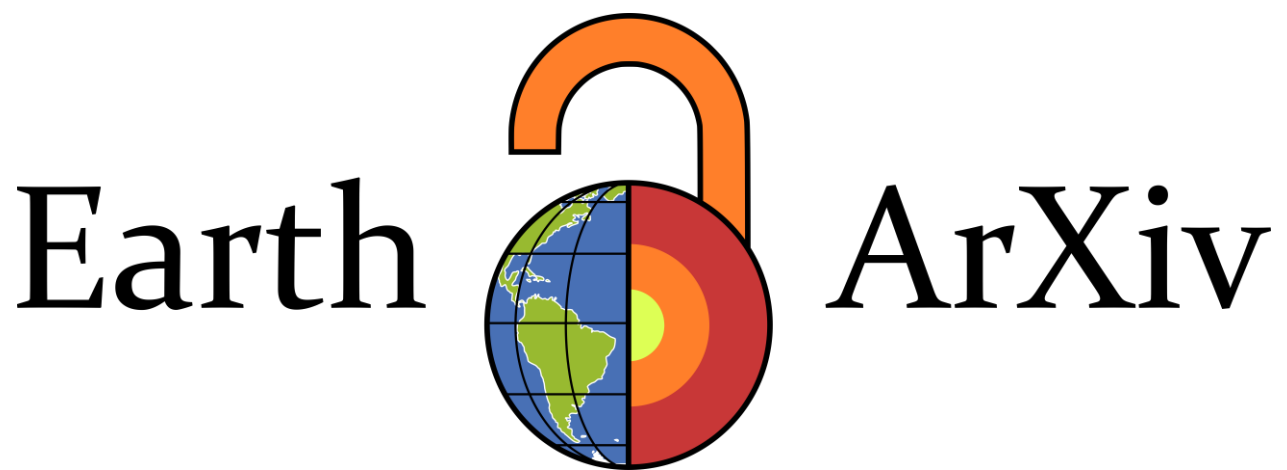




This is a non-peer-reviewed preprint submitted to EarthArXiv.

This manuscript has been submitted for publication in *Earth Science Reviews*. Please note the manuscript has yet to be formally accepted for publication. Subsequent versions of this manuscript may have slightly different content. If accepted, the final version of this manuscript will be available via the 'Peer-reviewed Publication DOI' link on the right-hand side of this webpage. Please feel free to contact any of the authors; we welcome feedback.

N₂O source partitioning and reduction progress quantified with isotopocule datasets – a review of the modelling strategies

Dominika Lewicka-Szczebak^{*1}, Maciej Piotr Lewicki², Caroline Buchen-Tschiskale³, Reinhard Well³

¹ Institute of Geological Sciences, University of Wrocław, Wrocław, Poland,

² Institute of Nuclear Physics, Polish Academy of Sciences, Krakow, Poland,

³ Thünen Institute of Climate-Smart Agriculture, Braunschweig, Germany

* Corresponding author: Dominika.lewicka-szczebak@uwr.edu.pl

Abstract

Nitrous oxide (N₂O) isotope signatures provide crucial information for partitioning microbial production pathways, including bacterial, fungal, and nitrifier denitrification, chemo-denitrification and nitrification, as well as for estimating the degree of N₂O reduction to N₂. However, interpreting the three-dimensional isotope data is highly challenging due to numerous production pathways and simultaneous occurrence of production and consumption. Here we comprehensively review the application of the Fractionation And Mixing Evaluation (FRAME) model, a Bayesian Markov-Chain Monte Carlo (MCMC) tool explicitly developed to resolve complex, underdetermined isotope mixing and fractionation dynamics.

The FRAME model shows widespread applicability across diverse global ecosystems, spanning agricultural soils, peatlands, groundwaters, rivers of Europe, America, Asia, and Africa, as well as marine environments. To address common interpretative challenges, standardized guidelines and best practices for configuring the model is established, and an overview of output quality checks and problem-solving ideas is provided. Most recent methodological enhancements of the model performance, including low-level ¹⁵N labelling, integration of natural abundance results with ¹⁵N tracing, and utilizing model extension for high-resolution time-series datasets are discussed.

Furthermore, we introduce FRAME v2.0, a major software update that features improved statistical treatment of source uncertainty and extended auxiliary variable priors, while maintaining full backward compatibility. The model's performance is discussed in the context of various cases studies and independent reference methods, like the ¹⁵N gas flux approach and microbial functional gene analysis. This review presents unified standardised and robust interpretation strategies supported by an enhanced computational tool to quantify N₂O production pathways and reduction across ecosystems.

Keywords: isotope mixing model, isotope fractionation model, N₂O isotopocules, FRAME, N₂O source partitioning, N₂O reduction, ¹⁵N site preference

1. Introduction

Nitrous oxide (N₂O) is a crucial compound in the environmental nitrogen (N) cycle, can be produced as a final or intermediate product of a several different N-transformations and further

reduced to di-nitrogen (N_2). The main processes of N_2O production can be divided into denitrification and nitrification pathways. During denitrification, N_2O is produced in anoxic conditions due to nitrate (NO_3^-) and nitrite (NO_2^-) reduction (Baggs, 2011). The process can be performed by bacteria, with possibility of further N_2O reduction to N_2 (*bD* – bacterial denitrification), or by fungi, with N_2O as a final product (*fD* – fungal denitrification). During nitrification, N_2O is produced as a byproduct of ammonium (NH_4^+) oxidation due to oxic hydroxylamine oxidation (*Ni* – nitrification) or in anoxic NO_2^- reduction (*nD* – nitrifier denitrification) (Butterbach-Bahl et al., 2013; Wrage-Mönnig et al., 2018). Moreover, there are numerous abiotic N_2O production pathways, mostly chemical NO_2^- reduction, often referred to as chemodenitrification (*chD*) (Heil et al., 2016; Xu et al., 2022). Hence, the N_2O pool integrates various pathways and its stable isotope signature reflects the processes of its production and simultaneously depends on the progress of its reduction. N_2O isotope datasets, although complex, can be diagnostic for the co-occurring N-transformations, but only when this complexity is properly taken into account. The information stored in the N_2O isotope signature can be applied both for the source partitioning, as well as for the estimation of the N_2O reduction progress. Such knowledge is especially important for identification of the origins of N_2O , a potent greenhouse and ozone layer depleting gas and testing the effectiveness of mitigation strategies (Ravishankara et al., 2009). Moreover, by quantifying the N_2O consumption, the N_2 emission can be indirectly estimated. N_2 flux is not measurable directly due to high atmospheric background, hence this indirect estimation is a unique way for the determination of the largely unknown loss of reactive N as N_2 , often called the main missing flux in the N cycle (Bai et al., 2012; Scheer et al., 2020).

Full isotope analysis of N_2O comprises not only the isotope signatures of oxygen ($\delta^{18}O$) and N ($\delta^{15}N$), but also the distinction of ^{15}N abundance in different positions of the linear N_2O molecule ($\delta^{15}N^{SP}$ – ‘site preference’) and are often referred to as *N_2O isotopocule analyses* (Yu et al., 2020). Discovered only in 1999, these unique measurements have opened novel interpretation perspectives (Brenninkmeijer and Röckmann, 1999; Toyoda and Yoshida, 1999) and the development of their adequate interpretation methods is still in progress (Deb et al., 2024; Denk et al., 2017; Ostrom and Ostrom, 2017; Toyoda et al., 2017; Yu et al., 2020). The $\delta^{15}N^{SP}$ is a very specific isotope signal, which is assumed independent of its substrate isotope signature, in contrast to all other isotope values, which strictly depend on their precursors (as $\delta^{15}N$, $\delta^{18}O$). Therefore, $\delta^{15}N^{SP}$ can be directly applied for identification of microbial pathways producing N_2O , whereas for $\delta^{15}N$ and $\delta^{18}O$, the isotope signatures of the precursors related with the specific process must be considered. The substrate for N isotopes in denitrification pathways (*bD* and *fD*) is NO_3^- , and in nitrification processes (*Ni* and *nD*) it is NH_4^+ . The oxygen (O) isotope signature in denitrification processes is governed by O-isotope exchange with water during *bD*, *fD* and *nD* and by isotope fractionation associated with this mechanism, which is similar for *bD* and *nD*, but differs significantly for *fD* (Lewicka-Szczebak et al., 2016; Rohe et al., 2017, 2014). Conversely, during *Ni*, O is incorporated from molecular O_2 and thus usually reflects the typical isotope signature of atmospheric O_2 (+23.5‰) (Snider et al., 2012). In *chD* NO_2^- is the direct precursor for both N and O isotopes in N_2O (Wei et al., 2019). Importantly, beside the different precursors also typical isotope fractionation differs for each of the microbial pathways, which allows their differentiation based on the isotope effects reported in pure culture studies for each process (Toyoda et al., 2017). Similarly, the N_2O reduction is also associated

with significant isotope fractionation, leaving the residual N₂O pool enriched in ¹⁸O and ¹⁵N in the central position (Well and Flessa, 2009a). The isotope effect of N₂O reduction was determined in controlled laboratory incubations with parallel application of reference methods for independent measurement of the N₂ flux (He incubations and ¹⁵N tracing) (Lewicka-Szczebak et al., 2015, 2014). The N₂O residual fraction (r_{N_2O} , R_{N_2O} or N₂O_i), defined as the (N₂O/(N₂O+N₂)) ratio of denitrification (often mentioned as product ratio) can be very variable; the entire spectrum of values from 0 to 1 has been reported in field and laboratory studies (Yu et al., 2020).

Summing up, the N₂O isotope signature is rich in information, but also challenging in interpretation due to: (i) three dimensional isotope data, (ii) multiple potential N₂O production pathways, including five main pathways (*bD*, *fD*, *nD*, *Ni* and *chD*), (iii) N₂O production and reduction processes of variable progress occurring simultaneously. Therefore, advanced calculation approaches are necessary to deal with this complex case. The **FRA**ctionation and **Mixing E**valuation (**FRAME**) model has been developed to provide a commonly available tool to deal with simultaneous isotope mixing and fractionation processes and to integrate all three isotope values. Previous isotope mixing models did not provide any option to integrate isotope fractionation with varying residual fraction (Moore and Semmens, 2008; Parnell et al., 2013; Phillips et al., 2014; Stock et al., 2018). By using FRAME, the fractionation equation with the relevant parameters can be defined by the user, hence it can be applied to all possible isotope systems with various fractionation patterns (Lewicki et al., 2022). The model applies Bayesian statistics (Markov Chain Monte Carlo) which can successfully deal with underdetermined systems and provides probability distribution for the possible outcomes, taking into account the uncertainties associated both with measurements and natural variability. It was originally constructed for the N₂O case study as 3DIM model (Lewicka-Szczebak et al., 2020; Well et al., 2025) and later, to make it widely applicable, a user-friendly software was developed, which does not require programming skills (Lewicki et al., 2022). This tool has been already applied for numerous N₂O case studies worldwide, both in field and experimental studies, including soil (Fang et al., 2024; Rummel et al., 2025a; Toyoda et al., 2024; Wei et al., 2024), groundwater (Deb et al., 2025), river (Chen et al., 2023; Shu et al., 2024; Wang et al., 2025b) and oceanic N₂O case studies (Wang et al., 2025). Thanks to these numerous modelling cases, we gained a lot of experience and together with all the authors, developed best procedures to gather the most reliable outputs. This review is a summary of all the common issues by applying FRAME for N₂O data interpretation and aims to build common standards and best rules for this application. Moreover, we present the current further developments allowing for optimising source partitioning of the overlapping pathways and increasing the overall modelling precision, i.e.: (i) increasing the $\delta^{15}\text{N}$ values of one mineral N pool (e.g. only NO₃⁻ or NH₄⁺) in order to shift the overlapping mixing endmember ranges (Deb et al., 2025), (ii) combining natural abundance N₂O results with ¹⁵N tracing (Micucci et al., 2025), (iii) applying statistical generalisation for time-series datasets (Harris et al., 2024).

This paper aims at providing a comprehensive and common interpretation strategy for N₂O isotope datasets, taking into account critical aspects of the model application. It includes the answers on the numerous questions asked by the users during the first years of FRAME

application since first published (Lewicki et al., 2022). We also provide here the new enhanced version of the software.

2. Model input data

2.1 N₂O datasets

2.1.1 Preparation of correct input values depending on case study

All measured N₂O isotope values should be normalised to the international isotope scale and expressed against the international standards. This should be performed with the intercalibrated working standards with at least two different values for $\delta^{15}\text{N}$, $\delta^{18}\text{O}$ and $\delta^{15}\text{N}^{\text{SP}}$, respectively, enabling the correction of measured values with the linear regression method (Mohn et al., 2014).

Importantly, the values of N₂O originating from the actual production and reduction processes must be calculated by isotope mass balance (Eq.1), if the measured sample constitute a mixture of background N₂O and the added flux of N₂O. An example of such a case are the chamber measurements of soil N₂O flux, where the background atmospheric N₂O is mixed with the soil emitted N₂O (Well et al., 2006) or the laboratory soil/slurry/water incubations with initial background N₂O in the headspace (Deppe et al., 2017). The following equation should be applied to determine the isotope characteristics of the actually emitted N₂O (δ_{emitted}),

$$\delta_{\text{emitted}} = \frac{(\delta_{\text{mix}} * c_{\text{mix}} - \delta_{\text{bgd}} * c_{\text{bgd}})}{(c_{\text{mix}} - c_{\text{bgd}})} \quad (\text{Eq. 1})$$

from the measured N₂O concentration and isotope signature (C_{mix} , δ_{mix}) and the background N₂O concentration and isotope signature (C_{bgd} , δ_{bgd}), which is determined from the outside air or the initial background gas collected parallelly with the samples collection. The precise determination of background gas isotope signature is crucial for precise calculation of the emitted N₂O, especially for the cases where the increase in N₂O accumulation is small (N₂O concentration increase from background to sample < 25 ppb). Usage of the mean tropospheric values as a background can be only justified for the high accumulation cases. Further discussion on the associated uncertainties and errors continues in Section 2.1.3.

This issue is more complex for aquatic studies where the N₂O dissolution and diffusion in water takes place, hence the dissolved portion of tropospheric N₂O is not stable and must be determined together with its isotope characteristics, also variable across ecosystems. Two approaches have been proposed in the literature: Keeling plot and two-endmember mixing models (Einzmann et al., 2026; Gu et al., 2025) and headspace mass-balance correction using atmospheric air (Barthel et al., 2026). The first approach assumes that the total observed N₂O concentration is a two-endmember mixture of a uniform and low-concentrated background N₂O from dissolved atmospheric air and an isotopically distinct source of newly, microbially produced N₂O, that cause increase in total N₂O concentration. Practically, the measured isotope signatures are plotted against the inverse of the observed N₂O concentration. Through this linear regression, the slope reflects the difference between the background and the source isotope

signatures scaled by the background concentration, while the y-intercept directly yields the corrected isotope signature of the newly produced microbial N₂O, effectively isolating it from the background (Einzmann et al., 2026; Gu et al., 2025). The second approach is based on experimental determination of the dissolved N₂O with headspace equilibration of water samples with ambient air, and direct analyses of the atmospheric air samples, for their concentration and isotope signatures. Similar isotope mass balance equation as in Eq.1 is applied to mathematically remove the background atmospheric component and determine isotope characteristic of dissolved N₂O (δ_{diss}):

$$\delta_{diss} = \frac{(\delta_{eq} * c_{eq} - \delta_{bgd} * c_{bgd})}{(c_{eq} - c_{bgd})} \quad (\text{Eq. 2})$$

from the measured in the equilibrated gas N₂O concentration and isotope signature (C_{eq} , δ_{eq}) and the background N₂O concentration and isotope signature (C_{bgd} , δ_{bgd}), which is determined from the outside air samples (Barthel et al., 2026).

