

High nitrous oxide isotopic variability during denitrification by *Pseudomonas* species bearing NirK and NirS

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Abstract

Nitrous oxide (N₂O) isotopocules provide key insights into microbial nitrogen cycling, but their interpretation requires well-constrained values for both oxygen isotope signatures ($\delta^{18}\text{O}\text{-N}_2\text{O}$) and intramolecular ¹⁵N site preference (SP) associated with N₂O production pathways. Site preference is widely used to distinguish N₂O formation pathways because bacterial denitrification is generally assumed to yield SP values near 0 ‰ through canonical NorB-mediated NO reduction. However, the extent to which SP remains stable across physiological states and changing NO reduction pathways remains poorly constrained. Likewise, interpretation of $\delta^{18}\text{O}\text{-N}_2\text{O}$ associated with denitrification requires understanding the relative contributions of branching kinetic isotope effects and oxygen atom exchange between nitrite and water during N₂O formation.

Here, we investigated N₂O isotopic signatures during denitrification by *Pseudomonas aureofaciens* (NirK-bearing) and *Pseudomonas chlororaphis* (NirS-bearing) under active-growth and resuspension conditions using quantum cascade laser absorption spectroscopy (QCLAS) and isotope ratio mass spectrometry (IRMS). SP tracked canonical NorB-mediated NO reduction but transiently increased above +10 ‰ during early N₂O production, suggesting-indicating temporary activity of alternative NO reductases. These dynamics were only resolved through continuous QCLAS measurements, highlighting the importance of time-resolved isotopic observations. While SP remains a useful indicator of NO reduction mechanisms, these results show that even within denitrification, shifts between NO reduction pathways may lead to variable SP signatures.

In parallel, we quantified oxygen atom exchange between nitrite and water using incubations prepared in natural-abundance and ¹⁸O-enriched water. Contrary to expectations from denitrifier-method studies, *P. aureofaciens* exhibited substantial and highly variable oxygen-atom exchange (38–100 ‰), far exceeding previously reported values (<9 ‰). In contrast, *P. chlororaphis* showed consistently high but less variable exchange (~66 ‰). Resuspension experiments reproduced the characteristic low- and high-exchange behavior reported for these strains under denitrifier-method conditions, demonstrating that these exchange values are specific to the methodological framework and not representative of actively growing systems. These results show that oxygen atom exchange is not governed solely by nitrite reductase identity (NirS vs. NirK) but is strongly modulated by physiological state and metabolic context, including active growth versus stationary-phase resuspension and differences in nitrite turnover. As a result, $\delta^{18}\text{O}\text{-N}_2\text{O}$ cannot be interpreted as a fixed tracer of denitrification pathways outside the constrained conditions of the denitrifier method.

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1 Together, these findings suggest that denitrifying bacteria may generate N₂O with a broader range of δ¹⁸O–
2 N₂O and SP than previously assumed. This calls for a reassessment of N₂O isotopocule interpretations and
3 emphasizes the need to integrate isotopic measurements with physiological and biochemical constraints.

4 1. Introduction

5 The intensification of agriculture and fossil fuel combustion over the past century has fundamentally altered
6 the global nitrogen (N) cycle, leading to increased emissions of nitrous oxide (N₂O), a potent greenhouse gas
7 and stratospheric ozone-depleting substance (IPCC, 2023; WMO, 2024). Microbial processes, particularly de-
8 nitrification – the sequential reduction of nitrate (NO₃[–]) to dinitrogen (N₂) via nitrite (NO₂[–]), nitric oxide (NO),
9 and N₂O – account for most anthropogenic N₂O emissions (Denman et al., 2007; Reay et al., 2012). However,
10 apportioning N₂O sources among microbial and abiotic pathways remains challenging due to the diversity of
11 processes involved (Yu et al., 2020).

12 Stable isotope analysis of N₂O provides an important tool to trace microbial processes and identify N₂O
13 sources. Bulk δ¹⁵N and δ¹⁸O values integrate information on substrate origin and isotope fractionation during
14 N₂O formation (Sutka et al., 2006; Toyoda et al., 2005; Denk et al., 2017). In multistep pathways such as deni-
15 trification, the apparent isotope effect reflects the combined influence of multiple enzymatic reactions
16 (Ostrom & Ostrom, 2011). The intramolecular site preference (SP), defined as the difference in ¹⁵N abundance
17 between the central (α) and terminal (β) positions in N₂O (Yoshida & Toyoda, 1999), is widely used to distin-
18 guish microbial formation pathways as it is considered to be largely substrate-independent. Bacterial denitri-
19 fication typically yields SP values near 0 ‰, whereas fungal denitrification ~~and~~, hydroxylamine oxidation, ~~and~~
20 ~~abiotic nitrite reactions, including chemodenitrification, can produce higher or more variable SP values pro-~~
21 ~~duce higher values (~20–35 ‰)~~ (Toyoda et al., 2005; Frame & Casciotti, 2010; Ostrom et al., 2011; ~~Jones et al.,~~
22 ~~2015; Toyoda et al., 2017; Wei et al., 2019; Yu et al., 2020).~~ ~~Recent work has further highlighted the importance~~
23 ~~of Fe(II)-mediated nitrite transformations and their potential contribution to abiotic N₂O formation under en-~~
24 ~~vironmentally relevant conditions (Visser et al., 2022).~~ These low SP values are generally attributed to NO re-
25 duction by the canonical nitric oxide reductase NorB. However, recent work has shown that alternative NO
26 detoxification pathways, particularly via flavohemoglobins (Fhp), can produce elevated SP values under high-
27 NO conditions (Wang et al., 2024). These reactions do not represent a separate environmental pathway, but
28 rather an alternative enzymatic fate of NO within denitrifying organisms. Together, these findings demon-
29 strate that while SP remains a powerful discriminator of broad N₂O formation mechanisms, its expression
30 may be more sensitive to which enzymes are involved and to physiological state than is typically assumed.

31 In contrast, the oxygen isotopic composition of N₂O (δ¹⁸O–N₂O) reflects both NO reduction by NorB and ~~the~~
32 ~~preceding transformations of nitrate and nitrite, upstream processes~~ and depends on the balance between
33 nitrate-derived oxygen, nitrite–water exchange, and branching isotope effects. The extent of oxygen atom
34 exchange between nitrite and water prior to NO reduction is a particular point of variation. Among denitrifi-
35 ers, two structurally distinct nitrite reductases are found: the cytochrome cd₁-type NirS and the copper-con-
36 taining NirK. Differences in oxygen isotope exchange between nitrogen oxide intermediates and ambient wa-
37 ter have often been attributed to Nir identity, largely based on observations from the ~~bacterial~~-denitrifier
38 method developed for nitrate ¹⁵N/¹⁴N and ¹⁸O/¹⁶O isotope analysis (Casciotti et al., 2002). Under these sta-
39 tionary-phase, washed-cell conditions, *Pseudomonas chlororaphis* subsp. *chlororaphis* (NirS) incorporates a

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1 large fraction of oxygen from water into N₂O (39–76 %), whereas the closely related *Pseudomonas chlororaphis* subsp. *aureofaciens* (NirK) shows minimal exchange (~6 %). ~~This contrast has often been interpreted as~~
2 ~~evidence that NirS and NirK differ systematically in their influence on $\delta^{18}\text{O}$ -N₂O expression (Kool et al., 2007;~~
3 ~~Gruber et al., 2022). This contrast has led to the widespread assumption that NirS and NirK impose funda-~~
4 ~~mentally different constraints on $\delta^{18}\text{O}$ -N₂O expression (Kool et al., 2007).~~
5
6 However, this inference is based on a narrow experimental framework and has not been systematically tested
7 under active growth conditions. Earlier microbiological studies (e.g., Ye et al., 1994) and more recent work in
8 soils and pure cultures report substantial variability in oxygen atom exchange behavior that cannot be ex-
9 plained by a simple NirK–NirS dichotomy (Lewicka-Szczebak et al., 2016; Rohe et al., 2017; Yu et al., 2020).
10 Whether observed $\delta^{18}\text{O}$ -N₂O variability reflects intrinsic enzyme properties or instead arises from physiologi-
11 cal state, substrate availability, and reaction dynamics therefore remains unresolved.

12 The ~~bacterial~~-denitrifier method not only provides well-established information on the behavior of NirS- and
13 NirK-bearing denitrifiers but also serves as a calibration framework for isotopic analyses. Complete conver-
14 sion of nitrate to N₂O allows direct determination of $\delta^{15}\text{N}$ -NO₃⁻ from $\delta^{15}\text{N}$ -N₂O (Sigman et al., 2001). For ox-
15 ygen isotopes, however only one of the six oxygen atoms originally present in the combined nitrate pool is
16 retained in N₂O, while the others are abstracted and transferred to water during reduction. Under controlled
17 conditions where oxygen atom exchange is low and invariant, the offset between $\delta^{18}\text{O}$ -NO₃⁻ and $\delta^{18}\text{O}$ -N₂O
18 remains constant, enabling correction of $\delta^{18}\text{O}$ -N₂O measurements (Casciotti et al., 2002). This approach im-
19 plicitly assumes that oxygen atom exchange remains stable across experimental conditions. If exchange var-
20 ies with physiological state or reaction dynamics, the $\delta^{18}\text{O}$ -N₂O offset is no longer constant, and $\delta^{18}\text{O}$ -N₂O
21 cannot be interpreted as a fixed tracer of nitrite reductase identity. Understanding the controls on oxygen
22 atom exchange during denitrification is therefore important both for interpreting $\delta^{18}\text{O}$ -N₂O signatures and
23 for evaluating the growth conditions under which the ~~bacterial~~-denitrifier method yields reliable results.

24 In this study, we investigate how nitrite reductase type, substrate availability, and growth phase influence
25 N₂O isotopic signatures in two closely related denitrifier strains, *P. aureofaciens* and *P. chlororaphis*, both
26 lacking NosZ and therefore accumulating N₂O. We combine (i) real-time measurements of $\delta^{15}\text{N}$, $\delta^{18}\text{O}$, and SP
27 using quantum cascade laser absorption spectroscopy (QCLAS) with (ii) isotope ratio mass spectrometry
28 (IRMS) analysis of the accumulated product in closed batch incubations to compare active growth and resus-
29 pension conditions. This experimental design allows us to separate the effects of physiological state during
30 active growth from those associated with washed-cell resuspension conditions during N₂O production.

31 Together, this approach addresses a central question: to what extent do N₂O isotopic signatures reflect in-
32 trinsic enzyme properties versus differences associated with physiological state and experimental conditions?
33 Resolving this distinction is critical for evaluating the validity and limitations of widely used interpretive
34 frameworks, including ~~the proposed relationship between nitrite reductase identity (NirK/NirS) and the de-~~
35 ~~gree of oxygen atom exchange with water the assumed NirK/NirS control on oxygen atom exchange with~~
36 ~~water~~ and the broader applicability of the ~~bacterial~~-denitrifier method, and the interpretation of SP as a sta-
37 ble proxy for NorB-mediated NO reduction.

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1 **2. Material & Methods**

2 **2.1 General experimental framework**

3 **2.1.1. Bacterial strains**

4 *Pseudomonas chlororaphis* subsp. *aureofaciens* (formerly *P. aureofaciens*, ATCC 13985) and *Pseudomonas*
5 *chlororaphis* subsp. *chlororaphis* (formerly *P. chlororaphis*, ATCC 9446) were obtained from the University of
6 Basel Aquatic and Isotope Biogeochemistry culture collection. Growth protocols were adapted from the deni-
7 trifier method (Sigman et al., 2001; Casciotti et al., 2002; Weigand et al., 2015).

8
9 **2.1.2. Overview of the experiments**

10 Two complementary incubation approaches were used to determine how species identity and physiological
11 state influence N₂O isotopic signatures. All experiments were performed in either natural-abundance water
12 (nat) or ¹⁸O-enriched water (en), and all media are described in Sect. 2.1.3.

13 Experiment codes follow the structure X_species_water, where X can be GF for gas flushed incubations or CB
14 denotes closed-batch incubation, CB-R denotes a resuspension assay, in which actively grown cultures were
15 harvested by centrifugation and resuspended in a defined nitrate-containing medium, following an approach
16 adapted from the bacterial-denitrifier method used to analyse the N and O isotopic composition of nitrate
17 (Sigman et al., 2001; Casciotti et al., 2002; Weigand et al., 2015). CB-R indicates a resuspension assay, with
18 nitrate (KNO₃), and CB-RS indicates a resuspension assay using nitrate reference standards. Species can be
19 *aur* or *chlor*, which denotes *P. aureofaciens* and *P. chlororaphis*, respectively, and water can be *nat* and *en* in-
20 dicating natural-abundance or ¹⁸O-enriched water.

21
22 **Table 1. Overview of experimental conditions for gas-flushed (GF) and closed-batch (CB) incubations.**

23 Experiments were conducted with *P. aureofaciens* (*P. aur*) and *P. chlororaphis* (*P. chlor*) using natural-abundance (nat) or
24 ¹⁸O-enriched (en) water. Gas flushed incubations (GF_aur_nat, GF_aur_en, GF_chlor_nat) were performed during active
25 growth under continuous N₂ stripping. Closed-batch incubations include: (i) active-growth experiments in natural-abun-
26 dace or ¹⁸O-enriched water (CB_aur_nat, CB_chlor_nat, CB_aur_en, CB_chlor_en); (ii) stationary-phase resuspension experi-
27 ments in natural-abundance or ¹⁸O-enriched water (CB-R_aur_nat, CB-R_chlor_nat, CB-R_aur_en, CB-R_chlor_en); and (iii)
28 nitrate-standard resuspension assays (CB-RS_aur, CB-RS_chlor) using USGS32, USGS34, IAEA-N3, and ¹⁸O-enriched IAEA-
29 N3 (IAEA-N3-spiked).

