In Situ Quantification of Biological N₂ Production Using Naturally Occurring ¹⁵N¹⁵N

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ABSTRACT: We describe an approach for determining biological N₂ production in soils based on the proportions of naturally occurring ¹⁵N¹⁵N in N₂. Laboratory incubation experiments reveal that biological N₂ production, whether by denitrification or anaerobic ammonia oxidation, yields proportions of ¹⁵N¹⁵N in N₂ that are within 1‰ of that predicted for a random distribution of ¹⁵N and ¹⁴N atoms. This relatively invariant isotopic signature contrasts with that of the atmosphere, which has ¹⁵N/¹⁴N proportions in excess of the random distribution by 19.1 ± 0.1‰. Depth profiles of gases in agricultural soils from the Kellogg Biological Station Long-Term Ecological Research site show biological N₂ accumulation that accounts for up to 1.6% of the soil N₂. One-dimensional reaction-diffusion modeling of these soil profiles suggests that subsurface N₂ pulses leading to surface emission rates as low as 0.3 mmol N₂ m⁻² d⁻¹ can be detected with current analytical precision, decoupled from N₂O production.

INTRODUCTION

Biological N₂ production constitutes the main mechanism through which fixed nitrogen is returned to the atmosphere. While many methods have been developed for measuring N₂ production in the field, obtaining accurate estimates of ecosystem fixed-nitrogen loss remains a challenge. Field-based techniques often require nutrient amendments (e.g., ¹⁵N-labeled nitrate), manipulation of biochemical pathways (e.g., C₃ inhibition of nitrous oxide reductase), or sampling and incubation of soil cores, all of which introduce poorly constrained uncertainties. For example, in nutrient amendment studies, the fraction of extant nitrogen substrate utilized must be accounted for, but it is often difficult to constrain. Moreover, biological N₂ production can be stimulated by substrate addition, biasing measurements based on this approach. Soil-core incubations to evaluate N₂ production may not require nutrient amendments, but instead require that the extant gases be replaced with a gas mixture to reduce or replace the ambient N₂ background. Ultimately, this suite of methods for quantifying N₂ production rates can only probe short-term and potential rates of denitrification and other nitrogen-loss processes. Importantly, they may not integrate variation in activity that occurs over longer time scales at a given sampling site. Passive in situ measurements are rare, and fraught with a different set of complications: a recent attempt to use N₂/Ar ratios to probe excess N₂ production in situ found that physical fractionation of gases, combined with insufficient sensitivity, would likely preclude its widespread application.

Stable isotopes of nitrogen at natural abundance levels could in principle be used to determine the amount of biologically produced N₂ in soil gases as well. Variations in the ¹⁵N/¹⁴N ratio of N₂, reported as a δ-value in per mil (‰) relative to atmospheric N₂,

\[ \delta^{15}N = \frac{^{15}R}{^{14}R} - 1 \]  

(1)

can be caused by variability in the chemistry of N₂ cycling, substrate δ¹⁵N, and physical transport. Nevertheless, a large isotopic contrast may exist between biological and atmospheric
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N₂: strong isotopic fractionation for N₂-yielding processes⁸⁻¹⁰ can result in local deviations in the δ¹⁵N value of N₂ relative to their substrates and the atmospheric background. However, closed-system and rate-dependent effects on isotopic fractionation,¹¹ the broad distribution of substrate δ¹⁵N values,¹² and physical fractionation affecting the elemental and isotopic composition of soil gases¹³ are rarely well-characterized, rendering the interpretation of bulk δ¹⁵N values in soil N₂ non-unique; disentangling the variations in δ¹⁵N of soil N₂ may not be possible without additional constraints.