2.1.2 Samples labelling and grouping strategy

The model software allows to group the individual samples. Creating of one group means that all the results are repetitions of exactly the same case and represent the same conditions, i.e. we expect the same contribution of sources and progress of reduction among all samples in one group. The model seeks the best common solution for all the points in the group, and deviations between points are propagated to the uncertainty of the model output. Therefore, the variation between the observed isotope signatures within one group should be minimal and only represent the possible analytical uncertainties and minor variations in isotope effects of particular processes. In cases where our theoretical repetitions indeed show large variations in their isotope signatures, it means that the conditions were actually not identical and we rather do not deal with the same sources' contribution and reduction progress of these samples. Then, rather each sample should be treated individually, because most probably each one represents different combination of N₂O production pathways and reduction.

Generally, samples collected in different time points should not be pooled together for the evaluation. This is not recommended because N₂O processes could be only constant in a steady state condition with continuous supply of substrates and equal growth and decay of microbes, which is very exceptional.

2.1.3 Defining errors

Standard deviation (σ) should be always determined for the samples' isotope signatures, it should never equal 0, since we always deal with some uncertainty, associated with analytical error, standardisation procedure, samples inhomogeneity and natural variations. All these errors origins must be taken into account and the propagated error should be calculated for each sample including all these uncertainties. The propagated error can be finally inserted into the model. This value is further applied in the model to calculate the probability distribution of the results, including given σ values for samples, sources (section 2.2) and fractionation factors (section 2.3). Neglecting any of the potential error origins and underestimation of the given σ for samples results in underestimated range of possible model outputs.

Depending on the samples characteristics and grouping strategy the following uncertainties should be considered:

- individual samples – analytical uncertainty (σ_{an}) should be included. The σ can be defined based on the repeated measurements of the standards and the same values can be applied for all samples,
- grouped samples (σ_{gr}) – propagated error of the heterogeneity within the particular group of samples (σ_{rep}) plus analytical uncertainty (σ_{an}):

$$\sigma_{gr} = \sqrt{(\sigma_{an}^2 + \sigma_{rep}^2)} \quad (\text{Eq. 3})$$

- when N₂O-emitted was calculated from the N₂O flux (σ_{flux}) added into the initial background samples containing background N₂O (N₂O_{bgd}) – the actual error depends on N₂O concentration increase, i.e. the magnitude of the added N₂O over the background determines the precision of the calculated emitted N₂O data. This must be included in the calculation of final σ for each sample individually taking into account the increase in N₂O concentration compared to background air (N₂O_{flux}):

$$\sigma_{flux} = \frac{\sqrt{(\sigma_{an}^2 + \sigma_{rep}^2)}}{(N_2O_{flux}/N_2O_{bgd})} \quad (\text{Eq. 4})$$

To estimate the error in the δ values ($\delta^{15}\text{N}^{\text{SP}}$, $\delta^{15}\text{N}$, $\delta^{18}\text{O}$) of the calculated N₂O-emitted (σ_{flux}) and to establish a threshold value for minimal N₂O flux for trustworthy $\delta_{emitted}$ values, a Monte Carlo simulation of error propagation can be used, based on the analytical uncertainty (σ_{an}) of the isotopic analysis and employing the Equation 1. In this approach, δ values from samples are calculated with errors, and these biased values are used to recalculate $\delta_{emitted}$. If δ_{mix} values are close to the δ_{air} values within the precision of the analysis and the propagated error in the calculation of $\delta_{emitted}$ values is large the values are rejected. Often, $\delta^{15}\text{N}^{\text{SP}}$ values are used for the simulation, as $\delta^{15}\text{N}^{\text{SP}}$ exhibits the highest σ_{an} values and is applied to set a threshold for N₂O concentration, e.g. as recently determined: minimal C_{mix} > 385 ppb for the target propagated error of $\delta_{emitted}$ < 4 ‰ (Buchen et al., 2018).

- For water dissolved N₂O studies with the background N₂O the error estimation must similarly include the uncertainty of background N₂O isotope signature determination and the mixing proportions of produced and background N₂O, the error estimation method depends on the approach to determine the N₂O background level (as summarized in 2.1.2).

2.2 Sources

2.2.1 Isotope characteristics and strategy for correction with precursors isotope signatures

Usually in the laboratory experiments or field studies only the N₂O isotope signature is directly measured and literature data of pure culture studies or controlled experiments is used as the

theoretical mixing endmember ranges for respective N₂O production pathways, i.e. *bD*, *fD*, *nD*, *Ni*, *chD*. However, these specific isotope values characteristic for particular processes have been determined with different precursors that impact the produced $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values of N₂O. The summary of all the isotope effects after consideration of the precursor isotope signatures and given in absolute value (in relation to 0, hence denoted as δ_0 in Table 1) have been described in detail and summarised in Yu et al., (2020) supplementary information. Actual values most typical for soil studies are summarised in Table°1. This summary provides a basic knowledge on the specific isotope effects which must be further adjusted to the particular case-study precursor data, ideally, measured together with the N₂O data, or if not available, to their best approximation (see section 2.1.6). The mixing endmember ranges should be never directly adopted from another study without addressing the issue of precursors. This summary mostly focuses on soil studies. Different values might be considered for aquatic environments, e.g. the $\delta_0^{15}\text{N}$ for *bD* shows typically less negative values in waters (i.e. smaller isotope effects) (Denk et al., 2017). For any particular case study, the applied isotope effects can be adjusted depending on the available literature or own experimental data (see also section 3.1.5). The given ranges are just the selected literature summary which should serve as a representative starting point, but the ranges can be narrowed or broadened according to the known experimental background.

Current best practise strategy is to correct the mixing endmember ranges (literature or experimental values) with the known relevant precursors for each process, both for $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ values, according to the Table 1 and apply that to the source file. The $\delta^{18}\text{O}$ value of *Ni*, governed by the atmospheric O₂ signature is generally stable (+23.5 ‰, (Luz and Barkan, 2011)), and does not require substrate correction, except in case of any known significant deviation from this global atmospheric value. This can be the case for both surface and groundwaters depending on their under or super saturation in O₂ (Trojanowska et al., 2008; Wassenaar and Hendry, 2007). Such correction should only account for deviation from the 23.5 ‰ value (Table 1).

However, initially, in older literature, not the mixing endmember values were corrected, but the measured $\delta_{\text{N}_2\text{O}}$ was shifted according to the value of assumed dominating precursor: $\delta^{15}\text{N}_{\text{NO}_3}$ and $\delta^{18}\text{O}_{\text{H}_2\text{O}}$. However, such approach can be only justified for the cases of the vast domination of N₂O production due to denitrification, like e.g. anoxic experiments. The advantage of such approach were the constant literature endmember ranges for each study. Nevertheless, this approach should be rather omitted, hence it shows shifted N₂O values and neglects the possible inputs from other precursors like NH₄⁺ or O₂ by nitrifying processes (Yu et al., 2020).

Importantly, $\delta^{15}\text{N}^{\text{SP}}$ must not be corrected because this value is independent of precursors isotope signatures, hence the literature ranges can be directly adopted for any case study.

Table 1: Summary of mixing endmembers isotope signatures of particular pathways (*bD*- bacterial denitrification, *nD*- nitrifier denitrification, *fD*- fungal denitrification, *Ni*- nitrification) and with respective references. Values given as mean±SD (calculated from all literature values) and as the range mean± $\Delta_{\text{min-max}}$ (defining the literature range from minimum (mean- Δ) to maximum (mean+ Δ)). Values are corrected with the substrates applied in the original experiments, hence the given δ_0 values refer to the theoretical substrate δ value of 0 permil. Source data available in the associated repository file (link).

For the model input each value must be corrected with the respective mean isotope signature of the substrate:
- for $\delta^{18}\text{O}$ – soil water ($\delta^{18}\text{O}_{\text{H}_2\text{O}}$) for *bD*, *nD* and *fD*, atmospheric/dissolved oxygen ($\delta^{18}\text{O}_{\text{O}_2}$) for *Ni*, nitrite oxygen ($\delta^{18}\text{O}_{\text{NO}_2}$) for *chD*;
- for $\delta^{15}\text{N}$ – respective substrate – NO_3^- for *bD* and *fD*, NH_4^+ for *nD* and *Ni*, NO_2^- for *chD*
- for $\delta^{15}\text{N}^{\text{SP}}$ no substrate correction is needed. The final model input values are marked with bold font.

	literature values (mean $\pm$ SD) (mean $\pm \Delta_{\text{min-max}}$)			substrates		final endmember values	
pathway	$\delta^{15}\text{N}^{\text{SP}}$	$\delta^{18}\text{O}$	$\delta^{15}\text{N}$	$\delta^{18}\text{O}$	$\delta^{15}\text{N}$	$\delta^{18}\text{O}$	$\delta^{15}\text{N}$
<i>bD</i> ^a	-2.4$\pm$2.8	19.2 $\pm$ 1.4	-47.6 $\pm$ 5.2	$\delta^{18}\text{O}_{\text{H}_2\text{O}}$	$\delta^{15}\text{N}_{\text{NO}_3}$	$\epsilon^{18}\text{O} + \delta^{18}\text{O}_{\text{H}_2\text{O}}$	$\epsilon^{15}\text{N} + \delta^{15}\text{N}_{\text{NO}_3}$
	-1.9$\pm$5.6	20.0 $\pm$ 3.3	-47.5 $\pm$ 8.3				
<i>nD</i> ^b	-2.1$\pm$4.9	15.9 $\pm$ 1.5	-56.9 $\pm$ 3.8	$\delta^{18}\text{O}_{\text{H}_2\text{O}}$	$\delta^{15}\text{N}_{\text{NH}_4}$	$\epsilon^{18}\text{O} + \delta^{18}\text{O}_{\text{H}_2\text{O}}$	$\epsilon^{15}\text{N} + \delta^{15}\text{N}_{\text{NH}_4}$
	-5.8$\pm$7.7	15.9 $\pm$ 3.5	n.a.*				
<i>fD</i> ^c	33.5$\pm$2.5	47.2 $\pm$ 3.8	-37.8 $\pm$ 6.6	$\delta^{18}\text{O}_{\text{H}_2\text{O}}$	$\delta^{15}\text{N}_{\text{NO}_3}$	$\epsilon^{18}\text{O} + \delta^{18}\text{O}_{\text{H}_2\text{O}}$	$\epsilon^{15}\text{N} + \delta^{15}\text{N}_{\text{NO}_3}$
	33.6$\pm$6.4	48.5 $\pm$ 6.6	-38.3 $\pm$ 7.4				
<i>Ni</i> ^d	34.0$\pm$2.0	23.5 $\pm$ 2.1	-56.6 $\pm$ 7.3	$\delta^{18}\text{O}_{\text{O}_2} - 23.5$	$\delta^{15}\text{N}_{\text{NH}_4}$	$\epsilon^{18}\text{O} + \delta^{18}\text{O}_{\text{O}_2} - 23.5$	$\epsilon^{15}\text{N} + \delta^{15}\text{N}_{\text{NH}_4}$
	34.7$\pm$4.0	23.5 $\pm$ 2.9	-55.5 $\pm$ 8.5				
<i>chD</i> ^e	23.0$\pm$3.7	18.6 $\pm$ 2.5	-24.5 $\pm$ 3.4	$\delta^{18}\text{O}_{\text{NO}_2}$	$\delta^{15}\text{N}_{\text{NO}_2}$	$\epsilon^{18}\text{O} + \delta^{18}\text{O}_{\text{NO}_2}$	$\epsilon^{15}\text{N} + \delta^{15}\text{N}_{\text{NO}_2}$
	24.1$\pm$6.3	17.7 $\pm$ 4.3	-25.4 $\pm$ 5.7				

^a – (Barford et al., 1999; Lewicka-Szczepak et al., 2016; Rohe et al., 2017; Sutka et al., 2006; Toyoda et al., 2005)

^b – (Frame and Casciotti, 2010; Sutka et al., 2006)

^c – (Maeda et al., 2015; Rohe et al., 2017, 2014; Sutka et al., 2008)

^d – (Frame and Casciotti, 2010; Mandernack et al., 2009; Sutka et al., 2006; Yoshida, 1988)

^e – (Wei et al., 2019) – sterilized soil data

* n.a. – full range not available because one study giving mean $\pm$ SD available only (Frame and Casciotti, 2010)

Table 1 presents a summary of the values that have been generally accepted for N_2O production pathways from soils. A critical examination of the literature led to the acceptance of the most coherent and substantiated values. The rationale for this selection is presented in Yu et al. (2020) (described in detail in the Appendix to that review paper). For example, for various fungal denitrification strains, a broad spectrum of $\delta^{15}\text{N}^{\text{SP}}$ values has been documented, but apparently the range of values exhibited by fungal species of significant N_2O production levels is comparatively narrow. Consequently, only fungal species with high N_2O production were considered, since they will have most impact on fungal denitrification isotope fingerprint in natural conditions. It has been shown later that some of these fungal strains are not denitrifiers, but produce low amount of N_2O from abiotically produced NO (Keuschnig et al., 2020). Similarly, for $\delta^{18}\text{O}$ of *bD* the complete exchange with soil water was assumed, in accordance with the findings of comprehensive soil experiments (Kool et al., 2007; Lewicka-Szczepak et al., 2016), although isolated bacterial strains show large diversity in the possible extent of O-isotope exchange (Rohe et al., 2017; Chénier et al., 2026). It was hypothesised that, the stable and consistent results obtained in whole soil studies are mostly representative of typical field conditions. However, given the strong dependence of the O-isotope exchange on pH (Casciotti et al., 2007), it is possible that this assumption may not be fulfilled in more acidic environments.

Besides biotic pathways, *chD* should often be considered as an additional potential source, especially under acidic conditions (Venterea, 2007). Defining the appropriate isotope range of *chD* is very challenging, as numerous distinct pathways can be understood under this process, including hydroxylamine oxidation (Heil et al., 2014), Fe(II) reactions (Buchwald et al., 2016; Grabb et al., 2017; Jones et al., 2015; Qi et al., 2024; Tischer et al., 2022; Visser et al., 2020), or lignin reactions (Wei et al., 2019). When summarising the overall isotope ranges of all these individual reactions together, a substantial range is obtained, overlapping with all the other biotic pathways, thereby precluding any reasonable N_2O source partitioning. Therefore, the

plausible range for *chD* needs to be defined depending on the environmental conditions and relevant literature data or own targeted experiments. This range should reflect the potential N₂O emissions resulting from *chD* in the environment under study. The typical soil *chD* range presented in Table 1 is based on the Wei et al. (2019) study, which examined the N₂O from sterilised soils as a product of abiotic NO₂⁻ reduction. The values obtained are highly coherent for different soils and demonstrate stability during continuous measurements and represent the closest conditions for natural soil case studies. However, a recent study of coastal saline soils has provided another comprehensive overview of possible *chD* pathways with different ranges applicable to this specific environment (Liang et al., 2026). The direct substrate for *chD* is NO₂⁻, and this compound should be applied for substrate-correction of *chD* range. It has been shown that with just comparing the $\delta^{18}\text{O}$ and $\delta^{15}\text{N}$ values produced by *chD* and biotic pathways no identification is possible, whereas precise correction with relevant substrate (NO₂⁻) and comparison of the isotope effect gives clear distinction of biotic and abiotic processes (Wei et al., 2019). However, since NO₂⁻ analyses are challenging due to chemical reactivity and usually low concentration in terrestrial environments (Lewicka-Szczebak et al., 2021; Deb and Lewicka-Szczebak, 2025), few field studies provide NO₂⁻ isotope signatures and the N₂O-NO₂⁻ isotope effects have not been determined for all pathways so far (Sebilo and Margalef-Marti, 2026).

2.2.2 Applying mean values or ranges for sources

The model enables the definition of the mixing endmembers ranges in two ways: (i) as mean values with standard deviation assuming Gaussian distribution of the values, or (ii) as the entire ranges with equal statistical distribution of all the literature data. The assumption of entire ranges frequently yields a substantially broader distribution of values (see Table 1: compare upper and lower values for δ_0 literature values), thereby also amplifying the probability distribution of the model outcomes.