Experiment ID	Variant	δ ¹⁸ O-H ₂ O (‰)	Replicates n
Gas flushed incubations (GF)			
GF-aur_nat	P. aur	-9.5	n = 6
GF-aur_en	P. aur	68.3	n = 1
GF3-chlor_nat	P. chlor	-9.5	n = 2
Closed batch incubations (CB)			
CB-aur_en	P. aur	73.5	n = 2
CB-chlor_en	P. chlor	73.5	n = 2
CB-aur_nat	P. aur	-9.5	n = 2

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CB-chlor_nat	P. chlor	-9.5	n = 2
CB-R-aur_nat	P. aur	-9.5	n = 2
CB-R-chlor_nat	P. chlor	-9.5	n = 2
CB-RS_aur	P. aur	-9.5 / 780 ^a	n = 2
CB-RS_chlor	P. chlor	-9.5 / 780 ^a	n = 1

^aFor ¹⁸O-enriched resuspension assays with IAEA-N3-spiked, incubation water was prepared by mixing natural water ($\delta^{18}\text{O} = -9.5\text{‰}$) with ¹⁸O-enriched water ($\delta^{18}\text{O} \approx +780\text{‰}$), yielding a final $\delta^{18}\text{O}\text{-H}_2\text{O}$ of +187.8 ‰ (see Sect. 2.1.4).

Gas-flushed (GF) incubations (GF_aur_nat, GF_aur_en, GF_chlor_nat) were conducted in a temperature-controlled bioreactor under continuous N₂ stripping, allowing real-time quantification of N₂O concentration and isotopic composition during active growth (Fig. 1).

Closed-batch (CB) incubations (CB_aur_nat, CB_chlor_nat, CB_aur_en, CB_chlor_en, CB-R_aur_nat, CB-R_chlor_nat, CB-RS_aur, CB-RS_chlor) were conducted in sealed vials and served three purposes. First, active-growth closed-batch incubations (CB_aur_nat, CB_chlor_nat, CB_aur_en, CB_chlor_en) were conducted in active-growth medium to generate cumulative N₂O for quantifying water–nitrite oxygen exchange during growth. ~~Second, for stationary-phase resuspension incubations (CB-R_aur_nat, CB-R_chlor_nat), used cultures were harvested by centrifugation (4700 rpm, 10 min) and resuspended in a defined nitrate medium prepared in natural-isotope-abundance water. Second, stationary-phase resuspension incubations (CB-R_aur_nat, CB-R_chlor_nat) used pelleted cultures transferred into defined nitrate medium prepared in natural-abundance water.~~ Third, nitrate-standard resuspension assays (CB-RS_aur, CB-RS_chlor) replaced KNO₃ with USGS32, USGS34, IAEA-N3, or ¹⁸O-enriched IAEA-N3 (see Sect. 2.1.4).

2.1.3. Media composition and resuspension procedure

Two distinct media formulations were used depending on experimental objective: an active-growth medium for gas-flushed and closed-batch incubations, and a defined resuspension medium for stationary-phase assays and nitrate-standard experiments. Active growth was carried out in a denitrifier-method medium formulation (Weigand et al., 2015) containing final concentrations of 10 mM KNO₃, 15 mM NH₄⁺ supplied as (NH₄)₂SO₄, 30 g L⁻¹ tryptic soy broth (TSB, Merck Germany), and 5 g L⁻¹ K₂HPO₄. Cultures grown in this medium reduced nitrate quantitatively and produced N₂O for isotopic analysis.

For stationary-phase resuspension incubations, actively grown cultures ~~were harvested by centrifugation and resuspended in a simplified medium were pelleted and transferred into a simplified medium~~ (Weigand et al., 2015) containing ~~final concentrations of~~ 7.5 mM NH₄⁺ supplied as NH₄Cl, 30 g L⁻¹ TSB, and 5 g L⁻¹ K₂HPO₄ ~~in the final solution~~. Cell pellets were resuspended in 3 mL of this medium in 20 mL crimp-sealed vials. Vials were purged with N₂ for ~1 h to establish an anoxic headspace and to remove pre-existing gaseous products (including N₂O) prior to initiating the incubation. ~~The resuspension protocol was adapted from the bacterial denitrifier method; a~~ ~~These conditions were designed to standardize the headspace and remove pre-existing gaseous products prior to incubation, rather than to reproduce the exact conditions of the bacterial denitrifier method.~~ After purging, 20 nmol KNO₃ was added, and cultures were incubated overnight to allow quantitative conversion to N₂O. Thereafter, N₂O was analyzed by GC-IRMS from the vial headspace.

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For CB_RS_aur and CB_RS_chlor (closed-batch resuspension assays using converting nitrate reference standards with *P. aureofaciens* and *P. chlororaphis*, respectively; experiment codes defined in Sect. 2.1.3 and Table 1), the same resuspension procedure was used, except that the nitrate substrate (20 nmol KNO₃) was replaced with 20 nmol of an international nitrate isotope reference standard (USGS32, USGS34, IAEA-N3, or ¹⁸O-enriched IAEA-N3-spike). The same resuspension procedure was used, except that the 20 nmol KNO₃ addition was replaced by 20 nmol of the respective nitrate isotope standard (USGS32, USGS34, IAEA-N3, or ¹⁸O-enriched IAEA-N3-spike).

2.1.4 Characterization of ¹⁸O-labelled water

Oxygen exchange between reaction intermediates and water in both GF and CB experiments was assessed using replicate incubations in natural-abundance and ¹⁸O-enriched water. The natural water used for all baseline incubations had a $\delta^{18}\text{O-H}_2\text{O} = -9.51 \pm 0.22 \text{ ‰}$. Two batches of ¹⁸O-enriched water were prepared and used: one with $\delta^{18}\text{O-H}_2\text{O} = +68.31 \pm 0.17 \text{ ‰}$, and a second with $\delta^{18}\text{O-H}_2\text{O} = +73.53 \pm 0.29 \text{ ‰}$ (Table 1). For nitrate-standard experiments (CB-RS-aur and CB-RS-chlor, Table 1), an ¹⁸O-enriched IAEA-N3 spike was prepared by mixing 1 mL of ¹⁸O-enriched water ($\delta^{18}\text{O-H}_2\text{O} \approx +780 \text{ ‰}$) with 3 mL of natural water ($\delta^{18}\text{O-H}_2\text{O} = -9.5 \text{ ‰}$), yielding a solution with $\delta^{18}\text{O-H}_2\text{O} \approx +187.8 \text{ ‰}$.

The ¹⁸O isotopic composition of both natural and ¹⁸O-enriched waters was measured using a cavity ring-down spectroscopy (CRDS) analyzer (L2130-i, Picarro Inc., USA). Measurements were calibrated on the VSMOW-SLAP scale using three international reference waters: VSMOW2 ($\delta^{18}\text{O} = 0.00 \pm 0.02 \text{ ‰}$), SLAP2 ($-55.50 \pm 0.02 \text{ ‰}$), and IAEA-607 ($+99.02 \pm 0.13 \text{ ‰}$), which were measured together with the samples during each analytical run to establish a three-point isotope calibration.

2.1.5 Endpoint N₂O isotopic analysis (IRMS)

Accumulated N₂O produced during incubations was analyzed using gas chromatography coupled to isotope ratio mass spectrometry (GC-IRMS; Delta V Plus coupled to a GasBench system for N₂O purification, Thermo Fisher Scientific, Germany). Isotopic composition was referenced to AIR-N₂ ($\delta^{15}\text{N}$) and VSMOW ($\delta^{18}\text{O}$) using three laboratory N₂O reference gases (STD1–STD3; ~90 ppm N₂O in N₂) measured alongside samples. Assigned delta values (in ‰) were: STD1: $\delta^{15}\text{N}^\alpha = -22.21 \pm 0.39$, $\delta^{15}\text{N}^\beta = -49.28 \pm 0.40$, SP = 27.08 ± 0.56 , $\delta^{15}\text{N}^{\text{bulk}} = -35.74 \pm 0.07$, $\delta^{18}\text{O} = 26.94 \pm 0.23$; STD2: $\delta^{15}\text{N}^\alpha = 1.71 \pm 0.47$, $\delta^{15}\text{N}^\beta = 94.44 \pm 0.70$, SP = -92.73 ± 0.84 , $\delta^{15}\text{N}^{\text{bulk}} = 48.09 \pm 0.23$, $\delta^{18}\text{O} = 36.01 \pm 0.27$; STD3: $\delta^{15}\text{N}^\alpha = 17.11 \pm 0.12$, $\delta^{15}\text{N}^\beta = -3.43 \pm 0.17$, SP = 20.54 ± 0.21 , $\delta^{15}\text{N}^{\text{bulk}} = 6.85 \pm 0.06$, $\delta^{18}\text{O} = 35.39 \pm 0.17$. Data processing, including scale normalization and correction for ion-source scrambling effects, was performed using the Pylisotopomer package (Kelly et al., 2023).

2.2 Gas-flushed (GF) incubations and online isotope monitoring

2.2.1 Experimental setup for gas-flushed incubations

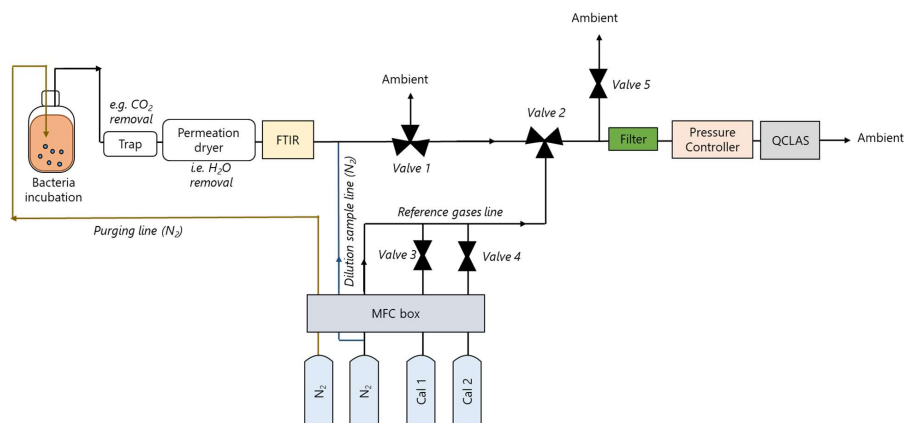


Figure 1. Schematic of the setup applied for bacterial incubations, continuous gas sampling, and real-time analysis of trace gases and N₂O isotopologues. N₂ is used to purge reaction products from the liquid phase via the headspace of the incubation vessel toward the analyzers. Trace gas concentrations (N₂O, NO, residual CO₂ and H₂O) are determined in the CO₂-free and dehumidified sample gas by FTIR spectroscopy. The N₂O concentration is used to set the MFC for N₂ dilution and stabilize the N₂O concentration for QCLAS analysis to 50 ppm. Downstream of the FTIR, the gas is directed to the QCLAS for isotopic analysis of N₂O. Calibration of the QCLAS is conducted via automated switching between sample gas and reference standards (valves 1-5). Calibration gas (Cal 1, Cal 2) flows are regulated with mass flow controllers (MFC). During calibration phases, the sample gas is vented to the atmosphere (valve 1); during measurement phases valves 1, 2 and 5 are switched in parallel, directing the sample gas toward the QCLAS. A pressure controller upstream of the QCLAS stabilizes the multipath cell pressure and ensures stable flow conditions.

For continuous N₂O isotope monitoring under cultivation conditions, strains were grown in the active-growth medium described in Sect. 2.1.2 (Fig. 1). Incubations were performed at 30 ± 1 °C for *P. aureofaciens* and 20 ± 1 °C for *P. chlororaphis*, reflecting optimal conditions for growth and N₂O production. Cultures were stirred using a magnetic stirrer to ensure homogeneous mixing, and maintained anaerobic by continuous N₂ flushing (100 mL min^{-1}). Dissolved oxygen, pH (Mettler-Toledo, Switzerland), and optical density (OD₅₅₀) were monitored throughout the experiments. Substrate consumption was monitored by daily nitrite measurements. Incubations were typically completed within 5–8 days. Isotopic measurements were stopped when N₂O concentrations dropped below 50 ppm, whereas concentration measurements by FTIR continued until N₂O levels dropped below detection limits ($0.0075 \text{ ppm N}_2\text{O}$), confirming complete turnover of nitrogen substrates (Fig. 2). Gas exiting the bioreactor was conditioned prior to analysis; carbon dioxide and water vapor were removed using two consecutive washing bottles containing 1 M NaOH, followed by permeation drying (Perma Pure, USA) (Fig. 1). The CO₂-free, dehumidified sample gas was subsequently filtered through a sintered metal filter ($2 \mu\text{m}$ pore size) and analyzed for trace gas concentrations (N₂O, NO) using an online FTIR analyzer (CX-4000 or CX-4015, Gaset Technologies, Finland) (Wunderlin et al., 2012). Three-minute average

N₂O concentration values were used to calculate dilution ratios and regulate the MFC settings to maintain an approximately constant N₂O concentration (~50 ppm) in the diluted sample gas, which minimized concentration effects on isotope ratio measurements by QCLAS (Wunderlin et al., 2013). Choosing 50 ppm as target concentration for N₂O isotope analysis was a compromise between data coverage and response time of the experimental setup but does not represent a lower threshold of the analytics. Automated sample gas dilution and calibration of the QCLAS system were controlled via a LabVIEW interface (National Instruments Corp., USA) connected to mass flow controllers (Vögtlin Instruments Inc., Switzerland) and electromagnetic valves (Series 9, Parker Hannifin, USA).

