

On-line analysis of N₂O isotopic composition during biological nitrogen removal in wastewater treatment to disentangle production and reduction processes

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Abstract. Nitrous oxide (N₂O) is a potent greenhouse gas, and emissions from wastewater treatment plants (WWTPs) represent a significant and highly variable source. Understanding the dynamics in microbial pathways of N₂O formation and reduction during biological nitrogen removal is essential for targeted mitigation strategies. Stable isotope analysis of N₂O ($\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, $\delta^{18}\text{O}$, and ^{15}N site preference) provides a powerful tool to disentangle and quantify N₂O production and reduction processes, yet conventional analytical approaches lack temporal resolution. Here, we present the first long-term application of an off-axis integrated cavity output spectrometer for real-time N₂O isotopic analysis at a pilot-scale WWTP over one year of operation. We developed a dynamic dilution system and implemented correction protocols for drift, N₂O mole fraction dependence, and gas matrix effects on isotopic results, achieving uncertainties of 0.8 ‰ ($\delta^{15}\text{N}^{\alpha}$), 1.1 ‰ ($\delta^{15}\text{N}^{\beta}$), 0.8 ‰ ($\delta^{15}\text{N}^{\text{bulk}}$), 0.5 ‰ ($\delta^{18}\text{O}$) and 1.1 ‰ (^{15}N site preference). Representative datasets demonstrate the system's capability to (i) identify dominant N₂O production pathways under standard WWTP operation, (ii) quantify N₂O reduction in relation to dissolved oxygen concentration, and (iii) trace nitrogen transformation during low-level ^{15}N -labelling experiments. Our results indicate nitrifier or heterotrophic denitrification as the main source of N₂O, and that N₂O reduction efficiency is strongly controlled by oxygen availability. This study highlights the potential of laser spectroscopy for continuous isotopic monitoring in real-world engineered systems and provides practical guidelines for uncertainty reduction and data interpretation. More specifically, our work forms a foundation for further investigations of the operational factors controlling N₂O formation and N₂O reduction in biological WWTPs and other complex anthropogenically-perturbed settings.

30 1. Introduction

Nitrous oxide is one of the most important greenhouse gases in the atmosphere, with a 100-year global warming potential of 273 CO₂ equivalents and a relative contribution to the total radiative forcing of about 6 % (IPCC, 2021). Anthropogenic activities enhance N₂O emissions, outweighing stratospheric destruction and leading to an increase in atmospheric N₂O mole fractions from 1750 to 2022 by about 25 % with an annual growth rate currently exceeding 1 ppb yr⁻¹ (WMO, 2024). On a global scale the largest share of man-made emissions is attributed to the agricultural sector, followed by fossil fuel combustion and industrial processes (Tian et al., 2024). N₂O emissions from wastewater treatment plants (WWTP) have long been underestimated, but long-term full-scale monitoring data have provided evidence for higher and more variable emission factors than had previously been assumed (Daelman et al., 2015; Gruber et al., 2021; Kosonen et al., 2016). As N₂O emission monitoring is not part of normal operation control at WWTPs, countries and WWTP facilities still rely on using emission factors for their reporting. Emission factors for N₂O from wastewater treatment were adjusted to 1.6 % of the total nitrogen load, as compared to a previously applied value as low as 0.035 % (EEA, 2017; IPCC, 2019). However, different emission factors are still implemented for reporting in European countries (EEA, 2023). Moreover, emissions were found to be related to plant operation (Gruber et al., 2021), and intermittency controlled by problematic situations, such as hampered microbial conversion or addition of supernatant from anaerobic digestion (Froemelt et al., 2025; Gruber et al., 2021). Applying a country-specific approach, Switzerland's National Greenhouse Gas Inventory reports wastewater treatment as the second largest source of N₂O accounting for about 20 % of national N₂O emissions, only topped by agriculture (BAFU, 2025). Consequently, the relevance of WWTPs as N₂O emission point-sources and their controlled operation makes them attractive targets for N₂O emission mitigation.

Nitrous oxide from WWTPs mainly originates from the biological nitrogen removal process (Kampschreur et al., 2009). In a conventional biological nitrogen removal process, N₂O is primarily produced by three microbial pathways mediated by two bacterial groups. Bacterial ammonium oxidizers emit N₂O during two processes, namely hydroxylamine oxidation (Hy) and nitrifier denitrification (nD). Hydroxylamine oxidation forms N₂O as a side product during NH₄⁺ oxidation to NO₂⁻. Nitrifier denitrification reduces NO₂⁻ to N₂O with NH₄⁺ as the electron donor. The second bacterial group, the ordinary heterotrophic organisms, couple oxidation of organic substrates to reduction of NO₃⁻, NO₂⁻, NO, and N₂O in heterotrophic denitrification (hD). During hD NO and N₂O can be emitted as obligatory intermediates. However, hD can also act as a N₂O sink process, reducing N₂O to N₂. The relative contributions and interactions of these pathways in a process workflow with variable control parameters and substrate availability remain poorly understood (Law et al., 2012). A comprehensive assessment of the active biological processes and their interplay is crucial for a targeted control and optimization of reactor and process design to minimize N₂O emissions, while maintaining efficient nitrogen removal.

Analyzing the stable isotopic composition (¹⁵N/¹⁴N, ¹⁸O/¹⁶O) of the asymmetric N₂O molecule has proven to be a powerful tool to disentangle N₂O production pathways and to quantify the extent of N₂O reduction to N₂ in laboratory and full-scale WWTP settings (Gruber et al., 2022; Harris et al., 2015; Tumendelger et al., 2016; Wunderlin et al., 2013; Toyoda et al.

2011). The relative abundances of the rare N₂O isotopologues ¹⁴N¹⁵N¹⁶O (¹⁵N in the central, α position), ¹⁵N¹⁴N¹⁶O (¹⁵N in the terminal, β position), and ¹⁴N¹⁴N¹⁸O, relative to the main isotopic species (¹⁴N¹⁴N¹⁶O) in a sample are expressed relative to standards (air-N₂ for ¹⁵N/¹⁴N, VSMOW for ¹⁸O/¹⁶O) in the δ-notation (Camin et al., 2025; Toyoda and Yoshida, 1999). Transformations during metabolic pathways (i.e. Hy, hD and nD) are associated with kinetic or equilibrium fractionation processes and leave their imprint on the isotopic composition of the emitted N₂O (Toyoda et al., 2015). An important characteristic of a N₂O formation process is the difference in ¹⁵N substitution in the central and terminal position, named site preference (SP = δ¹⁵N^α - δ¹⁵N^β), which is mainly controlled by the N-N bond formation (Toyoda et al., 2015). Data are often presented in a dual isotopic plot of SP versus δ¹⁸O or SP versus δ¹⁵N^{bulk} (δ¹⁵N^{bulk} = (δ¹⁵N^α + δ¹⁵N^β) / 2) and compared to isotopic signatures retrieved from pure culture studies to aid the identification of N₂O source processes (Yu et al., 2020). Furthermore, during N₂O reduction to N₂ by hD, N₂O molecules in which ¹⁴N is bound to ¹⁶O are preferentially reduced, leading to an enhancement in δ¹⁵N^{bulk}, SP, and δ¹⁸O in the remaining N₂O pool (Lewicka-Szczebak et al., 2017; Ostrom et al., 2007). This observation can be exploited to calculate the residual, non-reduced fraction of N₂O (f_{N2O}), if the isotopic signature of the initial unaffected N₂O pool for either δ¹⁵N^{bulk}, SP, or δ¹⁸O (i.e. δ₀) is known and the corresponding composition of the remaining N₂O fraction (δ_R) is measured (Lewicka-Szczebak et al., 2017; Mariotti et al., 1981; Ostrom et al., 2007):

$$\delta_R - \delta_0 = \varepsilon \ln f_{N2O} \quad (1)$$

This calculation utilizes literature estimates for ε, the enrichment factor for N₂O reduction, here defined so that a negative value corresponds to a mass-dependent isotope effect, i.e. the light isotope reacts faster than the heavier one (Yu et al., 2020; Ostrom et al. 2007). The fraction of reduced N₂O, i.e. the fraction of formed N₂ (f_{N2}), can be calculated by f_{N2} = 1 - f_{N2O}. To date, however, most N₂O isotope studies, whether using natural isotopic abundance or ¹⁵N or ¹⁸O-labelling, have quantified N₂O reduction using bag sampling and subsequent laboratory analysis, which is labour intensive and offers only limited temporal resolution, incapable of tracing process changes at timescales relevant for WWTPs that exhibit strong daily as well as seasonal dynamics (Domingo-Félez et al., 2024; Gruber et al., 2020).

Laser absorption spectroscopy offers the potential for continuous data on the isotopic composition of greenhouse gases, but apparent δ-values are dependent on the composition of the analyte gas and need correction schemes to produce accurate data (e.g. Sperlich et al., 2024). The fundamental reasoning behind these phenomena is the difference in pressure broadening caused by different bulk gases (e.g. N₂, O₂, Ar) and spectral interferences by non-target gaseous species with absorptions in the analysed spectral range. Furthermore, changes in analyte gas concentration can lead to inaccurate apparent δ-values due to detector non-linearities, residual baseline effects or other phenomena. It is therefore good practice to apply isotopic reference gases, which mimic the composition of the sample in target gas concentration, matrix composition and relevant trace gases. In this respect, process studies with strong changes in gas composition pose an inevitable challenge, as the reference gas can only be adapted to the most relevant or average sample gas composition, while residual variability has to be monitored and δ-values post-corrected, if changes pass a critical threshold. In recent years, different optical detection schemes, such as direct absorption spectroscopy, cavity ring-down spectroscopy and off-axis integrated cavity output

spectroscopy (OA-ICOS) have been implemented for analysis of N₂O isotopic composition (e.g. Harris et al., 2020). Analyser models from different manufacturers, including the OA-ICOS spectrometer model applied here, have been tested under laboratory settings (Harris et al., 2020) and the mathematical formalism for data processing has been implemented and validated (Havsteen et al., 2025). However, until now, very few long-term applications of laser spectroscopy for N₂O isotope analysis under field conditions have been realized.

Here, we implemented and tested a laser spectroscopic platform capable of real-time analysis of N₂O mole fractions and isotopic composition. We demonstrate the first on-line measurements during aeration phases at a pilot-scale WWTP over one year of operation to illustrate its potential for process identification. More specifically, we present three representative data sets: the first originates from periods of standard reactor operation that will help us distinguishing between the N₂O production pathways of Hy and denitrification (nD and hD); the second aims at exploring the influence O₂ availability on the N₂O reduction dynamics; and the third to demonstrate the systems potential for N₂O isotope analysis in low-level ¹⁵N labelling studies. We provide practical recommendation on how to reduce contributions to uncertainty in δ -values from variable gas composition and give guidelines on data analysis and uncertainty assessment.

2. Methods

2.1 Laser spectroscopic platform

An OA-ICOS (N₂OIA-30e-EP, model 914-0027, serial number: 14-0283, ABB – Los Gatos Research Inc., USA) was used to analyse the abundance of gaseous N₂O and its isotopic composition ($\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, $\delta^{18}\text{O}$). The analyser uses a quantum cascade laser emitting at 2192.1–2192.5 cm⁻¹, which covers the target species but also spectral lines of CO₂ and CH₄ (Harris et al., 2020). The analyser has a built-in sample pump which maintains the target pressure in the cavity (60.12 hPa) and a sample gas flow of about 200 mL min⁻¹. The analyser software displays the absorption signal and provides N₂O mole fractions and δ -values in real-time at maximum temporal resolution of one spectrum per second. Further information on the analytical platform is described in Baer et al. (2002).