For literature N₂O endmember ranges that have been determined in a few laboratory experiments, with different pure cultures or in controlled conditions favouring one of the pathways, the range determination is often based on very limited numbers of results, mostly too few to representatively determine the mean value and justify the Gaussian distribution. Therefore, when literature ranges are applied, it is reasonable to assume that the entire range is equally distributed (in FRAME ‘*spread*’).

2.2.3 Proper assumptions on precursor values

Best strategy is to directly analyse the main precursors isotope signatures: NO₃⁻, NH₄⁺ and H₂O, ideally alongside NO₂⁻, in the running field or experimental study, considering the possible heterogeneity and seasonality. For $\delta^{18}\text{O}_{\text{H}_2\text{O}}$, changes due to evaporation must be taken into account. For longer laboratory incubation with significant changes in soil water content, $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values of soil water should be determined at the beginning and end of an experiment. For field experiments it should be performed on a regular basis covering the annual cycle (at least four times per year) using interpolation to align $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values for intermediate N₂O samplings. In case of aquatic environments of significant dissolved O₂ consumption or production, $\delta^{18}\text{O}_{\text{O}_2}$ should be determined as O precursor for *Ni*. If the precursor values are not

available the best approximation should be applied. For water isotope signature the $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ values from the nearest GNIP station (Global Network of Isotopes in Precipitation, <https://www.iaea.org/services/networks/gnip>) for precipitation or surface water can be applied (Lewicka-Szczebak et al., 2015). $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ from tap water should be applied, if used for soil experiments irrigation (Rohe et al., 2014). Since the isotope signature of waters is a very well studied subject with numerous regular monitoring stations, this value can be assumed with high accuracy and the associated uncertainty has little impact on the final model outputs. In case of lacking data on dissolved O_2 in waters, O_2 consumption and the associated isotope effect can be applied for the estimation of the actual $\delta^{18}\text{O}_{\text{O}_2}$ (Barthel et al., 2026).

For N precursors it is important to keep in mind that the actual isotope signatures of the reaction substrate may differ for the analysed mean $\delta^{15}\text{N}$ values (e.g. obtained from analysing soil extracts), resulting e.g. from intensive substrate consumption in hot spots associated with high partial isotope fractionation (Lewicka-Szczebak et al., 2017).

Analyses of NO_2^- are so far rather rare, but could be also helpful - as a precursor for *chD* and intermediate product of all the biotic processes. In future, the application of NO_2^- as a common substrate for all processes can possibly improve the source partitioning potential (Sebilo and Margalef-Marti, 2026; Wei et al., 2019). Recent analytical enhancement allows for analyses of low-concentration NO_2^- samples, hence these analyses can become more routine (Deb and Lewicka-Szczebak, 2025).

In case the N-precursors were not analysed, the possible sources should be carefully considered and the respective values might be assumed (Deb et al., 2024). However, the true source values can be largely different due to variable origin and fractionation processes. Especially, NH_4^+ can be highly enriched due to rapid consumption after addition. The usual assumption of soil $\delta^{15}\text{N}_{\text{NH}_4}$ values of around 0‰ can be only justified, if we deal with native soil N without extra NH_4^+ amendments. By any NH_4^+ fertilizer addition the actual $\delta^{15}\text{N}_{\text{NH}_4}$ values can be vastly increased due to fast partial nitrification of the NH_4^+ pool. The ^{15}N enrichment of the residual NH_4^+ can be very pronounced, with the $\delta^{15}\text{N}_{\text{NH}_4}$ values reaching 41 ‰ in field studies and 79 ‰ in laboratory incubations (Lewicka-Szczebak et al., 2020). Similarly, the $\delta^{15}\text{N}_{\text{NO}_3}$ can be significantly enriched due to partial denitrification of the NO_3^- pool, the analysed mean $\delta^{15}\text{N}$ values of the residual NO_3^- pool in soils have reached 27‰ (Lewicka-Szczebak et al., 2020). Moreover, even higher enrichment can be noted locally in anaerobic microsites of most intensive denitrification. Considering all these factors it is very difficult to properly assess the N-precursor isotope signature and the errors of such assessment may be large, significantly impacting the final conclusions of the model calculations.

2.3 Fractionation equation

The most important unique feature of FRAME model is the ability of computing two simultaneous processes – production and consumption of N_2O . This co-existence can be described using the equations in the fractionation file, with defined parameters. Importantly, in FRAME software this fractionation equation describing N_2O reduction is not pre-defined and can be determined by the user. Depending on the case study, we can apply here the closed or open fractionation equation, different sequence of production and reduction or indicate which N_2O producing pathways may be followed by further reduction. The most common approach

for defining N₂O reduction is closed system fractionation equation (Rayleigh process) because the system is usually not in steady-state required for applying open system equations (Yu et al., 2020). However, in rare cases when the N₂O production and reduction is very stable with very rapid and constant exchange of substrates and products, open system may be applicable, e.g., by very high reduction rate and quite stable and small residual N₂O fraction (Decock and Six, 2013; Lewicka-Szczebak et al., 2015). Usually, the mixing followed by reduction is expected, i.e. the N₂O originating from all pathways can be reduced. Alternatively, it can be assumed that N₂O reduction is only possible in course of bacterial denitrification and only after this partial reduction the bacterial N₂O is further mixed with other pathways derived N₂O. Results of this scenario found better agreement with the reference methods of direct N₂ measurements (Wu et al., 2019). In reality, these both scenarios are probably co-existing, and potentially even more complex reduction scenarios can be also defined in FRAME. However, it has been shown, that in case of quite high uncertainty of exact N₂O production pathways contribution, the assumption of reduction associated with *bD* only will increase the possible modelling artefacts (Lewicka-Szczebak et al., 2020). Therefore, the reduction-mixing scenario, although found more realistic (Lewicka-Szczebak et al., 2014), can be applied only when sources are very well distinguished (Lewicka-Szczebak et al., 2017; Wu et al., 2019). If this is not the case applying mixing-reduction scenario should be preferred.

To define the isotope fractionation process in the model we determine the equation (examples presented in Table 2) and the fractionation factors for each isotope signature. The most actual values after (Yu et al., 2020) are summarised below (Table 3).

Table 2 Isotope fractionation equation for different scenarios with the way of their definition in FRAME

Fractionation scenario	Equation	Defined in FRAME*
Closed system mixing-reduction	$\delta = \delta_0 + \varepsilon * \ln(r)$	$M0[i] + A[i] * \log(r)$
Closed system reduction-mixing	$\delta = \delta_0 + f_{bD} * \varepsilon * \ln(r)$	$M0[i] + f[0] * A[i] * \log(r)$
Closed system reduction (<i>bD</i> + <i>nD</i>)-mixing	$\delta = \delta_0 + (f_{bD} + f_{nD}) * \varepsilon * \ln(r)$	$M0[i] + (f[0]+f[1]) * A[i] * \log(r)$
Open system mixing-reduction	$\delta = \delta_0 - \varepsilon * (1-r)$	$M0[i] - A[i] * (1-r)$
Open system reduction-mixing	$\delta = \delta_0 - f_{bD} * \varepsilon * (1-r)$	$M0[i] - f[0] * A[i] * (1-r)$

*equations given for the pathways defined as: *bD* – *f*[0], *nD* – *f*[1], *fD* – *f*[2], *Ni* – *f*[3] (numbers as sequenced in the source table); parameters (letters as defined by the user): A – isotope fractionation factor (ε_{p-s}); *r* – residual fraction; M₀ – isotope signature of initial mixture before fractionation

In most N₂O research, the N₂O reduction progress in the isotope fractionation equations is defined with residual unreacted fraction (*r*), usually denoted as *r*_{N₂O} (Yu et al., 2020), *R*_{N₂O} (Scheer et al., 2020) or as *N*₂*O*_{*i*} (Wei et al., 2024) representing the N₂O/(N₂O+N₂) ratio of denitrification (or just product ratio) mostly used in ¹⁵N traced approaches (eg. Pfülb et al., 2025). Both values are practically equal, but *N*₂*O*_{*i*} particularly applies to denitrification product ratio, since it takes into account only N₂ produced from the reduction of N₂O from bacterial denitrification, hence calculated in reduction-mixing scenario. This is because in ¹⁵N traced

studies by ^{15}N labelling NO_3^- pool, only N_2 originating from the NO_3^- pool can be detected and quantified. Dual or triple labelling approach of parallel treatments with different ^{15}N -labelled pools of NO_3^- , NH_4^+ , organic N can also identify N_2 originating from different N sources.

Table 3: Summary of isotope fractionation effects associated with N_2O reduction to N_2 .

literature isotope effects (mean $\pm$ SD)			
	$\epsilon^{15}\text{N}^{\text{SP}}$	$\epsilon^{18}\text{O}$	$\epsilon^{15}\text{N}$
N_2O reduction ^c	-6.0 $\pm$ 1.4	-15.9 $\pm$ 4.7	-7.0 $\pm$ 2.1

^c – (Jinuntuya-Nortman et al., 2008; Lewicka-Szczebak et al., 2015, 2014; Menyailo and Hungate, 2006; Ostrom et al., 2007; Well and Flessa, 2009b)

3 Modelling strategies

3.2 2D and 3D model

The model can be applied integrating all three N_2O isotope signatures ($\delta^{18}\text{O}$, $\delta^{15}\text{N}$, $\delta^{15}\text{N}^{\text{SP}}$), and, in case of complete data and sources characteristics, this is the preferred approach. The progress of the 3D compared to the 2D (e.g., $\delta^{15}\text{N}^{\text{SP}}$ - $\delta^{18}\text{O}$ or $\delta^{15}\text{N}^{\text{SP}}$ - $\delta^{15}\text{N}$) approach is, that the addition of the third isotope dimension (e.g., $\delta^{18}\text{O}$ or $\delta^{15}\text{N}$) theoretically allows calculation of additional unknown parameters from the isotope mass balance or better estimate the under-constrained analytical cases. But importantly, this third isotope parameter is only a valuable addition, if the precursor isotope values and proper source processes definitions can be included.

In case of problematic interpretation of one of the values, the model can be reduced to a 2D model. Usually, we deal with problematic interpretation of $\delta^{15}\text{N}$ values, due to lack of reliable precursor data and, in consequence, difficulties in proper determination of mixing endmember values. In case of large uncertainties, the 2D model applying $\delta^{15}\text{N}^{\text{SP}}$ and $\delta^{18}\text{O}$ should be preferred (Wei et al., 2024). The advantage of this simplified approach is the quite stable mixing endmember characteristics, namely – totally independent of precursors for $\delta^{15}\text{N}^{\text{SP}}$ and requiring a relatively small and easy correction with water isotope signatures for $\delta^{18}\text{O}$. As a result, the calculated pathways contributions and reduction progress may show low uncertainty, but for limited number of unknowns. Especially, for $\delta^{15}\text{N}^{\text{SP}}$ - $\delta^{18}\text{O}$ model the pathways *bD* and *nD* should be combined together, since there is almost no isotope distinction of these processes in the isotope 2D plot (Table 1) (Lewicka-Szczebak et al., 2020). Similarly, the *chD* pathway can overlap with *Ni* or *fD*, and will not be distinguishable in 2D model (Wei et al., 2019). The precision of such a model can be further enhanced if more limitations can be applied, like excluding of some pathways (e.g., no *Ni* in anoxic environments) (Senbayram et al., 2018) or some presumptions on N_2O reduction progress (Liang et al., 2026).

3.3 ^{15}N low-labelling strategy

To enhance the effective application of the 3D model, we can artificially change the isotope signatures of precursors to obtain a better processes distinction. For this purpose, low-level labelling can be applied with very low addition of ^{15}N tracer to one pool (e.g. only NO_3^-) but keeping the value within the natural abundance level (Deb et al., 2025). This allows for

unproblematic measurements of $\delta^{18}\text{O}$ and $\delta^{15}\text{N}^{\text{SP}}$ and applying the standard modelling approach including the isotope fractionation processes. The added ^{15}N tracer to one pool results in the shift of relevant pathways in the $\delta^{15}\text{N}$ values and clear distinction of denitrification processes (*bD* and *fD*) using NO_3^- as precursor and nitrification processes (*Ni*, *nD*) using NH_4^+ as precursor. (Deb et al., 2025). So far, the enrichment to $\delta^{15}\text{N}$ to ca. 100‰-200‰ was targeted to attain clear distinction of the endmember values and keep significant isotope fractionation effects (Deb et al., 2025). But it must be kept in mind that unknown isotope fractionation of NO_3^- and NH_4^+ pools can lead to bias in quantifying N_2O source contributions (Lewicka-Szczebak et al., 2020).

Potentially, the method of slightly higher labelling might also be a successful approach with even more clear processes distinction and reduction progress determined with $\delta^{18}\text{O}$ and $\delta^{15}\text{N}^{\text{SP}}$ values. The limit is the possible simultaneous precise measurement $\delta^{18}\text{O}$ and $\delta^{15}\text{N}^{\text{SP}}$ values by the elevated $\delta^{15}\text{N}$ values of produced N_2O . The upper limit for ^{15}N enrichment might depend on the measurement technique. When using laser absorption techniques, like cavity-ringdown spectroscopy (CRDS) method the sample measurements of $\delta^{15}\text{N} > 150\text{‰}$ may require additional correction of $\delta^{15}\text{N}^{\text{SP}}$ values. When using isotope ratio mass spectrometry (IRMS, coupled to a PreCon set up (Lewicka-Szczebak et al., 2014) it was possible to correct the measurements for all values up to at least 1 at% ^{15}N (Rohe and Well, 2026) when the theoretical formation of double- ^{15}N -substituted N_2O ($^{15}\text{N}^{15}\text{NO}$) was taken into account. But it still needs to be evaluated how this effect might differ between bacterial and fungal denitrifiers. To obtain precise results, suitable correction factors should be determined individually for the applied analytical approach.

3.4 ^{15}N combined model

Another recent idea is also the application of the N_2O modelling combining two datasets of parallel experiments: natural abundance and ^{15}N traced (Micucci et al., 2025). In this method the ^{15}N traced approach is applied to differentiate between N sources and natural abundance N_2O isotopes provide the further source partitioning. Mathematical integration of both parallel results in FRAME provides precise results with uncertainty estimation (Micucci et al., 2025). In this approach $r_{\text{N}_2\text{O}}$ is obtained from both approaches, hence can be combined and validated. The challenge here is the necessity to conduct two parallel treatments with comparable dynamics, that may be assumed identical, since they are treated as one set-up in the model.

3.5 Laboratory experiments for isotope effects determination

The example values of source processes given in Table 1, as the summary of isotope effects associated with particular pathways determined in pure culture studies or controlled incubations, can be modified according to each case study. Theoretically, the most proper way would be to analyse the isotope effects characteristic for the individual environment, like soil, water, sediment etc. This can be done with laboratory incubation experiments under controlled conditions for separation of oxic and anoxic processes, and with selecting specific substrates. For example, it has been shown that the anoxic soil incubations with acetylene (C_2H_2) addition for inhibition of N_2O reduction, favour *bD* and can be applied to determine the individual

isotope *bD* signal for the environment under study (Lewicka-Szczebak et al., 2017). Autotrophic nitrification could be inhibited by establishing 10 Pa acetylene (Well et al., 2008).

However, there are no similar easy methods for isolating other pathways. Selective inhibitors for fungal and bacterial denitrifiers can be applied, but the suitability of this approach to distinguish *bD* and *fD* has been shown to be questionable (Rohe et al., 2021).

The experimentally determined fractionation factors must be applied into the natural field studies with caution. Some experimental set-ups may produce significant experimental artefacts and provide isotope fractionation factors not related to the naturally occurring processes. Therefore, identifying of experimental artefacts is critical, e.g.:

- Determination of N₂O reduction isotope fractionation factor in static experiments appeared to be biased for ¹⁵N and ¹⁸O due to additional fractionation by N₂O diffusion having the opposite isotope effect. As a result, the final apparent isotope effect may differ from the conditions when N₂O is constantly escaping from the system and no back diffusion is possible (Lewicka-Szczebak et al., 2014). Hence, the dynamic experiments with constant gas flow provide rather more representative values of reduction isotope effect for typical open-system field studies.
- Incomplete N₂O reduction by C₂H₂ addition may underestimate the determined isotope effect (Lewicka-Szczebak et al., 2014).
- Very fast process rates can underestimate the determined isotope effects.
- Beginning of isotopic measurements of emitted N₂O before the equilibrium and isotope steady-state is established may result in observation of odd values for the first samples. This may be due to activation of NO detoxication pathways, as a reaction to highly increased NO concentrations and not sufficiently developed enzymes of further NO reduction (Chénier et al., 2026; Haslun et al., 2018).