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2.2.2. Online analysis of N₂O isotopic composition (QCLAS)

Temporally-resolved (1 Hz) N₂O isotopic analysis in the diluted sample gas was performed using a compact quantum cascade laser absorption spectrometer (mini-TILDAS, pathlength 76 m, Aerodyne Research Inc., USA), operated in flow-through mode at a sample pressure of 26.6 hPa (20 Torr) (Ibraim et al., 2018). The instrument simultaneously quantified the concentrations of the four most abundant N₂O isotopologues in the ν_3 absorption band (2203 cm⁻¹): ¹⁴N¹⁴N¹⁶O, ¹⁴N¹⁵N¹⁶O (¹⁵N^α), ¹⁵N¹⁴N¹⁶O (¹⁵N^β), and ¹⁴N¹⁴N¹⁸O. Raw concentrations were logged in ASCII format. Time series for each isotopologue were averaged to 60-second intervals as the first step in the data-processing workflow, prior to any correction or calibration. Isotopologue ratios (¹⁴N¹⁵N¹⁶O/¹⁴N¹⁴N¹⁶O, ¹⁵N¹⁴N¹⁶O/¹⁴N¹⁴N¹⁶O, and ¹⁴N¹⁴N¹⁸O/¹⁴N¹⁴N¹⁶O) were then calculated from these averaged concentrations using a customized R script. Allan deviations at a 60-second averaging time were used to propagate uncertainties in isotope delta values (see Appendix A).

Two N₂O isotope reference gases diluted in N₂ were used (Mohn et al., 2022); CG1 (RM1A, anchor gas, in ‰): $\delta^{15}\text{N}^\alpha = -0.22 \pm 0.46$, $\delta^{15}\text{N}^\beta = 0.84 \pm 0.46$, $\delta^{18}\text{O} = 39.22 \pm 0.15$; and CG2 (RM3A, span gas, in ‰): $\delta^{15}\text{N}^\alpha = 50.96 \pm 0.47$, $\delta^{15}\text{N}^\beta = 53.06 \pm 0.47$, $\delta^{18}\text{O} = 103.04 \pm 0.16$. CG1 was measured every 40 minutes for 10 minutes, to correct for instrumental drift, while the calibration span was determined twice daily analyzing CG1 and CG2. From the measured delta values, the following parameters were calculated: $\delta^{15}\text{N}\text{-bulk} = (\delta^{15}\text{N}^\alpha + \delta^{15}\text{N}^\beta)/2$ and $\text{SP} = \delta^{15}\text{N}^\alpha - \delta^{15}\text{N}^\beta$, following the nomenclature of Toyoda and Yoshida (1999). Data processing steps are detailed in Appendix A.

For validation an independent N₂O isotope reference gas (RM3B) with assigned values of $\delta^{15}\text{N}\text{-bulk} = 16.08 \pm 0.05$ ‰, $\delta^{18}\text{O} = 55.17 \pm 0.15$ ‰, and $\text{SP} = -0.68 \pm 0.91$ ‰, was diluted using a two-step diluter (METAS, Switzerland) to a target concentration of 50 ppm N₂O. Measurements were conducted during a short validation campaign in April–May 2024. RM3B was analyzed within the existing CG1/CG2 calibration framework, and the measured values agreed with assigned values within <0.5 ‰ across all isotope parameters.

2.2.3. Isotopic Fractionation Analysis (Rayleigh Model – QCLAS Time Series)

Isotope fractionation during denitrification was assessed using a Rayleigh-type cumulative product model, following the definitions of enrichment factors (ε) provided by Mariotti et al. (1981) and Sutka et al. (2006), which describe the isotopic discrimination between the substrate and the accumulated product over the course of the reaction.

Changes in the isotopic composition of N₂O were modeled as a function of nitrate reduction using the equation:

$$y = a + b \cdot [x] \quad (1)$$

where y is the isotopic composition (δ value) of the N₂O product, a and b are fitted coefficients, and $[x]$ represents the reaction progress. The variable $[x]$ was calculated using the Rayleigh equation (Sutka et al., 2006):

$$x = -f \cdot \ln(f) / (1 - f) \quad (2)$$

Here, f is the fraction of remaining nitrate, calculated as:

$$f = 1 - (2 \cdot n_{\text{N}_2\text{O}} / n_{\text{NO}_3^-}) \quad (3)$$

where n denotes the molar quantities of accumulated N₂O and initial NO₃⁻, respectively. The slope b corresponds to the apparent enrichment factor ϵ (in ‰), while the intercept a represents the initial isotopic composition of the product pool. This interpretation assumes Rayleigh-type fractionation behavior, i.e. a closed substrate pool with unidirectional consumption and negligible back-reaction. Microbial incubations featuring multistep processes may deviate from these conditions, particularly when O isotope exchange with water or partial recycling of intermediates occurs. Nevertheless, the Rayleigh model provides a useful first-order approximation for comparing fractionation patterns across experiments.

More complex Rayleigh formulations have been proposed to explicitly account for intramolecular isotope effects during N₂O formation, including site-specific treatments of $\delta^{15}\text{N}^\alpha$ and $\delta^{15}\text{N}^\beta$ (e.g., Rivett et al., 2025). In the present study, Rayleigh modeling was applied only to $\delta^{15}\text{N}$ -bulk, for which the standard cumulative product formulation is sufficient.

Fractionation calculations were performed using a custom R pipeline that included uncertainty propagation for N₂O gas concentration, sample gas flow, and the initial substrate amount (NO₃⁻). Full computational details are provided in the Appendix A.

2.2.4. Monitoring of nitrate and nitrite during GF incubations and nitrate isotope characterization

During gas-flushed incubations, NO₃⁻ and NO₂⁻ concentrations were monitored daily to track substrate depletion and the progression of nitrate reduction. Nitrite was measured spectrophotometrically immediately after sampling using the Griess assay (Hansen and Koroleff, 1999). For nitrate concentration analysis, 1 mL samples were collected daily, filtered through 0.22 μm syringe filters, and stored in Eppendorf tubes at -18 °C prior to analysis. Nitrate concentrations were determined by quantitative reduction to nitric oxide (NO) using acidic vanadium(III), followed by chemiluminescence detection (Braman and Hendrix, 1989).

The isotopic composition of the nitrate substrates used to prepare incubation media was determined prior to the experiments. In addition, selected samples from GF incubations were analyzed to assess changes in the isotopic composition of the residual nitrate pool during denitrification. Nitrate isotope analyses were not performed for closed-batch incubations.

For nitrate isotopic analysis, aliquots containing 20 nmol N were converted to N₂O using the denitrifier method (Sigman et al., 2001; Casciotti et al., 2002). The nitrate stock solution used for all active-growth media had a $\delta^{15}\text{N}\text{-NO}_3^- = +5.7 \pm 0.3 \text{ ‰}$ and a $\delta^{18}\text{O}\text{-NO}_3^- = +12.4 \pm 0.3 \text{ ‰}$ ($n = 3$).

Calibration was performed against international nitrate reference materials USGS32 (KNO_3 , $\delta^{15}\text{N} = +180 \text{ ‰}$, $\delta^{18}\text{O} = +25.4 \pm 0.2 \text{ ‰}$), USGS34 (KNO_3 , $\delta^{15}\text{N} = -1.8 \pm 0.1 \text{ ‰}$, $\delta^{18}\text{O} = -27.78 \pm 0.37 \text{ ‰}$), and IAEA-N3 (KNO_3 , $\delta^{15}\text{N} = +4.7 \pm 0.2 \text{ ‰}$, $\delta^{18}\text{O} = +25.6 \pm 0.4 \text{ ‰}$). Additional reference materials included UBN-1 ($\delta^{15}\text{N} = +14.15 \text{ ‰}$, $\delta^{18}\text{O} = +25.7 \text{ ‰}$), Deep Pacific nitrate ($\delta^{15}\text{N} \approx +5.0 \text{ ‰}$, $\delta^{18}\text{O} \approx +2.0 \text{ ‰}$), and an IAEA-N3-spike standard (IAEA-N3 dissolved in water with $\delta^{18}\text{O}\text{-H}_2\text{O} = +780 \text{ ‰}$, see Sect. 2.1.4).

2.3 Closed batch (CB) incubations

2.3.1 Active-growth incubations (natural-water and enriched-water)

Closed-batch incubations were used to generate cumulative N_2O under controlled growth conditions. All experiments were conducted in 500 mL Wheaton bottles containing 400 mL of active-growth medium (Sect. 2.1.2) and incubated at 23°C for 7 days. Experiments performed in natural-abundance water (CB_aur_nat, CB_chlor_nat) and ^{18}O -enriched-water (CB_aur_en, CB_chlor_en) differed only in the isotopic composition of the water used to prepare the medium. The enriched-water incubations produced $\delta^{18}\text{O}\text{-N}_2\text{O}$ endpoint values that were regressed against $\delta^{18}\text{O}\text{-H}_2\text{O}$ to quantify oxygen atom exchange between nitrite and water. After complete nitrate reduction, accumulated N_2O was analyzed by GC-IRMS (Sect. 2.1.5).

2.3.2 Resuspension incubations in defined nitrate medium (stationary phase)

To obtain stationary-phase $\delta^{18}\text{O}\text{-N}_2\text{O}$ endpoint values, actively grown cultures were pelleted and resuspended in the defined nitrate medium described in Section 2.1.2. These resuspension assays (CB-R_aur_nat, CB-R_chlor_nat) were performed with stationary-phase cultures resuspended in fresh nitrate medium. The experiments were conducted only in natural-abundance water. After overnight nitrate reduction, the accumulated N_2O was analyzed by GC-IRMS (Section Sect. 2.1.5).

2.3.3 Resuspension incubations with nitrate isotope standards (quantification of oxygen isotope exchange)

Additional stationary-phase incubations were performed to quantify oxygen-atom exchange between nitrite and water using nitrate standards with contrasting $\delta^{18}\text{O}\text{-NO}_3^-$ values (CB-RS_aur, CB-RS_chlor): USGS32, USGS34, IAEA-N3. These were accompanied by an ^{18}O -enriched IAEA-N3-spike to match the typical approach for quantifying oxygen-atom exchange in the bacterial-denitrifier method (Sect. 2.1.2). Following quantitative reduction of the added nitrate, $\delta^{18}\text{O}\text{-N}_2\text{O}$ was measured by GC-IRMS (Sect. 2.1.5). The resulting $\delta^{18}\text{O}\text{-N}_2\text{O}$ values were regressed against the known $\delta^{18}\text{O}\text{-NO}_3^-$ values of the standards. The $\delta^{18}\text{O}\text{-N}_2\text{O}$ versus $\delta^{18}\text{O}\text{-NO}_3^-$ relationship was applied to derive stationary-phase oxygen-exchange fractions.

This nitrate-standard approach provides an independent estimate of oxygen exchange complementary to the enriched-water experiments (Sect. 2.3.1). Whereas regressions of $\delta^{18}\text{O}\text{-N}_2\text{O}$ versus $\delta^{18}\text{O}\text{ H}_2\text{O}$ quantify exchange from the sensitivity of N_2O to water isotopic composition, regressions of $\delta^{18}\text{O}\text{-N}_2\text{O}$ versus $\delta^{18}\text{O}\text{-NO}_3^-$ quantify the O-atom exchange from the degree of scale compression relative to nitrate isotope standards. Under conditions of no oxygen exchange, $\delta^{18}\text{O}\text{-N}_2\text{O}$ scales directly with $\delta^{18}\text{O}\text{-NO}_3^-$ (slope ≈ 1), whereas increasing exchange reduces this dependence (slope < 1). The fraction of oxygen atoms in N_2O derived from

1 water can therefore be estimated from the regression slope (Casciotti et al., 2002; [Snider et al., 2009](#); [Lewicka-](#)
2 [Szczepak et al., 2016](#)).

3 3. Results and Discussion

4 3.1 Denitrifying growth and near-quantitative N₂O production in gas-flushed and closed- 5 batch incubations

6 Both *P. chlororaphis* (NirS-bearing) and *P. aureofaciens* (NirK-bearing) showed consistent denitrifying behav-
7 ior under anoxic conditions, with N₂O production closely coupled to nitrate reduction. Biomass accumulation
8 and substrate consumption followed the expected progression of anoxic denitrifying growth, indicating that
9 the incubation conditions supported stable and active metabolism throughout.