We recently developed methods to measure ¹⁵N/¹⁴N in N₂ with high precision at natural abundances, which offers a new approach to quantifying in N₂ production on local and global scales.¹⁴ Together with ¹⁹N/¹⁸N ratios, measurements of the ¹³N/¹⁴N ratio in N₂ yield a “clumped” isotope tracer, Δ₃₀, which is defined below and also reported in per mil:

\[
Δ₃₀ ≡ \frac{3₀ R_{sample}}{3₀ R_{random}} - 1
\]  

\[
3₀ R_{sample} = \frac{¹⁵N}{¹⁴N} / \frac{¹⁴N}{¹³N}
\]  

\[
3₀ R_{random} = (¹⁵N/¹⁴N)²
\]

Unlike the δ¹⁵N value, Δ₃₀ represents the proportional (rather than absolute) enrichment in ¹⁵N/¹⁴N, quantified relative to a random distribution of ¹⁵N and ¹⁴N atoms in N₂ molecules. The δ¹⁵N value of the substrate does not affect the Δ₃₀ value of a N₂-yielding process because the Δ₃₀ value is normalized against the bulk ¹⁵N/¹⁴N ratio (eqs 3 and 5). Moreover, the Δ₃₀ tracer is insensitive to physical fractionation and nitrogen fixation;¹⁴,¹⁵ these processes tend to preserve proportions of ¹⁵N/¹⁴N relative to ¹⁴N/¹⁴N. Furthermore, the Δ₃₀ values of the biological N₂ thus far identified cluster near zero, while the δ₃₀ value of atmospheric N₂ is 19.1 ± 0.1%—a signature of upper-atmospheric gas-phase reactions.¹⁶ It results in a large isotopic contrast between biological and atmospheric N₂. Local sub-atmospheric Δ₃₀ values in soils thus may reflect the presence of biological N₂, which can be quantified through a clumped-isotope mass balance if the Δ₃₀ signatures of different N₂-producing pathways are sufficiently similar. Δ₃₀ values may trace biological N₂ production in situ using the same principles first laid out by Hauck and co-workers,¹⁶,¹⁷ but without the need for nutrient amendments or isotopic labels.

Motivated by this potential application, we conducted a broader survey of Δ₃₀ values from biological processes. Specifically, we expanded our earlier characterization of Δ₃₀ values from denitrifying bacteria with new measurements of Δ₃₀ signatures from anaerobic ammonia-oxidizing (anammox) bacteria and incubations of natural soils. The narrow distribution of biological Δ₃₀ signatures that we find suggests that Δ₃₀ values can indeed be used to quantify biological N₂ production in soils, and possibly also other restricted environments. As a proof-of-principle application, we present two soil-gas depth profiles that show evidence for biological N₂ production, and evaluate the sensitivity of the approach.

**Experimental Methods**

Isotopic analyses were performed on the ultrahigh resolution Nu Instruments Panorama mass spectrometer at the University of California, Los Angeles according to methods described previously.¹⁴,¹⁸ The uniquely high resolution of the instrument allows the simultaneous measurement of ¹⁴N/¹⁵N and ¹⁵N/¹⁴N ratios at m/z = 29 and 30, with near-baseline resolution of ¹³N/¹⁴N from ¹⁴N/¹⁸O and ¹²C/¹⁸O at m/z = 30. N₂ gas samples (20–50 μmol) were isolated from experimental headspace and soil-derived gases using cryogenic purification on a high-vacuum sample preparation line followed by gas chromatographic separation from O₂ and Ar before isotopic analysis. Cryogenic purification removes condensable gases (e.g., CO₂ and some hydrocarbons) and was accomplished by passing the gas through a stainless-steel U-trap submerged in liquid nitrogen (−196 °C). The gas was then condensed onto silica gel pellets at −196 °C within the sample-injection loop of a gas-chromatographic system. N₂ gas was separated from O₂ and Ar using a molecular sieve SA column (3 m x 1/8” OD) followed by a HayeSep D column (2 m x 1/8” OD) inline, all with a 20 mL min⁻¹ He flow rate at 25 °C. The sample gases, air, and high-temperature standards of N₂ (which were heated at 800 °C for 24–48 h over strontium nitride) were purified the same way and analyzed during the same analytical sessions. Analytical precision for replicate air samples during these sessions was ±0.006‰ for δ¹⁵N and ±0.008‰ for Δ₃₀.