An excellent example of separating processes in the environment of the actual case study, municipal wastewater treatment processes, was recently presented by (Strubbe et al., 2026). Authors applied controlled incubation conditions and targeted the combination of substrates addition, dissolved O₂ levels, pH, and organic carbon availability to successfully separate *nD* and *bD* pathways. Importantly, in their experiments negligible contribution from hydroxylamine oxidation pathway (*Ni*) was documented. Such experiments allow to apply the isotope mixing model much more efficiently, since they allow a reasonable selection of the mixing endmembers and their actual isotope fingerprint.

3.5 Potential and relevant mixing sources

Table 1 shows potential main sources, for which the isotope effects are experimentally determined. Recently also some other processes have been isotopically characterised, like NO detoxifying pathway (Wang et al., 2024), DNRA (Xu et al., 2024) or *chD* pathways (Tischer et al., 2022; Visser et al., 2020). Although often neglected in the past studies they may play a significant role under certain conditions, hence need to be considered when relevant. In any case study, one should initially consider all the possible sources, but then critically analyse if each of the processes is really plausible in this environment. The less unknowns given for the model, the better precision of the model outputs. In case an additional pathway is needed, maybe

one of the routinely accepted pathways could be negligible for the environment under study. For example, in case of anoxic environment often oxic nitrification can be excluded (Einzmann et al., 2025). When the isotope study is combined with any supporting method, it can be used as a primary selection of the mixing pathways included in the model. For example, if the microbial methods have been co-applied and some of the relevant genes or enzymes show low abundances, this might indicate that this process is less relevant.

Another N_2O formation pathway that can further complicate the FRAME application are hybrid processes occurring during co-denitrification, where one N atom from NO_2^- or NO combines with second N atom from another compound, particularly organic N, NH_4^+ or hydroxylamine to form N_2O or N_2 (Spott et al., 2011). As a result, two nitrogen atoms in N_2O molecule originate from two different substrates. Two main problems arise in identifying and quantifying these processes with natural abundance isotope methods: (i) the isotope fingerprint of this process is so far unknown and (ii) the integration of two different substrates for one product was so far not supported in FRAME. Whereas the model equation modification to introduce two covariant sources is theoretically possible, there is probably still a long way to reveal isotope characteristics of this process. So far, the co-denitrification was identified and quantified in traced experiments, with ^{15}N multi-labelling of different substrates (Müller et al., 2014) or combined ^{18}O - ^{15}N approaches (He et al., 2026). Some studies have found significant contribution of co-denitrification of up to 18 % in grasslands (Müller et al., 2014) and even 33 % in grazed pastures after urea depositions (Rex et al., 2018), whereas other indicate minor input: below 7% in vegetable soils (Duan et al., 2019), and negligible contribution in intensively managed pasture soils (Friedl et al., 2021). Hitherto case studies show that co-denitrification may be especially active after drought periods (Harris et al., 2021), in alkaline soils (Duan et al., 2019), and under high N content, e.g. urine amendment (Li et al., 2022; Rex et al., 2018). Currently, this process can be only accounted by applying isotope traced studies.

3.6 Predefining N_2O reduction

A method for independent N_2 determination, as ^{15}N gas flux method or direct N_2 measurement in artificial He atmosphere, can be co-applied. If independent information on N_2O reduction is available, the model can be restricted with this value. It can be introduced into the model by giving the known value into the equation in the fractionation file (see 2.3, Table 2). In such case the model has a stable fractionation equation with fixed $r_{\text{N}_2\text{O}}$ and without unknown parameters and the sources contribution can be determined with better precision. This strategy was tested in the microcosm soil incubation study in He atmosphere, where N_2 flux, and hence the $\text{N}_2\text{O}/(\text{N}_2\text{O}+\text{N}_2)$ ratio was measured directly (Senbayram et al., 2018; Wei et al., 2024). First, the FRAME estimation of $r_{\text{N}_2\text{O}}$ was compared with direct measurement and the agreement was good, except for the highest $r_{\text{N}_2\text{O}}$ values (>0.8) (Wei et al., 2024). Then these directly measured values were inserted into FRAME and source partitioning was attempted, but no fit was found by the model, whereas full modelling without predefined reduction gave good modelled outputs. This indicates that for very high $r_{\text{N}_2\text{O}}$ values the direct N_2 measurements of the lowest fluxes, near the method detection limit, were biased. Nevertheless, for usual cases where we deal with significant N_2O reduction the independent estimation of $r_{\text{N}_2\text{O}}$ can be applied to reduce the unknown parameters.

3.7 Time FRAME

Based on the FRAME model, a further R-package TimeFRAME (Time-resolved FRactionation And Mixing Evaluation FRAME) has been build (Harris et al., 2024), incorporating statistical interpolations between sequential measurements. This software is intended to be applied for dense measurement series with high data resolution. Importantly, it assumes dependence on the following sampling points. The analysis can be automatised because it is implemented as an R package ([link](#)). TimeFRAME assumes the isotope fractionation based on closed system mixing-reduction case, and this cannot be easily modified in the software. So far, this tool has been applied for synthesis on source partitioning of the globally investigated N₂O (Li et al., 2026).

4 Model outputs interpretation

4.1 Check of proper model assumptions

Application of FRAME requires a few pre-assumptions, as discussed above in Chapter 3, i.e. on precursors isotope signatures, isotope fractionation factors associated with particular N₂O production pathways and its reduction to N₂, the model equation of N₂O reduction. The model outputs offer the graphics illustrating the modelling process that can help to properly assess the plausibility of the model assumptions.

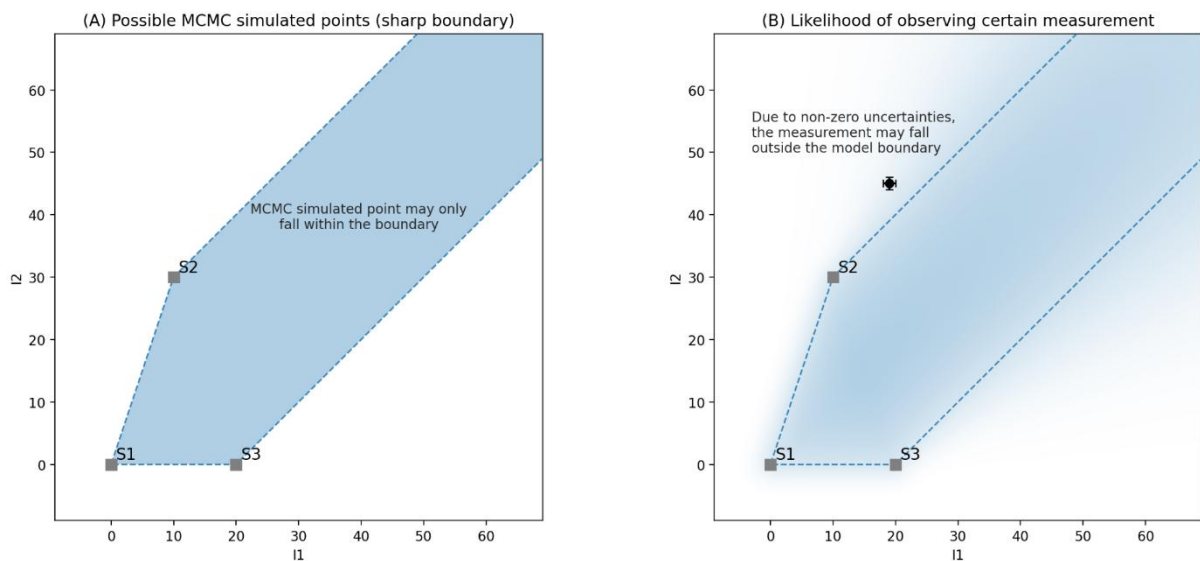


Fig. 1. MCMC phase space and measurement uncertainty in a fractionating three-source mixing model. (a) Hard boundary of admissible MCMC solutions (blue shading) spanned by end-members S1–S3 and fractionation along the dashed rays ($M = M_0 + A \ln r$). (b) Measurement likelihood envelope, broadened by Gaussian analytical uncertainty; dashed line shows strict model boundary. A measured composition may lie outside the MCMC region with exponentially decreasing probability.

First check is the sample's location on the initial graphs – 2D isotope maps (Fig.1) and its relation to the topology determined by the source processes and the N₂O reduction line representing the isotope enrichment due to progressive partial reduction of N₂O. If all the

assumptions are correct and all the potential sources are considered, the sample points must be situated within the polygon marked by between the mixing endmembers, and the shift associated with the fractionation due to reduction (diagonal translation), i.e. within the mixing-fractionation field (Fig. 1 – blue polygon). Importantly, the graphs produced in FRAME show the edge of the mixing-fractionation field with mean values of the border endmember ranges (Fig. 1A). But the further model calculations are performed taking into account associated uncertainties, so the actual range of plausible measurements extends wider than the rigid polygon boundary. The uncertainty of the mixing endmember values results in a blur of the edges of defined polygon, while the uncertainty of the reduction fractionation factor results additionally in the divergence of reduction line slopes (Fig.1B). The visualisation provided by FRAME (blue points in Fig. 2) simplifies this to the mean values. Thus, while the boundary polygons are marked using mean values for visual simplicity, the model computation accounts for all the associated uncertainties and the resulting likelihood distributions do not feature sharp boundaries, but rather a gradual fade-out, as displayed in Fig. 1B.

If the measured isotope signature falls outside the mixing fractionation field, the model will calculate this case assuming the nearest possible solution. Slight distinctions from the theoretical boundaries are possible and the model provides relevant results, however, the algorithm may provide a seemingly valid solution also for cases, when the measurement clearly violates the hypothesis encoded in the model equation. In the case of very large discrepancy between the measurement and the boundary of possible solutions, the model might take very long to converge or even fail the burnout process (Fig. 2B). This usually occurs when the samples are below the minimal slope of the reduction line or left from the *Ni* endmember box. Users may inspect the value of the “Z-score” statistical test outputted by the model, to verify if the distance between the MCMC simulated points and the measured data significantly exceeds the total uncertainty. For the measurements inside the polygon, the Z-score will be equal to zero. Values close to 1.0 should be regarded as absolutely plausible and the Z-score exceeding 3.0 signals one of the three problems:

- the model does not account for all the sources or processes present in the system (e.g., missing source),
- the uncertainties are not estimated correctly,
- the measured sample is faulty (e.g. contaminated or misplaced).

In case of samples with alarmingly high Z-scores, the following points should be double checked in order to understand the reason of this observation:

1. The characteristics of samples outside the box: Are these especially low or high N₂O concentration sample? Do our isotope normalisation procedure and measurement conditions provide proper values for these samples?
2. Are the N₂O samples from the starting point of an experiment? Possibly before the steady state has been reached? Or the last samples when maybe some new unexpected processes take place, or another N pool is activated?

3. The characteristics of mixing endmembers: Are all the possible sources taken into account? Are the assumed ranges relevant for the environment under study?
4. The procedure of sources correction with precursor values: Are the precursor values properly taken into consideration and properly estimated or measured?
5. In cases when the experimental conditions change significantly in course of the laboratory experiment or field study it might be necessary to evaluate each phase with a new model with specific sources newly defined for each phase.

If the above biases are checked and according to our best knowledge the sources are defined properly and the samples measured correctly, the results outside the mixing fractionation field should be reported and shown, with relevant information that they must have been disregarded from the modelling.

4.2 Check of model conformance

Very important step is the assessment of the model robustness, i.e. check if the output values result from a well determined model. This can be verified both visually, based on the graphics automatically provided by the FRAME software (*Paths* (Fig. 2), *Markov chain* (Fig. 3), *Correlations* (Fig. 4), *Results* (Fig. 5)) and with the provided numerical results (.xls output file):

1. The figure comparing modelled and measured values (*Paths* (Fig. 2)) indicates how well if the model is reflecting the reality. A robust model will produce the modelled values (blue points) closest to the measured points (black dot) (Fig. 2). A bad fit between measured and modelled values often indicates grouping of too different samples or inability to find a common good result for all the three isotope values in the 3D model.

2. The same can be checked by analysing the Z value defined by the model (provided in the model output summary - .xls output file), which indicates the distance between the modelled and measured values.

3. Time development of the model values (*Markov chain* (Fig. 3)) shows variation of the modelled values in time and illustrates if and when model stabilisation was achieved. In case the model did not find matching results, this graph illustrates why, possibly due to equal probability of all the possible variations or no solution meeting the modelling criteria. In such case, the model burnout phase is not reached (Fig. 3B). In MCMC simulations it is crucial that stabilization is indeed achieved, therefore such a plot is a key tool for quality assessment. In the example (Fig. 3), one simulation reached the stabilization (Fig. 3A) – and the second did not complete the burn-out phase (Fig. 3B).

4. Distribution estimation on the histograms (on the diagonal of *Correlations* (Fig. 4)) are calculated from the variables building the Markov chains, the wideness of these pools for each process represents the model uncertainty.

5. The same can be checked with σ values provided in the model output summary (.xls output file) for each mixing endmember and residual fraction.

6. Correlations between the modelled points are provided: as contour-plots (top-right) and correlation coefficients (bottom-left) in *Correlations* (Fig. 4). Very high correlation between two mixing endmembers combined with wide uncertainty ranges for them indicates poor distinguishing of these two mixing endmembers. Similarly, a correlation between source

contribution and the progress of fractionation process can indicate their opposite influence, e.g. increasing one mixing fraction (e.g. fD) results in higher δ values, while increasing r does the opposite due to negative value of the isotope fractionation factor.

7. Summary box-plots (*Results* (Fig. 5)) presenting mean, median, 1σ (~68% confidence interval), and 2σ (~95% confidence interval) directly illustrate the uncertainty of the model output.

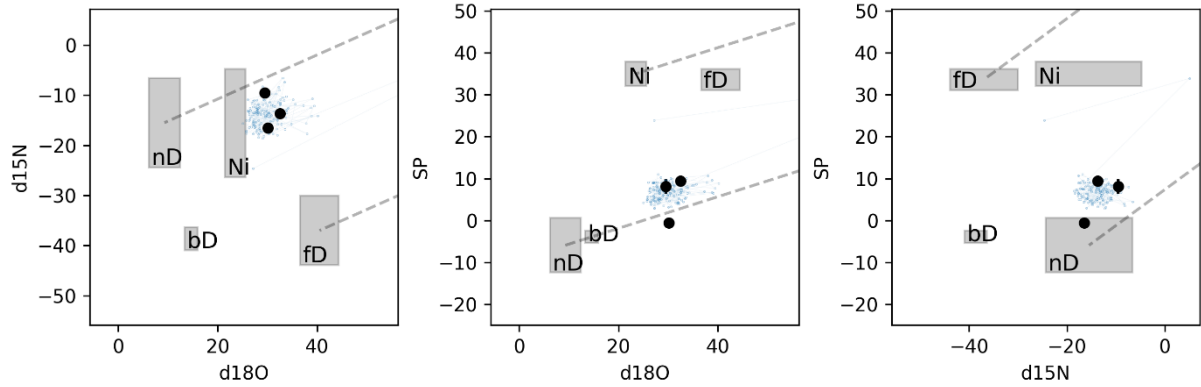


Fig. 2 *Paths* - simulation of 2D modelling paths – i.e. consecutive configurations accepted by the MCMC algorithm plotted in the dual isotope map (blue points) in relation to given sample data (black points), the relevant sources (grey rectangles: bD , nD , fD , Ni) and the relevant fractionation (dashed lines showing the direction and boundaries related to the fractionation process).