10 Biomass increased during the phase of active N₂O production and stabilized as nitrate became limiting, while
11 NO₃⁻ concentrations decreased in parallel with N₂O formation (Fig. 2C, 2D). This tight coupling indicates effi-
12 cient conversion of nitrate to N₂O, with only transient accumulation of nitrite (Fig. 2E). This tight coupling in-
13 dicates efficient conversion of nitrate to N₂O with no substantial accumulation of intermediates. [Accordingly,](#)
14 [cumulative N₂O yields indicated efficient denitrification \(Fig. 2A\), although yieldsthey varied between incuba-](#)
15 [tions, indicating that N₂O was not always the sole quantified-nitrogen oxide-containing producedt \(Appendix](#)
16 [B, Fig. B1\).](#) [Accordingly, cumulative N₂O yields approached quantitative conversion of the available nitrate](#)
17 [\(Fig. 2A\).](#)

18 Across gas-flushed incubations, N₂O yields ranged from 60–100 % for *P. aureofaciens* (n = 6) and 50–100 %
19 for *P. chlororaphis* (n = 2). [consistent with efficient denitrification under continuous N₂ stripping. These re-](#)
20 [sults demonstrate that both incubation approaches supported efficient denitrification and provided well-con-](#)
21 [strained systems for isotopic interpretation.](#) Dissolved O₂ concentrations remained below detection (<1 μM),
22 and pH varied only slightly (6.5–7.5), confirming stable anoxic conditions throughout the experiments. Nitrite
23 accumulated transiently during active nitrate reduction but did not persist, indicating rapid turnover within
24 the denitrification pathway.

25 While such growth dynamics are well established for denitrifying systems (Zumft, 1997), the present gas-
26 flushed setup enables direct observation of N₂O production dynamics under continuous stripping, providing
27 a temporally resolved view that is not accessible in conventional closed systems.

28 In closed-batch incubations, near-complete nitrate reduction was similarly observed, as indicated by the ab-
29 sence of residual NO₃⁻ and NO₂⁻ and the cessation of N₂O production. These results demonstrate that both
30 incubation approaches achieved near-quantitative conversion of nitrate to N₂O under the conditions applied,
31 providing well-constrained systems for isotopic interpretation. N₂O isotopic compositions were determined
32 from endpoint measurements and are discussed in Sect. 3.3 and 3.4.

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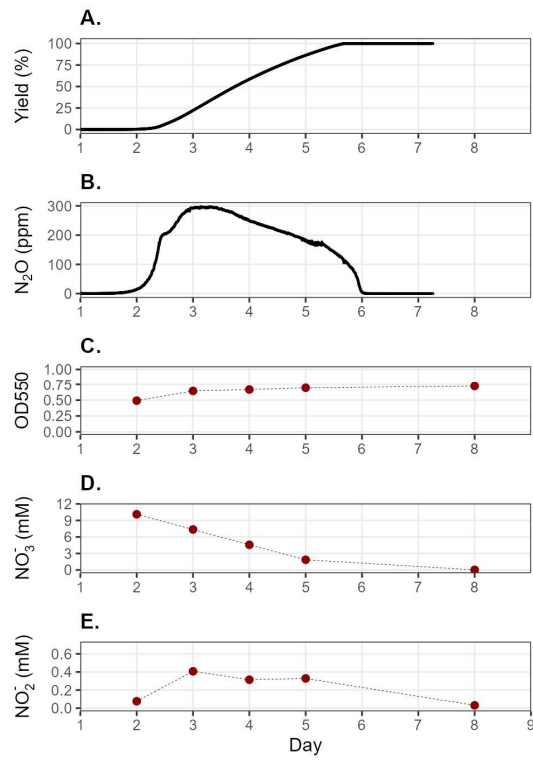


Figure 2. Representative temporal dynamics of N_2O production over days, cumulative N_2O yield, and culture characteristics during incubation of *P. aureofaciens* (GF_aur). A) Cumulative N_2O yield (% of initial nitrate converted to N_2O); B) Instantaneous N_2O formation, measured in N_2 purge gas; C) Optical density at 550 nm (OD_{550} , circles), as a proxy for biomass accumulation; D) Residual nitrate concentration; E) Nitrite concentration over the course of the experiment.

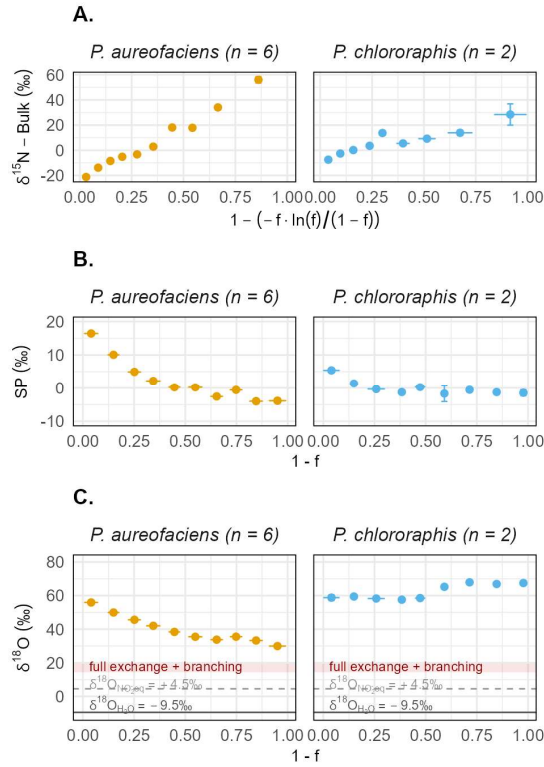


Figure 3. Temporal evolution of N_2O isotopic composition during gas-flushed incubations in natural-abundance water (GF_aur_nat, GF_chlor_nat). Panels show isotopic composition as a function of reaction progress for *P. aureofaciens* (n = 6) (Nirk) and *P. chlororaphis* (n = 2) (NirS). Reaction progress (f) was calculated from cumulative N_2O production and re-scaled for each incubation such that the first sampled point corresponds to $f = 0$ and the final point to $f = 1$, allowing comparison across experiments with different final yields. Measurements were grouped into equally spaced bins of reaction progress, and isotopic values were averaged within each bin. Points therefore represent bin-averaged values, with vertical error bars indicating within-bin variability and horizontal error bars indicating the spread in reaction progress of contributing measurements. A) $\delta^{15}\text{N}$ -Bulk versus the Rayleigh coordinate $1 - \frac{-f \cdot \ln(f)}{1 - f}$, which linearizes Rayleigh-type isotope fractionation during substrate consumption. B) Site preference (SP) versus reaction progress ($1 - f$). C) $\delta^{18}\text{O}$ - N_2O versus reaction progress ($1 - f$). Panels B and C use the simpler ($1 - f$) coordinate to directly visualize temporal trends, whereas panel A uses the Rayleigh transformation to resolve fractionation behavior. In panel C, horizontal lines indicate $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (-9.5‰ ; solid grey) and $\delta^{18}\text{O}_{\text{NO}_2, \text{eq}}$ ($+4.5\text{‰}$; dashed grey). The shaded red band ($+14.5$ to $+19.5\text{‰}$) represents the expected $\delta^{18}\text{O}$ - N_2O range under full nitrite-water equilibration with branching fractionation.

3.2 Rayleigh-type ^{15}N enrichment in the N_2O pool during nitrate reduction

The $\delta^{15}\text{N}$ -bulk of accumulated N_2O increased linearly over the course of nitrate reduction in all incubations when expressed against the reaction coordinate $1 - (-f \ln(f)/(1 - f))$ using the Rayleigh equation as presented in Mariotti et al. (1981) and Sutka et al. (2006) (Fig. 3A). Because the measurements represent the cumulative isotopic composition of the accumulated reaction product N_2O , N_2O was calculated from the amount and isotopic composition of instantaneously formed and analysed N_2O . product pool, the Rayleigh analysis follows the cumulative-product formulation of Mariotti et al. (1981) and Sutka et al. (2006). This pattern is consistent with Rayleigh-type enrichment expected during unidirectional substrate consumption under closed-system conditions. Enrichment factors ($\epsilon^{15}\text{N}$) were estimated for each incubation by linear regression of $\delta^{15}\text{N}$ -bulk against the reaction coordinate using the specific reaction progress of each experiment (individual experiment regressions are shown in Appendix B Fig. B1). The rescaling and binning shown in Fig. 3A serve only to visualize common trajectories across incubations.

Enrichment factors ($\epsilon^{15}\text{N}$) derived from this linear Rayleigh approximation ranged from +16 to +55 ‰ (35.5 ± 12 ‰) for *P. aureofaciens* ($n = 6$) and from +13 to +45 ‰ (25.4 ± 14 ‰) for *P. chlororaphis* ($n = 2$). Variability across replicate incubations likely reflects differences in nitrate turnover rates and additional biological variability, although no systematic relationship between nitrate reduction rate and $\epsilon^{15}\text{N}$ could be resolved with the present dataset.

The applicability of the linear Rayleigh model is supported by the consistent increase in $\delta^{15}\text{N}$ -bulk over the reaction coordinate, which reflects the expected kinetic isotope enrichment pattern during progressive substrate consumption (Mariotti et al., 1981). Nevertheless, the linear Rayleigh approximation represents an idealized description when applied to multistep microbial pathways such as denitrification. In such systems, intrinsic isotope effects may be modulated by intermediate pool dynamics, diffusion limitation, or partial reversibility, potentially leading to deviations from a single kinetic fractionation factor. These complexities have been discussed in detail by Haslun et al. (2018) and are not explicitly resolved here. Rather, the Rayleigh analysis is used as a first-order framework to derive apparent $\epsilon^{15}\text{N}$ values from the cumulative N_2O product pool, allowing comparison across strains. estimate apparent $\epsilon^{15}\text{N}$ values that describe bulk nitrogen isotope enrichment during nitrate reduction and allow comparisons across strains and incubation modes.

To place the $\epsilon^{15}\text{N}$ estimates obtained in this study in context, we compared them with a compilation of fractionation factors for N_2O production from a range of denitrifying organisms and environmental systems (Fig. 4). This dataset, largely derived from Denk et al. (2017), includes values enrichment factors determined under diverse experimental conditions and using different formulations of the Rayleigh model applied to either the instantaneous or accumulated product, or to the educt substrate and analytical approaches. Nonetheless, although these enrichment factors are not mathematically identical, they provide a context for comparing the magnitude of isotope fractionation observed in the present study. The $\epsilon^{15}\text{N}$ values obtained here fall within the broader range reported in the literature and align particularly well with previous results for *Pseudomonas* spp. under nitrate-reducing conditions. Additionally, we include instantaneous isotope effects derived from non-linear Rayleigh formulations, reported as $\eta^{15}\text{N}$ values by Haslun et al. (2018). Although expressed using a different notation, these values are conceptually analogous to $\epsilon^{15}\text{N}$ and allow comparison with the fractionation factors derived here. Taken together, these comparisons show that the magnitude of

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fractionation observed here falls well within the established range for denitrifying systems, indicating that the gas-flushed setup does not introduce atypical isotope effects.

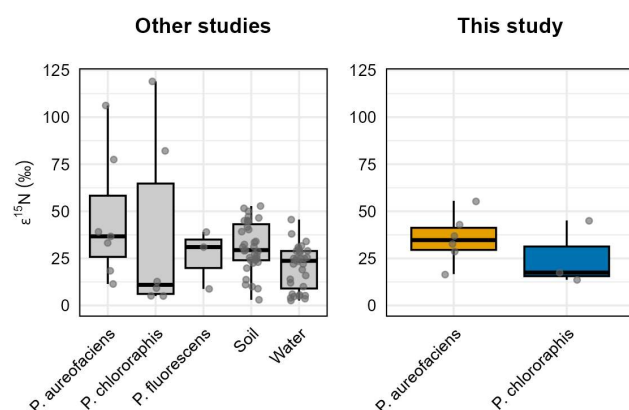


Figure 4. Isotopic enrichment factors ($\epsilon^{15}\text{N}$) for N_2O production across denitrifying microbial communities reported in the literature and observed in this study. Most literature values are compiled from Table S1 of Denk et al. (2017), which integrates enrichment factors derived from different experimental systems and using different mathematical Rayleigh formulations of the Rayleigh model (e.g., soils, aqueous environments, and pure-culture incubations), which integrates isotope effects derived using different experimental systems and modeling approaches (e.g., soils, aqueous environments, pure-culture incubations). Instantaneous isotope effects reported as $\eta^{15}\text{N}$ by Haslun et al. (2018) for *P. aureofaciens*, and *P. chlororaphis*, and as well as $\epsilon^{15}\text{N}$ for *P. fluorescens* (Toyoda et al., 2005) are also included for comparison. For clarity and comparability, all values shown here are expressed using a common sign convention, where positive $\epsilon^{15}\text{N}$ indicates normal isotope effects, i.e., preferential reaction of the light isotope.