To determine the Δ₃₀ signatures of N₂ produced by anammox bacteria, headspace outflows from several anammox bioreactors at Radboud University were sampled. Outflows from bioreactors containing enrichment cultures of the genera Candidatus Kuenenia and Ca. Brocadia (both freshwater genera), as well as Ca. Scalindua (a marine genus) were sampled using a 8 mL sampling loop made of a 1/4 in. OD stainless steel tube. The gas mixture was transferred cryogenically to a pre-evacuated sample finger filled with silica gel at −196 °C for 15 min before flame-sealing. All enrichment cultures at Radboud University were grown on the same NH₄SO₄ + NaNO₂ substrates, which had δ¹⁵N values of −0.5 ± 0.3‰ and −26.2 ± 0.3‰, respectively. Atmospheric contamination was monitored using gas chromatography—mass spectrometry of the outflow, using O₂ (m/z = 32) as a proxy. A correction for air-N₂ contamination in the bioreactor headspace was calculated from the O₂ signal and a proportionality coefficient determined through a series of volumetrically calibrated mixtures of air in the 95% Ar/5% CO₂ mixture used to flush the bioreactors. Measured air contamination varied between bioreactors, ranging from 0.6% for Kuenenia to 12.3% for Scalindua outflows, as a result of variable anammox activity compared to the flushing flow rate. After correction for background contamination (0.12–2.40‰ for δ¹³N and 0.1–2.3‰ for Δ₃₀), duplicate collections showed reproducibility in δ¹⁵N and Δ₃₀ within ±0.01‰ and ±0.3‰, respectively.

Incubations of natural soils were performed to determine the Δ₃₀ signatures of N₂ produced by natural biological communities. Soils from three agricultural treatments at the Kellogg Biological Station (KBS) Long-Term Ecological Research site were used for these experiments. Soils at the site belong to the Kalamazoo series, which are fine-loamy, mixed mesic Typic Hapludalfs.¹² Soils T1 and T2 are agricultural soils that have been under an annual corn–soybean–winter wheat rotation since 1989, with T1 conventionally tilled with a chisel plow and T2 being no-till. Soil T7 comes from a native early successional old field community (containing grasses, shrubs, and trees) that was established in 1989 and has been maintained by an annual spring burn since 1997. Incubations of 25-g soil samples were conducted in 125 mL glass serum bottles that were crimp-sealed using butyl rubber septa and sampled after 5, 10, and 15 days.
rubber stoppers (Geomicrobial Technologies, Inc., Ochelata, OK, U.S.A.). Initially, after saturating the dried soils, an anaerobic headspace was created by sparging with He. The soils were then allowed to denitify for 7–10 d to remove any initial oxidized N. At that point, the headspace was sparged again with He and then inoculated with glucose (0.3 mL, 1 M) and NaNO3 substrate (1 mL, 0.3 M; δ15N = 5.4‰). Production of N2 was allowed to proceed for 96 h to ensure enrichment had occurred.

For the in situ study, soil gas samples from the KBS Interactions Experiment site were obtained from a monolith soil lysimeter. The lysimeter is located 5 m from the edge of Plot 13 (27 × 40 m total width), which had followed an annual corn–soybean–winter wheat rotation (conventional tillage, no fertilizer) until spring 2016, when planting was changed to Cave-in-rock switchgrass (Panicum virgatum L.). Constructed of stainless steel, the 2.29 × 1.22 × 2.03 m (L × W × D) monolith lysimeter was installed with a minimum of disturbance to the soil column approximately 5 cm above the soil surface in 1986 as described in Brown et al. The origins of this correlation were not investigated, but deserve further scrutiny: the apparent difference in Δ30 value between freshwater and marine species may point to a different biochemistry related to the gene organization and subsequent expression of hydrazine synthase enzyme. In any case, the Δ30 values for N2 produced by freshwater anammox genera are close to that expected from combinatorial isotope effects: the contrast in isotopic compositions between the NaNO3 (δ15N = −26.2‰) and NH4NO3 (δ15N = −0.5‰) substrates, by itself, would yield Δ30 = −0.2‰, close to the mean measured values of −0.2 ± 0.1‰ and −0.5 ± 0.3‰ (1σ) for Ca. Kuenenia and Ca. Brocadia, respectively. Isotopic fractionation during biological uptake could may cause additional variability in the δ15N value of the assimilated substrates, but the Δ30 value of the N2 produced is not expected to deviate more than ~1‰ from zero because the combinatorial effect is a relatively weak function of the substrate δ15N contrast.