2.2 Identify optimal measurement conditions for wastewater treatment

In this study we target real-time data analysis using a practical instrumental approach, which can be implemented at WWTPs. Analysing N₂O from the undiluted source process for source attribution, we aim at a maximum uncertainty of 1 ‰ for $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{18}\text{O}$ and SP for final corrected δ -values. In the following sections, optimal averaging times, drift correction and calibration approaches, as well as uncertainty contributions from variable gas composition are evaluated. We followed the mathematical formalism and adapted the MATLAB algorithm provided by Havsteen et al. (2025).

130 In an initial test phase, important characteristics of the analyser performance were determined in accordance with Harris et al. (2020) to define a strategy for real-time measurements at WWTPs. Optimal averaging times to reach adequate precision levels (i.e. 0.2 ‰) and drift effects were determined using the Allan variance technique (Allan, 1966; Werle et al., 1993). For this, a gas mixture with known N₂O isotopic composition (Cal1, see Tab. 1) was dynamically diluted with N₂O-free synthetic air (SA) to a mole fraction of 12 ppm N₂O and a total flow of 300 mL min⁻¹ and measurements were recorded for more than 3.5 days. The Allan precision for δ -values for 5- and 10-minutes integration time were 0.08 – 0.09 ‰ and 0.06 – 0.07 ‰, respectively (Fig. 1). Instrumental drifts over 24-hour periods were estimated on basis of the Allan analysis and accounted for 0.26 ‰, 0.36 ‰, 0.05 ‰, 0.40 ‰, and 0.85 ‰ for $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{18}\text{O}$, and SP, respectively.

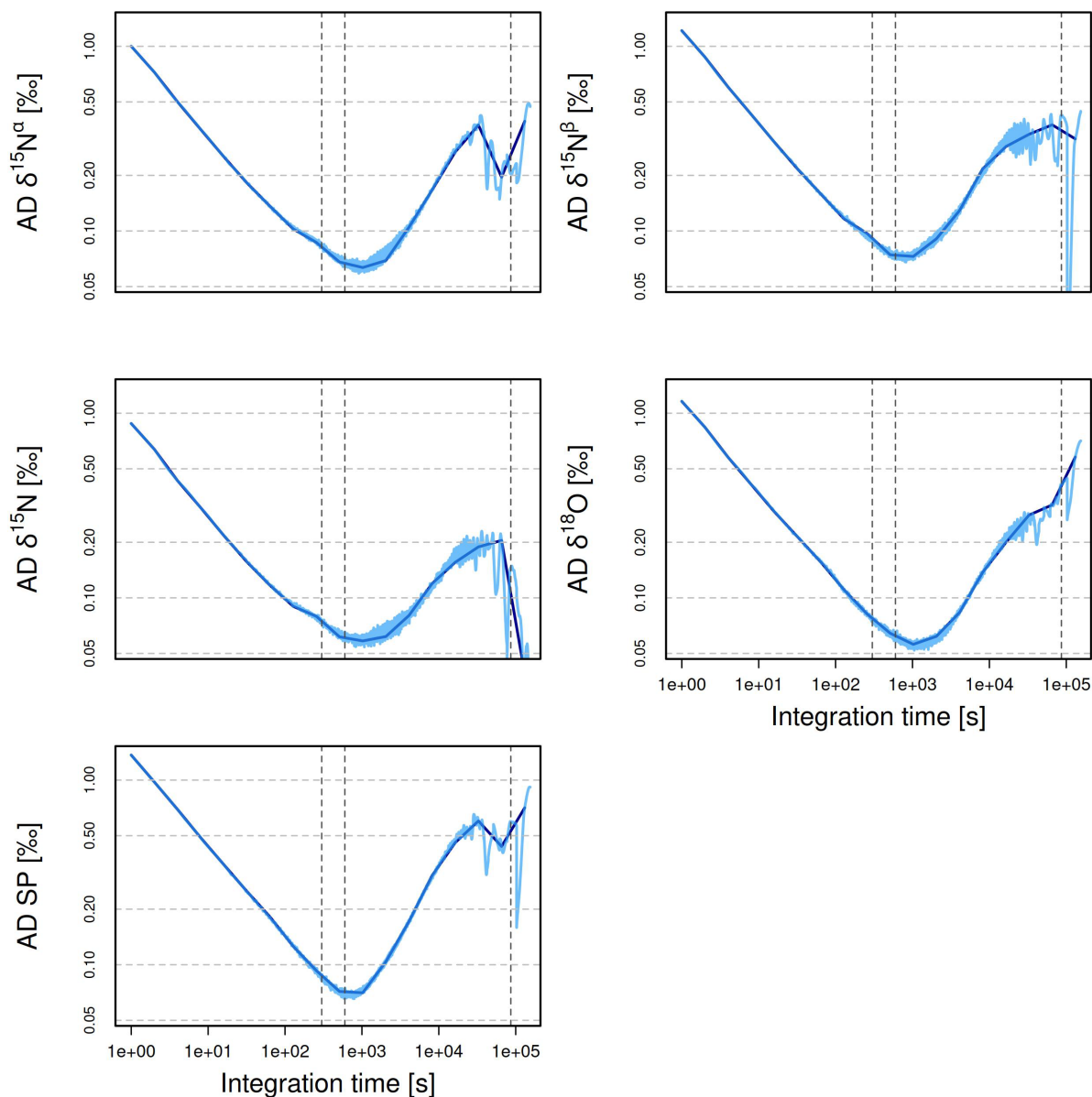


Figure 1: Allan deviations (AD), i.e. precision as function of integration time for $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{18}\text{O}$, and SP measured by an OA-ICOS analyser at 12 ppm N_2O . Dashed vertical lines are placed at 5 and 10 minutes and at 24 hours integration time.

2.2.2 Calibration procedure, drift correction and target gas measurements

From every 8 to 10-minute measurement interval the first three to five minutes were discarded to assure complete exchange of the analyte gas and only the last 5 minutes were averaged before further data processing. To reduce drift effects and enable offset correction, every analyte gas measurement was bracketed by a calibration gas (Call, Tab. 1) measurement. A

second calibration gas (Cal2, Tab. 1) was measured before and after each experiment or every eight hours during continuous measurements for two-point δ -calibration.

Table 1: N₂O mole fractions, isotopic composition and gas matrix of the calibration gases (Cal1, Cal2), the target gas and the synthetic air (SA) used in this study.

	N ₂ O [ppm]	$\delta^{15}\text{N}^{\alpha}$ [‰]	$\delta^{15}\text{N}^{\beta}$ [‰]	$\delta^{15}\text{N}^{\text{bulk}}$ [‰]	$\delta^{18}\text{O}$ [‰]	SP [‰]	Gas matrix ²⁾
Cal1 ¹⁾	98.3 (±1.9)	−0.74 (±0.19)	0.44 (±0.15)	−0.15 (±0.13)	38.74 (±0.23)	−1.2 (±0.23)	78.2 % N ₂ , 20.92 (±0.2) % O ₂ , 0.91 (±0.05) % Ar
Cal2 ³⁾	90.2 ⁵⁾	50.95 (±0.47)	55.09 (±0.47)	53.02 (±0.05)	103.04 (±0.16)	−4.13 (±0.93)	78.1 % N ₂ , 20.95 (±0.4) % O ₂ , 0.95 (±0.02) % Ar
Target gas ⁴⁾	90.1 ⁵⁾	−24.35 (±0.32)	−22.94 (±0.33)	−23.64 (±0.03)	31.79 (±0.12)	−1.41 (±0.46)	79.5 % N ₂ , 20.5 % O ₂ (±0.2)
SA ⁶⁾	< 0.001-	-	-	-	-	-	78.1 % N ₂ , 20.96 (±0.4) % O ₂ , 0.95 (±0.05) % Ar

¹⁾ Commercial gas mixture, procured from Linde Gas AG (Switzerland), isotopic composition analysed by Empa (Heil et al., 2014). The precision indicated is the standard deviation for replicate sample measurements and does not include the uncertainties of the calibration chain.

²⁾ Manufacturer's specifications.

³⁾ Empa cylinder D689516; N₂O isotopic composition of pure N₂O gas reported in (Mohn et al., 2022); the gas matrix contains additional trace gases: 400 (±8) ppm CO₂, 2 (±0.2) ppm CH₄, 200 (±40) ppb CO (manufacturer's specifications). Differences in gas matrix composition, i.e. presence of trace gases (CO₂, CH₄, CO) in Cal2, given the applied strong dilution with high purity SA are not expected to affect analytical results (see Harris et al., 2020).

⁴⁾ Empa cylinder CA06266; N₂O isotopic composition analysed by IRMS by Sakae Toyoda at Science Tokyo. The precision indicated is the standard deviation for replicate sample measurements and does not include the uncertainties of the calibration chain.

⁵⁾ N₂O mole fractions estimated from N₂O and SA volume used for production.

⁶⁾ Carbagas AG, Switzerland.

