Tracing N\textsubscript{2}O formation in full-scale wastewater treatment with natural abundance isotopes indicates control by organic substrate and process settings

Wenzel Gruber\textsuperscript{a,1,*}, Paul M. Magyar\textsuperscript{b,1}, Ivan Mitrovic\textsuperscript{a}, Kerstin Zeyer\textsuperscript{c}, Michael Vogel\textsuperscript{d}, Luzia von Känel\textsuperscript{d}, Lucien Biolley\textsuperscript{d}, Roland A. Werner\textsuperscript{e}, Eberhard Morgenroth\textsuperscript{a}, Moritz F. Lehmann\textsuperscript{b1}, Daniel Braun\textsuperscript{d}, Adriano Joss\textsuperscript{a}, Joachim Mohn\textsuperscript{a}

\textsuperscript{a} Eawag, Swiss Federal Institute for Aquatic Science and Technology, 8600 Dübendorf, Switzerland
\textsuperscript{b} Department of Environmental Sciences, Aquatic and Isotope Biogeochemistry, University of Basel, Basel 4056, Switzerland
\textsuperscript{c} Laboratory for Air Pollution / Environmental Technology, Empa, Dübendorf 8600, Switzerland
\textsuperscript{d} Department of Civil, Environmental and Geomatic Engineering, ETH, Zürich 8093, Switzerland
\textsuperscript{e} Department of Environmental Systems Science, ETH, Zürich 8092, Switzerland

* Corresponding author.
\textsuperscript{1} These authors contributed equally to this work.

Nitrous oxide (N\textsubscript{2}O) dominates greenhouse gas emissions in wastewater treatment plants (WWTPs). Formation of N\textsubscript{2}O occurs during biological nitrogen removal, involves multiple microbial pathways, and is typically very dynamic. Consequently, N\textsubscript{2}O mitigation strategies require an improved understanding of nitrogen transformation pathways and their modulating controls. Analyses of the nitrogen (N) and oxygen (O) isotopic composition of N\textsubscript{2}O and its substrates at natural abundance have been shown to provide valuable information on formation and reduction pathways in laboratory settings, but have rarely been applied to full-scale WWTPs.

Here we show that N-species isotope ratio measurements at natural abundance level, combined with long-term N\textsubscript{2}O monitoring, allow identification of the N\textsubscript{2}O production pathways in a full-scale plug-flow WWTP (Hofen, Switzerland). Heterotrophic denitrification appears as the main N\textsubscript{2}O production pathway under all tested process conditions (0–2 mgO\textsubscript{2}/l, high and low loading conditions), while nitrifier denitrification was less important, and more variable. N\textsubscript{2}O production by hydroxylamine oxidation was not observed. Fractional N\textsubscript{2}O elimination by reduction to dinitrogen (N\textsubscript{2}) during anoxic conditions was clearly indicated by a concomitant increase in site preference, $\delta^{18}$O(N\textsubscript{2}O) and $\delta^{15}$N(N\textsubscript{2}O). N\textsubscript{2}O reduction increased with decreasing availability of dissolved inorganic N and organic substrates, which represents the link between diurnal N\textsubscript{2}O emission dynamics and organic substrate fluctuations. Consequently, dosing ammonium-rich reject water under low-organic-substrate conditions is unfavorable, as it is very likely to cause high net N\textsubscript{2}O emissions.

Our results demonstrate that monitoring of the N\textsubscript{2}O isotopic composition holds a high potential to disentangle N\textsubscript{2}O formation mechanisms in engineered systems, such as full-scale WWTP. Our study serves as a starting point for advanced campaigns in the future combining isotopic technologies in WWTP with complementary approaches, such as mathematical modeling of N\textsubscript{2}O formation or microbial assays to develop efficient N\textsubscript{2}O mitigation strategies.

1. Introduction

Nitrous oxide is the third most important greenhouse gas and the dominant ozone depleting substance in the stratosphere (IPCC 2013; Ravishankara et al., 2009). Wastewater treatment plants are potent point sources and significant contributors to global anthropogenic N\textsubscript{2}O emissions (Tian et al., 2018; Vasilaki et al., 2019). N\textsubscript{2}O emissions from WWTP exhibit strong temporal dynamics (Gruber et al., 2020). The underlying drivers of these dynamics, however, remain partially unclear, and are likely linked to the complexity of the different nitrogen-cycle reactions involved in N\textsubscript{2}O production in wastewater treatment systems (Domingo-Félez and Smets 2020; Schreiber et al., 2018).
Three main metabolic pathways performed by two different groups of bacteria have been identified in WWTPs: (i) hydroxylamine (NH2OH) oxidation (Ni) and (ii) nitrifier denitrification (nD) by ammonia oxidizing bacteria (AOB), as well as (iii) heterotrophic denitrification (hd) by heterotrophic denitrifying bacteria (HET) (Ren et al., 2019; Wunderlin et al., 2012). Multiple other microbial and abiotic N2O production pathways have been described in literature for specific ecosystems (Butterbach-Bahl et al., 2013) but are not discussed here, to focus on the most relevant processes. However, given a sufficient supply of organic carbon, HET are also able to reduce N2O to N2, the target product of N elimination in WWTP (Conte et al., 2018; Fan et al., 2013).