An aerobic incubation of KBS soils yielded N2 with Δ30 values indistinguishable from the stochastic distribution of isotopes (i.e., all within 0.2‰; see Table 1). Unlike in previous axenic laboratory cultures of denitrifying bacteria, no statistically significant dependence on reaction extent or δ15N values was observed (p = 0.2–0.4 for a slope of zero, depending on the soil; see Table S1 of the Supporting Information, SI).

Compiling these results with those from earlier experiments on bacterial denitrifiers shows that biological N2 production yields Δ30 values between −0.7‰ and +1.4‰, with a weak dependence, if any, on bulk δ15N values (Table S1). Moreover, the lack of Δ30 fractionation during biological nitrogen fixation suggests that it preserves the Δ30 values of the N2 residue. Atmospheric N2, in contrast, is characterized by Δ30 values of ca. −19.1 ± 0.1‰ (Figure 1).

Using Δ30 Values to Detect Biological N2 Fraction in Soil Gas. Due to the large and relatively invariant Δ30 contrast between atmospheric and biologically produced N2, we suggest here that Δ30 values in N2 can be used to quantify biologically produced N2 in soils via mass balance. To illustrate this concept, we first write the two-component mixing equations for the N2 isotopologue ratios in soil, 29Rsoil and 30Rsoil, in terms of the biological N2 fraction (fbio) and the N2 isotopologue ratios of atmospheric and biological N2 (subscripts “atm” and “bio,” respectively):

\[ 29R_{\text{soil}} = (1 - f_{\text{bio}})29R_{\text{atm}} + f_{\text{bio}}29R_{\text{bio}} \]  

\[ 30R_{\text{soil}} = (1 - f_{\text{bio}})30R_{\text{atm}} + f_{\text{bio}}30R_{\text{bio}} \]  

Table 1. Clumped-Isotope Composition of N2 (±1σ) Derived from Experimental Cultures of Denitrifying or Anammox Bacteria

<table>
<thead>
<tr>
<th>substrate</th>
<th>Δ30 (‰)</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca. Brocadia spp.</td>
<td>NH4NO3 + NaNO3</td>
<td>−0.2 ± 0.1</td>
<td>this work</td>
</tr>
<tr>
<td>Ca. Kuenenia spp.</td>
<td>NH4NO3 + NaNO3</td>
<td>−0.5 ± 0.3</td>
<td>this work</td>
</tr>
<tr>
<td>Paracoccus denitrificans</td>
<td>NH4SO4 + NaNO3</td>
<td>1.0 ± 0.3</td>
<td>this work</td>
</tr>
<tr>
<td>Pseudomonas stutzeri</td>
<td>KNO3</td>
<td>0.9 ± 0.4</td>
<td>14</td>
</tr>
<tr>
<td>Scalindua spp.</td>
<td>KNO3</td>
<td>0.6 ± 0.2</td>
<td>14</td>
</tr>
</tbody>
</table>

The results of this correlation were not investigated, but deserve further scrutiny: the apparent difference in Δ30 value between freshwater and marine species may point to a different biochemistry related to the gene organization and subsequent expression of hydrazine synthase enzyme. In any case, the Δ30 values for N2 produced by freshwater anammox genera are close to that expected from combinatorial isotope effects: the contrast in isotopic compositions between the NaNO3 (δ15N = −26.2‰) and NH4NO3 (δ15N = −0.5‰) substrates, by itself, would yield Δ30 = −0.2‰, close to the mean measured values of −0.2 ± 0.1‰ and −0.5 ± 0.3‰ (1σ) for Ca. Kuenenia and Ca. Brocadia, respectively. Isotopic fractionation during biological uptake could may cause additional variability in the δ15N value of the assimilated substrates, but the Δ30 value of the N2 produced is not expected to deviate more than ~1‰ from zero because the combinatorial effect is a relatively weak function of the substrate δ15N contrast.