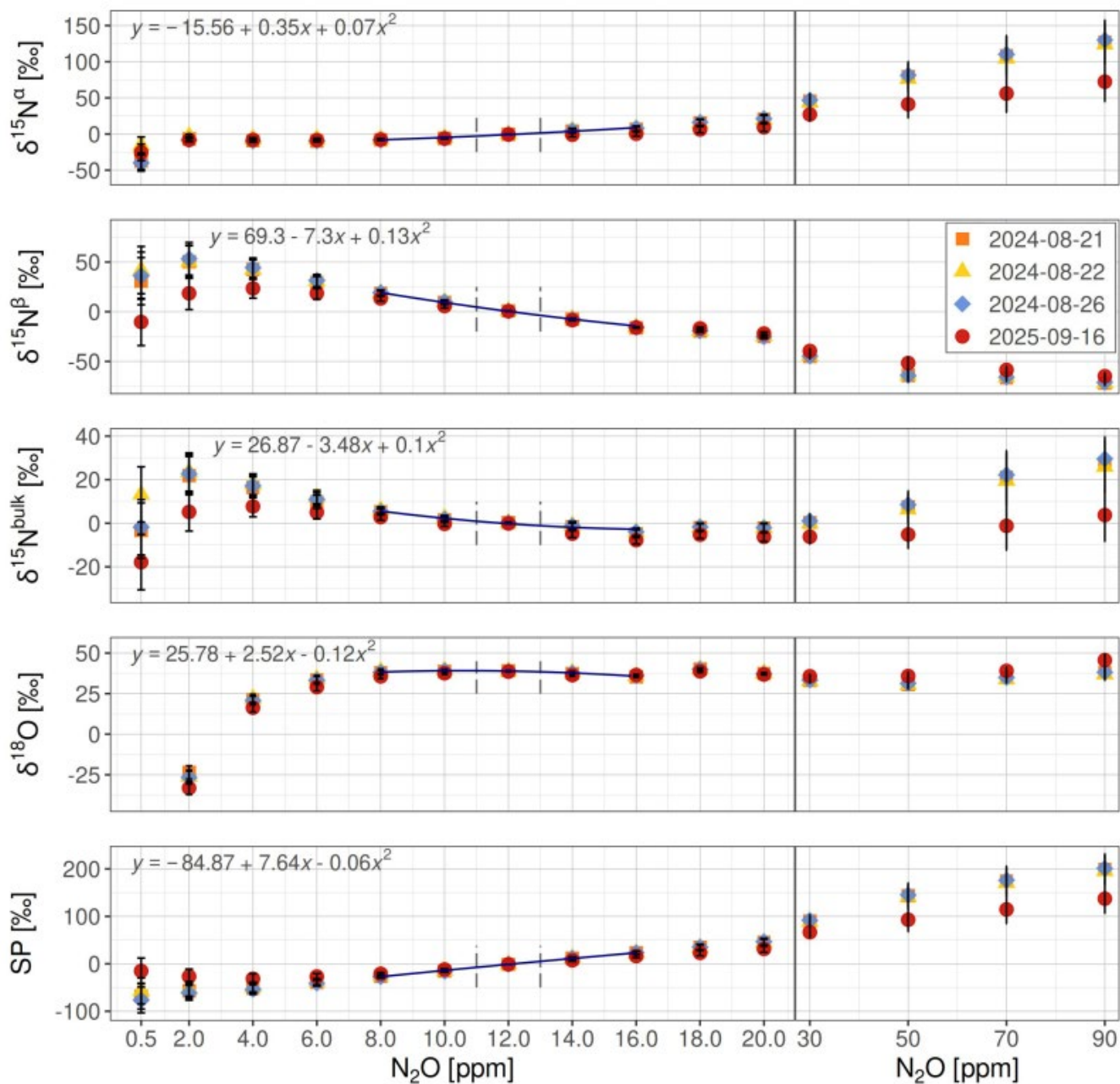
We acknowledge that δ -values of Cal1 and Cal2 do not cover most of the measurement range relevant for WWTP. The accuracy of measurement results, however, was assessed using target gas measurements (Tab. 1), at an isotopic composition typical for the WWTP application performed in four experiments distributed over the complete measurement period (24th September 2024, 30th and 31st January 2025, 11th September 2025). Each measurement block consisted of four 10-minute analyses of the target gas and was performed in an identical manner as any experimental measurements, i.e. with two-point calibration before and after each target gas measurement block and bracketed with Cal1 measurements. Average results were -25.51 ± 0.3 , -23.35 ± 0.9 , -24.43 ± 0.5 , 31.22 ± 0.2 and -2.15 ± 0.9 ‰ for $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{18}\text{O}$ and SP, respectively. Consequently, OA-ICOS results were systematically (0.4 to 1.2 ‰) lower than δ -values reported by IRMS (Tab. 1). This discrepancy is higher than differences observed in past inter-laboratory comparisons between Empa and

165 Science Tokyo (Mohn et al., 2014; Ostrom et al, 2018) was not further addressed as it is within the uncertainty targets of our study. It might be rationalized by the absence of Ar in the target gas tank, which was shown to lead to lower apparent δ -values for OA-ICOS (Harris et al., 2020) or a potential uncertainty introduced by extrapolating calibration scales.

2.2.3 Nitrous oxide mole fraction dependence

170 The analyser's dependence of reported δ -values on variations in N₂O mole fraction was assessed by dynamic dilution of a reference gas (Cal1) with N₂O free dilution air to a sequence of N₂O mole fractions ranging from 0.5 to 90 ppm. Gas mixtures were prepared at a flow rate of 300 mL min⁻¹ and measurements recoded for 10 minutes per mole fraction step. To account for analyser drift, in-between every mole fraction step the Cal1 gas was diluted to 12 ppm and measured as a reference point. This experiment was repeated three times on different days prior to the measurement period at the pilot reactors and once after the measurement period of 12 months. This enabled us to assess the analyser-specific variation in
175 N₂O mole fraction dependence in the short and long term.

Figure 2 displays apparent δ -values for Cal1 measured between 0.5 and 90 ppm N₂O. In accordance with earlier work (Harris et al., 2020) the mole fraction dependence (e.g. $\Delta\delta^{15}\text{N}^{\text{bulk}}$ per $\Delta[\text{N}_2\text{O}]$) showed largest nominal values towards low, i.e. ambient, mole fractions and less pronounced above 6 ppm N₂O. Only in a mole fraction range from 8 to 20 ppm N₂O could reported $\delta^{15}\text{N}^{\alpha}$ and $\delta^{15}\text{N}^{\beta}$ values be described by a quadratic function. For $\delta^{18}\text{O}$ the mole fraction range for which a
180 quadratic correction function applies was even smaller, 8 to 16 ppm N₂O. Like (Plouviez et al., 2026), we observed significant temporal changes in the N₂O non-linearity over time, necessitating a dynamic dilution as well as corrections for the non-linear mole fraction dependence (see section 2.4.1).

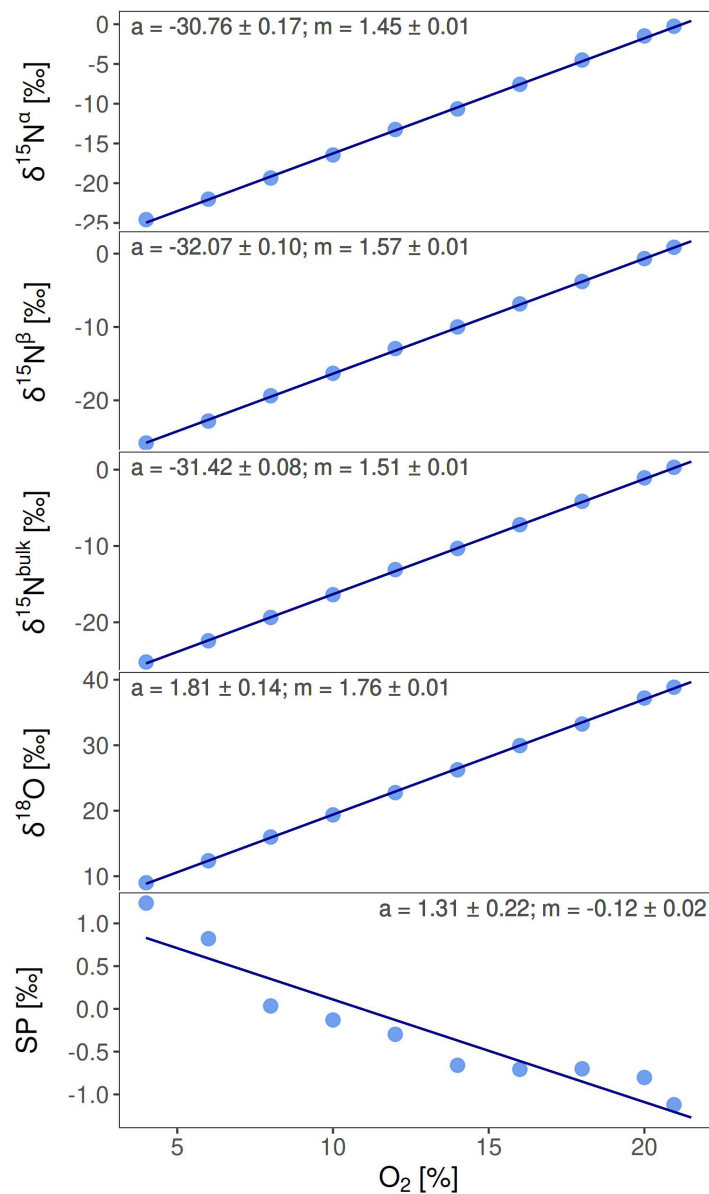


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Figure 2: N_2O mole fraction dependence of apparent δ -values, $\delta^{15}\text{N}^\alpha$, $\delta^{15}\text{N}^\beta$, $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{18}\text{O}$, and SP, for N_2O mole fractions ranging from 0.5 to 90 ppm as reported by the OA-ICOS analyser. The dotted lines indicate the range over which N_2O mole fraction corrections were performed (11 to 13 ppm N_2O). Note that for visual purposes the $\delta^{18}\text{O}$ values for the lowest mole fraction were removed from the plot (mean -371.1 ± 80.1 ‰).

190 2.2.4 Gas matrix effects of oxygen

In nitrifying zones of WWTPs, ambient air is injected to oxidize NH_4^+ to NO_2^- and NO_3^- as well as organic matter to CO_2 . Consequently, O_2 mole fraction in the off-gas are at sub-ambient levels and the volumetric content of persistent gas components such as N_2 and Ar is enhanced since any produced CO_2 is removed prior to analysis (see section 2.3.1 for details). We therefore tested the dependence of reported N_2O mole fraction and δ -values on O_2 mole fractions in the gas matrix. For this Cal1 was dynamically mixed with N_2O free SA, and high purity N_2 (99.999 %, Linde Gas AG, Switzerland) to incrementally vary the O_2 mole fraction in the analyte gas from 4 to 20.95 %, while N_2O mole fractions were held constant at 12 ppm. As N_2 did not contain Ar, in parallel with a decrease in O_2 by e.g. 16 % Ar dropped by 0.7 %. Earlier studies (Harris et al., 2020) indicate that the gas matrix effect attributed to O_2 for an OA-ICOS analyser as tested here might therefore be overestimated by around 10 %, and our uncertainty estimates (section 2.5.2) are rather conservative. Gas mixtures were prepared at a flow rate of 300 mL min^{-1} , and each sample was analysed for ten minutes. To account for any analyser drift, in between every O_2 mole fraction step, the Cal1 gas was measured at ambient O_2 mole fractions (20.95 % O_2). The O_2 mole fraction dependence of apparent δ -values was linear over the entire range of O_2 mole fractions (4 to 20.95 % O_2 ; Fig. 3), with values of 1.45, 1.57, 1.51, 1.76, and $-0.12 \text{ ‰ } [\% \text{ O}_2]^{-1}$ for $\delta^{15}\text{N}^a$, $\delta^{15}\text{N}^b$, $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{18}\text{O}$, and SP, respectively. Oxygen mole fractions in the reactors' off-gas ranged from 19.8 to 20.95 % over the entire 1-year experimental period (O_2 mole fractions were measured using a PG-350E, Horiba, Japan). The average O_2 mole fraction in the actual analyte gas is somewhat higher at $20.6 (\pm 0.2) \text{ ‰}$ as the off-gas is diluted with high-purity SA (see above) to set the N_2O mole fraction to 12 ppm. To account for a systematic reduction in O_2 mole fractions by 0.35 %, $\delta^{15}\text{N}^a$, $\delta^{15}\text{N}^b$, $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{18}\text{O}$, and SP values were corrected for 0.56, 0.53, 0.55, 0.83, and 0.04 ‰, respectively by using the linear models in Fig. 3. The variability in O_2 mole fractions was considered in the uncertainty budget.



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Figure 3: Oxygen mole fraction dependence of apparent δ -values, $\delta^{15}\text{N}^\alpha$, $\delta^{15}\text{N}^\beta$, $\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{18}\text{O}$, and SP in N_2O , for O_2 mole fractions in the gas matrix ranging from 4 to 20.95 %. Intercept (a) and slope (m) with corresponding standard errors. Presented O_2 gas matrix effects are conservative, i.e. overestimated, due to a parallel decrease in Ar mole fractions (0.5 % Ar per 10 % O_2).

