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The fate of N₂O consumed in soils

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Abstract. Soils are capable to consume N₂O. It is generally assumed that consumption occurs exclusively via respiratory reduction to N₂ by denitrifying organisms (i.e. complete denitrification). Yet, we are not aware of any verification of this assumption. Some N₂O may be assimilatorily reduced to NH₃. Reduction of N₂O to NH₃ is thermodynamically advantageous compared to the reduction of N₂. Is this an ecologically relevant process? To find out, we treated four contrasting soil samples in a flow-through incubation experiment with a mixture of labelled (98%) ¹⁵N₂O (0.5–4 ppm) and O₂ (0.2–0.4%) in He. We measured N₂O consumption by GC-ECD continuously and δ¹⁵N of soil organic matter before and after an 11 to 29 day incubation period. Any ¹⁵N₂O assimilatorily reduced would have resulted in the enrichment of soil organic matter with ¹⁵N, whereas dissimilatorily reduced ¹⁵N₂O would not have left a trace. None of the soils showed a change in δ¹⁵N that was statistically different from zero. A maximum of 0.27% (s.e. ±0.19%) of consumed ¹⁵N₂O may have been retained as ¹⁵N in soil organic matter in one sample. On average, ¹⁵N enrichment of soil organic matter during the incubation may have corresponded to a retention of 0.019 % (s.e. ±0.14%; n=4) of the ¹⁵N₂O consumed by the soils. We conclude that assimilatory reduction of N₂O plays, if at all, only a negligible role in the consumption of N₂O in soils.

1 Introduction

Nitrous oxide (N₂O) is produced in soils during the processes of nitrification and denitrification (Firestone et al., 1980). Since industrialisation, the global atmospheric N₂O concentration increased from about 270 ppb in 1800 to 319 ppb in

2005 (IPCC, 2006). Currently, more than one third of all N₂O emissions are of anthropogenic origin and primarily due to agriculture (IPCC, 2006). However, soils can also act as a sink for N₂O (reviewed in Chapuis-Lardy et al., 2007). In general, it is implicitly assumed that complete denitrification (reduction of N₂O to N₂) is the only process responsible for observed sink activity. Once produced by a soil organism, a molecule of N₂O is presumed to take one of the three known routes (Ostrom et al., 2007) (Fig. 1): (1) complete denitrification to N₂ within the cell prior to its escape into the gas phase (reviewed in Zumft, 1997); (2) escape from the cell into the gas phase of soil and potentially to the atmosphere; or (3) complete denitrification to N₂ upon re-entering a cell capable to reduce N₂O (e.g. Neftel et al., 2000; Clough et al., 2005). To the best of our knowledge other pathways have not been considered in natural soil so far. Here, we hypothesise a fourth pathway of assimilatory reduction to NH₃ may be responsible for some of observed N₂O consumption in soil (Fig. 1).

The only enzyme known to reduce N₂O to NH₃ is nitrogenase. First evidence was provided by Mozen and Burris (1954). Later studies confirmed that N₂O can be a substrate for nitrogenase which reduces N₂O to N₂ with subsequent reduction of N₂ to NH₃ (Hoch et al., 1960; Hardy and Knight, 1966; Jensen and Burris, 1986). However, one year later, reduction of N₂O to N₂ was already questioned by Yamazaki et al. (1987). They concluded from the stable isotope kinetics during N₂O fixation by *Azotobacter vinelandii* that N₂O fixation by nitrogenase must be an apparent one-step reaction transforming N₂O directly into NH₃, without the intermediary N₂. This conclusion was supported by a re-interpretation of the original results of Jensen and Burris (1986) by Burgess and Lowe (1996). The re-interpretation suggested there may be an additional pathway from N₂O to NH₃ which does not involve N₂ as an intermediary. Enzyme kinetics indicate a low affinity of N₂O to nitrogenase. A *k_m* value of 24 kPa for purified component proteins from *Klebsiella pneumoniae*



