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The fate of N_2O consumed in soils

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Abstract. Soils are capable to consume N_2O. It is generally assumed that consumption occurs exclusively via respiratory reduction to N_2 by denitrifying organisms (i.e. complete denitrification). Yet, we are not aware of any verification of this assumption. Some N_2O may be assimilatorily reduced to NH_3. Reduction of N_2O to NH_3 is thermodynamically advantageous compared to the reduction of N_2. 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%) $^{15}N_2O$ (0.5–4 ppm) and O_2 (0.2–0.4%) in He. We measured N_2O consumption by GC-ECD continuously and $\delta^{15}N$ of soil organic matter before and after an 11 to 29 day incubation period. Any $^{15}N_2O$ assimilatorily reduced would have resulted in the enrichment of soil organic matter with ^{15}N, whereas dissimilatorily reduced $^{15}N_2O$ would not have left a trace. None of the soils showed a change in $\delta^{15}N$ that was statistically different from zero. A maximum of 0.27% (s.e. ±0.19%) of consumed $^{15}N_2O$ may have been retained as ^{15}N in soil organic matter in one sample. On average, ^{15}N 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 $^{15}N_2O$ consumed by the soils. We conclude that assimilatory reduction of N_2O plays, if at all, only a negligible role in the consumption of N_2O in soils.

1 Introduction

Nitrous oxide (N_2O) is produced in soils during the processes of nitrification and denitrification (Firestone et al., 1980). Since industrialisation, the global atmospheric N_2O concentration increased from about 270 ppb in 1800 to 319 ppb in 2005 (IPCC, 2006). Currently, more than one third of all N_2O emissions are of anthropogenic origin and primarily due to agriculture (IPCC, 2006). However, soils can also act as a sink for N_2O (reviewed in Chapuis-Lardy et al., 2007). In general, it is implicitly assumed that complete denitrification (reduction of N_2O to N_2) is the only process responsible for observed sink activity. Once produced by a soil organism, a molecule of N_2O is presumed to take one of the three known routes (Ostrom et al., 2007) (Fig. 1): (1) complete denitrification to N_2 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 N_2 upon re-entering a cell capable to reduce N_2O (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 NH_3 may be responsible for some of observed N_2O consumption in soil (Fig. 1).

The only enzyme known to reduce N_2O to NH_3 is nitrogenase. First evidence was provided by Mozen and Burris (1954). Later studies confirmed that N_2O can be a substrate for nitrogenase which reduces N_2O to N_2 with subsequent reduction of N_2 to NH_3 (Hoch et al., 1960; Hardy and Knight, 1966; Jensen and Burris, 1986). However, one year later, reduction of N_2O to N_2 was already questioned by Yamazaki et al. (1987). They concluded from the stable isotope kinetics during N_2O fixation by *Azotobacter vinelandii* that N_2O fixation by nitrogenase must be an apparent one-step reaction transforming N_2O directly into NH_3, without the intermediary N_2. 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 N_2O to NH_3 which does not involve N_2 as an intermediary. Enzyme kinetics indicate a low affinity of N_2O to nitrogenase. A k_m value of 24 kPa for purified component proteins from *Klebsiella pneumoniae*

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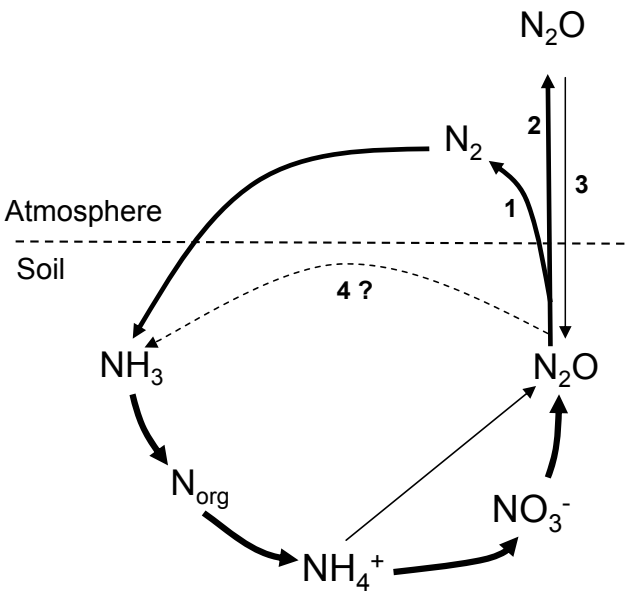