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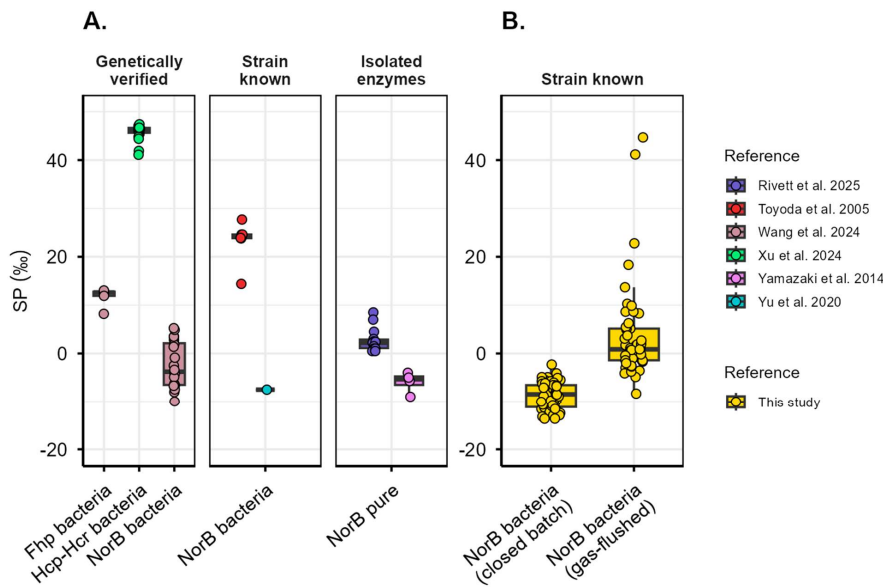
3.3 Transient elevated SP suggests variable NO reduction pathways

SP values measured at the endpoint of closed-batch incubations clustered near 0 ‰ for both *P. aureofaciens* and *P. chlororaphis* (Fig. 5B), within the canonical range reported for bacterial denitrification (−8 ‰ to +10 ‰; Yu et al., 2020). This outcome is consistent with the common assumption that SP during bacterial denitrification is invariant and reflects NO reduction by the nitric oxide reductase NorB, which yields values near 0 ‰. This interpretation is supported by measurements from intact denitrifying cultures and by direct assays of purified NorB enzyme (Sutka et al., 2006; Yamazaki et al., 2014; Wang et al., 2024; Rivett et al., 2025; Fig. 5A). Because these measurements integrate N_2O produced over the full course of the reaction, they represent time-averaged isotopic signatures. When assessed using these conventional accumulated-product approaches, N_2O formation appears broadly consistent with NorB-mediated denitrification, although such temporal integration may obscure short-lived deviations from canonical SP behavior.

In contrast to such endpoint observations, our time-resolved dataset shows that elevated SP values (+8 ‰ to +20 ‰) arise transiently but reproducibly during the early stages of N_2O formation under gas-flushed

1 conditions (Fig. 3B; Fig. 5B). Elevated SP values have occasionally been reported in denitrifying cultures.
 2 Toyoda et al. (2005), for example, observed SP values up to +22 ‰ in *P. fluorescens*, which they attributed to
 3 an abiotic pathway of unknown mechanism. Over the course of nitrate reduction, SP subsequently converged
 4 toward ~0 ‰ as the reaction progressed (Fig. 3B). When averaged over the full reaction progress, gas-
 5 flushed incubations yielded SP values near 0 ‰, with a slightly elevated median and broader variability com-
 6 pared to closed-batch endpoint values (Fig. 5B), yet still within the canonical range reported for bacterial de-
 7 nitrification. This convergence toward canonical SP values, despite pronounced early-stage elevations (Fig.
 8 3B), indicates that accumulated N₂O in closed-batch systems integrates over temporally variable production
 9 pathways and thereby masks transient deviations in SP. These transient SP elevations are most clearly ex-
 10 pressed in *P. aureofaciens* (n = 6). In *P. chlororaphis* (n = 2) they are less apparent in the binned averages due
 11 to limited replication and experiment-to-experiment variability, but they are evident in individual incubation
 12 trajectories (Appendix B Fig. B1).

13



14 **Figure 5.** Site preference (SP) of N₂O produced by three classes of N₂O-forming systems. A) Literature-derived SP values
 15 grouped by system type: left, intact denitrifiers with genetically verified N₂O-forming enzymes, where both strain identity
 16 and the active enzyme system (NorB, Hcp-Hcr, or Fhp) were confirmed (Xu et al., 2024; Wang et al., 2024); center, intact
 17 denitrifiers, strains are known but additional N₂O-forming pathways cannot be excluded (Toyoda et al., 2005; Yu et al.,
 18 2020); and right, isolated enzyme assays, in which purified NorB was studied directly, yielding enzyme-specific SP values
 19 (Yamazaki et al., 2014; Rivett et al., 2025). B) SP values obtained in this study for intact denitrifiers with known strain back-
 20 ground, shown separately for closed-batch and gas-flushed incubation modes. Boxplots show the distribution of SP val-
 21 ues within each category; horizontal bars indicate mean ± standard deviation. Gas-flushed incubations represent time-
 22 resolved SP measurements, whereas closed-batch incubations reflect SP integrated over the accumulated N₂O pool.

1
2 Although still within the broader isotopic space reported for bacterial denitrification and related enzyme sys-
3 tems (Fig. 5; Toyoda et al., 2005; Yamazaki et al., 2014; Haslun et al., 2018; Wang et al., 2024; Xu et al., 2024),
4 the consistent early-stage SP elevations suggest that NorB may not be the sole contributor to N₂O formation
5 during the onset of denitrification. One plausible explanation is that rapid NO accumulation during early
6 Nar/Nir activity initially exceeds the capacity of NorB, such that NO is partially reduced by alternative en-
7 zymes until NorB becomes transcriptionally or functionally dominant. Recent work by Wang et al. (2024) di-
8 rectly demonstrated that SP depends on NO reductase identity: NorB activity produced SP values near 0 ‰,
9 whereas flavohemoglobin (Fhp) activity under high-NO conditions yielded elevated SP values of approxi-
10 mately +10 ‰ (Fig. 5A). Using Δfhp mutants and inducible *norB* expression systems, the authors further
11 showed that the balance between these enzymes shifts with growth phase and NO availability. Although the
12 experimental conditions in that study differ from ours, it is plausible that early nitrate respiration in actively
13 growing cultures similarly produces transient NO accumulation combined with low NorB expression, allowing
14 temporary Fhp activity to elevate SP at the onset of denitrification. Future transcriptomic or proteomic anal-
15 yses would help confirm which NO reductases are expressed during this early phase.

16 Importantly, several of the transient SP values observed here (Fig. 5B) exceed not only the typical NorB range
17 but also the Fhp-associated values reported by Wang et al. (2024), motivating consideration of additional
18 NO-reducing pathways. Beyond Fhp, other NO detoxification systems may contribute to transient SP variabil-
19 ity. Xu et al. (2024) reported N₂O production during dissimilatory nitrate reduction to ammonium (DNRA) and
20 attributed it to the hybrid cluster protein–hybrid cluster reductase (Hcp–Hcr) system based on genomic and
21 transcriptomic evidence (Fig. 5A). While this attribution remains less firmly established than for NorB or Fhp,
22 it defines a distinct high-SP isotopic signature that broadens the reference space for interpreting transient SP
23 behavior. Flavodiiron proteins, which reduce NO to N₂O via asymmetric diiron intermediates (Caranto et al.,
24 2014a, 2014b), are also encoded in many *Pseudomonas* genomes, although their SP signatures remain un-
25 characterized.

26 Future work integrating SP-resolved N₂O measurements with transcriptomics, proteomics, or targeted inhibi-
27 tor assays will be essential to determine which NO reductases are active, when they operate, and how their
28 expression depends on metabolic state and environmental conditions. Taken together, these observations
29 indicate that SP expression during denitrification can be more dynamic than implied by static reference
30 ranges derived from endpoint measurements.

31 3.4 $\delta^{18}\text{O}$ systematics during denitrification across strains and incubation conditions

32 While site preference primarily reflects the identity of NO reductases operating during N₂O formation, the
33 oxygen isotopic composition of N₂O integrates upstream processes, particularly the extent of oxygen atom
34 exchange between nitrite and water prior to NO reduction. Because nitrite is the immediate precursor to NO,
35 its isotopic composition reflects both oxygen inherited from nitrate reduction and any subsequent equilibra-
36 tion with ambient water. As a result, $\delta^{18}\text{O}$ –N₂O does not represent a fixed enzymatic property, but rather a
37 composite signal that depends on the relative contributions of nitrate-derived oxygen, nitrite–water ex-
38 change, and branching isotope effects. Consequently, its interpretation as a tracer of nitrite reductase identity
39 requires that these processes remain constant – an assumption that has not been tested. Accordingly, $\delta^{18}\text{O}$ –

1 N₂O patterns are interpreted relative to reference scenarios spanning nitrate-dominated signatures, full ni-
2 trite–water equilibration, and intermediate exchange. In the following sections, we examine how $\delta^{18}\text{O}\text{--N}_2\text{O}$
3 varies across strains and incubation conditions as an observational framework, before explicitly quantifying
4 oxygen exchange in Sect. 3.4.2.

5
6

7 3.4.1 Strain-dependent $\delta^{18}\text{O}\text{--N}_2\text{O}$ patterns during active growth

8 A wide range of $\delta^{18}\text{O}\text{--N}_2\text{O}$ endpoint values was observed in natural-abundance water incubations under the
9 two active-growth modes (gas-flushed and closed-batch), as summarized in Fig. 6A. In closed-batch incuba-
10 tions, *P. aureofaciens* produced relatively low $\delta^{18}\text{O}\text{--N}_2\text{O}$ values ($\sim 9\text{‰}$), whereas *P. chlororaphis* yielded sub-
11 stantially higher values ($\sim 47\text{‰}$). Under gas-flushed conditions, $\delta^{18}\text{O}\text{--N}_2\text{O}$ endpoint values shifted in a strain-
12 specific manner: values for *P. aureofaciens* increased to an intermediate range ($26\text{--}42\text{‰}$), while *P. chlorora-*
13 *phis* produced consistently elevated values ($57\text{--}67\text{‰}$). These contrasts indicate a strong incubation-mode
14 sensitivity in *P. aureofaciens*, whereas *P. chlororaphis* maintained consistently elevated $\delta^{18}\text{O}\text{--N}_2\text{O}$ values with
15 a less pronounced response to incubation configuration. ~~whereas *P. chlororaphis* exhibits uniformly high~~
16 ~~$\delta^{18}\text{O}\text{--N}_2\text{O}$ expression with comparatively little sensitivity to incubation configuration.~~

17 Time-resolved $\delta^{18}\text{O}\text{--N}_2\text{O}$ trajectories during gas-flushed incubations further highlights this strain-dependent
18 behavior (Fig. 3C). In *P. aureofaciens*, $\delta^{18}\text{O}\text{--N}_2\text{O}$ starts at high values ($\sim 50\text{--}60\text{‰}$) early in nitrate reduction
19 and declines progressively toward $\sim 30\text{‰}$ as the reaction proceeds. In contrast, *P. chlororaphis* maintains ele-
20 vated $\delta^{18}\text{O}\text{--N}_2\text{O}$ values throughout the reaction, varying within a narrow band around 60‰ . Thus, under
21 identical conditions, *P. aureofaciens* shows strong variability in $\delta^{18}\text{O}\text{--N}_2\text{O}$, whereas *P. chlororaphis* maintains
22 consistently high values with comparatively little temporal change.

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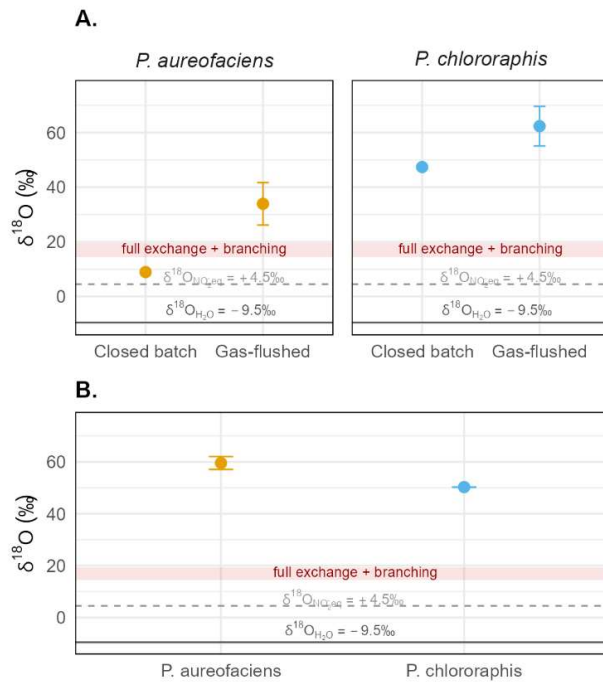


Figure 6. $\delta^{18}\text{O}$ – N_2O signatures for *Pseudomonas aureofaciens* (NirK) and *P. chlororaphis* (NirS) incubations in natural-abundance water. A) Active-growth incubations. Closed-batch experiments show $\delta^{18}\text{O}$ – N_2O of the accumulated N_2O pool ($n = 2$ per strain; mean $\pm$ SD). Gas-flushed experiments show final $\delta^{18}\text{O}$ – N_2O values from time-resolved incubations (*P. aureofaciens*, $n = 6$; *P. chlororaphis*, $n = 2$; mean $\pm$ SD). B) Resuspension closed-batch incubations following transfer of actively grown cells into fresh nitrate medium ($n = 2$ per strain; mean $\pm$ SD). Gas-flushed experiments show the final (endpoint) $\delta^{18}\text{O}$ – N_2O values from the time-resolved incubations (*P. aureofaciens*, $n = 6$; *P. chlororaphis*, $n = 2$; mean $\pm$ SD), whereas closed-batch experiments show the $\delta^{18}\text{O}$ – N_2O of the accumulated N_2O pool. Horizontal reference lines in both panels indicate $\delta^{18}\text{O}_{\text{H}_2\text{O}} (-9.5\text{‰})$ (solid grey) and $\delta^{18}\text{O}_{\text{NO}_2,\text{eq}} (+4.5\text{‰})$ (dashed grey). The shaded red band (+14.5 to +19.5‰) represents the expected $\delta^{18}\text{O}$ – N_2O range under assuming complete nitrite–water oxygen atom equilibration between nitrite and water, assuming a branching isotope effect of approximately 10–15‰ (Casciotti et al., 2002; Buchwald & Casciotti, 2010, 2007), full nitrite–water equilibration with branching fractionation.