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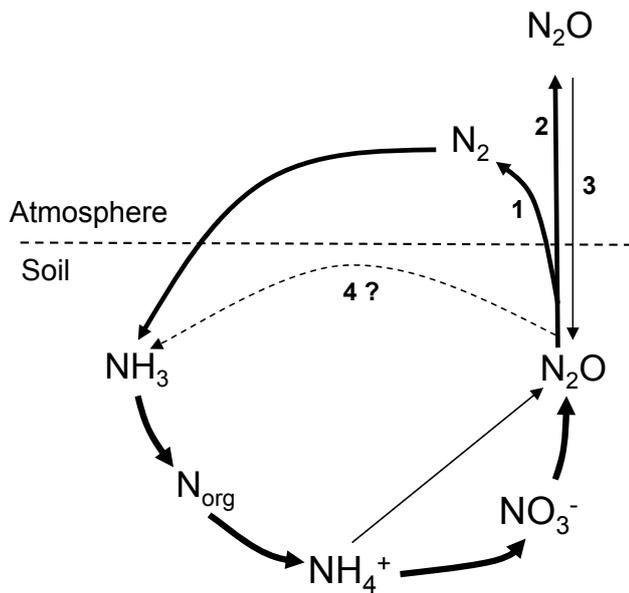


Fig. 1. Origin and possible fate of N₂O in soil. (1) Complete denitrification to N₂ before escape from the cell; (2) escape from cell; (3) re-entering a cell and subsequent reduction to N₂, or (4) assimilatory reduction to NH₃. The ecological relevance of pathway (4) in natural soil is unknown and the focus of this study.

has been determined by Jensen and Burris (1986). Yet, (apparent) substrate affinity can vary substantially between organisms and possibly methods. For N₂O consumption by anoxic soil incubations and denitrifiers, for example, k_m values differing by a factor of 2000 have been reported (Conrad, 1996). Even so, we would not expect nitrogenase to substantially contribute to N₂O consumption within the range of naturally occurring N₂O concentrations. Yet, other, unknown processes may account for some of the observed N₂O consumption in soil. Discovery of new processes and responsible organisms continues to our days (e.g., Strous et al., 1999). Strong support for the possible existence of assimilatory reduction of N₂O to NH₃ comes from thermodynamical considerations. Shestakov and Shilov (2001) concluded after the theoretical study of model reactions involving N₂O that a direct reduction of N₂O to NH₃ would be possible and thermodynamically advantageous to a reduction of N₂. The dissociation energy for the N-N bond in N₂O is only half that of the N₂ molecule (Herzberg, 1966). Our objective was to clarify whether such a reduction of N₂O is an ecologically relevant process occurring in soil.

2 Material and methods

Samples were obtained from the upper 10 cm of soil at three locations in Switzerland and one location in South-West Germany. The first site, A, is located at Zurich-Reckenholz (47°26' N, 8°32' E at 491 m a.m.s.l.), the sec-

ond site, B, is located in central Switzerland (47°17' N, 7°44' E at 450 m a.m.s.l.; for more details see Flechard et al. 2005). Both sites have soil types classified as cambisol and are experimental grassland sites of the Research Station ART (Agroscope Reckenholz-Tänikon), Switzerland. The third site, C, is located close to Basel (47°28' N, 7°42' E at 476 m a.m.s.l.) in a mixed deciduous forest on pseudogley over limestone; and the fourth site, D, is an open pine (*Pinus spp.*) forest on a peat bog in the Black Forest located 70 km north of Basel (47°52' N, 8°06' E at 975 m a.m.s.l.).

