

SCIENTIFIC REPORTS



OPEN

Disentangling gross N₂O production and consumption in soil

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Received: 15 June 2016

Accepted: 13 October 2016

Published: 04 November 2016

The difficulty of measuring gross N₂O production and consumption in soil impedes our ability to predict N₂O dynamics across the soil-atmosphere interface. Our study aimed to disentangle these processes by comparing measurements from gas-flow soil core (GFSC) and ¹⁵N₂O pool dilution (¹⁵N₂OPD) methods. GFSC directly measures soil N₂O and N₂ fluxes, with their sum as the gross N₂O production, whereas ¹⁵N₂OPD involves addition of ¹⁵N₂O into a chamber headspace and measuring its isotopic dilution over time. Measurements were conducted on intact soil cores from grassland, cropland, beech and pine forests. Across sites, gross N₂O production and consumption measured by ¹⁵N₂OPD were only 10% and 6%, respectively, of those measured by GFSC. However, ¹⁵N₂OPD remains the only method that can be used under field conditions to measure atmospheric N₂O uptake in soil. We propose to use different terminologies for the gross N₂O fluxes that these two methods quantified. For ¹⁵N₂OPD, we suggest using 'gross N₂O emission and uptake', which encompass gas exchange within the ¹⁵N₂O-labelled, soil air-filled pores. For GFSC, 'gross N₂O production and consumption' can be used, which includes both N₂O emitted into the soil air-filled pores and N₂O directly consumed, forming N₂, in soil anaerobic microsites.

N₂O is one of the most important long-lived greenhouse gases and is expected to be the single most important ozone-depleting substance throughout the 21st century¹. Soils account, globally, for about 60% of the total N₂O flux to the atmosphere, with 6.6 Tg N yr⁻¹ from natural ecosystems and 4.1 Tg N yr⁻¹ from agricultural systems². Although it is generally known that microbial nitrification and denitrification in soils are the major sources of atmospheric N₂O, it remains a struggle to disentangle and quantify gross rates of microbial N₂O production and consumption in soil which, in turn, determine the net N₂O flux across the soil-atmosphere interface.

Under anaerobic conditions, incomplete denitrification produces N₂O whereas the terminal step of denitrification (i.e. the reduction of N₂O to N₂) consumes N₂O. Hence, microbial N₂O production and consumption can occur simultaneously in soil via the activities of different microorganisms or even by a single denitrifying cell³. In addition, within the soil profile and in the soil air-filled pores, N₂O can be further reduced to N₂ during its transport to the soil surface⁴⁻⁶. Soil physical (e.g. water or oxygen content, temperature, porosity) and biochemical factors (e.g. pH, concentrations of electron donors and acceptors) influence the balance between soil N₂O production and consumption⁷, and consequently the net N₂O flux to the atmosphere. Soil net N₂O uptake has been compiled in a review⁸, which specifically refers to the net flux of N₂O from the atmosphere to the soil and can be detected only if soil N₂O consumption exceeds production. Soil N₂O consumption, however, is often ignored because it is prone to be masked by the much larger N₂O production⁴ and is difficult to measure directly (e.g. as soil N₂ flux) against a very high (78%) atmospheric background⁹.

The static chamber method, commonly used to measure net N₂O flux on the soil surface, cannot quantify the simultaneously occurring gross N₂O production and consumption within the soil. One possibility to measure gross N₂O production and consumption in soil is the ¹⁵N₂O pool dilution (¹⁵N₂OPD) technique, which entails adding ¹⁵N₂O to the chamber headspace and subsequently measuring the changes in ¹⁴N₂O and ¹⁵N₂O over time¹⁰. So far, this ¹⁵N₂OPD technique has been used in managed grassland and cropland soils and in salt marsh landscape, all located in northern California, by the same authors who first evaluated this method under field conditions¹⁰⁻¹².

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Site characteristics	Grassland	Cropland	Beech forest	Pine forest
Location	47.57°N, 11.03°E	48.19°N, 11.96°E	51.76°N, 9.58°E	43.72°N, 10.28°E
Mean annual temperature (°C)	6.7	8.5	7.3	14.1
Mean annual precipitation (mm)	1373	1029	1100	918
Elevation (m above sea level)	870	510	510	10
Vegetation/Crop	<i>Poaceae; Taraxacum</i>	<i>Zea mays</i>	<i>Fagus sylvatica</i>	<i>Pinus pinaster</i>
Soil type	Haplic Cambisol	Calcaric Cambisol	Dystric Cambisol	Calcareous Regosol
Soil texture (% sand/silt/clay)	10/68/23	30/52/18	12 / 54/34	93/3/4
Soil bulk density (g cm ⁻³)	0.59	1.17	0.64	1.30
Soil pH	7.1	6.7	3.8	5.7
Soil total organic carbon (g C kg ⁻¹)	135	20	127	10
Soil total nitrogen (g N kg ⁻¹)	8.0	1.7	6.6	0.7
Soil C:N ratio	16.9	11.8	18.9	13.5

Table 1. Site characteristics. Soil characteristics in the grassland, cropland and pine forest sites were measured in the top 10 cm of mineral soil^{19,21}; in the beech forest site, these were measured in the top 5 cm of mineral soil.