To interpret these variable the observed variability in $\delta^{18}\text{O}$ – N_2O patterns, three reference scenarios can be defined. Following the framework of Casciotti et al. (2002;) and (2007) Buchwald & Casciotti (2010), nitrate reduction to nitrite introduces a fractionation is associated with an oxygen isotope fractionations offset of approximately 25–30‰, while NO_2^- reduction to N_2O is associated with a branching isotope effect of approximately 10–15‰. In the absence of oxygen atom exchange between nitrite and water, $\delta^{18}\text{O}$ – N_2O is therefore expected to result in values around to be approximately ~50‰, equivalent to reflecting the nitrate-source signature ($\delta^{18}\text{O}$ – $\text{NO}_3^- = +12.4 \pm 0.3\text{‰}$) shifted by the combined oxygen isotope effects described

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1 ~~above branching isotope effect~~. If nitrite fully equilibrates with water prior to reduction, its isotopic composi-
2 tion approaches $\delta^{18}\text{O}\text{--NO}_2^-\text{eq} = \delta^{18}\text{O}\text{--H}_2\text{O} + \epsilon^{18}\text{eq}$ (here +4.5 ‰), resulting in $\delta^{18}\text{O}\text{--N}_2\text{O}$ values of +14.5 to
3 +19.5 ‰ after addition of the branching isotope effect (Casciotti et al., 2002, ~~2007~~ Buchwald & Casciotti,
4 2010). The $\delta^{18}\text{O}$ of water itself (−9.5 ‰) represents a conceptual lower limit approached only by direct incor-
5 poration of O atoms from water with no kinetic isotope effects or equilibrium exchange. Because oxygen ex-
6 change requires sufficient residence time of nitrite, the transient NO_2^- accumulation observed during active
7 growth (Fig. 2E) provides a mechanistic basis for such exchange.

8 Against this framework, the two strains exhibit contrasting behavior. *P. aureofaciens*, in closed-batch incuba-
9 tions, yields $\delta^{18}\text{O}\text{--N}_2\text{O}$ values below the full exchange plus branching range, indicating extensive oxygen ex-
10 change, while gas-flushed conditions produce intermediate values (26–42 ‰), consistent with reduced but
11 still substantial exchange. In contrast, *P. chlororaphis* yields consistently high $\delta^{18}\text{O}\text{--N}_2\text{O}$ values under both
12 incubation modes (≈47–67 ‰), clustering near the nitrate-dominated regime and indicating limited equili-
13 bration with water.

14 These patterns are consistent with previous observations under comparable active-growth conditions (Rohe
15 et al., 2017; Haslun et al., 2018), but here can be interpreted explicitly in terms of oxygen exchange dynamics.
16 Taken together, these results show that $\delta^{18}\text{O}\text{--N}_2\text{O}$ reflects the combined influence of nitrate-derived oxygen,
17 oxygen-atom exchange with water, and branching fractionation, which vary with strain and incubation mode.
18 To quantify these contributions, we derived oxygen exchange rates using ^{18}O -enriched water experiments
19 (Sect. 3.4.2).

20 21 3.4.2 Quantifying oxygen exchange with water during active growth

22 To quantify oxygen exchange between nitrite and water during active growth under gas-flushed conditions,
23 *P. aureofaciens* (NirK) was incubated in ^{18}O -enriched water (GF_aur_en; Table 1). $\delta^{18}\text{O}\text{--N}_2\text{O}$ values began near
24 ~40 ‰, well below the $\delta^{18}\text{O}$ of the enriched water (+68 ‰; Fig. 7). As nitrate reduction progressed, $\delta^{18}\text{O}\text{--}$
25 N_2O increased steadily, crossed the $\delta^{18}\text{O}\text{--H}_2\text{O}$ level, and approached 80 ‰ toward the end of the incubation.
26 Throughout the experiment, $\delta^{18}\text{O}\text{--N}_2\text{O}$ remained below values anticipated for $\delta^{18}\text{O}\text{--NO}_2^-$ under the nitrite–
27 water equilibrium ($\delta^{18}\text{O}_{\text{NO}_2\text{eq}} \approx +82$ ‰) and well below the expected range for $\delta^{18}\text{O}\text{--N}_2\text{O}$ derived from fully
28 equilibrated nitrite water combined with branching fractionation ($\delta^{18}\text{O}_{\text{NO}_2\text{eq}} + \epsilon^{18}\text{B} \approx +91$ to +97 ‰; Fig. 7).
29 This trajectory – beginning below $\delta^{18}\text{O}\text{--H}_2\text{O}$, rising above it, and stabilizing below $\delta^{18}\text{O}_{\text{NO}_2\text{eq}}$ – indicates that
30 the N_2O product contains a mixture of nitrate-derived and water-exchanged O atoms, with incomplete ap-
31 proach to full nitrite–water equilibration. The initial $\delta^{18}\text{O}\text{--N}_2\text{O}$ values reflect expression of the branching iso-
32 tope effect under minimal water exchange, whereas the later ^{18}O enrichment reflects the combined influence
33 of branching fractionation and increasing nitrite–water exchange. This behavior is consistent with the ele-
34 vated $\delta^{18}\text{O}\text{--N}_2\text{O}$ endpoints observed for gas-flushed incubations in natural-abundance water (Fig. 3C; Fig.
35 6A).

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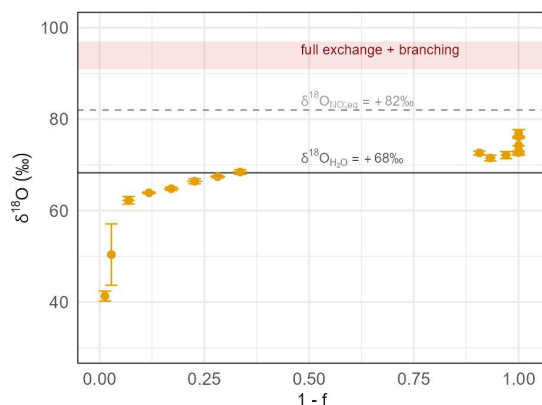


Figure 7. $\delta^{18}\text{O}\text{-N}_2\text{O}$ time series as a function of reaction progress ($1 - f$) for incubation of *P. aureofaciens* (NirK) in ^{18}O -enriched water. Horizontal reference lines indicate $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ (+68 ‰; solid grey) and $\delta^{18}\text{O}_{\text{NO}_2,\text{eq}}$ (+82 ‰; dashed grey). The shaded red band (+92 to +97 ‰) represents the expected $\delta^{18}\text{O}\text{-N}_2\text{O}$ range under full nitrite-water equilibration with branching fractionation. These references provide a framework to distinguish nitrate-derived oxygen, oxygen-atom exchange between nitrite-water, and branching-related ^{18}O enrichment.

To quantify these processes, $\delta^{18}\text{O}\text{-N}_2\text{O}$ values from natural-water and ^{18}O -enriched incubations were combined in $\delta^{18}\text{O}\text{-N}_2\text{O}$ vs. $\delta^{18}\text{O}\text{-H}_2\text{O}$ regressions (Fig. 8), following the framework of Casciotti et al. (2002). In this approach, the regression slope represents the fraction of oxygen atoms in N_2O derived from water during nitrite reduction, whereas the y-intercept at $\delta^{18}\text{O}\text{-H}_2\text{O} = 0$ ‰ isolates the component of the N_2O oxygen isotope signature independent of water isotopic composition. Under conditions of complete nitrite-water equilibration prior to reduction, the intercept approximates the sum of the nitrite-water equilibrium isotope effect and the branching isotope effect (i.e., $b \approx \epsilon^{18}_{\text{eq}} + \epsilon^{18}_{\text{B}}$; Casciotti et al., 2002, 2007). Deviations from this expectation indicate incomplete equilibration and/or retention of nitrate-derived oxygen in the N_2O product. Closed-batch incubations with *P. chlororaphis* (NirS) exhibited a slope of 0.67 ± 0.05 , consistent with partial water exchange (Fig. 8). Its intercept (53.7 ± 4.1 ‰) is strongly elevated relative to $\epsilon^{18}_{\text{eq}} + \epsilon^{18}_{\text{B}}$ (+24 to +30 ‰), reflecting combined contributions from nitrate-derived oxygen, incomplete exchange, and branching effects.

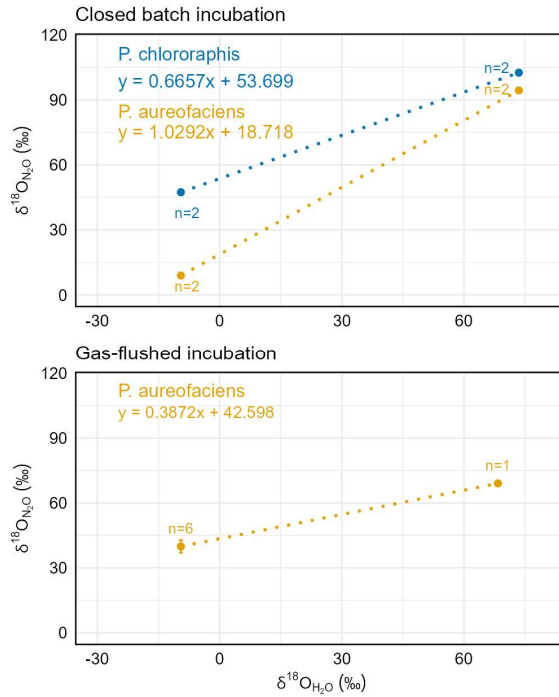


Figure 8. $\delta^{18}\text{O-N}_2\text{O}$ as a function of $\delta^{18}\text{O-H}_2\text{O}$ for both endpoint (closed batch) and gas-flushed incubations of *P. aureofaciens* (NirK) and *P. chlororaphis* (NirS). The regression slope from incubations in natural-abundance ($\delta^{18}\text{O-H}_2\text{O} \approx -9.5$ ‰) and ^{18}O -enriched water ($\delta^{18}\text{O-H}_2\text{O} \approx +68.3$ ‰ or $+73.5$ ‰) reflects the extent of oxygen-atom exchange between nitrite and water during stepwise N_2O formation. The intercept represents the $\delta^{18}\text{O-N}_2\text{O}$ value expected for incubation in water with $\delta^{18}\text{O-H}_2\text{O} = 0$ ‰, and thus reflects the combined effects of the nitrite–water equilibrium isotope effect and the branching isotope effect for the reaction of $\text{NO}_2^- \rightarrow \text{N}_2\text{O}$, plus any contribution from nitrate-derived oxygen that is not removed by nitrite–water exchange. The slope for *P. aureofaciens* in gas-flushed incubation (0.38) indicates partial exchange (38 %), whereas endpoint incubations for *P. aureofaciens* and *P. chlororaphis* suggest near-complete (≈ 100 %) and partial exchange (≈ 66 %), respectively.

For *P. aureofaciens*, closed-batch incubations yielded a slope of 1.03 ± 0.06 , indicating complete oxygen-atom exchange of nitrite with water during active growth. The corresponding intercept (18.7 ± 4.5 ‰) is at the lower end of values expected for $\epsilon^{18}_{\text{eq}} + \epsilon^{18}_{\text{B}}$. In contrast, gas-flushed incubation of *P. aureofaciens* yielded a slope of 0.38 ± 0.01 , indicating only partial oxygen-atom exchange. The intercept (42.6 ± 1.4 ‰) is substantially higher than both the $\epsilon^{18}_{\text{eq}} + \epsilon^{18}_{\text{B}}$ expectation and the fully exchanged closed-batch intercept, indicating strong retention of nitrate-derived oxygen and enhanced expression of branching fractionation under gas-flushed conditions.

1 These results demonstrate that oxygen isotope exchange in *P. aureofaciens* during active growth is highly
2 sensitive to incubation conditions, spanning from complete to strongly limited exchange. *In contrast, P. chlo-*
3 *roraphis maintained consistently high oxygen-atom exchange across the experimental conditions investi-*
4 *gated.* These contrasts suggest that the canonical low-exchange behavior attributed to NirK is not an intrinsic
5 property of the enzyme, but instead a condition-specific outcome of the denitrifier method.

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3.4.3 Stationary-phase resuspension incubations recover canonical Nir-dependent oxygen exchange

8 To assess whether exchange patterns observed during active growth reflect intrinsic enzyme behavior or ex-
9 perimental conditions, we performed resuspension experiments in which actively grown cultures were pel-
10 leted and transferred into a defined nitrate medium. This setup places both strains into a stationary-phase
11 reduction regime closely resembling the *bacterial* denitrifier method (Casciotti et al., 2002; Weigand et al.,
12 2015).

13 Resuspension in natural-abundance water produced high and strain-specific $\delta^{18}\text{O}$ - N_2O endpoints (Fig. 6B):
14 $59.6 \pm 2.5 \text{ ‰}$ for *P. aureofaciens* and $50.3 \pm 0.04 \text{ ‰}$ for *P. chlororaphis*. These values lie well above the range
15 expected under full nitrite-water equilibration (~ 14.5 – 19.5 ‰), indicating limited oxygen exchange and
16 strong retention of nitrate-derived oxygen, consistent with denitrifier-method observations.