215 2.3 Implementing real-time nitrous oxide isotope analysis at a wastewater treatment plant

2.3.1 Dynamic dilution system

As the apparent δ -values reported by the OA-ICOS analyser are subject to a strong, time-variant N_2O mole fraction dependence, as discussed in sect. 2.2.3, we decided to implement a dynamic dilution system (Fig. 4) using N_2O -free SA ($\text{N}_2\text{O} < 1$ ppb) as dilutant to limit uncertainty contributions from N_2O non-linearity corrections. The target mole fraction of the dilution system was set to 12 ppm N_2O and data with N_2O mole fractions outside the range of 11 to 13 ppm were discarded (53 % of data). Data passing this criterion were corrected for non-linearity using average correction functions (Fig. 2). The target N_2O mole fraction (12 ppm) represents a compromise for analyser sensitivity and non-linearity, which both degrade data quality at low N_2O concentrations and temporal coverage, i.e. sufficiently low N_2O target concentration to cover relevant emissions.

The custom-built dilution system consisted of five mass flow controllers (MFC; Vögtlin Instruments GmbH, Switzerland), automated by a customized LabView programme (National Instruments, USA). Four MFCs were used to control flows of the analyte gas, Cal1, Cal2 and N_2O -free SA to dilute the analyte gas to the target N_2O mole fraction. One additional MFC set to 400 mL min^{-1} was used to maintain a constant flow of analyte gas during Cal1 and Cal2 measurements to reduce lag times during subsequent analyte gas measurements (Fig. 4). The dilution ratio and MFC flow rates were calculated based on a 20 second running average of the actual N_2O mole fraction in the off-gas of the reactor provided by a NDIR analyser (X-STREAM X2XF, Rosemount Emerson, USA). The analyte gas was provided to the regulating MFC at 2.5 bar overpressure using a membrane pump (N86, KNF Holding AG, Switzerland). The intake of the pump was controlled with a needle valve, while the overpressure downstream of the membrane pump was monitored and manually adjusted with a pressure relief valve. To maintain stable operation of the MFC a critical orifice (bore diameter $150 \mu\text{m}$) was installed in between membrane pump and MFC dampening pressure oscillations. The analyte gas was dehumidified by permeation drying (MD-070, Perma Pure, USA), CO_2 removed by absorption (Ascarite, mesh size 20-30; Sigma Aldrich, USA) and particles retained using an in-line sintered metal filter (pore size $2 \mu\text{m}$; Swagelok, USA). Part of the analyte gas was supplied to the OA-ICOS spectrometer (about 200 mL min^{-1}), and the remainder (about 200 mL min^{-1}) exhausted to ambient air passing a NDIR CO_2 sensor (Sensair HPP, Sweden), to detect breakthrough of the CO_2 trap.

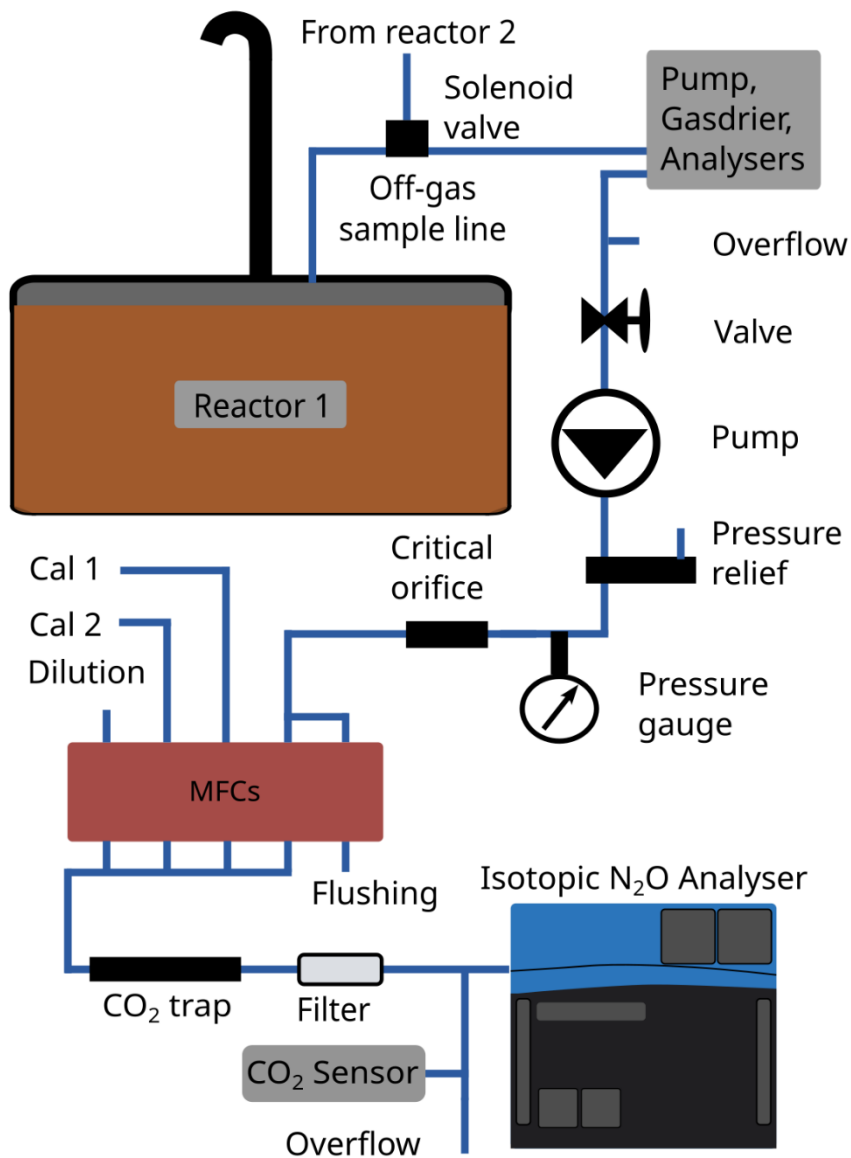


Figure 4: Scheme of the experimental set-up applied for alternate sampling the off-gas of two wastewater treatment reactors and real time analysis of N_2O mole fraction and isotopic composition using an OA-ICOS spectrometer. The analyte gas treatment includes sequential dehumidification, dilution using MFCs (mass flow controllers), CO_2 removal and particle filtering. The CO_2 sensor is implemented to monitor quantitative CO_2 removal and thus the absence of CO_2 spectral interferences.

2.4 Experiments at a pilot-scale wastewater treatment plant

Real-time analysis of N₂O mole fraction and isotope δ -values was performed at two pilot-scale wastewater treatment reactors (volume per reactor: 8 m³) operated by Eawag in Dübendorf, Switzerland. The reactors were fed with local municipal wastewater taken directly from the sewer and were run as sequencing batch reactors in parallel. Each cycle consisted of an anoxic feeding phase, an aeration phase with duration controlled by NH₄⁺ concentration, a settling phase, and a discharge period with 25 to 55 % volume exchange. After each cycle, the remaining sludge of both reactors was mixed to ensure a comparable microbiome between the two reactors (Strubbe et al., 2026). To distinguish between N₂O production pathways, we analysed the N₂O isotopic composition in the reactors' off-gas. During aeration periods the air volume in the reactors head space was constantly replaced by the aeration gas bubbling through the reactors liquid phase (Fig. 4). A subsample of the off-gas was constantly drawn from the emission duct to the analytic equipment and analysed for isotopic signatures. Only data from the aeration phases were used for further analysis. As O₂ exchange between nitrogenous oxides and H₂O can occur in abiotic systems but also mediated by microbes (Kool et al 2007), the $\delta^{18}\text{O}$ of N₂O partly reflects the $\delta^{18}\text{O}$ of the dissolution water. This dependency was considered by subtracting the $\delta^{18}\text{O}$ signature of the local tap water ($\delta^{18}\text{O}(\text{H}_2\text{O}) = -11.2 \text{ ‰}$) from the $\delta^{18}\text{O}$ of N₂O before further interpretation. Experiments were conducted over a period of 12 months with 998 hours of measurement time. While this study focuses on performance and usability of the described analytical setup and corresponding methodological aspects based on a selection of representative data sets, follow up manuscripts will address more specific wastewater treatment related research questions in detail (e.g. Strubbe et al., 2026). The following representative datasets were obtained under three different experimental conditions and are used to demonstrate the usability and applicability of our analytical setup:

Experiment 1: To determine the predominant N₂O biological production pathway, we analysed the N₂O isotopic composition over four months (September to December 2024) under standard WWTP operation, i.e. at a dissolved O₂ (DO) concentration setpoint of 2 mg L⁻¹ ($\pm 0.5 \text{ mg L}^{-1}$) and without N-substrate additions.

Experiment 2: To assess the usability of our on-line isotopic measurement setup in capturing relevant process dynamics, we designed experiments in which the DO concentration was varied between reactors while all other parameters remained constant. This allowed us to investigate effects of different DO concentrations on microbial pathways and N₂O reduction. In short, we ran one reactor at a DO concentration of 2 mg L⁻¹, while the second was run at a DO concentration of 0.5 mg L⁻¹. At the start of the aeration phase, each reactor received 118 g NaNO₂ (targeting 3 mg N L⁻¹ in the reactor) to stimulate N₂O production. Beforehand, the $\delta^{18}\text{O}\text{-NO}_2^-$ of the NaNO₂ solutions was set by equilibrating (74 hours at 40°C, 74 hours at reactor temperature) with the local tap water ($\delta^{18}\text{O}(\text{H}_2\text{O}) = -11.2 \text{ ‰}$).

Experiment 3: As a third application of our on-line isotopic measurement setup, we investigated its usability for low-level ¹⁵N-labelling, a novel methodology that uses the addition of small amounts of labelled substrate to increase δ -values to a level above natural abundance but low enough to still allows the use of natural abundant fractionation factors as well as

standard isotopic measurement methods (Deb et al., 2025). This method is very valuable to investigate N-transformation processes and nD and hD activity during different operational conditions of WWTPs. We therefore performed a low-level ^{15}N -labelling experiment in one of the two reactors. In short, the following procedure was applied: At the beginning of the aeration phase the reactor was depleted of NH_4^+ , by setting the DO concentration to 6 mg L^{-1} until the NH_4^+ concentration was below detection limit of the NH_4^+ sensor (COS61, ISEmax CAS40, Endress+Hauser, Switzerland). Then, the DO concentration setpoint was reduced to 4 mg L^{-1} , and the NO_3^- concentration increased to 10 mg N L^{-1} by adding NaNO_3 . Additionally, NH_4^+ concentrations were increased to 10 mg N L^{-1} by adding ^{15}N -labelled NH_4^+ , to set $\delta^{15}\text{N}_{\text{NH}_4^+}$ in the reactor to approx. 100 ‰. The duration of the aeration phase was manually set to 3 hours, during which the N_2O isotopic composition was monitored. During the subsequent cycle, i.e. after an exchange of 60 % of the reactor content with fresh municipal wastewater, the DO setpoint was reduced to 2 mg L^{-1} and no substrates were added. Again, the isotopic composition was analysed during the aeration phase.

2.5 Data processing

Data processing was performed using a MATLAB script adapted from Havsteen et al. (2025) (MATLAB version R2022b Update 3). This script was used to import spectrometer data as well as combine measurement data with labels of the analyte gas identity (reactor 1 / 2, Cal1, Cal2). Analyte labels provide start/end times for each measurement interval. The last 5 minutes of each interval, when mole fractions and δ -values reached a plateau were used to calculate average N_2O mole fractions ($[\text{N}_2\text{O}]_{\text{S,Av}}$), δ -values, and their standard deviations. For further data quality assurance, any averaged δ -values with a standard deviation > 5 ‰ were disregarded from further data processing (4.1 % of total data). To correct for N_2O mole fraction dependence, and for data visualization RStudio (2025.05.1, R version 4.5.1, R Core Team, 2025) and the packages ggplot2 and datatable were used (Barrett et al., 2025; Wickham, 2016).