The soils of these sites range in texture from sandy loam to clay loam and organic, in pH from 2.9 to 7.0, in the C:N ratio from 9.4 to 28.8 and in the soil moisture from 23.0 to 93.6% (Table 1). Fresh samples were broken into aggregates of ≤ 6.2 mm within 5 h after collection from the field sites and 200 g were placed into an incubation vessel (glass, 415 cm³) at 20°C for a 24 h pre-incubation period. To minimise entry of atmospheric N₂ into the sample environment, we placed the incubation vessel during the entire experiment in an aluminium coated foil bag (volume about 2 l), which was continuously flushed with He (200 ml min⁻¹). During the incubation, the samples were exposed to a mixture of labelled (98%) ¹⁵N₂O (0.5–4 ppm) and O₂ (0.2–0.4%) in He (with a purity of 99.9999%). Labelled ¹⁵N₂O had been produced by the following thermal method. An amount of 0.1 g fully (98%) labelled NH₄NO₃ (Cambridge Isotope Laboratories, Inc., Andover, USA) was gently heated with 5 ml 6M HNO₃ and 0.05 g NaCl. The arising ¹⁵N₂O was collected in a syringe. Later it was transferred with a stream of He into a 10 l He bottle filled to 1.1 bar pressure and topped up with He to 6 bar. Close to 100% labelling of N₂O with ¹⁵N was confirmed by mass spectrometry (T. Blunier, personal communication). In the incubation experiment, a gas stream of the labelled ¹⁵N₂O, O₂ and He was mixed together and monitored by mass-flow controllers. It passed a humidifier and was divided into two equal streams with flow rates around 30 ml min⁻¹. One of the gas streams passed through the incubation vessel containing the soil sample before entering a 6-port selection valve (Valco Instruments Co. Inc., Houston, Texas, USA). The second gas stream arrived directly at the selection valve. This valve selected alternating one of the gas streams and sent it through a Nafion dryer (MD™ Series Gas dryer, Perma Pure LLC., Toms River, N.J., USA) to a 2 ml sample loop on an injection valve (10-port selection valve, Valco Instruments Co. Inc., Houston, Texas, USA) for 5 min, while the other gas stream was vented to the atmosphere. Concentrations of N₂O were measured by GC-ECD (SRI8610C Gas Chromatograph; SRI Instruments Inc., Las Vegas, N.V., USA). By varying the concentration of N₂O in the gas mixture, we were able to determine rates of gross production and gross consumption of N₂O from a linear regression fitted through the measured net N₂O fluxes against N₂O concentrations (Vieten et al., 2007). The $\delta^{15}\text{N}$ of soil organic matter (SOM) was measured (on three or more sub-samples) before and after an 11 to 29 day incubation period (Table 1)

Table 1. Summary of soil sample properties and the results of the incubation experiments with ¹⁵N labelled N₂O.

Sample	A	B	C	D
Ecosystem	Grassland	Grassland	Forest	Forest
Soil type	Cambisol	Cambisol	Pseudogley	Histosol
Texture	Sandy loam	Clay loam	Loam	Organic
C:N ratio	11.2	9.4	14.6	28.8
pH in 0.01 M CaCl ₂	6.1	5.7	7.0	2.9
Moisture [%]	23.0	33.9	33.3	93.6
dry weight of sample [g]	154	132.2	133.4	12.8
Initial mass of ¹⁵ N in sample [mg]	1.02	1.55	1.91	0.82
Duration of incubation [days]	11	20	12	29
¹⁵ N ₂ O offered to sample [mg ¹⁵ N]	1.23	1.86	0.81	1.84
¹⁵ N ₂ O consumed [mg ¹⁵ N]	0.21	0.18	0.29	0.20
Shift in δ ¹⁵ N during incubation [‰]	0.12	−0.23	−0.09	0.66

on the Flash Elemental Analyser (Thermo Finnigan; Milano, Italy) connected to a CF-IR-MS (DELTA^{plus}XP; Thermo Finnigan MAT; Bremen, Germany).

3 Results and discussions

During the incubation period of 11 to 29 days, between 0.81 and 1.86 mg of fully labelled ¹⁵N₂O were flowing through each soil sample, containing a background of 0.82 mg to 1.91 mg of ¹⁵N in organic matter (Table 1). During this time, 0.18 mg to 0.29 mg of the offered ¹⁵N₂O was consumed (Table 1). Figure 2 presents the fraction of consumed ¹⁵N₂O-N that might have been retained as part of the soil organic matter. This fraction was between −0.20% (±0.11%) and 0.27% (±0.19%) with an average across all four soils of 0.019% (±0.12%). None of the measured values were significantly different from zero ($p > 0.05$). Thus, we can consider them as measurement noise. Still, their presentation is useful insofar as it serves to illustrate the sensitivity of our method. This sensitivity was about one order of magnitude larger than that of the classical assay for N₂ fixation by acetylene reduction, as for example described by Weaver and Danso (1994). This sensitivity was brought about by the long duration of the incubation, the large atom fraction of ¹⁵N in the consumed N₂O (>0.98) and the high rates of N₂O consumption by the soil samples. During the incubation period, an equivalent of 11.6% to 24.4% of ¹⁵N initially present in the soil samples was consumed as ¹⁵N₂O (Table 1).