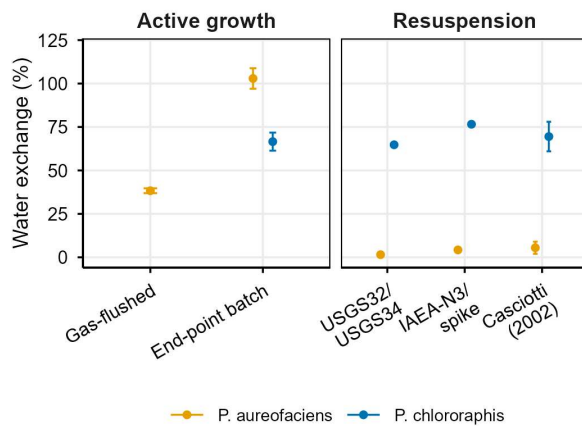
17 Because endpoint values do not directly constrain exchange, nitrate standards spanning a range of $\delta^{18}\text{O}$ -
18 NO_3^- values can be applied to quantify exchange from $\delta^{18}\text{O}$ - N_2O vs. $\delta^{18}\text{O}$ - NO_3^- regressions. Across both ni-
19 trate-standard pairs (USGS32–USGS34 and IAEA-N3– ^{18}O -enriched IAEA-N3), the two strains reproduced the
20 canonical low- versus high-exchange behavior reported previously (Casciotti et al., 2002; Appendix B Fig. B2).
21 *P. aureofaciens* exhibited low oxygen exchange (1.5–4.3 %), whereas *P. chlororaphis* showed substantially
22 higher exchange (64.7–76.6 %), in close agreement with published ranges. These results confirm that resus-
23 pension experiments recover the expected strain-specific exchange behavior under denitrifier-method condi-
24 tions.

3.5 Physiological controls on N_2O isotopic signatures: implications for the denitrifier method and in- 27 terpretation of N_2O isotopocules

28 The combined isotopic evidence presented here demonstrates that $\delta^{18}\text{O}$ - N_2O is strongly controlled by physi-
29 ological state and experimental conditions, rather than reflecting fixed enzyme-specific properties. Oxygen
30 isotope signatures are modulated by the balance between nitrate-derived oxygen, nitrite-water equilibration,
31 and branching processes, all of which vary with metabolic activity and reaction environment.

32 A direct comparison across incubation regimes (Fig. 9) reveals a clear contrast. During active growth, oxygen
33 exchange is highly variable: *P. aureofaciens* spans from partial to near-complete exchange ($\sim 38 \text{ ‰}$ under gas-
34 flushed conditions to $\sim 100 \text{ ‰}$ in closed-batch incubations), whereas *P. chlororaphis* exhibits consistently
35 higher but more stable exchange ($\approx 66 \text{ ‰}$). In contrast, under stationary-phase resuspension conditions, both
36 strains reproduce the *canonical-characteristically* low- versus high-exchange behavior reported for the deni-
37 trifier method, with *P. aureofaciens* showing low exchange (≈ 2 – 4 ‰) and *P. chlororaphis* high exchange (~ 65 –
38 75 ‰).

1 This contrast demonstrates that the widely used $\delta^{18}\text{O}$ - N_2O signatures commonly associated with NirK and
 2 NirS are not intrinsic enzyme properties, but arise under the constrained conditions of the denitrifier method.
 3 Outside this experimental framework, oxygen-atom exchange is dynamic and reflects physiological state ra-
 4 ther than nitrite reductase identity alone.



6 **Figure 9.** Compilation of water exchange (%) for *P. aureofaciens* (NirK) and *P. chlororaphis* (NirS) under active growth and
 7 resuspension conditions. Exchange during active growth was derived from $\delta^{18}\text{O}$ - N_2O versus $\delta^{18}\text{O}$ - H_2O relationships us-
 8 ing ^{18}O -enriched water (Fig. 8). Under resuspension conditions, exchange was quantified from $\delta^{18}\text{O}$ - N_2O versus $\delta^{18}\text{O}$ -
 9 NO_3^- regressions using nitrate standards spanning a range of $\delta^{18}\text{O}$ values (USGS32/34, IAEA-N3 ± spike), based on the
 10 degree of scale compression. These two independent approaches provide consistent constraints on oxygen exchange.
 11 For active growth, *P. aureofaciens* showed 38.3 ± 1.4 % (gas-flushed) and 102.9 ± 5.9 % (end-point batch) exchange,
 12 whereas *P. chlororaphis* showed 66.6 ± 5.2 % (end-point batch). Closed triangles indicate reference values from Casciotti
 13 et al. (2002), plotted at the midpoint of reported ranges (2–9 % for *P. aureofaciens*, 61–78 % for *P. chlororaphis*).
 14

16 A mechanistic explanation for this variability likely lies in the dynamics of the nitrite intermediate pool (Cas-
 17 ciotti et al., 2007). Oxygen exchange between nitrite and water requires sufficient residence time of NO_2^- to
 18 approach isotopic equilibration. During active growth, differences in nitrate reduction rates, nitrite accumula-
 19 tion, and consumption can alter both the size and lifetime of this intermediate pool, thereby modulating the
 20 extent of exchange. In addition, because actively growing cultures simultaneously undergo biomass synthesis
 21 and enzyme expression, nitrate reduction and downstream nitrite reduction may be less tightly coupled than
 22 in resuspension assays, allowing nitrite to persist longer before reduction. In contrast, uUnder resuspension
 23 conditions, fully induced cells rapidly reduce the supplied nitrate, and tighter coupling between nitrate and
 24 nitrite reduction likely limits nitrite accumulation and, consequently, constrains oxygen isotope exchange.
 25 Additional physiological factors, such as pH-dependent exchange kinetics (Buchwald and Casciotti, 2013), or
 26 changes in the cellular microenvironment, might also contribute but could not be resolved in the present

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1 ~~study and therefore warrant future investigation.~~ These observations indicate that oxygen exchange reflects
 2 system-level metabolic dynamics rather than fixed enzymatic behavior.

3 Consistent strain-specific differences in $\delta^{18}\text{O}$ - N_2O under active-growth conditions have been reported previ-
 4 ously (Rohe et al., 2017; Haslun et al., 2018; Sect. 3.4.1). Their agreement with the patterns observed here
 5 suggests that such behavior may be more ~~general, but~~ general but has not been systematically evaluated
 6 within a process-based framework.

7 These findings have important implications for the interpretation of N_2O isotopocules. Application of fixed
 8 $\delta^{18}\text{O}$ - N_2O endmember ranges assumes that oxygen exchange is stable and pathway-specific. The results pre-
 9 sented here demonstrate instead that $\delta^{18}\text{O}$ - N_2O is condition-dependent, and that variability in oxygen iso-
 10 tope signatures reflects changes in exchange dynamics rather than differences in nitrite reductase type. As a
 11 result, $\delta^{18}\text{O}$ - N_2O does not provide a reliable tracer of nitrite reductase identity (NirK vs. NirS) outside the
 12 constrained conditions of the denitrifier method. Consequently, exchange characteristics derived from deni-
 13 trifier-method experiments should not be extrapolated to microbial systems without accounting for physio-
 14 logical context. ~~These findings do not imply that existing isotopic signatures currently applied used to identify~~
 15 ~~N_2O produced by denitrification endmembers should be replaced by a single, much broader universal inter-~~
 16 ~~val range of delta values range. Instead, Rather, they demonstrate that physiological state of the microbial~~
 17 ~~community should be considered explicitly when defining and applying isotopic endmembers signatures for~~
 18 ~~environmental interpretation. Failure to do so risks systematic misinterpretation of N_2O production pathways.~~
 19 More generally, accurate interpretation of $\delta^{18}\text{O}$ - N_2O requires frameworks that explicitly incorporate meta-
 20 bolic state, intermediate dynamics, and environmental conditions, rather than relying on static enzyme-based
 21 classifications.

22 4. Conclusion

23 The results presented here demonstrate that N_2O isotopic signatures are strongly controlled by physiological
 24 state and experimental conditions, rather than reflecting fixed enzyme-specific properties. In particular, $\delta^{18}\text{O}$ -
 25 N_2O varies extensively with oxygen atom exchange between nitrite and water, which depends on metabolic
 26 activity and reaction context. Only under stationary-phase resuspension conditions do enriched-water and
 27 nitrate-standard approaches reproduce the characteristic exchange behavior reported in classical denitrifier-
 28 method studies. This shows that the widely used $\delta^{18}\text{O}$ - N_2O exchange rates are specific to the denitrifier
 29 method and do not represent general physiological behavior, but instead reflect the constrained conditions
 30 under which the method operates. As a result, $\delta^{18}\text{O}$ - N_2O cannot be interpreted as a reliable tracer of nitrite
 31 reductase identity outside these conditions.

32 Site preference (SP) measurements provide complementary, time-resolved insight into N_2O formation path-
 33 ways during active growth. SP trajectories exhibit a reproducible pattern, with early high values (+10 to +20
 34 ‰) followed by a transition toward values near 0 ‰. These dynamics ~~indicate suggest~~ transient activity of
 35 alternative NO reductases, such as flavohemoglobins, during the onset of denitrification, before canonical
 36 NorB-mediated NO reduction dominates. Importantly, these shifts occur within the same denitrifying micro-
 37 organisms and suggest that temporary engagement of NO detoxification pathways may contribute to N_2O
 38 formation during phases of elevated NO stress.

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1 These findings have important implications for environmental interpretation of N₂O isotopocules. The appli-
2 cation of fixed $\delta^{18}\text{O}$ -N₂O or SP endmember ranges assumes that isotopic signatures are stable and pathway-
3 specific constants. Our results demonstrate instead that isotopic expression is conditional. For example, SP
4 values in the range of ~5–15 ‰, ~~are~~ often interpreted as ~~an~~ evidence for mixed or non-denitrification
5 sources when using fixed endmember frameworks (e.g., Opdyke et al., 2008). ~~However, they, could~~ arise
6 entirely from denitrification through shifts in NO reduction pathways. Similarly, variability in $\delta^{18}\text{O}$ -N₂O may
7 reflect changes in oxygen exchange dynamics rather than differences in nitrite reductase type, and denitrifi-
8 cation may give rise to N₂O with a broader range in $\delta^{18}\text{O}$ -N₂O than is typically assumed. ~~Moreover, abiotic~~
9 ~~nitrite reactions, including chemodenitrification and reactions between nitrite and organic matter, have also~~
10 ~~been shown to produce a broad range of SP values overlapping those of biological pathways (Jones et al.,~~
11 ~~2015; Visser et al., 2022; Wei et al., 2019). Together, these findings further emphasize that N₂O isotopocules~~
12 ~~should be interpreted within their physiological and environmental context rather than as unique process-~~
13 ~~specific fingerprints.~~

14 Accurate interpretation of N₂O isotopocules therefore requires frameworks that explicitly account for physio-
15 logical state, intermediate dynamics, and environmental context, rather than relying on static metabolism or
16 enzyme-based classifications. ~~Instead of Rather than treating~~ defining and interpreting isotopic signatures
17 ~~denitrification endmembers as fixed constants, our results support~~ to consider ~~of that they~~
18 should be interpreted in the context of defining and interpreting isotopic endmembers within the physiologi-
19 cal and environmental conditions under which they were established. Constraining process signatures under
20 more realistic environmental conditions (Strubbe et al., 2026) will be essential for improving the interpreta-
21 tion of N₂O isotopic signatures in natural systems. Integrating isotopocule measurements with approaches
22 that resolve metabolic activity and enzyme expression may further help link isotopic signatures to specific
23 microbial processes.

24 **Data availability**

25 The datasets generated and analysed during this study are provided as a supplementary Excel file accompa-
26 nying this article. Custom analysis scripts are available from the corresponding author upon request.

27 **Competing interests.** The authors declare that they have no conflict of interest.

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32 and figure generation, as well as for text editing, paragraph polishing, and grammar correction during the
33 manuscript preparation. All scientific interpretation, analytical decisions, and final manuscript content were
34 reviewed and verified by the authors.

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Appendix A

A1. Calibration of isotopic data provided by Quantum Cascade Laser Absorption Spectroscopy (QCLAS)

Isotopic composition ($\delta^{15}\text{N}^\alpha$, $\delta^{15}\text{N}^\beta$, $\delta^{18}\text{O}$) of N₂O was derived from uncorrected QCLAS output values (TDL Wintel, Aerodyne Research, USA) using a dedicated R script (IsotopeRead 2.0, N. Chénier, Empa). The processing pipeline includes (i) temporal averaging, (ii) extraction of sample and calibration-gas intervals via external command language (ECL) indices, (iii) drift correction to reference gas CG1, (iv) calibration-factor

1 determination from CG1–CG2 differences, and (v) uncertainty propagation based on Allan deviation and sig-
2 nal intensity.

3 **Data averaging and ratio calculation**

4 QCLAS output data are reported using HITRAN-style shorthand (e.g., 446 equivalent to $^{14}\text{N}^{14}\text{N}^{16}\text{O}$) and aver-
5 aged over 1-minute time intervals. Isotopologue ratios (e.g. R448) are computed from the concentration of
6 the minor isotopologue (e.g. i448) to the 446 isotopologue:

- 7 • $R448 = i448/i446$
- 8 • $R456 = i456/i446$
- 9 • $R546 = i546/i446$

10 Temperature stability of the laser and optical cell was verified through automated plotting.

11 **Identification of gas sample and calibration intervals**

12 ECL indices assigned during acquisition were used to identify sample blocks and the two calibration gases
13 (CG1 and CG2). Only intervals with the correct predefined length were retained to ensure consistent calibra-
14 tion.