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\[ 29R_{\text{soil}} = (1 - f_{\text{bio}})29R_{\text{atm}} + f_{\text{bio}}29R_{\text{bio}} \]  

\[ 30R_{\text{soil}} = (1 - f_{\text{bio}})30R_{\text{atm}} + f_{\text{bio}}30R_{\text{bio}} \]
While the soil-gas $^{29}\text{R}_{\text{soil}}$ and $^{30}\text{R}_{\text{soil}}$ values can be measured (as $^{15}\text{N}_{\text{soil}}$ and $\Delta_{30,\text{soil}}$ values) and $^{29}\text{R}_{\text{atm}}$ and $^{30}\text{R}_{\text{atm}}$ are known, this system of equations remains under-constrained. However, the proportionality between $^{29}\text{R}_{\text{bio}}$ and $^{30}\text{R}_{\text{bio}}$ coming from a relatively invariant biological clumped-isotope signature ($\Delta_{30,\text{bio}}$) provides a way forward.

Two-component mixing is linear in $\Delta_{30}$ values if the biologically produced $\text{N}_2$ has the same $^{15}\text{N}/^{14}\text{N}$ ratio as that of the atmosphere, i.e., $\Delta^{15}N_{\text{bio}} = \Delta^{15}N_{\text{atm}}$ yielding eqs 8, 14, 27:

$$\Delta_{30,\text{soil}} = (1 - f_{\text{bio}})\Delta_{30,\text{atm}} + f_{\text{bio}}\Delta_{30,\text{bio}}$$

(8)

In that case, the soil-gas $\Delta_{30}$ value ($\Delta_{30,\text{soil}}$) would be simply related to $f_{\text{bio}}$ and the atmospheric ($\Delta_{30,\text{atm}}$) and biological clumped-isotope signatures. Measurements of $\Delta_{30,\text{soil}}$ would allow one to solve for $f_{\text{bio}}$:

$$f_{\text{bio}} = \frac{\Delta_{30,\text{atm}} - \Delta_{30,\text{soil}}}{\Delta_{30,\text{atm}} - \Delta_{30,\text{bio}}}$$

(9)

Unknown and variable $\Delta^{15}N_{\text{bio}}$ values lead to deviations from this relationship, and uncertainty in $f_{\text{bio}}$. However, for $\Delta_{30,\text{soil}}$ values close to $\Delta_{30,\text{atm}}$ (i.e., mixtures dominated by atmospheric $\text{N}_2$), eqs 8 and 9 retain much of their accuracy over a wide range of $\Delta^{15}N_{\text{bio}}$ values (Figure 2). For example, when $\Delta^{15}N_{\text{bio}}$ is 20‰ different from $\Delta^{15}N_{\text{atm}}$ the $f_{\text{bio}}$ value derived from eq 9 is within 6% of the true $f_{\text{bio}}$ value (e.g., calculated $f_{\text{bio}}$ of 0.094 when the true $f_{\text{bio}}$ is 0.1). The expected range of $\Delta_{30,\text{bio}}$ values coming from natural communities of ±1‰—i.e., the range observed in laboratory experiments—results in an additional ±6‰ relative uncertainty in $f_{\text{bio}}$ (e.g., an error of ±0.006 for $f_{\text{bio}} = 0.1$). Both errors are similar to that contributed by analytical uncertainty for $f_{\text{bio}} = 0.1$ (resulting in a cumulative uncertainty of ±10‰ if added in quadrature), but they quickly decrease in importance as $f_{\text{bio}}$ decreases: for $f_{\text{bio}} = 0.01$, analytical uncertainty of ±0.08‰ in $\Delta_{30}$ results in an asymmetrical uncertainty of ±36‰ and ±56‰ $f_{\text{bio}}$ i.e., $f_{\text{bio}} = 0.010^{+0.004}_{-0.005}$. Therefore, analytical uncertainty dominates $\Delta_{30}$-based estimates of $f_{\text{bio}}$ for $f_{\text{bio}} < 0.1$. Current analytical uncertainties suggest that soil gas containing ≥1‰ $\text{N}_2$ will be detectable in $\Delta_{30,\text{soil}}$ values.