In 2013, when the first ¹⁵N₂OPD measurements were reported¹⁰, a debate emerged as to what extent this technique is able to quantify gross N₂O production and consumption in soil. Well & Butterbach-Bahl¹³ questioned the key assumptions of the ¹⁵N₂OPD technique: the exchange and mixing of soil-derived N₂O and ¹⁵N₂O label between aerobic and anaerobic soil microsites. They argued that gross N₂O production and consumption in soil would be underestimated if produced N₂O was immediately reduced to N₂ without first mixing with the ¹⁵N₂O-labelled air in interconnected soil pore spaces. This may occur within denitrifier cells and between different microorganisms³ in anaerobic microsites, which here we infer to include not only microsites saturated with water but also isolated pores filled with or enclosed by water and water-entrapped N₂O¹⁴. Yang *et al.*¹⁵ replied that such constraints could only occur when the soil has a high proportion of anaerobic microsites, and argued that the ¹⁵N₂O label and soil-derived N₂O are likely distributed homogeneously in the chamber headspace from which the calculation of gross N₂O fluxes is derived. In summary, the efficacy of the ¹⁵N₂OPD technique to estimate gross N₂O production and consumption is still not settled, and so far this technique has only been compared with results from acetylene inhibition and ¹⁵N tracing methods. These latter methods, however, have their own limitations for determining gross N₂O production and consumption in soil since they either modify the entire denitrification process as well as its single steps (acetylene inhibition method) or require the addition of ¹⁵N-labelled substrate (¹⁵N tracing method) with the need to label the soil homogeneously including its anaerobic microsites^{9,16}.

To date, the enigmatic lack of measurements of gross N₂O production and consumption in soil impedes our ability to predict N₂O dynamics across the soil-atmosphere interface. Our study aimed to disentangle gross N₂O production and gross N₂O consumption in soil by comparing measurements from ¹⁵N₂OPD technique and gas-flow soil core (GFSC) method. The latter is an established method that directly measures gross N₂O production and consumption in soil by simultaneously quantifying N₂O and N₂ fluxes¹⁷ without the use of an inhibitor or ¹⁵N labelling of substrate^{9,16}. We hypothesized that if the assumption of the ¹⁵N₂OPD method (i.e. exchange and mixing of soil-derived N₂O and ¹⁵N₂O label between aerobic and anaerobic soil microsites) is attained, then the ¹⁵N₂OPD and GFSC methods should yield comparable estimates of gross N₂O production and consumption in soil. We tested this hypothesis using different soils from four ecosystems: grassland, cropland, beech and pine forests (Table 1), covering a range of soil biochemical characteristics as well as soil aeration status (e.g. water content and soil texture) and N availability.

Results

From the ¹⁵N₂OPD measurements, gross N₂O production and consumption rates and net N₂O flux (Fig. 1a–c) were higher ($p = 0.01–0.03$) in the silty loam Cambisol soil in manured grassland than in the sandy Regosol soil in unmanaged pine forests, and neither differed from the sandy loam Cambisol soil in cropland or the silty loam Cambisol soil in unmanaged beech forest. For the grassland, cropland and beech forest, net N₂O emissions accounted for 66–79% of gross N₂O production (Fig. 1d). For the pine forest, net N₂O uptake (Fig. 1c) was paralleled by larger gross N₂O consumption (Fig. 1b) than gross N₂O production (Fig. 1a); these fluxes were very small but still above our detection limit.

From the GFSC measurements, gross N₂O production (Fig. 1a) was higher ($p = 0.02$) in the beech forest than in the cropland and pine forest and intermediate in the grassland. Gross N₂O consumption ($p = 0.37$; Fig. 1b) and net N₂O fluxes ($p = 0.06$; Fig. 1c) did not differ among sites. Net N₂O fluxes accounted, on average, for only 24% of gross N₂O production (Fig. 1d), and hence most (76%) of the produced N₂O was further reduced to N₂.