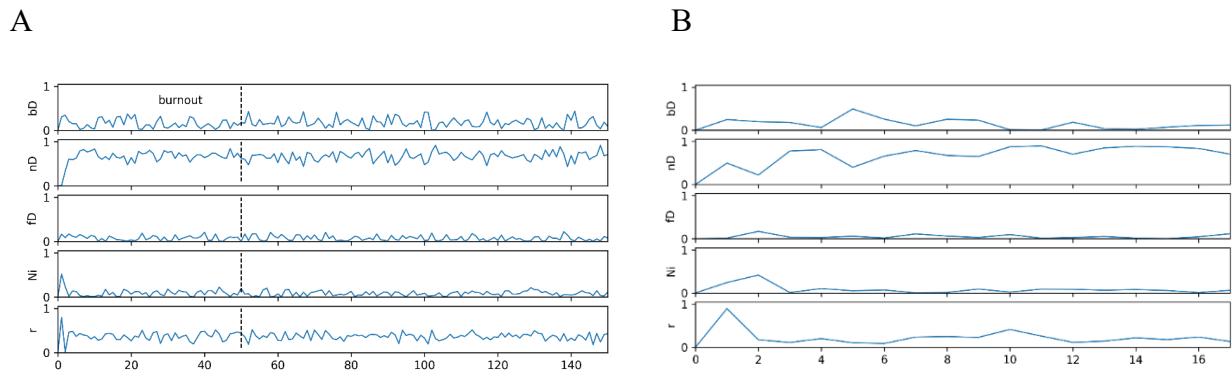


Fig. 3 *Markov chain* – Simulated values from all model iterations that fulfilled the Metropolis condition—The Markov chain. The number of initial iterations required for the model stabilisation is discarded (burnout, left to the dashed line), reaching the region of maximum likelihood (panel A). When the simulation cannot reach the stabilization the burn-out phase is not completed (panel B).

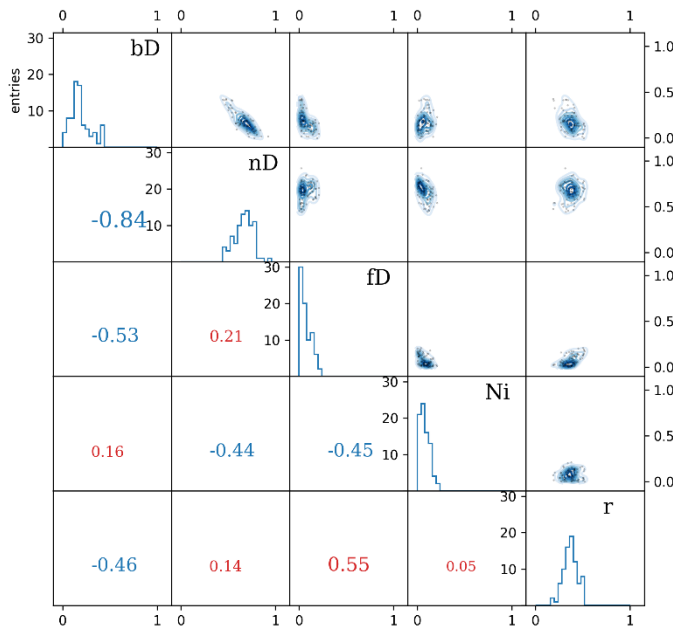


Fig. 4 *Correlations* – the probability distributions of mixing fractions (bD , nD , fD , Ni) and fractionation parameter r . Histograms (on the diagonal) are calculated from the variables building the Markov chains along with their cross-correlations as contour-plots (top-right) and correlation coefficients between the two analysed unknown (bottom-left).

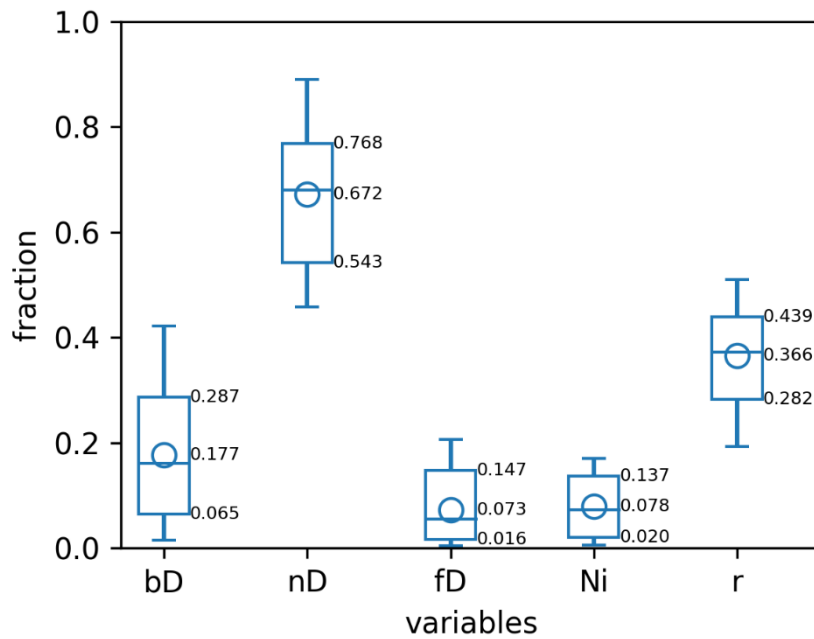


Fig. 5 *Results* – Summary statistics on the simulation results of mixing fractions (bD , nD , fD , Ni) and fractionation parameter r : The circle stands for the mean value, the horizontal line represents the median, the box encloses 68% confidence interval (CI) and the whiskers stand for 95% CI.

For good results documentation the model graphics and output files should be available in supplementary materials or repository databases.

5 Actualised model release

FRAME v2.0 is a major update of the Bayesian MCMC mixing and fractionation model (<https://malewick.github.io/frame/>) first published in 2022 (Lewicki et al., 2022). The core workflow is unchanged: users supply measured isotope data, end-member source signatures, and (optionally) a fractionation model; FRAME then estimates source contributions and auxiliary parameters by Metropolis–Hastings sampling. The update improves statistical treatment of source uncertainty, extends prior specification for fractionation variables, and brings practical gains in speed and accessibility.

5.1 Likelihood for uniform source uncertainty

In v1.0, sources described by a flat range (using non-zero values of spread parameters) were handled with an approximation obtained by linearly combining spreads. This was convenient but could bias results when several such sources were used or when spreads were large relative to measurement uncertainty. Version 2.0 replaces this approximation with an exact numerical treatment. For each isotope dimension and MCMC step, the Probability Density Function (PDF) is built as the convolution of (i) a Gaussian term combining all “*stdev*” contributions and data uncertainty, and (ii) one symmetric uniform distribution per source with nonzero spread. Convolutions are computed with Fast Fourier Transform (FFT) and the resulting PDF is evaluated at the measured value. Models using only Gaussian source uncertainties are unchanged and retain the fast analytical likelihood computation. While the previous approximation remains robust for most applications, upgrading to v2.0 is recommended for maximum accuracy when modelling multiple spread-based sources.

5.2 Configurable priors for auxiliary variables

Fractionation models in FRAME include auxiliary variables (commonly denoted r , e.g. residual fraction or reaction progress). In v1.0, these were always sampled from Uniform(0, 1). Version 2.0 allows the sampling prior to be chosen explicitly:

- uniform: $r \sim \text{Uniform}[r_{\min}, r_{\max}]$ (default; identical to v1.0 when bounds are [0, 1]);
- log-uniform: $\log(r) \sim \text{Uniform}[\log(r_{\min}), \log(r_{\max})]$, suitable when r may span orders of magnitude.

This is available in the graphical interface (prior type and bounds per auxiliary variable), in batch mode via appropriate execution arguments (`--aux_prior_type`, `--aux_r_min`, and `--aux_r_max`) and in Python through an instruction `set_aux_prior`.

5.3 Performance and interface

Long MCMC runs benefit from precompiled model expressions (replacing repeated `eval()` calls). Additional optimisations in the convolution and PDF evaluation routines reduce per-iteration cost. The batch runner and Windows executable gained fuller command-line control; documentation and a Google Colab notebook now allow users with basic Python knowledge to run example analyses in a browser without local installation.

5.4 Compatibility note

Input file formats and the scientific model structure are preserved – full backward compatibility is ensured. The main behavioural change affecting published results concerns spread-based source uncertainty; prior choice affects fractionation models whenever r is not fixed to Uniform(0, 1).

5.5 Model parameters: MCMC and auxiliary-variable prior settings

FRAME is controlled through a compact set of run-time options available in the graphical interface and in headless batch mode (equivalent command-line flags in parentheses). These settings govern chain length and convergence, file output, computational efficiency, and—when fractionation or related processes are modelled—the prior distribution of unknown auxiliary variables.

Maximum iterations (`--niter`) - Upper limit on MCMC proposals per sample group; the run terminates earlier once the target chain length is reached. Increase if the chain is not filled (e.g. low acceptance, measurements near mixing-space boundaries, or strongly correlated fractionation models). GUI default: 10^5 ; batch mode: 10^6 .

Burn-in (`--burnout`) - Number of accepted steps discarded before posterior recording begins, allowing the adaptive acceptance threshold to stabilise. Default: 100; increase to 200–500 if initial chain traces show drift.

Chain length (`--chain_length`) - Number of accepted post-burn-in steps retained for inference and diagnostic plots. Default: 500; increase to 1000–5000 for smoother posterior histograms or models with several correlated unknowns.

Prior type (`--aux_prior_type`) - Sampling distribution for each unknown auxiliary variable r . uniform:

- $r \sim \text{Uniform}[r_{\min}, r_{\max}]$ (default; appropriate for bounded fractions).
- loguniform: $\log(r)$ uniform in log-space; suitable when r may span orders of magnitude (requires $r_{\min} > 0$).

Prior range minimum and maximum are controlled with $r_{\min}$ (`--aux_r_min`) and $r_{\max}$ (`--aux_r_max`).

6 Examples from case studies

6.1 Applicability to different environments

Thanks to the flexibility of FRAME in defining the number and isotope characteristics of N_2O mixing sources and the systematics of further N_2O reduction, this modelling tool is applicable across all environments and has been so far applied for N_2O source partitioning in both laboratory microcosm (Deb et al., 2024; Einzmann et al., 2025; Gu et al., 2025; Guo et al., 2026; Stephens et al., 2026; Wei et al., 2024) and mesocosm (Götze et al., 2025; Masta et al., 2026; Rummel et al., 2025a, 2025b; Zhu et al., 2026) incubation experiments, as well as in field studies across the globe: in African lake in Kongo (Barthel et al., 2026), in stratified mountain lake in Switzerland (Einzmann et al., 2026), on Pacific research cruise (Gu et al., 2025), Tibetan

rivers (Chen et al., 2023), China rivers (Wang et al., 2025b), agricultural wheat/sugar beet fields in Switzerland (Rainford et al., 2026), wheat/corn fields in Germany (Well et al., 2025), soybean field in Japan (Toyoda et al., 2024), semi-arid savannah in Kenya (Fang et al., 2024; Zhu et al., 2026) and in one marine samples meta-analysis (Wang et al., 2025a). These various case studies investigated terrestrial environments: agricultural soils (Götze et al., 2025; Guo et al., 2026; Rainford et al., 2026; Rummel et al., 2025a; Toyoda et al., 2024), post-wildfire chaparral soils (Stephens et al., 2026), coastal saline soils (Liang et al., 2026), livestock enclosures in sub-Saharan Africa (Fang et al., 2024; Zhu et al., 2026), aquatic environments: polluted groundwaters (Deb et al., 2025), lakes (Barthel et al., 2026; Einzmann et al., 2026, 2025), rivers (Chen et al., 2023; Shu et al., 2024; Wang et al., 2026, 2025b) and marine environments (Gu et al., 2025; Wang et al., 2025a).

6.2 Different application strategies

The unique opportunity provided by FRAME is the integration of three isotope values ($\delta^{15}\text{N}^{\text{SP}}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$) to find one best solution. First study where 3D N_2O isotope model (3DIM) was applied and validated was the agricultural field and laboratory experiment (Lewicka-Szczebak et al., 2020), continued in more frequent field study of different agricultural management systems at two field sites (Well et al., 2025). Application of 3D N_2O modelling allowed for better source partitioning and denitrification product ratio estimation and hence provided insight into underlying processes associated with different farming strategies and soil types. In further research studies the 3D modelling was successfully implemented with FRAME software to differentiate the *bD*, *fD*, *nD* and *Ni* production pathways and quantifying N_2O reduction where the $\delta^{15}\text{N}$ values of mixing endmembers were precisely analysed (Deb et al., 2025; Fang et al., 2024; Gu et al., 2025; Masta et al., 2026; Zhu et al., 2026) or assumed based on best literature estimates for similar environment and conditions (Barthel et al., 2026).

In numerous of the case studies applying FRAME the $\delta^{15}\text{N}$ of the precursors is missing or too dynamically changing, hence the 2D model is applied. For example, this strategy was chosen for the agricultural soil experiments amended with mineral, organic and mixed fertilizers (Wei et al., 2024). The various N pools were dynamically changing, and the isotope signatures of different soil N pools were not analysed. Similarly, in the Tibetan River study very low NH_4^+ concentrations disabled the isotope analysis of this pool and the assumption on equal $\delta^{15}\text{N}$ was tested but indicated as potentially biased and rejected by the authors (Chen et al., 2023). A 2D model was also chosen when the 3D model fails to yield reliable results due to deviations between modelled and measured $\delta^{15}\text{N}$ values and no well-fitted solution found by the model in 3D space (Rummel et al., 2025b).

Reduction was calculated assuming closed system (Rayleigh equation) in most of the above case studies, however, especially for water environments this model may appear too much simplified (Deb et al., 2025) and some modified approach applicable also to open systems has been proposed (Einzmann et al., 2026). Some studies applied and compared both scenarios: reduction-mixing and mixing-reduction. The observed differences between their outcomes depend largely on the specific characteristics of the studied environment and the proportion of dominant microbial pathways. In studies of agricultural soils (Götze et al., 2025) significant differences were observed in estimating the level of N_2O reduction to N_2 . The $r_{\text{N}_2\text{O}}$ was

estimated at 47–70% using the R-M scenario, while for the M-R scenario, these values were distinctly higher, ranging from 73–83%. Regarding source contributions, the estimated *Ni* share in total N₂O production was slightly more stable in the R-M scenario (42–48%) compared to a wider range in the M-R scenario (40–57%). However, in studies of dominating *bD* pathway (Well et al., 2025) and large N₂O reduction (Chen et al., 2023) the difference between both scenarios was minimal, within the model uncertainty.

6.3 Selection of source pathways

In most studies the standard main pathways are assumed as mixing endmember: *bD*, *fD*, *nD*, *Ni*. However, some studies included more individualised contributing pathways basing on specific environments. In marine and oceanic studies, *Ni* was explicitly divided into archaeal ammonia oxidation (*aN/AOA*) and bacterial ammonia oxidation (*bN/AOB*), alongside *bD* and *nD* (Gu et al., 2025; Wang et al., 2025a). In agricultural soybean fields, *bD* was subdivided to distinguish between standard soil denitrifiers (*sBD*) and specific nodule rhizobia (*nBD*) (Toyoda et al., 2024). Because *bD* and *nD* often exhibit weak isotopic separation, several studies chose to aggregate them into a single combined fraction (*bD+nD*) to improve model reliability (Well et al., 2025; Zhu et al., 2026).

Lack of *chD* included in the potential sources was pointed out as a weakness of the model (Deb et al., 2025). Only in recent studies *chD* pathway was also included (Einzmann et al., 2025; Liang et al., 2026). First, in anoxic lake environments, *chD* was introduced as a potential alternative source, while *Ni* was excluded entirely (due to the lack of oxygen) (Einzmann et al., 2025). Later, *chD* was introduced as the fifth mixing endmember and the robustness of the model taking into account large uncertainties of isotope characteristics of this N₂O source were tested. Only minor changes and very indivertible general conclusions of the model in this case study were documented (Liang et al., 2026). The model results were also combined with microbial data that further confirmed its robust output (Liang et al., 2026).