To test this concept, we obtained two depth profiles of $\delta^{15}N$ and $\Delta_{30}$ values in $\text{N}_2$, along with $\text{N}_2\text{O}$ concentrations, from a monolith lysimeter installed in the KBS Interactions site. We found that many $\Delta_{30,\text{soil}}$ values were less than or equal to $\Delta_{30,\text{atm}}$ (Figure 3 and Table S2), ranging from 18.8‰ to 19.1‰. One sample analysis (34 cm depth on 10/11/17) was rejected based on apparent contamination that resulted in an abnormally elevated $\Delta_{30}$ value (4σ above the mean atmospheric value measured during the analytical session). The largest $\Delta_{30,\text{soil}}$ depletions (−0.3 ± 0.1‰ relative to $\Delta_{30,\text{atm}}$), observed in both profiles, correspond to 1.6%−0.5% of soil $\text{N}_2$ at those depths being derived from biological processes. Soil-$\text{N}_2$ $\delta^{15}N$ values were equal to or slightly lower than the atmospheric value, although they differed between profiles: the profile obtained in July 2018 had $\delta^{15}N$ values close to the atmospheric value, while the profile obtained in October 2017 had subatmospheric $\delta^{15}N$ values ranging from −0.4 to −0.6‰. $\text{N}_2\text{O}$ concentrations increased nearly monotonically with increasing depth, with values exceeding 1000 parts per billion (ppb) at 170 cm depth (Figure 4). Taken together, these data imply an active nitrogen cycle and the presence of biological $\text{N}_2$ in these soils.

Gas Diffusion and Denitrification Hot-Spots Can Explain Observed Soil $\Delta_{30}$ Profiles. A further understanding of the chemical and isotopic signatures measured in the soil gas can be obtained using a one-dimensional diffusion-reaction model based on Fick’s second law:

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial z^2} + J(z, t)$$

(10)

where $D$ is the effective gas diffusivity and $J(z, t)$ is the production rate of a gas, which may be depth-($z$) and time-($t$) dependent. We treat the soil-gas system as a diffusive column ventilated to the atmosphere at the top ($z = 0$) and with zero permeability at the bottom ($z = 180$ cm). At steady state ($\partial C/\partial t = 0$) the depth profile is described by $\partial^2 C/\partial z^2 = -J/ D$; because $J$ and $D$ are positive as defined, concentration depth profiles at steady state should monotonically decrease toward the atmospheric value. Isotopic tracers may increase or
decrease toward the top depending how they are defined, but the change with depth should be monotonic toward the atmospheric value.

The depth profiles are not in steady state with respect to N$_2$. At steady state, deeper soil-gas would have accumulated low-$\Delta_{30}$ biological signals over time, resulting in $\Delta_{30}$,soil values increasing from depth to the surface. The N$_2$O depth profiles show accumulation at depth, but the N$_2$ profiles do not (Figures 3 and 4). Instead, $\Delta_{30}$,soil values are close to atmospheric values at depth, decrease at mid-depths, and return to atmospheric values at the surface. Pulsed biological N$_2$ production over a limited depth range is required to reproduce these mid-depth minima in $\Delta_{30}$,soil values. Specifically, a quiescent period with respect to N$_2$ production, which ventilates the soil down to 170 cm, must precede the pulse. Quantitative ventilation is not necessary, however; the quiescent period need only be long enough to dilute remnant $\Delta_{30}$,soil signals from earlier events beyond the limits of detection ($\sim$5 days for the expected diffusivities; see below). Denitrification “hot moments” related to heterogeneities in soil moisture and organic carbon availability 28,29 have the appropriate temporal and spatial variability. The contrast between N$_2$ and N$_2$O depth profiles suggest that their production during these hot moments can be temporally decoupled. Moreover, the accumulation of N$_2$O at depth argues against ventilation via gas exchange at the lysimeter–soil interface as the origin of the nonsteady-state $\Delta_{30}$,soil depth profile.