Although significant differences in gross N₂O production and consumption between the ¹⁵N₂OPD technique and GFSC method were only found in the grassland site ($p = 0.02$ for both; Fig. 1a,b), the fluxes measured by the GFSC method were up to two orders of magnitude larger than those measured by the ¹⁵N₂OPD technique (Fig. 1a,b). The large spatial variation within each site (indicated by the large standard errors) resulted in non-statistically detectable differences between these two methods. However, for gross N₂O production, rates measured by the ¹⁵N₂OPD technique were on average 10% of those measured by the GFSC method (Fig. 1a). For gross

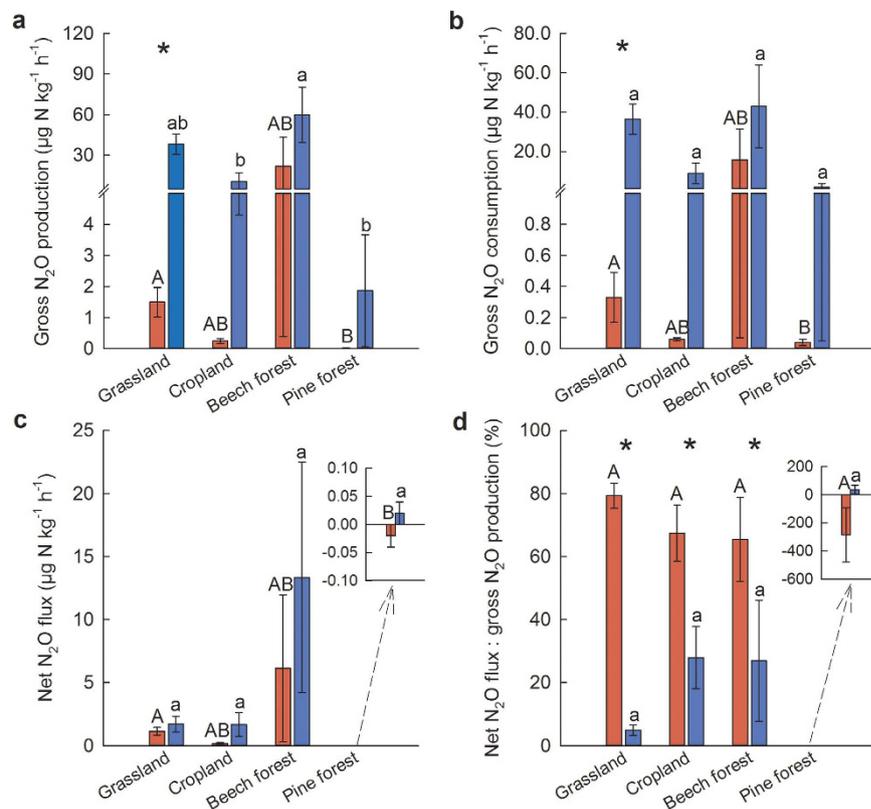


Figure 1. Soil gross and net N₂O fluxes. Gross N₂O production (a), gross N₂O consumption (b), net N₂O flux (c), and the ratio of net N₂O flux to gross N₂O production (d), measured by ¹⁵N₂O pool dilution (¹⁵N₂OPD; red bars) and gas-flow soil core (GFSC; blue bars). For each method, means (± s.e., n = 4 replicate sampling points) with different capital (for ¹⁵N₂OPD) and small letters (for GFSC) indicate significant differences among sites (one-way ANOVA with Fisher's LSD test at $p \leq 0.05$ or Kruskal-Wallis ANOVA with multiple comparisons of mean ranks at $p \leq 0.05$). For each site, asterisks above the bars indicate significant differences between the two methods (paired t test at $p \leq 0.05$).

N₂O consumption, rates measured by the ¹⁵N₂OPD technique were on average 6% of those measured by the GFSC method (Fig. 1b). Net N₂O fluxes from the soil cores used for the ¹⁵N₂OPD measurement were on average 94% of those measured by the GFSC method, which did not differ in any of the sites ($p = 0.11$ – 0.61 ; Fig. 1c). In three sites, except in the pine forest that had very low fluxes, the ratios of net N₂O flux to gross N₂O production measured by the ¹⁵N₂OPD technique were higher ($p < 0.01$ – 0.05) than those measured by the GFSC method (Fig. 1d).

Soil water-filled pore space (WFPS), microbial C and N, and denitrification enzyme activity (DEA) were generally higher ($p \leq 0.02$) in the grassland than in the pine forest (Table 2). Soil NH₄⁺ concentrations were higher ($p < 0.01$) in the grassland and beech forest compared to the cropland, whereas soil NO₃⁻ concentrations were higher ($p = 0.02$) in the cropland than in the grassland and pine forest (Table 2). Gross N₂O production and consumption, measured by either the ¹⁵N₂OPD technique or the GFSC method, showed positive correlations with WFPS, NH₄⁺, microbial C and N, and DEA ($R = 0.56$ – 0.93 , $p < 0.05$; Supplementary Table S1). Net N₂O fluxes from the soil cores used for the ¹⁵N₂OPD measurements correlated positively with the same soil properties ($R = 0.64$ – 0.92 , $p < 0.01$; Supplementary Table S1), whereas no correlation was found with net N₂O flux measured by the GFSC method.