Fig. 1. Origin and possible fate of N_2O in soil. (1) Complete denitrification to N_2before escape from the cell; (2) escape from cell; (3) re-entering a cell and subsequent reduction to N_2, or (4) assimilatory reduction to NH_3. The ecological relevance of pathway (4) in natural soil is unknown and 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 N_2O 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 N_2O consumption within the range of naturally occurring N_2O concentrations. Yet, other, unknown processes may account for some of the observed N_2O 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 N_2O to NH_3 comes from thermodynamical considerations. Shestakov and Shilov (2001) concluded after the theoretical study of model reactions involving N_2O that a direct reduction of N_2O to NH_3 would be possible and thermodynamically advantageous to a reduction of N_2. The dissociation energy for the N-N bond in N_2O is only half that of the N_2 molecule (Herzberg, 1966). Our objective was to clarify whether such a reduction of N_2O 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 second 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 cm^3) at 20°C for a 24 h pre-incubation period. To minimise entry of atmospheric N_2 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%) $^{15}N_2O$ (0.5–4 ppm) and O_2 (0.2–0.4%) in He (with a purity of 99.9999%). Labelled $^{15}N_2O$ had been produced by the following thermal method. An amount of 0.1 g fully (98%) labelled NH_4NO_3 (Cambridge Isotope Laboratories, Inc., Andover, USA) was gently heated with 5 ml 6M HNO_3 and 0.05 g NaCl. The arising $^{15}N_2O$ 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 N_2O with ^{15}N was confirmed by mass spectrometry (T. Blunier, personal communication). In the incubation experiment, a gas stream of the labelled $^{15}N_2O$, O_2 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 N_2O were measured by GC-ECD (SRI8610C Gas Chromotograph; SRI Instruments Inc., Las Vegas, N.V., USA). By varying the concentration of N_2O in the gas mixture, we were able to determine rates of gross production and gross consumption of N_2O from a linear regression fitted through the measured net N_2O fluxes against N_2O concentrations (Vieten et al., 2007). The $\delta^{15}N$ 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 ^{15}N labelled N_2O.

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
$^{15}N_2O$ offered to sample [mg ^{15}N]	1.23	1.86	0.81	1.84
$^{15}N_2O$ consumed [mg ^{15}N]	0.21	0.18	0.29	0.20
Shift in $\delta^{15}N$ 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 $^{15}N_2O$ were flowing through each soil sample, containing a background of 0.82 mg to 1.91 mg of ^{15}N in organic matter (Table 1). During this time, 0.18 mg to 0.29 mg of the offered $^{15}N_2O$ was consumed (Table 1). Figure 2 presents the fraction of consumed $^{15}N_2O$-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 N_2 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 ^{15}N in the consumed N_2O (>0.98) and the high rates of N_2O consumption by the soil samples. During the incubation period, an equivalent of 11.6% to 24.4% of ^{15}N initially present in the soil samples was consumed as $^{15}N_2O$ (Table 1).

No significant N_2O production by the soil samples themselves was detected during these experiments. Therefore $^{15}N_2O$ would have been the principal source for potential N_2O assimilation. During our experiments, we incubated the soils with He (purity of 99.9999%) plus small amounts of O_2 and $^{15}N_2O$. Since we flushed the aluminium-coated foil bag that was around the incubation vessel continuously with He, we reduced the potential influx of atmospheric N_2 into

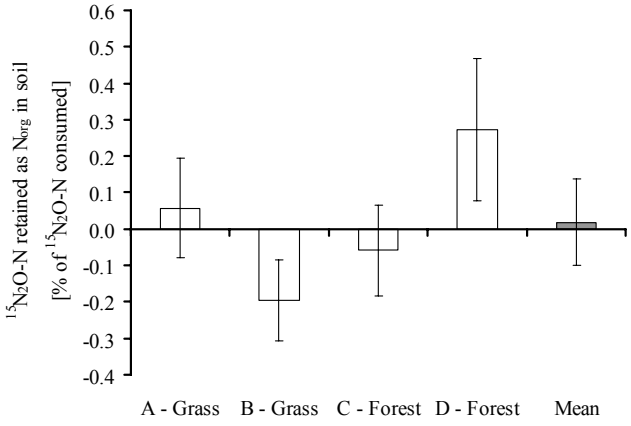

Fig. 2. Proportion of consumed $^{15}N_2O$-N that might have been retained in four soil samples. Error bars indicate ±1 standard error of the measurement as determined by the measurements of $\delta^{15}N$ in soil organic matter on replicate sub-samples before and after the incubation period. The proportion retained has been calculated as: Shift in $\delta^{15}N$ during incubation [‰]/1000 [‰] * initial mass of ^{15}N in sample [mg]/$^{15}N_2O$ consumed [mg] *100 [%].

the sample. We did not measure N_2 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 N_2O (here: 0.5–4 ppm) relative to N_2 to be assimilated had been shifted by orders of magnitude in favour of N_2O. Thus, the likelihood for soil organisms to reduce N_2O instead of N_2 to NH_3 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 N_2O consumed in soil is assimilated, by whatever reduction pathway possible. Our method of choice ($^{15}N_2O$ labelling) would be unable to tell us anything specific about the nature of the pathway. We still chose

this method because detection of ^{15}N 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 N_2O assimilation is unexpected, considering the observed large rates of N_2O consumption, the thermodynamical advantage of N_2O over N_2 as a substrate for the production of NH_3 (Shestakov and Shilov, 2001), and the evidence of direct N_2O to NH_3 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 N_2O rather than N_2. We can not completely rule out that N_2O 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, N_2O assimilation is an ecologically irrelevant pathway in N_2O consumption by soil. Thus, the general assumption that N_2O is exclusively consumed by dissimilatory reduction to N_2 (i.e. complete denitrification) almost certainly applies in these environments.

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