The shapes of the $\Delta_{30}$,soil depth profiles can be reproduced by solving eq 10 using a 0.1-day N$_2$ production pulse of Gaussian shape (1 cm full width at half-maximum), followed by solving eq 6 with N$_2$O diffusion coefficients of 0.0026 cm$^2$ s$^{-1}$.31,32

<table>
<thead>
<tr>
<th>sampling date</th>
<th>center depth (cm)</th>
<th>pulse peak (nmol N$_2$ cm$^{-3}$ s$^{-1}$)</th>
<th>sampling lag (h)</th>
<th>N$_2$ production (mmol N$_2$ m$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/11/17</td>
<td>59</td>
<td>1.2</td>
<td>1.9</td>
<td>5.7</td>
</tr>
<tr>
<td>7/18/18</td>
<td>37</td>
<td>2.6</td>
<td>26.4</td>
<td>12.9</td>
</tr>
</tbody>
</table>

“Pulses are Gaussian (1 cm full width at half maximum), occurring for 0.1 days.
by a small time lag between the $N_2$ pulse and sampling (see Table 2). Here, we assume an air-filled porosity, $\varepsilon$, of 0.05—resulting in a calculated $^{15}$N soil diffusivity of $D_z = 0.0039$ cm$^2$ s$^{-1}$ for $N_2$—and $\Delta_{30,soil} = 0$. The assumed $\varepsilon$ value is within the plausible range for these soils$^{13}$ so it is appropriate for illustrative purposes. Using these parameters, the modeled $\Delta_{30,soil}$ depth profile for July 2018 (the best-fit curve using a least-squares algorithm) reflects a depth-integrated gross production of 10.4 mmol $N_2$ m$^{-2}$ remaining in the soil after a 12.9 mmol $N_2$ m$^{-2}$ pulse (Figure 3A). The $\delta^{15}$N values of $N_2$ in that profile can be reproduced if the biological $N_2$ has $\delta^{15}$N = $-11\%_e$, on average. Note that the particular pulse shape, duration, and sampling lag used here (Table 2) is likely one of many that can explain the data and therefore not meant to be diagnostic; consequently, the profile is considered a local (rather than global) best fit. The depth-integrated gross production, however, should be robust for a given air-filled porosity. For example, the model can also yield a satisfactory fit of the data using a 10-fold longer initial $N_2$ pulse length of 1 day with a correspondingly weaker peak pulse peak of 0.3 mmol $N_2$ cm$^{-3}$ s$^{-1}$ (instead of 2.6 mmol $N_2$ cm$^{-3}$ s$^{-1}$). Both scenarios yield scaled-up $N_2$ pulse magnitudes (3–4 kg N ha$^{-1}$) that are consistent with peak $N_2$ fluxes observed in previous in lab$^{13}$ and field$^{13}$ experiments.

The modeled $\Delta_{30}$ depth profile for October 2017 shown in Figure 3 implies a depth-integrated gross production of 5.7 mmol $N_2$ m$^{-2}$ using a pulse centered at 59 cm (Figure 3B, Table 2). Unlike for the July 2018 profile, the $\delta^{15}$N values of $N_2$ in that profile cannot be explained by biological $N_2$ production alone. Gravitational fractionation over this depth range would increase $\delta^{15}$N values by $<0.01\%_e$, so other physical mechanisms such as diffusive fractionation and/or water vapor flux fractionation$^{13}$ may be especially important for this profile. Sampling took place the morning after a heavy overnight precipitation event (>40 mm), implicating a physical isotope effect such as a hydrologically driven diffusive influx of atmospheric $N_2$. These physical mechanisms will not affect $\Delta_{30,soil}$ values significantly because they fractionate proportionately over a small $\delta^{15}$N range.$^{14,15}$ In addition, solubility fractionation does not seem to affect clumped-isotope compositions of sparingly soluble gases$^{14,35}$ despite its effects on both elemental$^{36}$ and bulk-isotope composition.$^{37}$ Consequently, the $\Delta_{30}$ tracer shows a clearer measure of biological $N_2$ production than the $\delta^{15}$N value of $N_2$.