Discussion

Both the ¹⁵N₂OPD and GFSC methods have been proposed to be able to measure gross N₂O production and consumption in soils^{9,10}. The comparable net N₂O fluxes determined by these methods (Fig. 1c) suggest that both methods can yield similar results in terms of the net effect of concurrently occurring production and consumption of N₂O. However, the measured gross N₂O production and consumption rates (Fig. 1a,b), and thus the ratios of net N₂O flux to gross N₂O production (Fig. 1d), differed between the two methods. Hence, we reject our hypothesis that ¹⁵N₂OPD technique and GFSC method yield comparable estimates of gross N₂O fluxes.

When using the ¹⁵N₂OPD technique, gross N₂O production is determined from the dilution of ¹⁵N₂O label by ¹⁴N₂O produced in the soil¹⁰. An implicit assumption of this approach is that the headspace-labelled ¹⁵N₂O that diffuses into the soil results in a homogeneous mixture of ¹⁵N₂O with soil-derived N₂O in the soil air-filled pores, which also imply that these pores must be interconnected to the soil surface for homogenous mixing to occur. Our conservative calculations of diffusive transport of ¹⁵N₂O into interconnected soil air-filled pores suggest that ¹⁵N₂O must have diffused into these pores and back to the headspace within 0.5 h. However, there may be two situations when gross N₂O production and consumption will be underestimated by this method: 1) produced N₂O

Soil characteristics	Grassland	Cropland	Beech forest	Pine forest
Water-filled pore space (%)	79 ± 1 a	57 ± 2 ab	70 ± 14 ab	25 ± 1 b
NH ₄ ⁺ (mg N kg ⁻¹)	4.34 ± 0.97 a	0.66 ± 0.12 b	2.35 ± 0.37 a	1.30 ± 0.18 ab
NO ₃ ⁻ (mg N kg ⁻¹)	1.00 ± 0.14 b	5.42 ± 0.60 a	4.17 ± 2.14 ab	0.71 ± 0.38 b
Microbial C (g C kg ⁻¹)	3.26 ± 0.13 a	0.76 ± 0.03 c	2.68 ± 0.24 ab	1.72 ± 0.10 bc
Microbial N (mg N kg ⁻¹)	211.02 ± 4.84 a	69.22 ± 0.90 c	160.90 ± 11.35 ab	98.70 ± 5.37 bc
Denitrification enzyme activity (g N kg ⁻¹ h ⁻¹)	5.16 ± 0.64 a	0.21 ± 0.07 bc	0.83 ± 0.17 ab	0.00 ± 0.00 c

Table 2. Soil physical and biochemical characteristics in the top 5 cm, determined from the soil cores immediately after the measurement of gross N₂O fluxes. Means ± s.e. (n = 4) within each row followed by different letter indicate significant differences among sites (one-way ANOVA with Fisher's LSD test at $p \leq 0.05$ or Kruskal-Wallis ANOVA with multiple comparisons of mean ranks at $p \leq 0.05$).

is immediately consumed within denitrifier cells³, and 2) produced N₂O diffuses out of denitrifier cells and is consumed by other microorganisms, which may have N₂O reductase but cannot act on the preceding substrates of the denitrification pathway¹⁸, without being mixed first with the ¹⁵N₂O label during the 3-hour measurement period. Both situations can occur in anaerobic microsites, which here we infer to microsites saturated with water, isolated pores filled with or enclosed by water forming a diffusion barrier, and water-entrapped N₂O as expounded by Clough *et al.*¹⁴. If these situations happen, disparity between ¹⁵N₂OPD and GFSC measurements would be large in a fine-textured soil with high water content whereas they would be comparable in a coarse-textured soil with low water content. The fact that our results showed the large differences between the ¹⁵N₂OPD and GFSC measurements in the silty loam soil of grassland with high WFPS and they were particularly comparable in the sandy soil of pine forest with low WFPS (Fig. 1a,b; Table 2) suggest that the ¹⁵N₂OPD technique was not able to quantify gross N₂O production in these above-mentioned two situations. With the GFSC method, gross N₂O production is measured as the sum of emitted N₂O and N₂, and thus those immediately consumed N₂O to N₂ within denitrifier cells and between different microorganisms in anaerobic microsites are included in this measurement.