No significant N₂O production by the soil samples themselves was detected during these experiments. Therefore ¹⁵N₂O would have been the principal source for potential N₂O assimilation. During our experiments, we incubated the soils with He (purity of 99.9999%) plus small amounts of O₂ and ¹⁵N₂O. Since we flushed the aluminium-coated foil bag that was around the incubation vessel continuously with He, we reduced the potential influx of atmospheric N₂ into

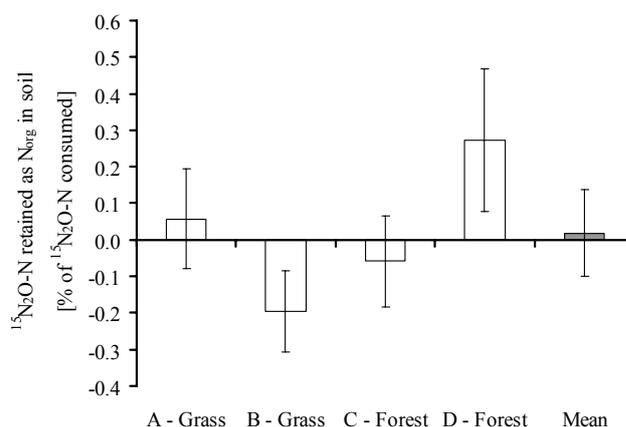


Fig. 2. Proportion of consumed ¹⁵N₂O-N that might have been retained in four soil samples. Error bars indicate ±1 standard error of the measurement as determined by the measurements of δ¹⁵N in soil organic matter on replicate sub-samples before and after the incubation period. The proportion retained has been calculated as: Shift in δ¹⁵N during incubation [‰]/1000 [‰] * initial mass of ¹⁵N in sample [mg]/¹⁵N₂O consumed [mg] * 100 [%].

the sample. We did not measure N₂ concentrations in the sample air but estimate that it has been in the order of ppm rather than per mil. Compared to the natural environment, the competitive advantage of N₂O (here: 0.5–4 ppm) relative to N₂ to be assimilated had been shifted by orders of magnitude in favour of N₂O. Thus, the likelihood for soil organisms to reduce N₂O instead of N₂ to NH₃ was substantially increased. Maybe, we should underline here that it was not our objective to measure nitrogenase activity but to find out whether some proportion of N₂O consumed in soil is assimilated, by whatever reduction pathway possible. Our method of choice (¹⁵N₂O labelling) would be unable to tell us anything specific about the nature of the pathway. We still chose

this method because detection of ¹⁵N in tissues of biological systems exposed to labelled N gas is the only direct, unequivocal method for demonstrating that its assimilation occurred (Weaver and Danso, 1994).

The negative result regarding N₂O assimilation is unexpected, considering the observed large rates of N₂O consumption, the thermodynamical advantage of N₂O over N₂ as a substrate for the production of NH₃ (Shestakov and Shilov, 2001), and the evidence of direct N₂O to NH₃ reduction as a biological process (Yamazaki et al., 1987) albeit only for a low affinity enzyme (Jensen and Burris, 1986) so far. One would expect natural selection to have favoured organisms assimilating N₂O rather than N₂. We can not completely rule out that N₂O assimilation may have some ecological importance, at least in N limited soils. Still, our results strongly indicate that in environments similar to those studied here, N₂O assimilation is an ecologically irrelevant pathway in N₂O consumption by soil. Thus, the general assumption that N₂O is exclusively consumed by dissimilatory reduction to N₂ (i.e. complete denitrification) almost certainly applies in these environments.

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