If these biological $N_2$ pulses are isolated in time, then equivalent surface $N_2$ fluxes $F$ can be derived from the reaction-diffusion models, and the results compared to previous measurements of KBS soils. For one-dimensional diffusion, the equation $F = [N_2]_{20cm} \times D_z/z$ describes the instantaneous surface gas flux, where $[N_2]_{20cm}$ is the concentration of biological $N_2$ and $z$ is the depth from the surface. For $z = 5$ cm, the biological $N_2$ flux from the top 5 cm of soil, are shown in Figure 5. The flux $F$ for the two profiles ranges from 0.1–2.9 mmol $N_2$ m$^{-2}$ d$^{-1}$ (3–81 mg N m$^{-2}$ d$^{-1}$) during the first 10 days after the pulse events, with a prolonged period of low, but nonzero flux lasting several times longer (e.g., $F = 0.1–0.2$ mmol $N_2$ m$^{-2}$ d$^{-1}$ for the 7/18/18 profile between 10 and 20 days after the pulse). These estimates are comparable to previous amendment-stimulated $N_2$ production rates from these soils.$^{38,39}$ In particular, Bergsma et al. (2001) reported surface $N_2$ fluxes of 0.2–2.0 mmol $N_2$ m$^{-2}$ d$^{-1}$ (6–55 mg N m$^{-2}$ d$^{-1}$) during a four-day experiment utilizing a surface flux chamber and an amendment of $^{15}$N-labeled KNO$_3$.$^{38}$ The model-derived fluxes strongly depend on the assumed air-filled porosity $\varepsilon$—which was not measured directly and can vary in time and space—so this agreement may be coincidental. Nevertheless, the two methods appear to yield results on the same order of magnitude. More well constrained in situ soil-atmosphere fluxes can be obtained with concurrent measurements of soil physical properties.

The only comparable in situ method for quantifying biological $N_2$ production in soils is the $N_2/$Ar method. Yang and Silver (2012) reported a relatively high detection limit of 3.9 mmol $N_2$ m$^{-2}$ d$^{-1}$ for surface-flux measurements,$^{14}$ larger than the calculated peak surface fluxes shown in Figure 5. While the method can analytically resolve $N_2$ excesses of less than 0.1%,$^{40}$ physical fractionation of $N_2$ and Ar in soils presents substantial systematic uncertainties in these environments. We hypothesize that measurements of $N_2/$Ar soil profiles may yield limited improvements in uncertainty because the physical mechanisms complicating the interpretation of $\delta^{15}$N values of $N_2$ (e.g., the water vapor flux fractionation)$^{13}$ fractionate $N_2/$Ar ratios to a greater degree, offsetting any analytical sensitivity advantages. Soil $\Delta_{30,soil}$ depth profiles, in contrast, are insensitive to physical fractionation, revealing evidence for biological $N_2$ production in soil profiles despite the lower analytical sensitivity of the method.

$N_2$ fluxes into the atmosphere can be derived from $\Delta_{30,soil}$ profiles if soil physical properties (i.e., air-filled porosity and diffusivity) are determined independently. The method could be used to compare in situ production rates to incubation- and amendment-based methods in field studies, or to obtain independent estimates using an array of spatially dispersed observations across soil types and conditions. Time series of soil-gas profiles similar to those shown here, sampled through
lysimeters or air-permeable tubing, would provide a long-term perspective on soil N₂ production dynamics, which is presently difficult to access without perturbing soil biogeochemistry and is useful for models. Analytical throughput (2–3 samples/day) and availability of instrumentation are currently limiting factors for the Δ₃₀ approach, but the relatively long ventilation time scales of certain soils may still allow weekly to-monthly sampling to capture the impacts of hot moments. The initial results reported here suggest that Δ₃₀,atm signals are sufficiently large that the approach can be used in future assessments of site- and ecosystem-scale loss of fixed nitrogen. Furthermore, the approach can also be applied to marine environments to investigate both the magnitude and mechanisms of fixed-nitrogen loss in low-oxygen zones. Finally, constraining biological N₂ production globally using Δ₃₀,atm appears possible in principle if the terms related to upper-atmospheric chemistry in the global Δ₃₀ budget—both the isotopic reordering rates and Δ₃₀ endmembers—can be refined.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.est.9b00812.

Compilation of isotopic composition data for N₂ produced during pure- and enrichment-culture experiments reported here and in ref 14; isotopic composition data for N₂ and concentrations of N₂O in soil gases (PDF).

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### Notes

The authors declare no competing financial interest.

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