We summarize our results into a conceptual model in order to illustrate two decoupled pathways of N₂O production and consumption in soil (Fig. 2). In the first pathway, N₂O is produced in anaerobic microsites and reduced immediately to N₂ without first mixing with the ¹⁵N₂O label. Based on our results, only the GFSC method but not the ¹⁵N₂OPD technique was able to quantify this pathway. The second pathway covers the soil-derived N₂O that diffuses into the interconnected soil air-filled pores and mixes with the ¹⁵N₂O label, which was captured by the ¹⁵N₂OPD technique. Even if the N₂O that has moved into the soil air-filled pores is being consumed during its diffusion towards the soil-atmosphere interface⁴, as long as the produced N₂O mixes with the ¹⁵N₂O label this can be included in the ¹⁵N₂OPD calculations of gross N₂O production. It is clear that both ¹⁵N₂OPD and GFSC methods yield complementary important information, and thus a differentiation in the use of terminologies is needed. Since the ¹⁵N₂OPD technique reflects the N₂O dynamics in the gas phase of the soils and its exchange with the atmosphere, we propose to use the terms 'gross N₂O emission' and 'gross N₂O uptake' to denote the gross N₂O fluxes in interconnected soil air-filled pores measured by this method. Since the GFSC method measures gross N₂O fluxes not only in interconnected soil air-filled pores but also in anaerobic microsites, we propose that the terms 'gross N₂O production' and 'gross N₂O consumption' be used (Fig. 2). Below we will use these proposed terminologies to distinguish between the processes measured by these two methods.

It is important to point out that the ¹⁵N₂OPD technique is able to yield information on gross N₂O uptake from the atmosphere to the soil. For years there has been a discussion on the importance of N₂O uptake in the soil from the atmosphere and substantial progress has been hampered because until now only the net N₂O fluxes on the soil surface can be routinely measured with inexpensive static chamber method. With the ¹⁵N₂OPD technique, we now have an operational approach that can be used for field measurements and can separate the net N₂O fluxes across the soil-atmosphere interface into gross N₂O emission and gross N₂O uptake. It is a significant advancement since this technique will allow us to investigate the factors that control N₂O uptake by soils under actual field conditions, which is a commonly unquantified sink of ecosystem N budgets.

Moreover, our results contrast to the notion that substantial N₂O uptake only happens in soils with net negative N₂O flux. This was shown by the larger gross N₂O uptake (measured by ¹⁵N₂OPD technique) in the grassland that had larger net N₂O emissions than in the pine forest that had a net negative N₂O flux (Fig. 1b,c). The positive correlations of gross N₂O uptake with soil biochemical characteristics (Supplementary Table S1) suggest that high gross N₂O uptake occurs in soils with high microbial activity and high substrate availability (Table 2). The ratios of net to gross N₂O emissions (66–79% in grassland, cropland and beech forest; Fig. 1d) were similar to the values reported by Yang *et al.*¹⁰ and Yang and Silver¹² from managed grassland and cropland in California (net to gross N₂O emission ratio of 68–70%). These generally comparable ratios may open the possibility of making estimates of gross N₂O emissions and uptake based on measured net N₂O emissions.

The large fraction of gross N₂O production that was consumed to N₂ (measured by GFSC method) suggests that gross N₂O production and consumption were closely coupled, which is in line with our aforementioned deduction (i.e. most N₂O was immediately reduced to N₂ in anaerobic microsites). Hence, the similar correlations found for gross N₂O production and consumption with soil biochemical characteristics (Supplementary Table S1) as those found for gross N₂O emission and uptake (measured by ¹⁵N₂OPD technique) suggests that these gross N₂O fluxes were regulated by the same process, denitrification⁴.

Our findings show that whereas the ¹⁵N₂OPD technique is a valuable tool to separate net N₂O flux across the soil-atmosphere interface into gross N₂O emission and uptake, it did not allow measuring a large part of gross