6.4 Validations with independent methods

In Lewicki et al. (2022) the model performance with theoretical values was tested and validated. Before, the performance of the FRAME precursor - 3DIM model - was validated in detail applying ¹⁵N gas-flux approach both in field and laboratory experiments (Lewicka-Szczebak et al., 2020). 3DIM applied mathematically the same idea and same calculation script and was applied before release of FRAME software. For model estimation of *r*_{N₂O} very good agreement with reference method was documented and the typical accuracy for *r*_{N₂O} determination was 0.15 (Lewicka-Szczebak et al., 2020). Source partitioning could be only partially validated in that study, by comparing the NO₃⁻-pool derived and NH₄⁺-pool derived N₂O. This processes distinction was positively validated for experiments showing significant N₂O flux, but showed significant bias for low N₂O fluxes, near analytical detection limit for ¹⁵N gas flux method.

Further studies provided comparisons of N₂O isotope modelling outputs with microbial methods applying DNA extraction and qPCR analyses. In groundwater incubations microbial and isotope method indicated similar shift in time from nitrification to denitrification dominated

pathways (Deb et al., 2025). In saline soil incubations, an agreement in N₂O source partitioning has been shown, too, but the observed relative enrichment in N₂O reduction genes (*NosZ*) was accompanied by actual decrease in N₂O consumption, indicating decoupled genetic potential from actual enzymatic activity (Liang et al., 2026). Importantly, the applied microbial approach (DNA extraction) provides information on gene abundances associated with particular microbial pathways, not the gene expression. Hence, the information obtained relates only to potential of N₂O production, but do not show if these genes are transcribed and expressed (Esenberg et al., 2018). Including mRNA extraction to quantify gene expression would show the production of enzymes which can be seen as direct evidence for initiation of a reaction step leading to N₂O production or reduction. However, such analyses are much more challenging and therefore so far were not combined with isotope results (Baumann et al., 2024). So far, close correlation between gene expression and N₂O transformation rates over time has never been proven and is questionable, since the transformation processes may keep active when gene expression declines, because the enzymes are present at a certain level, which needs to be maintained only (Rohe et al., 2020).

Masta et al. (2026) indicated contrasting results of FRAME modelling, microbial analysis and ¹⁵N tracing. This was most probably due to very dynamic experimental setup of the mesocosm incubations comprised with changing conditions of water content and O₂ concentration resulting in rapid fluctuations between oxic and hypoxic conditions. Consequently, each approach provided a different perspective, e.g. microbial analysis of top soil layer responding instantaneously on changed conditions vs. N₂O flux from deeper soil layer showing delay in change of N₂O production pathways. This indicated that parallel validation methods do not always provide good reference values, and microbial and isotope results should be rather treated as complementing not validating approaches.

On the other hand, a very good agreement on partitioning of bacterial and fungal denitrification was obtained in soil microcosm incubations applying organic and mineral fertilization (Wei et al., 2024). Importantly, in this study significant correlation between gene abundances and the modelled *fD* contribution has been indicated. This is so far the only partial verification of fungal contribution determined by the isotope models, however only quasi-quantitative, since the gene abundances allow only relative quantification of N₂O fluxes. NO₂⁻ induced transcription of *p450nor* during denitrification by the fungal denitrifier *Fusarium oxysporum* has been shown to correlate with the production of N₂O with a high δ¹⁵N^{SP} typical for fungal denitrifiers (Rohe et al., 2020). *p450nor* gene expression can thus be promising to validate the occurrence of active *fD*. But results also showed that gene expression declined during constant N₂O production, supporting the assumption that gene expression can only identify the existence of a certain process, but not its actual rate.

The validation of source partitioning between *bD* and *fD* was also aimed by application of selective inhibition of fungal or bacterial growth (Rohe et al., 2021). However, due to large fraction of uninhibited N₂O production and low contribution of *fD* in studied soils this approach was not suited for validating the isotope modelling results (Rohe et al., 2021).

7. Conclusions and outlook

The FRAME model was introduced to enable the standardised, fast and robust interpretation of the complex N₂O isotopocule datasets. By integrating three isotope values ($\delta^{15}\text{N}^{\text{SP}}$, $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$) and simultaneous isotope mixing and fractionation processes, it provides quantitative information on mixing pathways contribution and N₂O reduction progress with estimation of uncertainty and detailed documentation of the modelling process and outputs. The N₂O isotope community thus received a tool for well unified and comparable approach for data evaluation. Simultaneously, the model remains flexible, allowing users to make individual modifications according to specific cases. Due to open availability of the modelling code further developments of the model by wide research community are facilitated. Moreover, open discussion forum on the FRAME website allows reporting and discussing all the issues with model authors, which resulted in new ideas and improvements, which should also further continue.

Further work is needed on the quality of input data that are introduced into the model. The less uncertainty in the measured isotope values and defined sources, the more robust the output values. However, some of the sources are still poorly determined and their further characterisation for particular environments is still needed. The model outputs are already well validated for the estimation of N₂O reduction ($r_{\text{N}_2\text{O}}$), while a proper validation for source partitioning is still missing, only plausibility of the modelled contribution of selected pathways was indicated by molecular biology results. There is definitely a need for more robust validation by combining FRAME with well-tailored high or low level ¹⁵N labelling or with gene expression approach.

An important aspect of N₂O isotope studies is their relation to substrates and the awareness of this has definitely grown in recent years, most authors now provide measured isotope signatures of N substrates (NO₃⁻, NH₄⁺) and ambient water (H₂O). In future perspectives, possibly addition of further N-compounds, central for various N-transformations, like NO and NO₂⁻, may open new, more precise interpretation strategies by allowing to link various compounds of the N cycle.

AI disclosure

The original FRAME package (v1.0, 2022) was developed entirely without generative artificial intelligence: model design, statistical methods, code, graphical interface, documentation, and validation were human work ((Lewicki et al., 2022)).

The v2.0 update (2026) followed the same principle for scientific and design decisions. The authors identified required changes—correction of the likelihood for uniform (spread/delta) source uncertainty, configurable priors for auxiliary variables, associated GUI and batch-interface extensions, documentation, and release infrastructure—and specified them in detail. Implementation was AI-augmented: a coding agent (Cursor) was instructed to draft, refactor, and debug code and documentation under direct human supervision. In the public GitHub repository (github.com/malewick/frame), some commits are attributed to Cursor; this attribution faithfully reflects the realistic scope of AI contribution, which is limited to software implementation and documentation. All changes were reviewed and accepted by the authors

1000 before integration. No generative AI was used in hypothesis formulation, model selection,
1001 interpretation of results, or in writing the scientific manuscript.

1002 **CRedit author statement**

1003 DLS: Conceptualization, Methodology, Supervision, Project administration and funding,
1004 Writing - Original Draft; MPL: Methodology, Software, Formal analysis, Writing - Original
1005 Draft; CBT: Writing - Review & Editing; RW: Writing - Review & Editing

1006 **Funding statement**

1007 This study was financially supported by the grant Opus-2021/41/B/ST10/01045 (PI: Dominika
1008 Lewicka-Szczebak) of the National Science Centre Poland.

1009
1010 **Bibliography:**
1011

1012 Baggs, E.M., 2011. Soil microbial sources of nitrous oxide: recent advances in knowledge,
1013 emerging challenges and future direction. *Curr. Opin. Environ. Sustain.* 3, 321–327.
1014 <https://doi.org/10.1016/J.COSUST.2011.08.011>

1015 Bai, E., Houlton, B.Z., Wang, Y.P., 2012. Isotopic identification of nitrogen hotspots across
1016 natural terrestrial ecosystems. *Biogeosciences* 9, 3287–3304. [https://doi.org/10.5194/bg-](https://doi.org/10.5194/bg-9-3287-2012)
1017 [9-3287-2012](https://doi.org/10.5194/bg-9-3287-2012)

1018 Barford, C.C., Montoya, J.P., Altabet, M.A., Mitchell, R., 1999. Steady-State Nitrogen Isotope
1019 Effects of N₂ and N₂O Production in *Paracoccus denitrificans*. *Appl. Environ. Microbiol.*
1020 65, 989–994. <https://doi.org/10.1128/AEM.65.3.989-994.1999>

1021 Barthel, M., Drake, T.W., de Clippele, A., de Groot, L.W., Engelhardt, M., Haghipour, N., Hou,
1022 Y., Kueter, N., Lewicka-Szczebak, D., Ludjwera Bahati, A., Tschumbu, C.L., Van Oost,
1023 K., Wabakanghanzi, J.N., Werner, R.A., Zambo Mande, J., Six, J., Hemingway, J.D.,
1024 2026. Constraining Greenhouse Gas Cycling and Emissions in Africa's Largest Humic
1025 Lake. *J. Geophys. Res. Biogeosci.* 131. <https://doi.org/10.1029/2025JG009218>

1026 Baumann, K.B.L., Mazzoli, A., Salazar, G., Ruscheweyh, H.J., Müller, B., Niederdorfer, R.,
1027 Sunagawa, S., Lever, M.A., Lehmann, M.F., Bürgmann, H., 2024. Metagenomic and -
1028 transcriptomic analyses of microbial nitrogen transformation potential, and gene
1029 expression in Swiss lake sediments. *ISME Communications* 4.
1030 <https://doi.org/10.1093/ismeco/ycae110>

1031 Brenninkmeijer, C.A.M., Röckmann, T., 1999. Mass spectrometry of the intramolecular
 1032 nitrogen isotope distribution of environmental nitrous oxide using fragment-ion analysis.
 1033 Rapid Communications in Mass Spectrometry 13, 2028–2033.
 1034 [https://doi.org/10.1002/\(SICI\)1097-0231\(19991030\)13:20<2028::AID-](https://doi.org/10.1002/(SICI)1097-0231(19991030)13:20<2028::AID-RCM751>3.0.CO;2-J)
 1035 [RCM751>3.0.CO;2-J](https://doi.org/10.1002/(SICI)1097-0231(19991030)13:20<2028::AID-RCM751>3.0.CO;2-J)

1036 Buchen, C., Lewicka-Szczebak, D., Flessa, H., Well, R., 2018. Estimating N₂O processes
 1037 during grassland renewal and grassland conversion to maize cropping using N₂O
 1038 isotopocules. Rapid Communications in Mass Spectrometry 32, 1053–1067.
 1039 <https://doi.org/10.1002/rcm.8132>

1040 Buchwald, C., Grabb, K., Hansel, C.M., Wankel, S.D., 2016. Constraining the role of iron in
 1041 environmental nitrogen transformations: Dual stable isotope systematics of abiotic NO₂–
 1042 reduction by Fe(II) and its production of N₂O. Geochim. Cosmochim. Acta 186, 1–12.
 1043 <https://doi.org/10.1016/J.GCA.2016.04.041>

1044 Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Butterbach-bahl, K., Baggs,
 1045 E.M., Dannenmann, M., Kiese, R., Zechmeister-boltenstern, S., Butterbach-bahl, K., 2013.
 1046 Nitrous oxide emissions from soils : how well do we understand the processes and their
 1047 controls ? Nitrous oxide emissions from soils : how well do we understand the processes
 1048 and their controls ? Philosophical Transactions of the Royal Society 368, 20130122.

1049 Casciotti, K.L., Böhlke, J.K., McIlvin, M.R., Mroczkowski, S.J., Hannon, J.E., 2007. Oxygen
 1050 isotopes in nitrite: Analysis, calibration, and equilibration. Anal. Chem. 79, 2427–2436.
 1051 <https://doi.org/10.1021/ac061598h>

1052 Chen, X., Zhang, S., Liu, J., Wang, J., Xin, Y., Sun, S., Xia, X., 2023. Tracing Microbial
 1053 Production and Consumption Sources of N₂O in Rivers on the Qinghai-Tibet Plateau via
 1054 Isotopocule and Functional Microbe Analyses. Environ. Sci. Technol. 57, 7196–7205.

1055 Chénier, N., Magyar, P.M., Zopfi, J., Frey, C., Kuhn, T., Lehmann, M.F., Mohn, J., 2026. High
 1056 nitrous oxide isotopic variability during denitrification by Pseudomonas species bearing
 1057 NirK and NirS. EGU sphere. <https://doi.org/10.5194/egusphere-2026-1824>

1058 Deb, S., Espenberg, M., Well, R., Bucha, M., Jakubiak, M., Mander, Ü., Edrysek, M.O.J.,
 1059 Lewicka-Szczebak, D., 2025. N transformations in nitrate-rich groundwaters: combined
 1060 isotope and microbial approach. Biogeosciences 22, 5535–5556.
 1061 <https://doi.org/10.5194/bg-22-5535-2025>

1062 Deb, S., Lewicka-Szczebak, D., 2025. Simplified bacterial denitrification method using
 1063 *Stenotrophomonas nitritireducens* for nitrite dual isotope analysis in low-concentration
 1064 environmental samples. *Front. Environ. Sci.* 13.
 1065 <https://doi.org/10.3389/fenvs.2025.1536882>

1066 Deb, S., Lewicka-szczebak, D., Rohe, L., 2024. Microbial nitrogen transformations tracked by
 1067 natural abundance isotope studies and microbiological methods : A review. *Science of the*
 1068 *Total Environment* 926, 172073. <https://doi.org/10.1016/j.scitotenv.2024.172073>

1069 Decock, C., Six, J., 2013. How reliable is the intramolecular distribution of ^{15}N in N_2O to
 1070 source partition N_2O emitted from soil? *Soil Biol. Biochem.* 65, 114–127.
 1071 <https://doi.org/10.1016/j.soilbio.2013.05.012>

1072 Denk, T.R.A., Mohn, J., Decock, C., Lewicka-Szczebak, D., Harris, E., Butterbach-Bahl, K.,
 1073 Kiese, R., Wolf, B., 2017. The nitrogen cycle: A review of isotope effects and isotope
 1074 modeling approaches. *Soil Biol. Biochem.* 105, 121–137.
 1075 <https://doi.org/10.1016/j.soilbio.2016.11.015>

1076 Deppe, M., Well, R., Giesemann, A., Spott, O., Flessa, H., 2017. Soil Biology & Biochemistry
 1077 Soil N_2O fluxes and related processes in laboratory incubations simulating ammonium
 1078 fertilizer depots. *Soil Biol. Biochem.* 104, 68–80.
 1079 <https://doi.org/10.1016/j.soilbio.2016.10.005>

1080 Duan, P., Zhou, J., Feng, L., Jansen-Willems, A.B., Xiong, Z., 2019. Pathways and controls of
 1081 N_2O production in greenhouse vegetable production soils. *Biol. Fertil. Soils* 55, 285–297.
 1082 <https://doi.org/10.1007/s00374-019-01348-9>

1083 Einzmann, T., Lehmann, M., Bourbonnais, A., Zopfi, J., Lepori, F., Frey, C., 2026. Nitrous
 1084 oxide cycling and isotopic overprinting in lake basins with contrasting mixing regimes.
 1085 <https://doi.org/10.26434/chemrxiv-2026-5pnpg>

1086 Einzmann, T., Lehmann, M.F., Zopfi, J., Frey, C., 2025. Non-canonical reductive nitrous oxide
 1087 production pathways in a seasonally stratified lake basin.
 1088 <https://doi.org/10.1101/2025.08.15.670496>

1089 Espenberg, M., Truu, M., Mander, Ü., Kasak, K., Nõlvak, H., Ligi, T., Oopkaup, K., Maddison,
 1090 M., Truu, J., 2018. Differences in microbial community structure and nitrogen cycling in
 1091 natural and drained tropical peatland soils. *Sci. Rep.* 8, 1–12.
 1092 <https://doi.org/10.1038/s41598-018-23032-y>

1093 Fang, X., Harris, S.J., Leitner, S.M., Butterbach-Bahl, K., Conz, R.F., Merbold, L.,
1094 Dannenmann, M., Oyugi, A., Liu, S., Zou, J., Six, J., Barthel, M., 2024. Mechanisms
1095 behind high N₂O emissions from livestock enclosures in Kenya revealed by dual-isotope
1096 and functional gene analyses. *Soil Biol. Biochem.* 196.
1097 <https://doi.org/10.1016/j.soilbio.2024.109505>