The raw sample δ -values provided by the analyser (δ_S^{Raw}) were corrected for drift ($\Delta\delta_{\text{Drift}}$), N_2O mole fraction dependence ($\Delta\delta_{\text{N}_2\text{O}}$) and the dependence of apparent δ -values on the O_2 mole fraction ($\Delta\delta_{\text{O}_2}$):

$$\delta_S^{\text{Corr}} = \delta_S^{\text{Raw}} - \Delta\delta_{\text{Drift}} - \Delta\delta_{\text{N}_2\text{O}} - \Delta\delta_{\text{O}_2} \quad (2)$$

Since CO_2 was systematically removed prior to analysis, no correction for CO_2 spectral interferences relevant for the applied OA-ICOS analyser (Harris et al., 2020) was implemented. Analyser drift was monitored and corrected by regular analysis of reference gas Cal1 (Havsteen et al., 2025). For each sample interval at time t_S , the drift-related offset was determined by subtracting the overall mean based on all Cal1 intervals recorded during an experiment ($\delta_{\text{Cal1}}^{\text{Mean,Raw}}$) from the linear interpolation of δ -values of the two nearest bracketing Cal1 intervals ($\delta_{\text{Cal1}}^{\text{int}(+)}$ and $\delta_{\text{Cal1}}^{\text{int}(-)}$) during time $t_{\text{Cal1}}^{\text{int}(+)}$ and $t_{\text{Cal1}}^{\text{int}(-)}$:

$$\Delta\delta_{\text{Drift}} = \frac{\delta_{\text{Cal1}}^{\text{int}(-),\text{Raw}} \cdot (t_S - t_{\text{Cal1}}^{\text{int}(-)}) + \delta_{\text{Cal1}}^{\text{int}(+),\text{Raw}} \cdot (t_{\text{Cal1}}^{\text{int}(+)} - t_S)}{t_{\text{Cal1}}^{\text{int}(+)} - t_{\text{Cal1}}^{\text{int}(-)}} - \delta_{\text{Cal1}}^{\text{Mean,Raw}} \quad (3)$$

The N_2O mole fraction dependence of δ -values was corrected using quadratic correction functions (Fig. 2):

$$\Delta\delta_{\text{N}_2\text{O}} = b([\text{N}_2\text{O}]_S - [\text{N}_2\text{O}]_{\text{Cal1}}) + c([\text{N}_2\text{O}]_S^2 - [\text{N}_2\text{O}]_{\text{Cal1}}^2) \quad (4)$$

where b and c represent fitting parameters of the quadratic correction function and $[N_2O]_s$ the observed N_2O mole fraction of the sample and $[N_2O]_{Cal1}$ the true mole fraction of the Cal1 gas.

With respect to the O_2 mole fraction correction of δ -values an offset correction to average O_2 mole fractions in the analyte gas (20.6 % O_2) was applied over the entire measurement period, instead of a correcting for actual O_2 mole fraction values (Fig. 3):

$$\Delta\delta_{O_2} = m_{O_2}([O_2]_{S,avg} - [O_2]_{Cal1}) \quad (5)$$

where m_{O_2} represents the slope of the respective linear correction function and $[O_2]_{S,avg}$, $[O_2]_{Cal1}$ are the average O_2 mole fraction of the analyte gas and Cal1.

Corrected δ -values (δ_S^{Corr}) were calibrated ($\delta_S^{Corr,Calib}$) as follows:

$$\delta_S^{Corr,Calib} = y \cdot (\delta_S^{Corr} - \delta_{Cal1}^{Mean,Corr}) + \delta_{Cal1}^{True} \quad (6)$$

$$y = \frac{(\delta_{Cal1}^{True} - \delta_{Cal2}^{True})}{\delta_{Cal1}^{Mean,Corr} - \delta_{Cal2}^{Mean,Corr}} \quad (7)$$

where δ_{Cal1}^{True} and δ_{Cal2}^{True} represent the “true” δ -values of the applied reference gases.

2.5.1 Fraction of reduced nitrous oxide

Based on the isotopic signatures, the fraction of residual, non-reduced N_2O (f_{N_2O}) for each measurement (δ_i) was estimated quantitatively by reformulating Eq. 1 to:

$$f_{N_2O} = e^{(\delta_R - \delta_0)/\varepsilon} \quad (8)$$

where δ_R is the isotopic signature (SP) of the residual N_2O fraction, δ_0 represents the SP end-member value of the initial N_2O , i.e. N_2O not yet affected by reduction, and ε is the enrichment factor for SP (i.e. ε -5.9 ‰; Yu et al., 2020).

As relevant δ_0 values for SP reported in literature span from -7.5 to 1.9 ‰ (hD: -7.5 to 3.7 ‰; nD: -13.6 to 1.9 ‰; Yu et al. 2020), we estimated our system specific δ_0 value based on our dataset. First, it was ensured that the SP- $\delta^{18}O$ regression slope fell within the expected range reported in literature (0.23 to 0.98; Yu et al. 2020), then the value of δ_0 was estimated for SP by ranking observations according to the sum of their $\delta^{18}O$ and SP values of which we retained the five lowest-ranked points. Further, the perpendicular distance of each of the lowest-ranked observations in the SP and $\delta^{18}O$ space to the regression line was calculated and the observations with the smallest distance selected. The corresponding SP value was defined as δ_0 (Fig. 5).

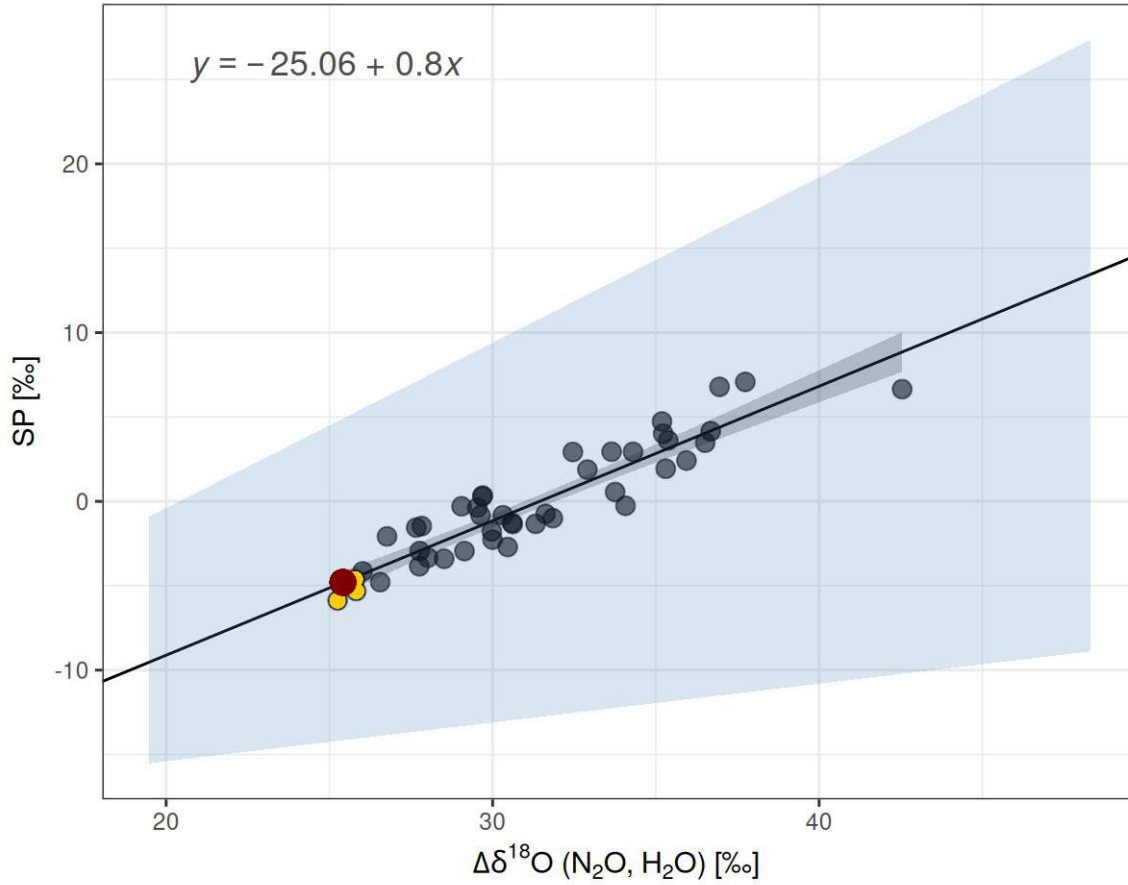


Figure 5: SP plotted against $\Delta\delta^{18}\text{O} (\text{N}_2\text{O}, \text{H}_2\text{O})$ with the linear regression line representing the study-specific reduction line. Grey area covers the 99 % confidence interval; the blue area indicates the range of reduction slopes reported in the literature (Yu et al., 2020); the red dot represents δ_0 and the yellow dots the five lowest-ranked points as estimated by the method described in section 2.3.1. Data from February 5th to February 21st 2025; n = 45.

The fraction of reduced N_2O (f_{N_2}) was calculated as $f_{\text{N}_2} = 1 - f_{\text{N}_2\text{O}}$. Here, we used SP as input variables to calculate f_{N_2} , as SP is independent of the isotopic signature of the source. Signatures of $\delta^{15}\text{N}^{\text{bulk}}$ however, are dependent on the isotopic signature of the substrate and may vary over the course of the aeration time and was therefore not used to calculate f_{N_2} .

2.5.2 Uncertainty assessment

The total uncertainty associated with the corrected δ -values (σ_i) was assessed by combining uncertainty contributions from (i) the N₂O mole fraction correction ($\sigma_{N_2O_i}$), (ii) the correction for the gas matrix effect of O₂ ($\sigma_{O_2_i}$), and (iii) the repeatability of the instrument ($\sigma_{rep\ i}$). To do so, the law of error propagation was applied on the respective correction functions.

The uncertainty of the N₂O mole fraction correction was estimated from the uncertainty in the quadratic correction function. For this the 95th confidence interval of the fit was chosen at a mean N₂O mole fraction of 12 ppm. In contrast, the uncertainty related to the correction of the O₂ gas matrix effect was assumed to be dominated by the variability of O₂ mole fractions in the actual analyte gas ($20.6 \pm 0.2\%$). Individual uncertainty contributions are shown in Tab. 2. To obtain the total uncertainty of the corrected isotope values the individual contributions were combined in quadrature:

$$\sigma_i = \sqrt{(\sigma_{N_2O_i}^2 + \sigma_{O_2_i}^2 + \sigma_{rep\ i}^2)} \tag{9}$$

The combined uncertainty (σ_i) represents the 1-sigma uncertainty of the final corrected δ -value. Combined uncertainties were 0.85, 1.08, 0.81, 0.48, and 1.09 ‰ for $\delta^{15}N^\alpha$, $\delta^{15}N^\beta$, $\delta^{15}N^{bulk}$, $\delta^{18}O$, and SP respectively (see Tab. 2).

The uncertainty estimate for the reduced N₂O fraction ($\sigma_{f_{N_2}}$) was provided, propagating the uncertainty of SP (σ_i), accounting for the number of samples (n) following first-order Taylor expansion:

$$\sigma_{f_{N_2}} = \frac{\sigma_i}{|\varepsilon| \cdot \sqrt{n}} \tag{10}$$

Table 2: Uncertainty contributions to the total uncertainty of corrected δ -values originating from N₂O mole fraction correction, gas matrix effects and repeatability.