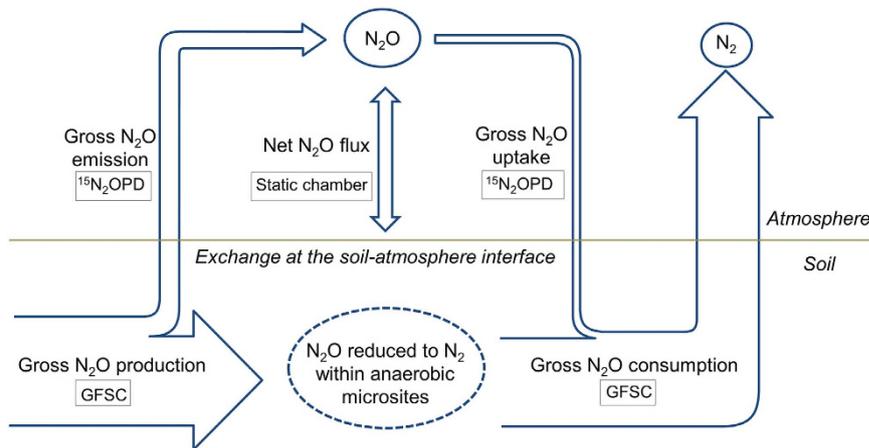


Figure 2. Conceptual diagram of gross N_2O fluxes. Gross N_2O emission and gross N_2O uptake, measured by $^{15}\text{N}_2\text{O}$ pool dilution ($^{15}\text{N}_2\text{OPD}$), which largely includes gas exchange in interconnected air-filled pores in the soil; gross N_2O uptake = gross N_2O emission – net N_2O flux. Gross N_2O production and gross N_2O consumption, measured by gas-flow soil core (GFSC), which encompasses the soil air-filled pores as well as anaerobic microsites (e.g. soil micro spots saturated with water, isolated pores filled with or enclosed by water, and water-entrapped N_2O); gross N_2O consumption = N_2 emission, and gross N_2O production = gross N_2O consumption + net N_2O flux.

N_2O production and consumption in anaerobic microsites. In order to avoid misinterpretations of terminologies, we propose that the terms ‘gross N_2O emission and uptake’ should be used for gross N_2O fluxes measured with the $^{15}\text{N}_2\text{OPD}$ technique and ‘gross N_2O production and consumption’ should be used for gross N_2O fluxes measured with the GFSC method.

Methods

Study sites and soil sampling.

Soil samples were collected from four ecosystems: grassland, cropland, beech and pine forests, covering different vegetation, soil types and climatic conditions (Table 1). The montane grassland is manured 2–3 times a year and cut for hay three times a year¹⁹. The cropland is a conventional corn-winter wheat rotation. The unmanaged beech forest (*Fagus sylvatica*) is 163 years old²⁰, and the unmanaged Mediterranean pine forest (*Pinus pinaster*) is 52 years old²¹.

At each site, we selected four sampling points as replicates with a minimum distance of 25 m from each other. At each replicate, eight intact soil cores (250 cm³ each) were taken using stainless-steel cores (8 cm diameter, 5 cm height): four of which were used for the $^{15}\text{N}_2\text{OPD}$ measurement and the other four for the GFSC measurement. The $^{15}\text{N}_2\text{OPD}$ measurement was conducted concurrently with the GFSC measurement, such that the soil cores for these two methods were handled similarly in all aspects. Neither soil moisture nor substrate level was adjusted.

$^{15}\text{N}_2\text{O}$ pool dilution. Four intact soil cores were placed in an incubation glass (6.6 L volume), equipped with Luer-lock stopcock for gas sampling. Upon closure of the incubation vessel, we injected into the chamber headspace 7 mL of $^{15}\text{N}_2\text{O}$ label gas, containing 100 ppmv of 98% single labelled $^{15}\text{N}-\text{N}_2\text{O}$, 275 ppbv sulfurhexafluoride (SF_6 , as a tracer for physical loss of N_2O) and the rest as synthetic air. This injected amount increased the N_2O concentration in the headspace by approx. 106 ppbv N_2O with 12.5 atom% ^{15}N enrichment and SF_6 concentration of 292 pptv. At 0.5, 1, 2, and 3 h following label gas injection, 100 mL and 12 mL gas samples were taken out and stored in pre-evacuated 100 mL glass bottles and 12 mL glass tubes (Exetainer; Labco Limited, Lampeter, UK), respectively, with rubber septa. The sampled air volume was then replaced with 112 mL of a gas mixture (80% helium and 20% oxygen) to maintain the headspace at atmospheric pressure and oxygen concentration, without altering the isotopic composition of the headspace N_2O . The dilution that this replacement caused was accounted for in the calculations. The 100 mL gas samples were used to analyze isotopic composition using an isotope ratio mass spectrometer (IRMS) (Finnigan Delta^{plus} XP, Thermo Electron Corporation, Bremen, Germany). The 12 mL gas samples were used to measure N_2O and SF_6 concentrations using a gas chromatograph equipped with an electron capture detector (GC 6000 Vega Series 2, Carlo Erba Instruments, Milan, Italy). The detection limit of the entire measurement set-up and instrument precision was <0.9 ppbv N_2O h⁻¹.

We modeled the vertical diffusive transport of $^{15}\text{N}_2\text{O}$ label through the 5 cm long soil cores, using the diffusion equation $\frac{\partial C}{\partial t} = \frac{\partial^2 C}{\partial x^2}$ in which C, t and x denote concentration, time and path length, respectively²². The free-air N_2O diffusion coefficient at 15 °C, 0.1582 cm s⁻¹, was used and adjusted for soil tortuosity based on the air-filled porosity²³, which was calculated using the measured bulk density and gravimetric moisture contents. Our most conservative calculations, using the lowest air-filled porosity and assuming an impervious boundary condition at bottom of the soil cores, showed that the $^{15}\text{N}_2\text{O}$ label had diffused into the 5 cm long soil cores and back to the headspace within 0.5 h. Thus, our sampling interval during the 3-hour measurement period was sufficient to allow mixing of the label gas with the soil-derived N_2O in interconnected air-filled pores and to quantify the changes in N_2O concentrations and $^{15}\text{N}_2\text{O}$ enrichments in the headspace.