1098 Frame, C.H., Casciotti, K.L., 2010. Biogeochemical controls and isotopic signatures of nitrous
1099 oxide production by a marine ammonia-oxidizing bacterium. *Biogeosciences* 7, 2695–
1100 2709. <https://doi.org/10.5194/bg-7-2695-2010>

1101 Friedl, J., Scheer, C., De Rosa, D., Müller, C., Grace, P.R., Rowlings, D.W., 2021. Sources of
1102 nitrous oxide from intensively managed pasture soils: The hole in the pipe. *Environmental*
1103 *Research Letters* 16. <https://doi.org/10.1088/1748-9326/abfde7>

1104 Götze, H., Buchen-Tschiskale, C., Eder, L., Pacholski, A., 2025. Effects of inhibitors and slit
1105 incorporation on NH₃ and N₂O emission processes after urea application. *Agric. Ecosyst.*
1106 *Environ.* 378. <https://doi.org/10.1016/j.agee.2024.109307>

1107 Grabb, K.C., Buchwald, C., Hansel, C.M., Wankel, S.D., 2017. A dual nitrite isotopic
1108 investigation of chemodenitrification by mineral-associated Fe(II) and its production of
1109 nitrous oxide. *Geochim. Cosmochim. Acta* 196, 388–402.
1110 <https://doi.org/10.1016/J.GCA.2016.10.026>

1111 Gu, T., Chen, Z., Hutchins, D.A., Sun, J., 2025. Urea-driven nitrification contributes to N₂O
1112 production in the oligotrophic euphotic ocean. *ISME Journal* 19.
1113 <https://doi.org/10.1093/ismejo/wraf281>

1114 Guo, S., Lin, H., Li, Z., Han, Z., Wu, J., Bo, X., Shen, M., Zhang, Z., Liu, S., Wang, J., Zou, J.,
1115 2026. Divergent legacy effects of biochar on nitrous oxide emissions in acidic soils driven
1116 by altered microbial N pathways. *Biochar* 8, 40. [https://doi.org/10.1007/s42773-025-](https://doi.org/10.1007/s42773-025-00558-9)
1117 [00558-9](https://doi.org/10.1007/s42773-025-00558-9)

1118 Harris, E., Diaz-Pines, E., Stoll, E., Schlöter, M., Schulz, S., Duffner, C., Li, K., Moore, K.L.,
1119 Ingrisch, J., Reinthaler, D., Zechmeister-Boltenstern, S., Glatzel, S., Brüggemann, N.,
1120 Bahn, M., 2021. Denitrifying pathways dominate nitrous oxide emissions from managed
1121 grassland during drought and rewetting. *Sci. Adv.* 7.
1122 <https://doi.org/10.1126/sciadv.abb7118>

- 1123 Harris, E., Fischer, P., Lewicki, M.P., Lewicka-Szczebak, D., Harris, J., Perez-cruz, F., 2024.
1124 Technical Note : TimeFRAME - A Bayesian Mixing Model to Unravel Isotopic Data and
1125 Quantify Trace Gas Production and Consumption Pathways for Timeseries Data.
1126 Biogeosciences.
- 1127 Haslun, J.A., Ostrom, N.E., Hegg, E.L., Ostrom, P.H., 2018. Estimation of isotope variation of
1128 N₂O during denitrification by *Pseudomonas aureofaciens* and *Pseudomonas chlororaphis*:
1129 Implications for N₂O source apportionment. Biogeosciences 15, 3873–3882.
1130 <https://doi.org/10.5194/bg-15-3873-2018>
- 1131 He, X., Yan, Z., Wang, S., Comer-Warner, S.A., Zhu-Barker, X., 2026. Revealing microbial
1132 pathways of N₂O production in agricultural ditches. Water Res. 288, 124732.
1133 <https://doi.org/10.1016/J.WATRES.2025.124732>
- 1134 Heil, J., Vereecken, H., Brüggemann, N., 2016. A review of chemical reactions of nitrification
1135 intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil.
1136 Eur. J. Soil Sci. 67, 23–39.
- 1137 Heil, J., Wolf, B., Brüggemann, N., Emmenegger, L., Tuzson, B., Vereecken, H., Mohn, J.,
1138 2014. Site-specific ¹⁵N isotopic signatures of abiotically produced N₂O. Geochim.
1139 Cosmochim. Acta 139, 72–82. <https://doi.org/10.1016/j.gca.2014.04.037>
- 1140 Jinuntuya-Nortman, M., Sutka, R.L., Ostrom, P.H., Gandhi, H., Ostrom, N.E., 2008.
1141 Isotopologue fractionation during microbial reduction of N₂O within soil mesocosms as a
1142 function of water-filled pore space. Soil Biol. Biochem. 40, 2273–2280.
1143 <https://doi.org/10.1016/j.soilbio.2008.05.016>
- 1144 Jones, L., Peters, B., Pacheco, J., Casciotti, K., Fendorf, S., 2015. Stable Isotopes and Iron
1145 Oxide Mineral Products as Markers of Chemodenitrification. Environ. Sci. Technol. 49,
1146 3444–3452.
- 1147 Keuschnig, C., Gorfer, M., Li, G., Mania, D., Frostegård, Å., Bakken, L., Larose, C., 2020. NO
1148 and N₂O transformations of diverse fungi in hypoxia: evidence for anaerobic respiration
1149 only in *Fusarium* strains. Environ. Microbiol. 22, 2182–2195.
1150 <https://doi.org/10.1111/1462-2920.14980>
- 1151 Kool, D.M., Wrage, N., Oenema, O., Dolfing, J., Van Groenigen, J.W., 2007. Oxygen exchange
1152 between (de)nitrification intermediates and H₂O and its implications for source

1153 determination of NO₃⁻ and N₂O: A review. *Rapid Communications in Mass Spectrometry*.
 1154 <https://doi.org/10.1002/rcm.3249>

1155 Lewicka-Szczebak, D., Augustin, J., Giesemann, A., Well, R., 2017. Quantifying N₂O
 1156 reduction to N₂ based on N₂O isotopocules-validation with independent methods (helium
 1157 incubation and ¹⁵N gas flux method). *Biogeosciences* 14, 711–732.
 1158 <https://doi.org/10.5194/bg-14-711-2017>

1159 Lewicka-Szczebak, D., Dyckmans, J., Kaiser, J., Marca, A., Augustin, J., Well, R., 2016.
 1160 Oxygen isotope fractionation during N₂ O production by soil denitrification.
 1161 *Biogeosciences* 13, 1129–1144. <https://doi.org/10.5194/bg-13-1129-2016>

1162 Lewicka-Szczebak, D., Jansen-Willems, A., Müller, C., Dyckmans, J., Well, R., 2021. Nitrite
 1163 isotope characteristics and associated soil N transformations. *Sci. Rep.* 11, 1–11.
 1164 <https://doi.org/10.1038/s41598-021-83786-w>

1165 Lewicka-Szczebak, D., Lewicki, M.P., Well, R., 2020. N₂O isotope approaches for source
 1166 partitioning of N₂O production and estimation of N₂O reduction-validation with the ¹⁵N
 1167 gas-flux method in laboratory and field studies. *Biogeosciences* 17, 5513–5537.
 1168 <https://doi.org/10.5194/bg-17-5513-2020>

1169 Lewicka-Szczebak, D., Well, R., Bol, R., Gregory, A.S., Matthews, G.P., Misselbrook, T.,
 1170 Whalley, W.R., Cardenas, L.M., 2015. Isotope fractionation factors controlling isotopocule
 1171 signatures of soil-emitted N₂O produced by denitrification processes of various rates.
 1172 *Rapid Communications in Mass Spectrometry* 29, 269–282.
 1173 <https://doi.org/10.1002/rcm.7102>

1174 Lewicka-Szczebak, D., Well, R., Köster, J.R., Fuß, R., Senbayram, M., Dittert, K., Flessa, H.,
 1175 2014. Experimental determinations of isotopic fractionation factors associated with N₂O
 1176 production and reduction during denitrification in soils. *Geochim. Cosmochim. Acta* 134,
 1177 55–73. <https://doi.org/10.1016/j.gca.2014.03.010>

1178 Lewicki, M.P., Lewicka-Szczebak, D., Skrzypek, G., 2022. FRAME—Monte Carlo model for
 1179 evaluation of the stable isotope mixing and fractionation. *PLoS One* 17, e0277204.
 1180 <https://doi.org/10.1371/journal.pone.0277204>

1181 Li, T., Harris, E., Niu, Z., Yu, L., 2026. Synthesis of Global Soil-Emitted N₂O Isotopic
 1182 Signatures: Geoclimatic Patterns and Influential Factors. *Geophys. Res. Lett.* 53.
 1183 <https://doi.org/10.1029/2025GL116045>

1184 Li, Z., Tang, Z., Song, Z., Chen, W., Tian, D., Tang, S., Wang, X., Wang, J., Liu, W., Wang, Y.,
 1185 Li, J., Jiang, L., Luo, Y., Niu, S., 2022. Variations and controlling factors of soil
 1186 denitrification rate. *Glob. Chang. Biol.* 28, 2133–2145. <https://doi.org/10.1111/gcb.16066>

1187 Liang, X., Wu, Y., Toyoda, S., Lewicka-Szczebak, D., Ji, Y., Zheng, Y., Hou, L., 2026. Dual
 1188 inhibition of biotic and abiotic pathways drives nitrous oxide mitigation in ameliorated
 1189 coastal saline soils 2.

1190 Luz, B., Barkan, E., 2011. The isotopic composition of atmospheric oxygen 25, 1–14.
 1191 <https://doi.org/10.1029/2010GB003883>

1192 Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M.-C., Bizouard, F., Toyoda, S.,
 1193 Yoshida, N., Steinberg, C., Philippot, L., 2015. N₂O production, a widespread trait in
 1194 fungi. *Sci. Rep.* 5, 9697. <https://doi.org/10.1038/srep09697>

1195 Mandernack, K.W., Mills, C.T., Johnson, C.A., Rahn, T., Kinney, C., 2009. The $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$
 1196 values of N₂O produced during the co-oxidation of ammonia by methanotrophic bacteria.
 1197 *Chem. Geol.* 267, 96–107. <https://doi.org/10.1016/J.CHEMGEO.2009.06.008>

1198 Masta, M., Kazmi, F.A., Espenberg, M., Visnapuu, T., Sennett, L.B., Eving, L., Lewicka-
 1199 Szczebak, D., Deb, S., Khanongnuch, R., Kuusemets, L., Kupper, P., Butterbach-Bahl, K.,
 1200 Mander, Ü., 2026. Transitional hypoxia during peatland water table fluctuations drives
 1201 high N₂O fluxes via shifting microbial pathways. *Environ. Res.* 305.
 1202 <https://doi.org/10.1016/j.envres.2026.124922>

1203 Menyailo, O. V., Hungate, B.A., 2006. Tree species and moisture effects on soil sources of N₂O:
 1204 Quantifying contributions from nitrification and denitrification with ¹⁸O isotopes. *J.*
 1205 *Geophys. Res. Biogeosci.* 111, 1–8. <https://doi.org/10.1029/2005JG000058>

1206 Micucci, G., Lewicka-Szczebak, D., Sgouridis, F., Well, R., Buchen-Tschiskale, C., McNamara,
 1207 N.P., Krause, S., Lynch, I., Roos, F., Ullah, S., 2025. Combining the ¹⁵N Gas Flux Method
 1208 and N₂O Isotopocule Data for the Determination of Soil Microbial N₂O Sources. *Rapid*
 1209 *Communications in Mass Spectrometry* 39. <https://doi.org/10.1002/rcm.9971>

1210 Mohn, J., Wolf, B., Toyoda, S., Lin, C.T., Liang, M.C., Brüggemann, N., Wissel, H., Steiker,
 1211 A.E., Dyckmans, J., Szvec, L., Ostrom, N.E., Casciotti, K.L., Forbes, M., Giesemann, A.,
 1212 Well, R., Doucett, R.R., Yarnes, C.T., Ridley, A.R., Kaiser, J., Yoshida, N., 2014.
 1213 Interlaboratory assessment of nitrous oxide isotopomer analysis by isotope ratio mass

1214 spectrometry and laser spectroscopy: Current status and perspectives. *Rapid*
1215 *Communications in Mass Spectrometry* 28, 1995–2007. <https://doi.org/10.1002/rcm.6982>

1216 Moore, J.W., Semmens, B.X., 2008. Incorporating uncertainty and prior information into stable
1217 isotope mixing models. *Ecol. Lett.* 11, 470–480. [https://doi.org/10.1111/j.1461-](https://doi.org/10.1111/j.1461-0248.2008.01163.x)
1218 [0248.2008.01163.x](https://doi.org/10.1111/j.1461-0248.2008.01163.x)

1219 Müller, C., Laughlin, R.J., Spott, O., Rütting, T., 2014. Quantification of N₂O emission
1220 pathways via a ¹⁵N tracing model. *Soil Biol. Biochem.* 72, 44–54.
1221 <https://doi.org/10.1016/j.soilbio.2014.01.013>

1222 Ostrom, N.E., Ostrom, P.H., 2017. Mining the isotopic complexity of nitrous oxide: a review
1223 of challenges and opportunities. *Biogeochemistry* 132, 359–372.
1224 <https://doi.org/10.1007/s10533-017-0301-5>

1225 Ostrom, N.E., Piit, A., Sutka, R., Ostrom, P.H., Grandy, A.S., Huizinga, K.M., Robertson, G.P.,
1226 2007. Isotopologue effects during N₂O reduction in soils and in pure cultures of
1227 denitrifiers. *J. Geophys. Res. Biogeosci.* 112, 1–12.
1228 <https://doi.org/10.1029/2006JG000287>

1229 Parnell, A.C., Phillips, D.L., Bearhop, S., Semmens, B.X., Ward, E.J., Moore, J.W., Jackson,
1230 A.L., Grey, J., Kelly, D.J., Inger, R., 2013. Bayesian stable isotope mixing models.
1231 *Environmetrics* 24, 387–399. <https://doi.org/10.1002/env.2221>

1232 Pfülb, L., Elsgaard, L., Dörsch, P., Fuß, R., Well, R., 2025. Impact of liming and maize residues
1233 on N₂O and N₂ fluxes in agricultural soils: an incubation study. *Biol. Fertil. Soils* 61, 575–
1234 594. <https://doi.org/10.1007/s00374-024-01825-w>

1235 Phillips, D.L., Inger, R., Bearhop, S., Jackson, A.L., Moore, J.W., Parnell, A.C., Semmens,
1236 B.X., Ward, E.J., 2014. Best practices for use of stable isotope mixing models in food-web
1237 studies. *Can. J. Zool.* 92, 823–835. <https://doi.org/10.1139/cjz-2014-0127>

1238 Qi, M., Gao, D., Dong, H., Liang, X., Song, Z., Liu, M., Hou, L., Li, X., 2024. Nitrous oxide
1239 production from biological and chemodenitrification by Fe(II) in estuarine and coastal
1240 sediments. *Applied Geochemistry* 161, 105884.
1241 <https://doi.org/10.1016/J.APGEOCHEM.2023.105884>

1242 Rainford, S.K., Havsteen, J.C., Mohn, J., Ammann, C., Keel, S.G., 2026. Coupling
1243 observational methods and the DayCent model to improve examinations of N₂O

1244 production pathways. *Nutr. Cycl. Agroecosyst.* 133. <https://doi.org/10.1007/s10705-026->
1245 10504-1

1246 Ravishankara, A., Daniel, J., Portmann, R., 2009. Nitrous Oxide (N₂O): The Dominant Ozone-
1247 Depleting Substance Emitted in the 21st Century. *Science* (1979). 326, 123–125.