	$\delta^{15}N^\alpha$ [‰]	$\delta^{15}N^\beta$ [‰]	$\delta^{15}N^{bulk}$ [‰]	$\delta^{18}O$ [‰]	SP [‰]	Comment
N ₂ O mole fraction correction	0.7	0.5	0.6	0.2	0.6	Dominated by uncertainty in correction function
Gas matrix effects of O ₂	0.3	0.3	0.3	0.4	0.02	Uncertainty in O ₂ mole fraction
Repeatability	0.3	0.9	0.5	0.2	0.9	Derived from repeated measurements of the target gas
Total uncertainty	0.8	1.1	0.8	0.5	1.1	

2.6

3. Results and Discussion

3.1 Performance of the measurement setup

The dynamic dilution system reliably diluted the reactor off-gas to a target mole fraction of approximately 12 ppm (Fig. 6, note that the figure shows raw data, i.e. prior to calibration and corrections). It performed particularly well if N₂O mole fractions in the reactor off-gas were consistent throughout the measurement interval. Initially, under rapid mole fraction changes, particularly at high N₂O mole fractions (above approx. 100 ppm), however, the off-gas transfer from the N₂O mole fraction analyser to the dilution system occasionally lagged. This led to enhanced variability in the analyte N₂O mole fraction and partly to invalid isotope data due to N₂O mole fractions outside the 11 to 13 ppm N₂O target range. The situation was improved by increasing the gas flow rate and implementing an additional mass flow controller to flush the sample stream during Cal1 measurements between each sample analysis (Fig. 6). Another challenge throughout the experimental period was maintaining consistent CO₂ removal rates, as the absorbent traps regularly showed CO₂ breakthrough before the trap capacity was reached. We assume the occurrence of preferential flow paths to be responsible for this. As all data acquired during breakthrough periods were discarded, data quality was not compromised. A possible strategy to avoid future data loss could be the more frequent automated change of traps as integrated by Ibraim et al., (2019) or the development of an alternative dual-trap system with regenerative adsorbent. A further possible constraint is the necessity to use relatively large volumes of N₂O free dilution air and the need for frequent drift corrections and calibrations, especially as necessary isotopic calibration standards at the required process mole fractions and in the appropriate gas matrix are not yet commercially available (Mohn et al., 2022; Ostrom et al., 2018). However, the resulting efforts and costs need to be evaluated against the alternatives. Intermittent sampling protocols bear significant labour costs as the samples need to be collected manually, the sample containers need to be cleaned and prepared beforehand and the analytics adapted for the target application. In addition, procedures for sample storage need to be carefully tested to avoid sample loss or increased uncertainty due to fractionation effects. Furthermore, off-line analytics requires a similar analytical setup, dilution gas and isotopic calibration standards. Furthermore, the setup described in this study is scalable, i.e. the measurement frequency can be increased significantly if higher temporal resolution is needed or for sequential analysis of multiple reactors. This enables high-frequency monitoring to capture short-term fluctuations and dynamic processes. Resulting data enables more accurate characterisation of N₂O emission patterns like diurnal cycles and reduces uncertainty associated with interpolation between sparse data points. With minor adaptations the introduced isotopic measurement setup presented here will be capable of performing long-term measurements at full-scale WWTPs to provide on-line data that enables direct control in on-site mitigation efforts. Thus, the isotopic data are actionable, enabling the identification of the root source of emissions and their underlying drivers in near real-time. This information can improve the understanding of the emission dynamics and support evidence-based targeted mitigation strategies, thereby enhancing the ability of WWTP operators to reduce N₂O emissions effectively.

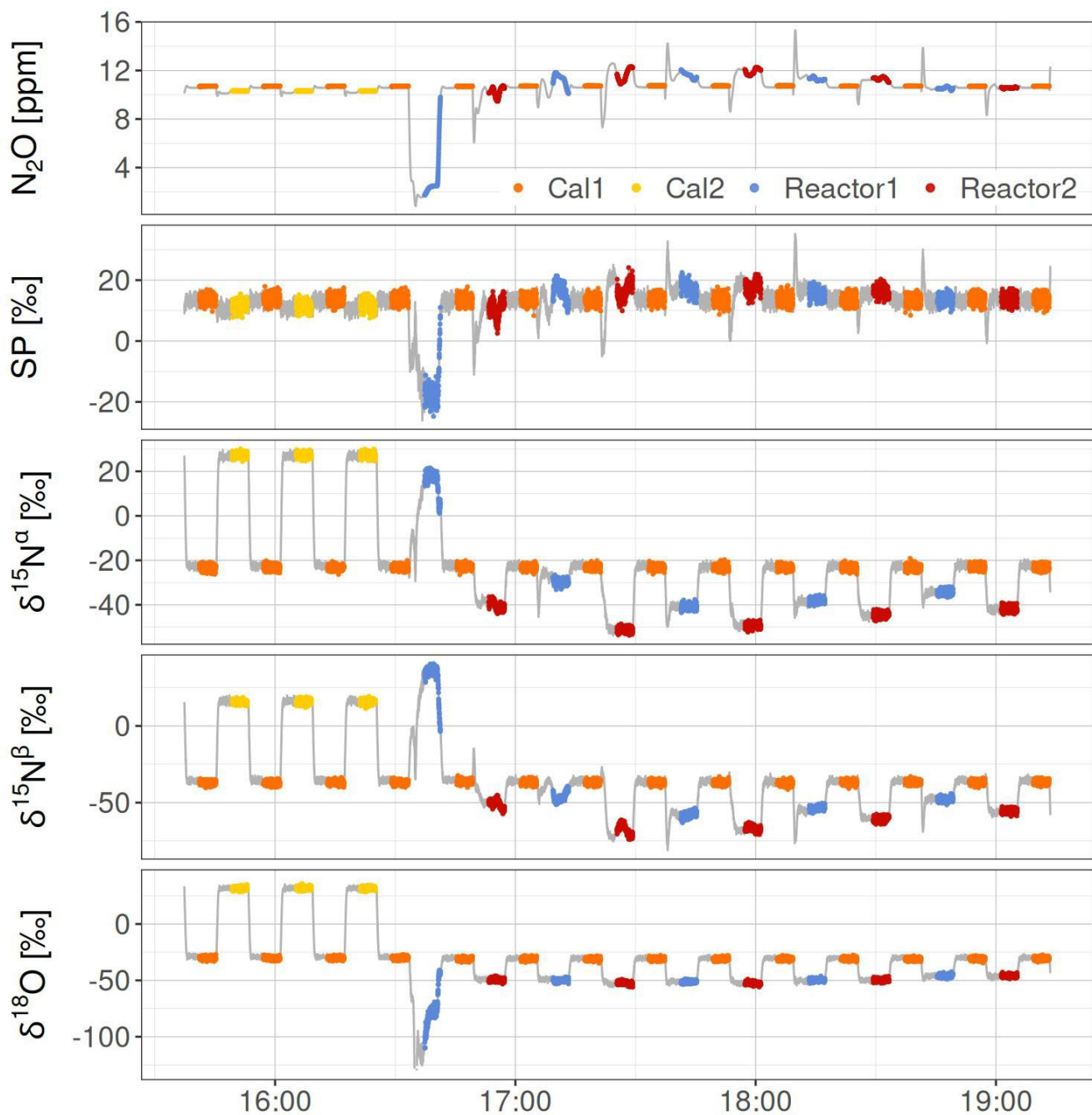


Figure 6: N₂O mole fraction data and SP, $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, $\delta^{18}\text{O}$ values as provided by the laser spectrometer, i.e. prior to corrections and calibration. Data from June 12th 2025 under standard WWTP operation conditions. A measurement interval of 8 minutes was chosen and only the last five minutes of each interval were used for further analysis to assure complete exchange of the analyte gas in the gas pretreatment and analyser. Cal1 measurements are shown in orange, Cal2 measurements in yellow and sample measurements in blue (reactor 1) and red (reactor 2).

3.2 Representative data sets demonstrating system applicability

3.2.1 Experiment 1: Constraining nitrous oxide production pathways under standard WWTP operation

To determine the predominant microbial N₂O production pathway, we analysed the N₂O isotopic composition over several months (September to December 2024) under standard WWTP operation, i.e. at a dissolved O₂ concentration setpoint of 2 mg L⁻¹ (± 0.5 mg L⁻¹) and without N-substrate additions. Figure 7 displays the N₂O isotopic composition during standard operation of the two wastewater treatment reactors presented as a dual isotopic plot of SP versus $\delta^{18}\text{O}$ (N₂O) values, after data processing (section 2.3). Isotopic signatures of relevant production processes (Hy, nD, and hD) derived from laboratory studies are indicated as rectangular shaded areas for comparison (Yu et al., 2020). Concurrent changes in SP and $\Delta\delta^{18}\text{O}$ (N₂O, H₂O) along a straight line with a slope of 0.37 indicate successive reduction of N₂O to N₂ as the slope falls within a range defining the reduction line (spanning from 0.23 to 0.98, see Yu et al., 2020) and therefore validating the activity of N₂O reduction. From Fig. 7 we can infer nD or hD as the dominant N₂O production process, as measurements fall within the range of isotopic signatures expected for denitrification processes with a minimal effect of N₂O reduction (Yu et al., 2020) and are in accordance with other studies on wastewater treatment (Gruber et al., 2022; Wunderlin et al., 2013). Furthermore, the absence of elevated SP values (> 30‰) suggests that Hy was not a source term during our measurements (Frame and Casciotti, 2010; Sutka et al., 2006; Wunderlin et al., 2013; Yu et al., 2020). This interpretation may strike the reader as odd, as the data points so obviously lay out-side of the shaded source signature areas that are traditionally visualised as boxes in dual isotope plots. These source signature areas (see Fig. 7 and Fig. 8A) are based on a limited number of pure culture studies and laboratory incubations (Yu et al., 2020) and therefore cannot be perceived as hard limits but require refinement to fully capture the dynamics of complex mixed microbial communities as are found in wastewater treatment reactors. Thus, the isotopic source signatures and their overlaps will be subject to change with increasing understanding of the complexities of managed and natural N₂O source systems. To determine system-specific source signature areas and to robustly differentiate between the contributions of nD and hD to N₂O formation requires further dedicated experiments that aim at stimulating both processes independently. A series of such experiments applying the analytical toolkit developed in this study is described in Strubbe et al. (2026). Controlling the concentration of ammonium, nitrite, and DO nitrifier denitrification was successfully isolated from heterotrophic denitrification and became more active at lower DO concentrations. Lower DO, higher organic carbon availability, and lower pH increased N₂O production by heterotrophic denitrification during aeration. These new insights provide a systematic framework for understanding N₂O dynamics and support the development of mitigation strategies at full-scale.