15 **Calibration gases and normalization**

16 Two N_2O isotope reference gases diluted in N_2 and calibrated to international isotope-ratio scales (Air– N_2 for
17 $^{15}\text{N}/^{14}\text{N}$, VSMOW for $^{18}\text{O}/^{16}\text{O}$) by several expert laboratories were used (Mohn et al., 2022):

18 **CG1 (RM1A; anchor gas):**

19 $\delta^{15}\text{N}^\alpha = -0.22 \pm 0.46 \text{ ‰}$,

20 $\delta^{15}\text{N}^\beta = 0.84 \pm 0.46 \text{ ‰}$,

21 $\delta^{18}\text{O} = 39.22 \pm 0.15 \text{ ‰}$

22 **CG2 (RM3A; span gas):**

23 $\delta^{15}\text{N}^\alpha = 50.96 \pm 0.47 \text{ ‰}$,

24 $\delta^{15}\text{N}^\beta = 53.06 \pm 0.47 \text{ ‰}$,

25 $\delta^{18}\text{O} = 103.04 \pm 0.16 \text{ ‰}$

26 For each sample block, isotopologue ratios were normalized by dividing the sample ratio by the mean CG1
27 ratio measured immediately before and after the sample:

28
$$R_X = \frac{R_{X,\text{sample}}}{R_{X,\text{CG1}}}$$

29 **Calibration-factor determination**

30 Calibration factors (a_x) were calculated from the measured ratio differences between CG1 and CG2 and the
31 known isotopic differences:

32
$$a_x = \frac{\Delta\delta_x^{(\text{true})}}{\Delta R_{X,\text{meas}}}$$

33 Uncertainty in a_x incorporates both the assigned uncertainty of CG1 and CG2 and the variance of their meas-
34 ured ratios.

35 Corrected delta values were computed as:

$$\delta X = \left(\frac{R_x}{R_{x_{GG1}}} - 1 \right) \times 1000 \times \alpha x + \delta X_{GG1}$$

Uncertainty estimation

Instrumental precision was assessed using the Allan deviation at 60 s spectral averaging and was better than 0.1 ‰ for all δ values.

Uncertainty in $\delta^{15}\text{N}^\alpha$, $\delta^{15}\text{N}^\beta$, and $\delta^{18}\text{O}$ was assessed by propagating contributions from (i) Allan deviation of the relevant isotopologue ratios, (ii) signal-intensity variability (i446 and minor isotopologues), and (iii) variability in calibration-factors.

The uncertainty for derived quantities was calculated using standard error-propagation rules. For example, site preference ($\text{SP} = \delta^{15}\text{N}^\alpha - \delta^{15}\text{N}^\beta$) uncertainty was calculated as:

$$\sigma_{\text{SP}} = \sqrt{\sigma_\alpha^2 + \sigma_\beta^2}$$

Uncertainty in $\delta^{15}\text{N}$ -bulk and $\delta^{18}\text{O}$ was propagated analogously from their respective isotopologue components.

Data output

Corrected delta values with propagated uncertainties were exported as .csv files. Diagnostic plots (SP , $\delta^{15}\text{N}$ -bulk, $\delta^{18}\text{O}$) were automatically generated for quality assurance.

All processing was performed in RStudio (RStudio Team, 2020). The full script (IsotopeRead 2.0) is available upon request.

A2. Calculation of N_2O yield and fractionation factors

N_2O yield and isotopic fractionation as well as their uncertainties were calculated using a customized R script (FractionationFactors 1.0, developed by N. Chénier, Empa).

The molar amounts of N_2O ($n\text{N}_2\text{O}$) produced during each time interval ΔT were calculated from FTIR-derived concentrations (C_{gas} in ppm; 10^{-6} L L^{-1}) and nitrogen gas flow rates (Q in mL min^{-1}), using a molar volume of 22.4 L mol^{-1} . NO was treated analogously (n_{NO}), considering concentrations which exceed the analytical detection limit. For intervals where NO remained below detection, n_{NO} was not calculated, and no NO yield was reported.

$$n\text{N}_2\text{O}_{\text{gas}} = \left(\frac{\left(\frac{Q \times \Delta T}{22.4} \right) \times \left(\frac{C_{\text{gas}}}{10^6} \right)}{1000} \right)$$

Uncertainties in N_2O production were calculated by propagation through partial derivatives of the above expression, incorporating both flow and concentration uncertainties. The uncertainty of the measured N_2O and NO concentrations was attributed to either the instrumental detection limit (0.0075 ppm for N_2O and 0.133 ppm for NO), or a relative uncertainty of 10 % for both N_2O and NO, with the larger value used at each time point. Uncertainty in the nitrogen flow rate was assumed to be 0.3 % based on mass flow controller specifications (Vögtlin Instruments Inc., Switzerland).

1 The N₂O yield (in %) was calculated from the molar amount of N₂O produced over the experiment related to
 2 the molar amount of nitrogen substrate (KNO₃, molar mass 101.1 g mol⁻¹). Associated uncertainty was propa-
 3 gated from balance precision (±0.01 g) and purity (≥99 %, conservatively estimated at ±0.5 %).

4 To calculate Rayleigh-type enrichment factors (ε), the isotopic composition of the accumulated N₂O product
 5 gas (δ_{cum}) is required. This was calculated as a weighted average from temporal trends in delta values (δ_i)
 6 weighted by the molar amount of N₂O (n_{iN2O}) emitted in the respective time interval. Calculations were per-
 7 formed on synchronized data averaged over 15-minute time intervals:

$$\delta_{cum} = \frac{\sum(\delta_i \times n_{iN2O})}{\sum n_{iN2O}}$$

8
 9 Uncertainties in δ_{cum} were computed from uncertainties in individual δ-values and the associated molar
 10 amount of N₂O. Uncertainties in δ_{cum} were propagated by summing the individual variances associated with
 11 δ-values and their corresponding N₂O fluxes. At each time step, the square root of the cumulative variance
 12 yielded the standard error used to construct time-resolved confidence intervals for δ¹⁵N-bulk, δ¹⁸O, and SP.
 13 The fraction of remaining substrate (f) in the Rayleigh reaction progress variable $x = -f \cdot \ln(f) / (1 - f)$ was calcu-
 14 lated as:

$$f = 1 - \left(\frac{2 \times n_{NO3-}}{n_{N2O}} \right)$$

15
 16 Uncertainties in f and x were propagated from uncertainties in the molar amount of substrate (n_{NO3-}), calcu-
 17 lated from the nitrate mass and its molecular mass, and the cumulative molar amount of N₂O.

18 Enrichment factors (ε) were obtained by Rayleigh-type regressions, plotting δ values of the accumulated N₂O
 19 product gas against the transformed progress variable.

20 Daily averages, cumulative profiles, and model fits were exported as CSV files. Summary statistics include re-
 21 gression intercept, slope, standard errors, and confidence intervals for each isotopic parameter.

22

1 Appendix B

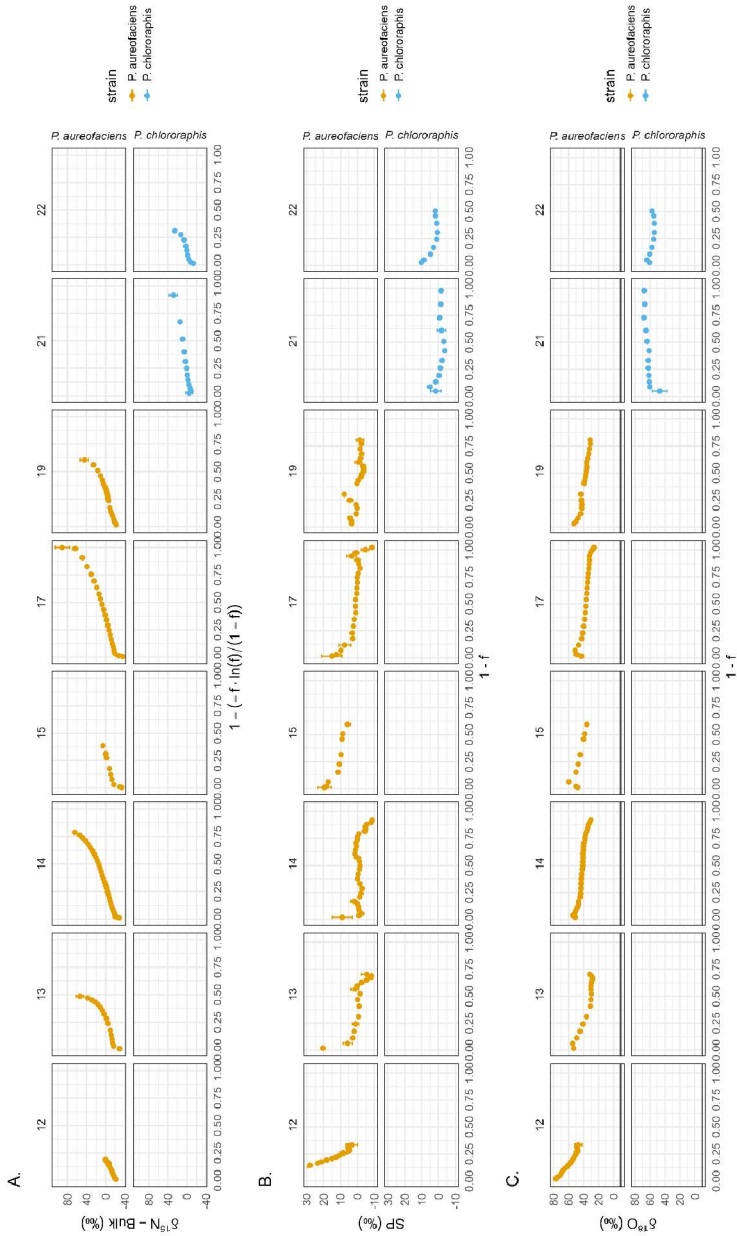


Figure B1. Individual time series for all gas-flushed active-growth incubation experiments shown in Fig. 3. Panels are faceted by strain and by experiment number (numbers at the top of each panel correspond to individual incubation IDs). A. $\delta^{15}\text{N}$ -bulk, B. site preference (SP), and C. $\delta^{18}\text{O}$ are plotted as a function of reaction progress, expressed as either $1 - f$ or $1 - (-f \ln f)/(1 - f)$, as indicated on the x-axis. Each point represents an individual measurement; vertical error bars denote analytical uncertainty, and horizontal error bars reflect uncertainty in reaction progress. No binning or normalization across experiments has been applied. In panel C, the short horizontal line in each subplot marks the $\delta^{18}\text{O}$ of water (-9.5‰). These plots illustrate experiment-to-experiment variability underlying the binned trends presented in the main text.

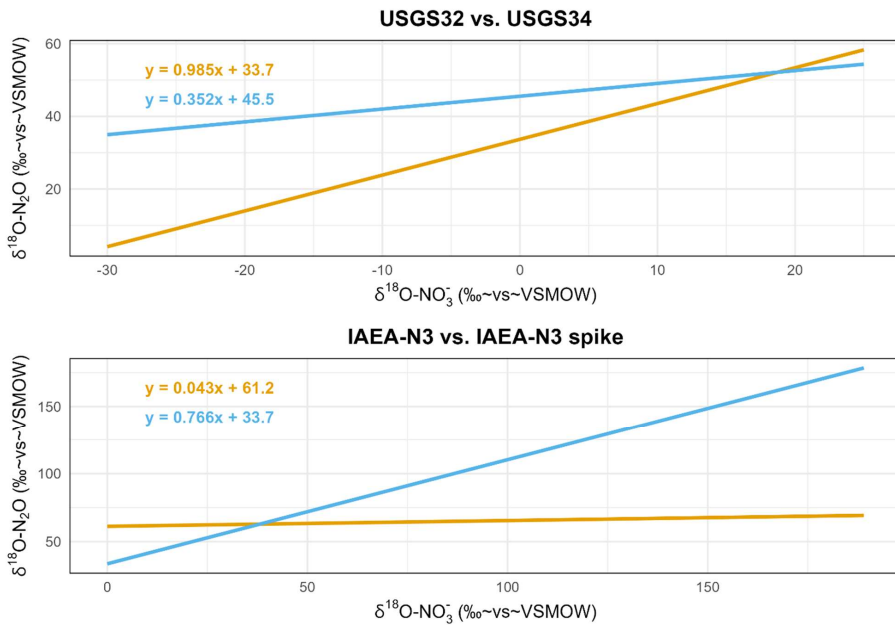


Figure B2. Relationship between $\delta^{18}\text{O-N}_2\text{O}$ and $\delta^{18}\text{O-NO}_3^-$ in resuspension incubations with international nitrate standards (USGS32/USGS34; IAEA-N3 vs. $\delta^{18}\text{O}$ -enriched IAEA-N3 spike) for *P. aureofaciens* (orange) and *P. chlororaphis* (blue). Regression slopes quantify the fraction of nitrate-derived oxygen retained in N_2O . For USGS32/USGS34 comparisons, water exchange is calculated as $1 - \text{slope}$, yielding 1.54 % exchange for *P. aureofaciens* (slope = 0.9846) and 64.8 % for *P. chlororaphis* (slope = 0.3524). For IAEA-N3/spike comparisons, water exchange is estimated directly from the slope, yielding 4.26 % exchange for *P. aureofaciens* (slope = 0.043) and 76.6 % for *P. chlororaphis* (slope = 0.766).