1248 Rex, D., Clough, T.J., Richards, K.G., de Klein, C., Morales, S.E., Samad, M.S., Grant, J.,
1249 Lanigan, G.J., 2018. Fungal and bacterial contributions to codenitrification emissions of
1250 N₂O and N₂ following urea deposition to soil. *Nutr. Cycl. Agroecosyst.* 110, 135–149.
1251 <https://doi.org/10.1007/s10705-017-9901-7>

1252 Rohe, L., Anderson, T., Braker, G., Flessa, H., Gieseemann, A., Wrage-mönnig, N., Well, R.,
1253 2014. Fungal oxygen exchange between denitrification intermediates and water 377–384.
1254 <https://doi.org/10.1002/rcm.6790>

1255 Rohe, L., Anderson, T.-H., Flessa, H., Goeske, A., Lewicka-Szczebak, D., Wrage-Mönnig, N.,
1256 Well, R., 2021. Comparing modified substrate-induced respiration with selective
1257 inhibition (SIRIN) and N₂O isotope approaches to estimate fungal contribution to
1258 denitrification in three arable soils under anoxic conditions. *Biogeosciences* 18, 4629–
1259 4650. <https://doi.org/10.5194/bg-18-4629-2021>

1260 Rohe, L., Oppermann, T., Well, R., Horn, M.A., 2020. Nitrite induced transcription of p450nor
1261 during denitrification by *Fusarium oxysporum* correlates with the production of N₂O with
1262 a high ¹⁵N site preference. *Soil Biol. Biochem.* 151, 108043.
1263 <https://doi.org/10.1016/j.soilbio.2020.108043>

1264 Rohe, L., Well, R., 2026. Combining low level labelling with ¹⁵N and ¹⁵N site preference to
1265 distinguish N₂O production by nitrification and fungal denitrification, in: *EGU General*
1266 *Assembly 2023*. pp. EGU23-4990.

1267 Rohe, L., Well, R., Lewicka-Szczebak, D., 2017. Use of oxygen isotopes to differentiate
1268 between nitrous oxide produced by fungi or bacteria during denitrification. *Rapid*
1269 *Communications in Mass Spectrometry* 31, 1297–1312. <https://doi.org/10.1002/rcm.7909>

1270 Rummel, P.S., Englert, P., Beule, L., Pausch, J., 2025a. N₂O flux dynamics and production
1271 pathways modulated by soil organic matter and litter turnover. *Biol. Fertil. Soils* 61, 1235–
1272 1251. <https://doi.org/10.1007/s00374-025-01925-1>

1273 Rummel, P.S., Oyeogbe, A.I., Wang, H., Urich, T., Wrage-Mönnig, N., 2025b. Nitrous oxide
1274 fluxes, their sources, and soil microbial communities depend more on carbon availability
1275 than long- and short-term phosphorus addition. *Biol. Fertil. Soils* 61, 955–970.
1276 <https://doi.org/10.1007/s00374-025-01909-1>

1277 Scheer, C., Fuchs, K., Pelster, D.E., Butterbach-Bahl, K., 2020. Estimating global terrestrial
1278 denitrification from measured $\text{N}_2\text{O}:(\text{N}_2\text{O} + \text{N}_2)$ product ratios. *Curr. Opin. Environ.*
1279 *Sustain.* <https://doi.org/10.1016/j.cosust.2020.07.005>

1280 Sebilo, M., Margalef-Martí, R., 2026. Ideas and perspectives: Nitrite turnover controls nitrogen
1281 fate across redox gradients. *Biogeosciences* 23, 4711–4718. [https://doi.org/10.5194/bg-23-](https://doi.org/10.5194/bg-23-4711-2026)
1282 [4711-2026](https://doi.org/10.5194/bg-23-4711-2026)

1283 Senbayram, M., Well, R., Bol, R., Chadwick, D.R., Jones, D.L., Wu, D., 2018. Interaction of
1284 straw amendment and soil NO_3^- content controls fungal denitrification and denitrification
1285 product stoichiometry in a sandy soil. *Soil Biol. Biochem.* 126, 204–212.
1286 <https://doi.org/10.1016/J.SOILBIO.2018.09.005>

1287 Shu, W., Zhang, Q., Audet, J., Li, Z., Leng, P., Qiao, Y., Tian, C., Chen, G., Zhao, J., Cheng, H.,
1288 Li, F., 2024. Non-negligible N_2O emission hotspots: Rivers impacted by ion-adsorption
1289 rare earth mining. *Water Res.* 251, 121124.
1290 <https://doi.org/10.1016/J.WATRES.2024.121124>

1291 Snider, D.M., Venkiteswaran, J.J., Schiff, S.L., Spoelstra, J., 2012. Deciphering the oxygen
1292 isotope composition of nitrous oxide produced by nitrification. *Glob. Chang. Biol.* 18,
1293 356–370. <https://doi.org/10.1111/j.1365-2486.2011.02547.x>

1294 Spott, O., Russow, R., Florian, C., 2011. Soil Biology & Biochemistry Formation of hybrid N_2O
1295 and hybrid N_2 due to codenitrification: First review of a barely considered process
1296 of microbially mediated N-nitrosation. *Soil Biol. Biochem.* 43, 1995–2011.
1297 <https://doi.org/10.1016/j.soilbio.2011.06.014>

1298 Stephens, E.Z., Pulido Barriga, M.F., Greene, A.C., Krichels, A.H., Kargul, M., Larios, L.,
1299 Glassman, S.I., Homyak, P.M., 2026. Wildfire alters nitrogen cycling to increase soil
1300 emissions of nitric oxide (NO) and the heterogeneity of nitrous oxide (N_2O) in California
1301 chaparral. *Geoderma* 469, 117807. <https://doi.org/10.1016/j.geoderma.2026.117807>

1302 Stock, B., Jackson, A., Ward, E., Parnell, A., Phillips, D.L., Semmens, B., 2018. Analyzing
 1303 mixing systems using a new generation of Bayesian tracer mixing models. *PeerJ*
 1304 Publishing 6, e5096. <https://doi.org/10.7717/peerj.5096>

1305 Strubbe, L., Keck, H., Magyar, P.M., Mohn, J., Joss, A., Froemelt, A., 2026. Activating specific
 1306 N₂O production pathways to understand emission dynamics in wastewater treatment.
 1307 *Water Res.* 296. <https://doi.org/10.1016/j.watres.2026.125580>

1308 Sutka, R.L., Adams, G.C., Ostrom, N.E., Ostrom, P.H., 2008. Isotopologue fractionation during
 1309 N₂O production by fungal denitrification. *Rapid Communications in Mass Spectrometry*
 1310 22, 3989–3996. <https://doi.org/10.1002/rcm.3820>

1311 Sutka, R.L., Ostrom, N.E., Ostrom, P.H., Breznak, J.A., Gandhi, H., Pitt, A.J., Li, F., 2006.
 1312 Distinguishing Nitrous Oxide Production from Nitrification and Denitrification on the
 1313 Basis of Isotopomer Abundances. *Appl. Environ. Microbiol.* 72, 638–644.
 1314 <https://doi.org/10.1128/AEM.72.1.638-644.2006>

1315 Tischer, J., Zop, J., Frey, C., Magyar, P.M., Brand, A., Oswald, K., Jegge, C., Frame, C.H.,
 1316 Miracle, M.R., Sòria-perpinyà, X., Vicente, E., Lehmann, M.F., 2022. Isotopic signatures
 1317 of biotic and abiotic N₂O production and consumption in the water column of meromictic
 1318 , ferruginous Lake La Cruz (Spain) 1760–1775. <https://doi.org/10.1002/lno.12165>

1319 Toyoda, S., Damak, F., Hattori, S., Takeda, M., Akiyama, H., Sasaki, Y., Minamisawa, K., 2024.
 1320 Dynamics of N₂O production and reduction processes in a soybean field revealed by
 1321 isotopocule analyses. *Soil Biol. Biochem.* 191, 109358.
 1322 <https://doi.org/10.1016/J.SOILBIO.2024.109358>

1323 Toyoda, S., Mutoh, H., Yamagishi, H., Yoshida, N., Tanji, Y., 2005. Fractionation of N₂O
 1324 isotopomers during production by denitrifier. *Soil Biol. Biochem.* 37, 1535–1545.
 1325 <https://doi.org/10.1016/j.soilbio.2005.01.009>

1326 Toyoda, S., Yoshida, N., 1999. Determination of Nitrogen Isotopomers of Nitrous Oxide on a
 1327 Modified Isotope Ratio Mass Spectrometer. *Anal. Chem.* 71, 4711–4718.
 1328 <https://doi.org/10.1021/ac9904563>

1329 Toyoda, S., Yoshida, N., Koba, K., 2017. Isotopocule analysis of biologically produced nitrous
 1330 oxide in various environments. *Mass Spectrom. Rev.* 36, 135–160.
 1331 <https://doi.org/10.1002/mas.21459>

- 1332 Trojanowska, A., Lewicka-szczebak, D., Kurasiewicz, M., Wassenaar, L.I., Izydorczyk, K.,
1333 Canada, E., 2008. Diurnal variations in the photosynthesis-respiration activity of a
1334 cyanobacterial bloom in a freshwater dam reservoir: An isotopic study. *Isotopes Environ.*
1335 *Health Stud.* 44, 163–175. <https://doi.org/10.1080/10256010802066240>
- 1336 Venterea, R.T., 2007. Nitrite-driven nitrous oxide production under aerobic soil conditions:
1337 Kinetics and biochemical controls. *Glob. Chang. Biol.* 13, 1798–1809.
1338 <https://doi.org/10.1111/j.1365-2486.2007.01389.x>
- 1339 Visser, A., Wankel, S.D., Niklaus, P.A., Byrne, J.M., Kappler, A.A., Lehmann, M.F., 2020.
1340 Impact of reactive surfaces on the abiotic reaction between nitrite and ferrous iron and
1341 associated nitrogen and oxygen isotope dynamics 4355–4374.
- 1342 Wang, R.Z., Lonergan, Z.R., Wilbert, S.A., Eiler, J.M., Newman, D.K., 2024. Widespread
1343 detoxifying NO reductases impart a distinct isotopic fingerprint on N₂O under anoxia.
1344 *Proc. Natl. Acad. Sci. U. S. A.* 121. <https://doi.org/10.1073/pnas.2319960121>
- 1345 Wang, S., Huang, J., Wu, Z., Li, S., Zhu, X., Liu, Y., Ji, G., 2025a. Global mapping of flux and
1346 microbial sources for oceanic N₂O. *Nature Communications* 16.
1347 <https://doi.org/10.1038/s41467-025-58715-4>
- 1348 Wang, S., Li, S., Chen, Y., Dang, Z., Guo, R., Zhu, X., Zhi, W., Ji, G., 2026. Four typical N₂O
1349 emission regimes in temperate monsoon-type urban rivers unraveled via quantified
1350 microbial sources under coupled natural hydrological and anthropogenic perturbations.
1351 *Water Res.* 300, 125920. <https://doi.org/10.1016/J.WATRES.2026.125920>
- 1352 Wang, S., Zhi, W., Li, S., Lyu, T., Ji, G., 2025b. Sustainable management of riverine N₂O
1353 emission baselines. *Natl. Sci. Rev.* 12. <https://doi.org/10.1093/nsr/nwae458>
- 1354 Wassenaar, L.I., Hendry, M.J., 2007. Dynamics and Stable Isotope Composition of Gaseous and
1355 Dissolved Oxygen. *Groundwater* 45, 447–460.
- 1356 Wei, J., Ibraim, E., Brüggemann, N., Vereecken, H., Mohn, J., 2019. First real-time isotopic
1357 characterisation of N₂O from chemodenitrification. *Geochim. Cosmochim. Acta* 267, 17–
1358 32. <https://doi.org/10.1016/j.gca.2019.09.018>
- 1359 Wei, Z., Well, R., Ma, X., Lewicka-Szczebak, D., Rohe, L., Zhang, G., Li, C., Ma, J., Bol, R.,
1360 Xu, H., Shan, J., Yan, X., Senbayram, M., 2024. Organic fertilizer amendment decreased
1361 N₂O/(N₂O+N₂) ratio by enhancing the mutualism between bacterial and fungal

denitrifiers in high nitrogen loading arable soils. *Soil Biol. Biochem.* 198.
<https://doi.org/10.1016/j.soilbio.2024.109550>

Well, R., Flessa, H., 2009a. Isotopologue enrichment factors of N₂O reduction in soils. *Rapid Communications in Mass Spectrometry* 23, 2996–3002. <https://doi.org/10.1002/rcm.4216>

Well, R., Flessa, H., 2009b. Isotopologue signatures of N₂O produced by denitrification in soils. *J. Geophys. Res. Biogeosci.* 114, 1–11. <https://doi.org/10.1029/2008JG000804>

Well, R., Flessa, H., Xing, L., Xiaotang, J., Römheld, V., 2008. Isotopologue ratios of N₂O emitted from microcosms with NH₄⁺ fertilized arable soils under conditions favoring nitrification. *Soil Biol. Biochem.* 40, 2416–2426.
<https://doi.org/10.1016/j.soilbio.2008.06.003>

Well, R., Kurganova, I., De Gerenyu, V.L., Flessa, H., 2006. Isotopomer signatures of soil-emitted N₂O under different moisture conditions - A microcosm study with arable loess soil. *Soil Biol. Biochem.* 38, 2923–2933. <https://doi.org/10.1016/j.soilbio.2006.05.003>

Well, R., Ruoss, N., Grosz, B., Brunotte, J., Buchen-Tschiskale, C., Lewicka-Szczebak, D., Schäfer, B.C., 2025. Effect of agricultural management system (“cash crop”, “livestock” and “climate optimized”) on nitrous oxide and ammonia emissions. *Biol. Fertil. Soils* 61, 469–488. <https://doi.org/10.1007/s00374-024-01843-8>

Wrage-Mönnig, N., Horn, M.A., Well, R., Müller, C., Velthof, G., Oenema, O., 2018. The role of nitrifier denitrification in the production of nitrous oxide revisited. *Soil Biol. Biochem.* 123, A3–A16. <https://doi.org/10.1016/J.SOILBIO.2018.03.020>

Wu, D., Well, R., Cárdenas, L.M., Fuß, R., Lewicka-Szczebak, D., Köster, J.R., Brüggemann, N., Bol, R., 2019. Quantifying N₂O reduction to N₂ during denitrification in soils via isotopic mapping approach: Model evaluation and uncertainty analysis. *Environ. Res.* 179. <https://doi.org/10.1016/j.envres.2019.108806>

Xu, C., Qi, M., Lin, W., Li, X., 2022. Nitrous Oxide from Abiotic Processes of Hydroxylamine and Nitrite in Estuarine and Coastal Ecosystems: A Review. *J. Mar. Sci. Eng.* <https://doi.org/10.3390/jmse10050623>

Xu, Z., Hattori, S., Masuda, Y., Toyoda, S., Koba, K., Yu, P., Yoshida, N., Du, Z.J., Senoo, K., 2024. Unprecedented N₂O production by nitrate-ammonifying *Geobacteraceae* with distinctive N₂O isotopocule signatures. *mBio* 15. <https://doi.org/10.1128/mbio.02540-24>

1392 Yoshida, N., 1988. ^{15}N -depleted N_2O as a product of nitrification. *Nature* 335, 528–529.
1393 <https://doi.org/10.1038/335528a0>

1394 Yu, L., Harris, E., Lewicka-Szczebak, D., Barthel, M., Blomberg, M.R.A., Harris, S.J., Johnson,
1395 M.S., Lehmann, M.F., Liisberg, J., Müller, C., Ostrom, N.E., Six, J., Toyoda, S., Yoshida,
1396 N., Mohn, J., 2020. What can we learn from N_2O isotope data? – Analytics, processes and
1397 modelling. *Rapid Communications in Mass Spectrometry* 34, 1–14.
1398 <https://doi.org/10.1002/rcm.8858>

1399 Zhu, Y., Merbold, L., Oduor, C.O., Barthel, M., Fang, X., Six, J., Butterbach-Bahl, K., Ntinyari,
1400 W., Leitner, S.M., 2026. Manure management in African smallholder farms affects
1401 greenhouse gas emissions and leachate losses. *Resources, Environment and Sustainability*
1402 26. <https://doi.org/10.1016/j.resenv.2026.100352>

1403