Gross N₂O emission rate was calculated using the following equations modified from Yang *et al.*¹⁰:

$$[^{14}\text{N}_2\text{O}]_t = \frac{F_{14} \times P}{(k_{14} + k_l)} - \left\{ \frac{F_{14} \times P}{(k_{14} + k_l)} - [^{14}\text{N}_2\text{O}]_0 \right\} \times \exp\{-(k_{14} + k_l) \times (t - t_0)\} \quad (1)$$

$$[^{15}\text{N}_2\text{O}]_t = \frac{F_{15} \times P}{(k_{15} + k_l)} - \left\{ \frac{F_{15} \times P}{(k_{15} + k_l)} - [^{15}\text{N}_2\text{O}]_0 \right\} \times \exp\{-(k_{15} + k_l) \times (t - t_0)\} \quad (2)$$

where $[^{14}\text{N}_2\text{O}]_t$ is the concentration of $^{14}\text{N}_2\text{O}$ at time t , calculated as the product of N₂O concentration and ^{14}N -N₂O atom%; $[^{15}\text{N}_2\text{O}]_t$ is the concentration of $^{15}\text{N}_2\text{O}$, calculated as the product of N₂O concentration and ^{15}N -N₂O atom% excess, assuming that the ^{15}N isotopic composition of background N₂O is 0.3688 atom%¹⁰; t represents the time of gas sampling from the headspace; F_{14} represents the $^{14}\text{N}_2\text{O}$ mole fraction (0.997) and F_{15} represents the $^{15}\text{N}_2\text{O}$ mole fraction (0.003) of emitted N₂O; k_{14} and k_{15} represent the first-order rate constants of $^{14}\text{N}_2\text{O}$ and $^{15}\text{N}_2\text{O}$ reduction to N₂, respectively, calculated based on the fractionation factor ($\alpha = k_{15}/k_{14}$) that has an average value of 0.9924 ± 0.0036 in literature¹⁰; k_l represents the first-order rate constant for loss of inert transport tracer, SF₆; P is gross N₂O emission rate. The k_{14} and k_{15} represent the biological loss, and k_l represents the physical loss. Since the changes of their concentrations in the headspace are simultaneously affected by biological consumption and physical loss, we used the sum of these constants ($k_{14} + k_l$ or $k_{15} + k_l$) in the above equations.

We estimated the parameters for P and k_{15} by simultaneously fitting the measured $[^{14}\text{N}_2\text{O}]_t$ and $[^{15}\text{N}_2\text{O}]_t$ with equation (1) and (2). The best fit of $[^{14}\text{N}_2\text{O}]_t$ and $[^{15}\text{N}_2\text{O}]_t$ was found using the least square approach and minimizing the following error function:

$$E = \sum_{t=1}^n \frac{(Y_{\text{predicted}}(t) - Y_{\text{observed}}(t))^2}{SD} + \sum_{t=1}^n \frac{(Z_{\text{predicted}}(t) - Z_{\text{observed}}(t))^2}{SD} \quad (3)$$

where E is minimal weighted error (E); Y , Z and n indicate $^{14}\text{N}_2\text{O}$, $^{15}\text{N}_2\text{O}$ concentrations, and the number of measurements, respectively; SD refers to the standard deviation of the observed concentrations over the course of measurements^{24,25}. Equation (3) was minimized using the 'fminsearchbnd' function in MATLAB (MathWorks, Version R2011b, USA). Gross N₂O uptake was calculated as the difference between gross N₂O emission and net N₂O flux¹⁰.