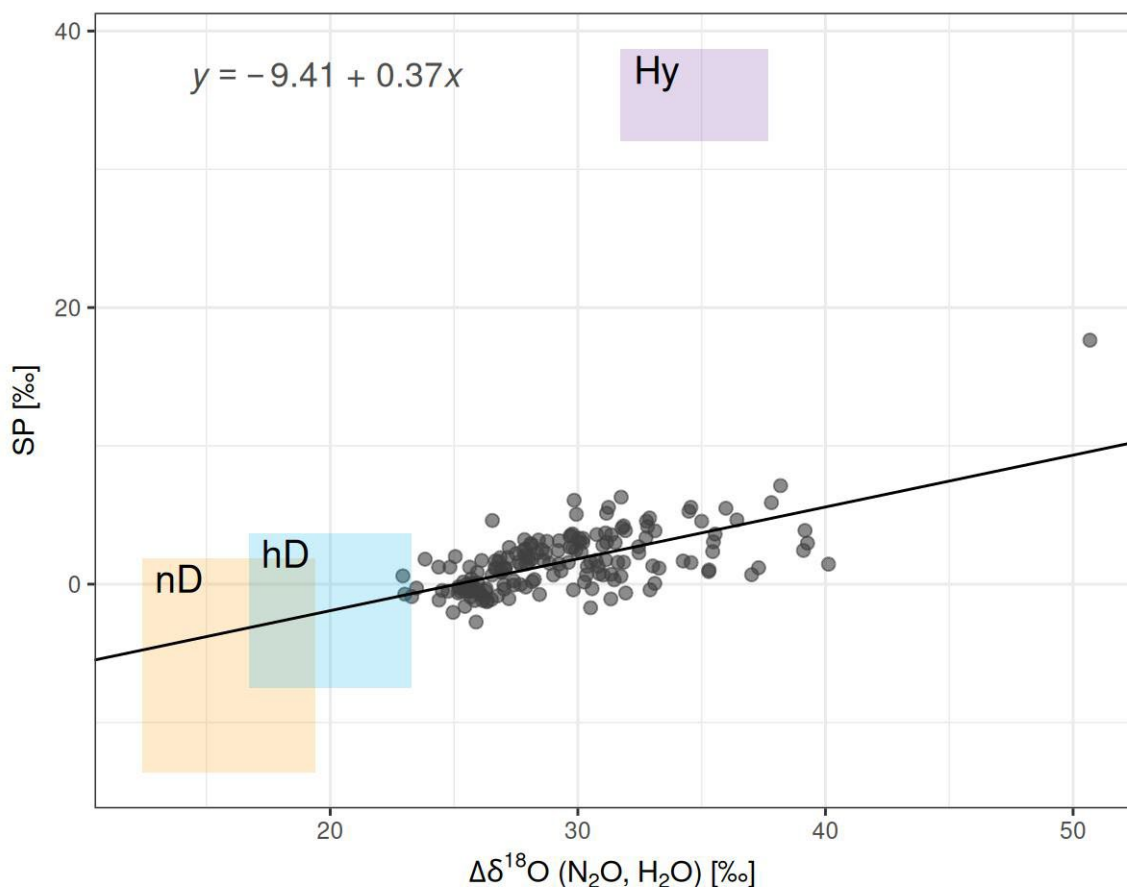


Figure 7: Dual-isotope plot of SP and $\Delta\delta^{18}\text{O}$ (N_2O , H_2O) measured during standard operations of wastewater treatment (data from September to December 2024, $n = 170$). Total uncertainties are 1.09 ‰ for SP and 0.48 ‰ for $\delta^{18}\text{O}$ (N_2O). Coloured boxes indicate expected source signatures of N_2O production pathways without fractionation effects from partial N_2O to N_2 reduction (Yu et al., 2020). The linear regression (black line; slope of 0.37) represents the so-called "reduction line", i.e. the progressive increase in SP and $\delta^{18}\text{O}$ with progressive heterotrophic N_2O reduction. Differences in reduction slopes observed for different experiments are likely due to variations in the microbial community. (Hy: hydroxylamine oxidation, nD: nitrifier denitrification, hD: heterotrophic denitrification).

3.2.2 Experiment 2: Assessing the degree of nitrous oxide reduction as a function of dissolved oxygen concentration

During heterotrophic N_2O reduction to N_2 , the N_2O molecule is preferentially split between the N-O bond of lighter isotopes, leading to an enhancement in $\delta^{15}\text{N}^{\text{bulk}}$, SP, and $\delta^{18}\text{O}$ in the remaining N_2O pool (Lewicka-Szczebak et al., 2017; Ostrom et al., 2007). The enrichment of SP, and $\delta^{18}\text{O}$ caused by N_2O reduction in experiment 2 is characterised by a slope of 0.72 (Fig.

8A), which falls within the data range defined by Yu et al. (2020) for such a process, supporting the validity of the here reported N₂O reduction fractions. As the two experiments (1 and 2) are separated in time, the difference in the reduction line slopes is likely related to a shift in the active microbial community over time. This variability is within the range seen in
445 scientific literature under similar settings (Gruber et al., 2022; Yu et al., 2020, Strubbe et al., 2026) but still represents an open research question worthwhile exploring in more detail. Dissolved O₂ concentration was identified as an important driver of N₂O to N₂ reduction by hD (Fig. 8B). In general, higher N₂O reduction was seen in the reactor set to a lower DO concentration. This can be seen in Fig. 8A, as the data from the reactor with a lower DO have progressed further along the SP/ $\delta^{18}\text{O}$ line compared to those from the other reactor. Intuitively, this can be explained by the enzymes responsible for N₂O
450 reduction preferentially cleaving the bonds between lighter isotopes, i.e. between ¹⁴N-¹⁶O rather than between ¹⁵N-¹⁶O or ¹⁴N-¹⁸O, which leads to an accumulation of molecules with ¹⁸O and ¹⁵N in the central molecular position in the residual, unreduced N₂O fraction. When applying Eq. 8 to calculate the f_{N_2} for both scenarios, we can quantify the difference in f_{N_2} . The low DO concentration setpoint of 0.5 mg L⁻¹ led to an increase in f_{N_2} by 40 % (± 2 %) compared to the DO setpoint of 2 mg L⁻¹. Therefore, on-line isotopic analysis can highlight periods during which the DO set-points can be optimised for N₂O
455 emission reduction.

During the aeration phases both reactors showed a considerable dynamic in f_{N_2} over time leading to a difference in f_{N_2} of 10 to 15 % over the course of the experiment. In detail, the wastewater treatment reactor set to low DO showed an increasing trend in f_{N_2} from 35 to 50 % followed by a decline to 40 % over the course of the experiment. In contrast, the reactor set to high DO displayed a steady decrease in the f_{N_2} from 10 to 0 %. The substantial difference between the two treatments can be
460 explained by the O₂-sensitive nature of the enzyme responsible for N₂O reduction (Pomowski et al., 2011). These findings align with previous studies demonstrating that low DO concentrations enhance N₂O reduction efficiency and vice-versa (Morley et al., 2008; Suenaga et al., 2018; Tang et al., 2022; Zhou et al., 2021). Different DO concentration thresholds were found to trigger N₂O reduction, Tang et al. (2022) found the N₂O reduction rate to increase exponentially with decreasing DO concentration below a threshold of 1.6 mg O₂ L⁻¹ in estuarine waters. In contrast, Rees et al. (2021) reported that in
465 marine water samples, N₂O consumption was observed at DO concentrations as high as 8 mg O₂ L⁻¹ and Körner and Zumft (1989) reported a threshold of 5 mg O₂ L⁻¹ below which N₂O reductase expression was enhanced. This high variability of reported thresholds may be related to microbial species or community dependencies in the response of N₂O reduction to DO concentrations (Cavigelli and Robertson, 2001; Suenaga et al., 2018; Zhou et al., 2021). The type and availability of organic carbon represents another factor parameter affecting N₂O reduction, which likely controlled the decrease in N₂O reduction
470 over time, particularly at the high DO setpoint (Azam et al., 2002; Liu et al., 2022; Morley and Baggs, 2010). This suggests that a higher availability of organic carbon enhances N₂O reduction. Isolating the N₂O-to-N₂ reduction step by on-line isotopic analysis for the first time provides insights into its key drivers, helping identify operational levers to reduce production and enhance consumption of N₂O, supporting the design of net-zero emission treatment systems. The detailed interpretation of positive or negative effects of operational factors on N₂O reduction beyond data shown here, i.e. pH,
475 temperature, total air consumption, total suspended solids, will be published elsewhere.

