 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 O₂ concentrations than in the bulk liquid. Therefore, caution is required in the interpretation of N-oxide production later on during the incubation while O₂ was still present in the liquid.

The early (0–20 h) NO accumulation in proportion to the NO₂⁻ concentration at 80 $\mu\text{M O}_2$ (Figs 2b and 3b) could in theory be due to chemical decomposition of NO₂⁻ 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 NO₂⁻ concentration). NO emission in response to NO₂⁻ addition to autoclaved soil is normally found to be higher than that in response to NO₂⁻ 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 N₂OR became active as well (thus causing the observed depletion of NO), without being detected as an increase in N₂O and N₂ since the detection limit for N₂O and N₂ accumulation is much higher than that for NO (approximate detection limits are 150–200 nmol N₂ per flask, 2.5 nmol N₂O 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 NO₂⁻ treatment (Fig. 3), and possibly somewhat higher if NoR and N₂OR were active from the very beginning of the incubation. The absence of NO accumulation in the treatment without NO₂⁻ 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 NO₂⁻ 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 expres-

sion of NiR, which has been reported for denitrifying bacteria isolated from soil (Ka *et al.*, 1997), and previous findings that the *cd₁*-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 N₂O 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 C₂H₂, in order to check this contribution. The results prove that ammonia oxidation was an insignificant source of N₂O during the oxic phase, as judged by the absence of any C₂H₂ effect on gas production (Fig. 7). Ammonia oxidation cannot possibly have been significant during the first anoxic phase either, as judged by the higher N₂O accumulation rate with than without C₂H₂ (Fig. 4). The only result which could be taken as an evidence for a significant role of AO is the higher N₂O production rate in the absence than in the presence of 10 Pa C₂H₂ during the second anoxic phase (Fig. 5 vs. Fig. 6). If this difference was due to AO, however, this source of N₂O 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 C₂H₂ on the relative N₂OR activity during the second incubation could be ascribed to an overexpression during the late phase of the first anoxic period in the presence of C₂H₂, due to its apparent partial inhibition of N₂OR. Partial inhibition of N₂OR by 10 Pa C₂H₂ is not unlikely, although the concentration is three orders of magnitude lower than that used to achieve complete inhibition in the C₂H₂ inhibition assay of denitrification. We acknowledge that this is only a speculative interpretation of the somewhat surprising effect of C₂H₂ 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 N₂OR.

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 NO₂⁻ 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 O₂ supply is partly or fully restored. And this aerobic/microaerobic denitrification would be a much stronger N₂O emitter than that occurring during anoxia, due to the apparently high O₂ inactivation of N₂OR. More soils should be tested to confirm the community response we observed. Our results are of significance to soil N₂O emission models, which do not explicitly include the inhibition of N₂O reductase by O₂ and the unbalanced denitrification process as a result of O₂ fluctuations.

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