Gas-flow soil core. The GFSC method is a fully automated, direct and sensitive quantification of the change of N₂O and N₂ concentrations in the headspace above the soil cores. The soil air of the four soil cores and the headspace of the incubation vessel were completely replaced by a gas mixture consisting of 20% O₂ (purity grade of 5.5), 80% He (purity grade of 5.0), N₂O (400 ppbv) and N₂ (25 ppmv). This complete exchange was done by automated repeated cycles of evacuation and gas purging, achieved through a built-in purging system in an extremely air-tight chamber that is connected directly to a gas chromatograph (Shimadzu GC-17A, Shimadzu, Munich, Germany)^{17,26–28}. Eighteen hours of evacuation-purging cycles ensure a complete removal of the background atmospheric air²⁷, after which the headspace and tubing connections to the gas chromatograph were further purged for three hours. Subsequently, the system switched to a static chamber mode, and the headspace air of the incubation vessel was analyzed hourly over four hours through a directly connected gas chromatograph with an electron capture detector for N₂O analysis and a pulse discharge He ionization detector (Vici AG, Schenkon, Switzerland) for N₂ analysis²⁶. To sample the headspace, a slight overpressure was created by injecting 40 mL of the He-based gas mixture to the headspace, directing headspace air to the sampling loops²⁶. The dilution of this non-intrusive overpressure sampling technique was accounted for in the calculation of N₂O and N₂ concentrations²⁶. In order to achieve the best possible tightness of the incubation system against intrusion of atmospheric N₂, all tubing connections, valves as well as the entire incubation vessel were placed under water. Before starting the N₂O and N₂ measurements, the air-tightness of the system was always checked with an empty incubation vessel, which was connected in parallel with the measuring vessel. Based on the sensitivity and repeatability of the gas chromatograph measurements, the detection limits were <0.03 ppmv h⁻¹ for N₂ and <0.45 ppbv h⁻¹ for N₂O. The measured N₂ flux from the soil equals to gross N₂O consumption whereas the sum of N₂ and N₂O fluxes equals to gross N₂O production^{17,26–28}.

Soil controlling factors. Soil water content (one-day oven-drying at 105 °C and expressed as WFPS using 2.65 g cm⁻³ as particle density and the measured bulk density; Table 1), NH₄⁺ and NO₃⁻ concentrations (0.5 M K₂SO₄ extraction), and microbial biomass C and N (CHCl₃ fumigation-extraction) were determined from the soil cores immediately after the gas measurements. NH₄⁺ and NO₃⁻ concentrations in the soil extract were determined using continuous flow autoanalyzer (Skalar Scan plus system, Skalar Analytical B.V., Breda, Netherlands). Microbial biomass C and N were determined as the difference in 0.5 M K₂SO₄-extractable organic C and N (analyzed using persulfate oxidation with an infrared detector; Multi N/C 3100 TOC/TNb-Analysator, Analytik Jena, Jena, Germany) between the fumigated and unfumigated soils divided by $k_{\text{EC}} = 0.45$ and $k_{\text{EN}} = 0.68$ ²⁹. DEA was determined from the N₂O produced during an anaerobic incubation with glucose and NO₃⁻ added in excess and acetylene inhibited N₂O reduction of to N₂³⁰.

Statistical analysis. The above soil properties, determined separately from the soil cores used for $^{15}\text{N}_2\text{OPD}$ and GFSC measurements, did not differ between these two measurements ($p > 0.05$; paired t test); thus, the values from the two measurements were averaged to represent a replicate sampling point. Data sets were first tested for

normal distribution (Shapiro-Wilk's test) and equality of variance (Levene's test). We used log-transformation for variables with non-normal distributions or unequal variances and assessed the differences in gross N₂O fluxes and soil properties among sites using one-way analysis of variance (ANOVA) with Fisher's least significant difference test. When none of the data transformations were able to attain normal distribution and equality of variance, differences among sites were tested using the Kruskal-Wallis ANOVA with multiple comparisons test. The differences in gross and net N₂O fluxes between the ¹⁵N₂OPD and GFSC methods for each site were assessed using the paired t test. Relationships of gross N₂O fluxes with soil properties were assessed using Spearman rank correlation test. Statistical significance was set at $p \leq 0.05$. Statistical analyses were conducted using SPSS (SPSS, Chicago, Illinois, USA).

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Acknowledgements

This work was funded by the Deutsche Forschungsgemeinschaft (DFG, Co 749/1-1). Yuan Wen was supported by China Scholarship Council. Zhe Chen was supported by Alexander von Humboldt Foundation and National Natural Science Foundation of China (21107109). Further funding was provided by the Helmholtz/BMBF TERENO initiative, BMBF SUSALPS project, DFG BU 1173/17-1, DFG SFB 990/2 (project A05), DFG VE 219/14-1, and BMBF SIGNAL project.

Author Contributions

M.D.C., E.V., M.D., B.W. and K.B.-B. designed the study; Y.W. and Z.C. carried out the measurements and analyzed data; B.W. modeled the diffusive transport of ¹⁵N₂O label in soil; A.C. and Y.W. solved the ¹⁵N₂OPD equations in MATLAB and experimentally tested them; G.W., Z.C., B.W., R.K., M.D. and K.B.-B. established the GFSC method; M.D. and Z.C. conceptualized Fig. 2; M.D.C., Y.W. and E.V. wrote most parts of the manuscript; all authors reviewed and rewrote parts of the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Wen, Y. *et al.* Disentangling gross N₂O production and consumption in soil. *Sci. Rep.* **6**, 36517; doi: 10.1038/srep36517 (2016).

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