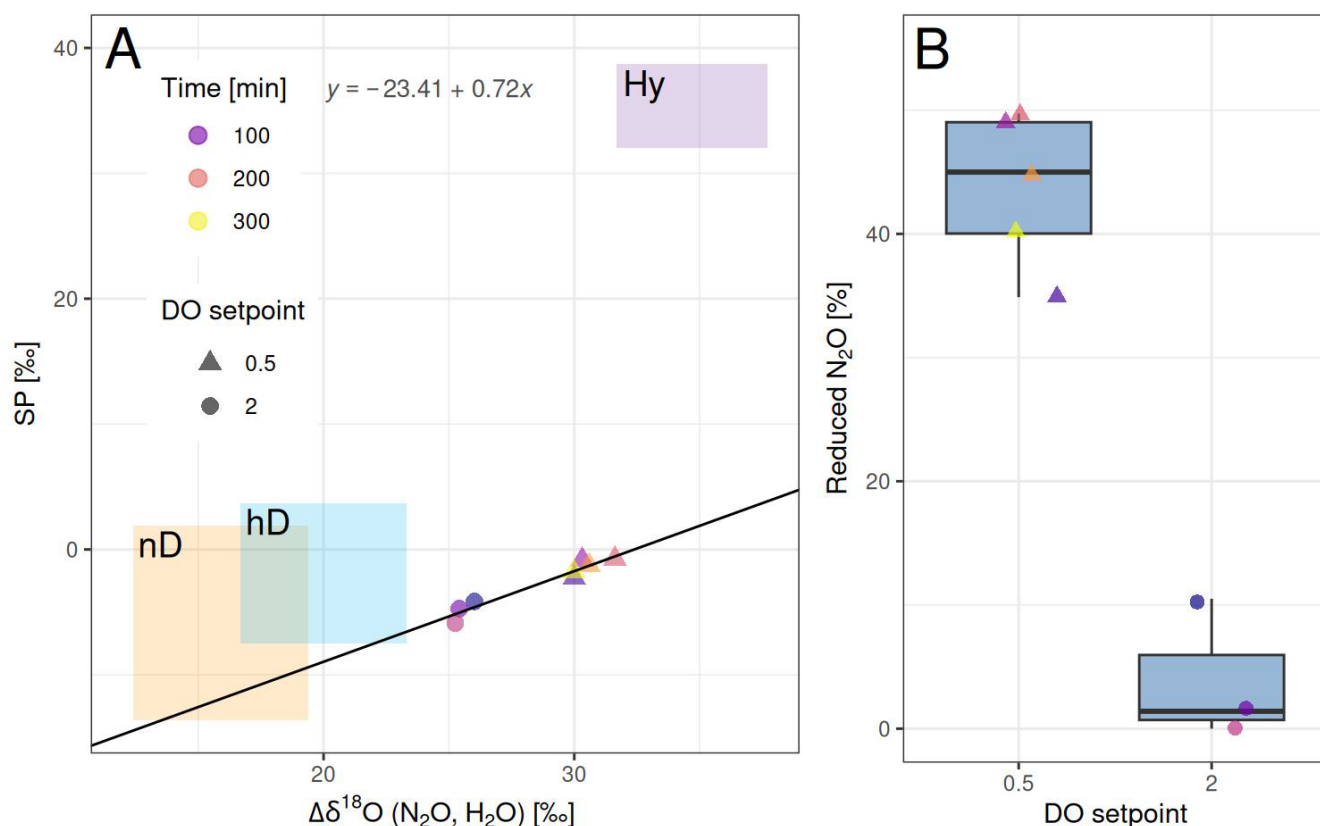
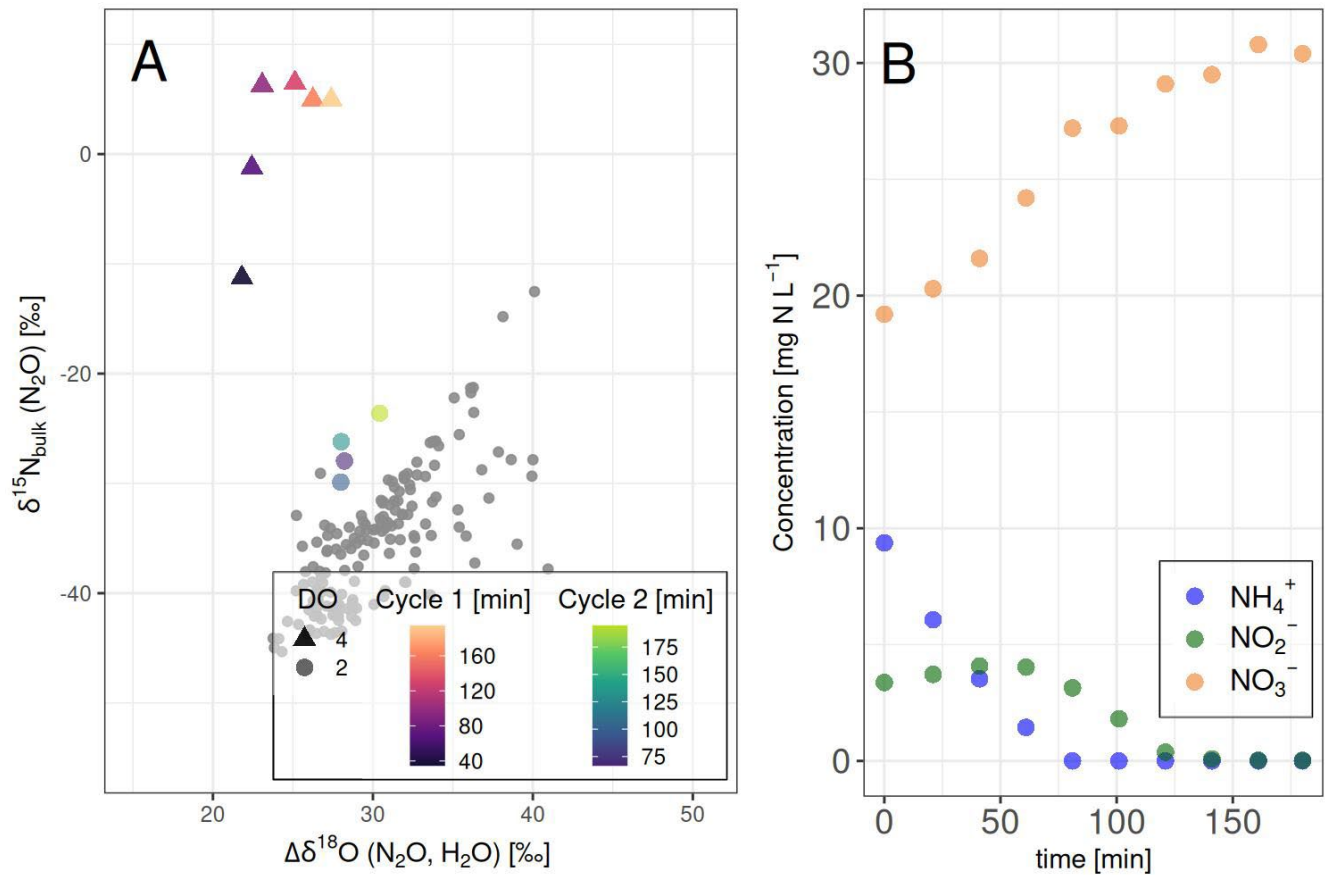


Figure 8: A: Dual-isotope plot of SP and $\Delta\delta^{18}\text{O}(\text{N}_2\text{O}, \text{H}_2\text{O})$ measured during experiments with distinctly different dissolved O_2 (DO) content in both reactors. Coloured boxes indicate expected source signatures of N_2O production pathways without N_2O to N_2 reduction (Yu et al., 2020), the linear regression (black line; slope of 0.72) represents the "reduction line". (Hy: hydroxylamine oxidation, nD: nitrifier denitrification, hD: heterotrophic denitrification). B: comparison of the fraction of reduced N_2O (f_{N_2}) for the two DO setpoints. Uncertainties for f_{N_2} are estimated to be 2 %. The colour gradient of the symbols in A and B indicates the time passed since aeration started. The different number of data points for the low DO scenario (n=5) and the high DO scenario (n=3) is due to a longer N_2O production phase at low DO. Data from February 21st 2025.

3.2.3 Experiment 3: ^{15}N -labelling

Low-level ^{15}N -labelling is a novel methodology that uses the addition of labelled substrate to slightly increase $\delta^{15}\text{N}$ values to a level above natural abundance (100 to 200 ‰) to achieve clear traceability through biogeochemical reactions, but within a range that still allows the use of natural abundant fractionation factors as well as standard isotopic measurement methods (Deb et al., 2025). Here, the addition of $^{15}\text{N}\text{-NH}_4^+$ ($\delta^{15}\text{N}\text{-NH}_4^+ = 100$ ‰ in the total NH_4^+ pool) at the onset of the aeration phase 1 results in N_2O emissions with distinctly higher $\delta^{15}\text{N}^{\text{bulk}}$ values compared to the bulk of our measurements outside of the low-level labelling experiments (Fig. 9A). An initial increase in $\delta^{15}\text{N}^{\text{bulk}}$ to 6.5 ‰ as compared to around -40 ‰ under standard WWTW operation is consistent with progressive conversion of ^{15}N -labelled NH_4^+ into NO_2^- and NO_3^- , which act as substrates for N_2O formation (Fig. 9B). After the NH_4^+ is depleted, $\delta^{15}\text{N}^{\text{bulk}}$ of N_2O stagnates. In the second cycle, after a

volume exchange of ca. 38 % and an anoxic feeding phase, denitrification likely converted a large portion of the labelled
 inorganic ^{15}N nitrogen substrate to N_2O and N_2 of which most was removed from the system but the $\delta^{15}\text{N}^{\text{bulk}}$ values of -27
 495 ‰ are still slightly elevated compared to standard WWTP operation. These results demonstrate the suitability of the on-line
 isotopic measurement setup for low-level ^{15}N -labelling studies as well as the benefit of labelled substrate addition to isolate
 contributions from specific microbial conversions to the N_2O that is produced. A more complete quantitative analysis of
 these results requires considering the $\delta^{15}\text{N}$ of aqueous nitrogen species and will be published independently.



500 Figure 9: A: Dual-isotope plot of $\delta^{15}\text{N}^{\text{bulk}}$ and $\Delta\delta^{18}\text{O} (\text{N}_2\text{O}, \text{H}_2\text{O})$ measured in a ^{15}N labelling experiment for two consecutive
 aeration cycles, the first cycle after $^{15}\text{N}\text{-NH}_4$ addition ($\delta^{15}\text{N}\text{-NH}_4^+ = 100$ ‰) and the second cycle are printed in green and
 blue, respectively (data from June 10th 2025). Grey dots represent isotopic values during standard WWTP operation (data
 from September to December 2024, n = 170), given for comparison. The colour gradient indicates the time passed since
 aeration started. B: Temporal trend of ammonium (NH_4^+), nitrate (NO_3^-), and nitrite (NO_2^-) concentration over the course of
 505 the first cycle.

4. Conclusion

This study demonstrates the feasibility and advantage of real-time N₂O isotopic analysis using off-axis integrated cavity output spectroscopy in a pilot-scale wastewater treatment setting. By implementing a dynamic dilution system and robust correction protocols, we achieved accurate measurements of $\delta^{15}\text{N}^{\alpha}$, $\delta^{15}\text{N}^{\beta}$, $\delta^{15}\text{N}$, $\delta^{18}\text{O}$, including SP, under variable process conditions, most importantly analyte gas composition. Applying a fully-automated setup, with intermittent drift correction, up to three 5-minute averaged gas sample measurements were realised per hour. Continuous monitoring in intermittent campaigns was demonstrated over a one-year study period, pointing out heterotrophic denitrification or nitrifier denitrification as the dominant N₂O source and no signs indicative of hydroxylamine oxidation. Furthermore, the system enabled the assessment of N₂O reduction dynamics and its dependence on process parameters, in our study DO variation were exemplarily tested. In a prototype application the analytical setup also proved suitable for ¹⁵N labelling experiments, offering new opportunities to study nitrogen transformation dynamics in complex environments with mixed microbial populations at high temporal resolution.

Beyond these initial applications, the platform offers significant potential for in-depth pathway characterisation by isolating contributions from individual microbial processes through targeted stimulation. It can support optimisation strategies by identifying the balance between N₂O production and reduction in near real time and quantifying environmental constraints such as pH, carbon availability, micronutrient supply, or microbial composition. Importantly, the approach is not limited to pilot-scale reactors; it can be adapted for on-line monitoring at full-scale wastewater treatment plants, enabling integration into operational control and mitigation frameworks. Thus, the isotopic data are actionable and directly relevant for improved process understanding and optimization to support evidence-based operational decisions targeting the reduction of N₂O emissions from WWTPs.

These findings underline the potential of laser spectroscopy as a practical tool for process optimisation and emission mitigation in wastewater treatment. Future work should focus on extending this approach to full-scale plants and incorporating isotopic data into advanced control strategies for greenhouse gas emission reduction.

5. Code and data availability

All raw data and code can be provided upon request to the corresponding author.

6. Author contributions

HK tested and implemented the on-line isotopic analytic system guided by JM, contributed to the conceptualization of the study and the experimental strategy, performed data acquisition and analysis, and drafted the manuscript. LS operated the pilot reactors, contributed to the experimental strategy, to the interpretation of the results, and by editing the manuscript.

535 PMM supported the conceptualization of the study and the formation of the experimental strategy and contributed to the manuscript by writing and editing. AJ provided the experimental wastewater treatment pilot setup, discussed the experimental strategy and contributed to writing and editing. AF discussed the experimental strategy and contributed to the manuscript by writing and editing. AK developed a solution for automatic calibration and dynamic dilution of the off-gas. KHK made the OA-ICOS available for this study and contributed to the manuscript by writing and editing. JM provided the
540 framework to this study, prepared the calibration gases used in this study and made significant contributions to the conceptualisation as well as to the manuscript by writing and editing.

7. Competing interests

The authors declare no competing interests.

8. Acknowledgements

545 We thank Marco Kipf, Martin Breitenstein and the team at Eawag's pilot wastewater treatment plant for maintaining reactor operation and for their support during implementation of our on-line analytic system, Roland Werner for $\delta^{18}\text{O}$ analysis of our water samples, and Pascal Rubli for providing us with a CO_2 sensor. Christoph Hüglin, Stephan Henne, Liu Ye, Kristie Boering, and Lukas Emmenegger provided valuable input to conceptualization, data interpretation and analysis.

9. Financial support

550 The project is part of Empa's and Eawag's contribution to the Swiss Center of Excellence on Net-Zero Emissions (SCENE), a joint initiative of all six institutions of the ETH Domain, which is partly funded by the ETH Board.

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