The systematic and efficient mitigation of N2O emissions in WWTPs is a challenging task and requires both long-term monitoring of emissions to identify emission peaks as well as a mechanistic understanding of N2O formation mechanisms in the wastewater treatment process (Vasilaki et al., 2019). A number of approaches have been applied successfully in full-scale WWTPs to reduce N2O emissions, such as the control of the dissolved oxygen (DO) through different aeration rates and timing (Rodriguez-Caballero et al., 2015; Sun et al., 2017), or different feeding regimes (e.g., step / intermittent feeding) maintaining low in situ ammonium concentrations (Hu et al., 2013). However, given the intricacy of N2O production and turnover, methods to quantify and to mechanistically understand the pathways involved are essential to explain emission dynamics and develop robust mitigation strategies (Duan et al., 2021).

Differences in stable isotopic substitution of the N2O molecule and the bulk isotopic composition of reactive nitrogen substrates ammonium (NH4+), nitrite (NO2−), and nitrate (NO3−), provide valuable information on N2O transformation processes, since distinct microbial and/or abiotic pathways exhibit characteristic isotopic signatures (Sutka et al., 2006; Yoshida and Toyoda, 2000). Quantifying the four most abundant N2O isotope profiles, 14N18O (15N at central, α position), 15N18O (15N at terminal, β position), and 14N18O (Toyoda and Yoshida, 1999) provides three distinct constraints: the bulk 15N/14N (δ15Nbulk) and the 18O/16O (δ18O) isotope composition as well as the 15N site preference (SP). The N and O isotopic compositions of N2O are controlled by (1) the composition of the substrate, (2) kinetic isotope effects that occur during N2O formation, and (3) kinetic isotope effects associated with N2O reduction to N2 (Denk et al., 2017; Toyoda et al., 2017; Yu et al., 2020). In addition, the O isotope ratio in the N2O pool is influenced by O-atom exchange reactions between water and N intermedium molecules, especially NO2− (Casciotti et al., 2002; Lewicka-Szczebak et al., 2016). SP is independent of the substrate isotopic composition and, therefore, an especially sensitive tool for distinguishing mechanisms of N2O production and consumption. A powerful way to use the isotopic composition of N2O to constrain its formation and processing is the dual isotope mapping approach, where SP values are plotted against either δ15Nbulk(N2O, substrate) or δ18O(N2O, H2O) and compared to the isotope signatures known for a given process (Yu et al., 2020). Despite the potential that natural abundance N2O isotope measurements offer for pathway characterization, past applications have been almost exclusively limited to laboratory scale reactors (Wunderlin et al., 2013, Tumendelger et al. (2016)).

In this study, we tested, for the first time, whether natural abundance stable isotope measurements in a full-scale WWTP can be applied to characterize N2O production pathways under changing inflow composition and process operation. In particular, we evaluated the influence of organic substrate availability and aeration strategies on the N2O formation pathways. To further support the estimated contributions of different production pathways and N2O reduction, we used measurements of the 15N/14N and 18O/16O isotope ratios of N substrates, NH4+, NO2−, and NO3−. Additionally, we performed both spatially and temporally resolved process monitoring of N2O emissions and aqueous nitrogen species to interpret the process dynamics during the experiments. Finally, we propose reduction strategies based on the observed emission patterns and attributed pathways.

2. Materials & methods

2.1. Field site

The Hofen WWTP (Switzerland, 47°27’57.3″N 9°23’49.1″E) treats the wastewater of roughly 70,000 population equivalents. After mechanical treatment by screening, grit chambers, and primary clarification, the wastewater enters the biological treatment stage, consisting of six activated-sludge plug-flow reactors, each comprising three cascaded stirred reactors (3 × 530 m3, Fig. 1). While organic compounds and N are removed biologically, phosphorus is removed through chemical precipitation using iron(III). This biological treatment scheme represents a standard activated sludge configuration (Tchobanoglous et al., 2014). The average COD and nitrogen load of the treatment plant are 9700 kgCOD/d and 860 kgN/d with average removal rates of 95% and 65%, respectively.

The biological treatment is equipped with multiple liquid-phase sensors for continuous DO (LDO sc, Hach, USA) monitoring (Fig. 1). Effluent concentrations for various nitrogen species (NH4+, NO3−, and NO2−) are measured daily in 24 h composite samples.

The wastewater is evenly distributed over the six treatment lanes. The N removal process is anoxic – oxic, i.e., anaerobic denitrification to N2 and aerobic NH4+ oxidation. The DO concentration is controlled at distinct set-points for each compartment. The first zones are generally operated anoxically and stirred, but can be aerated, as soon as the air consumption in Zone 3 exceeds a defined threshold. This primarily happens during wet weather conditions and in the winter seasons at low temperatures. The second and the third zone are obligatory oxic, i.e. are continuously aerated. Even under aerated conditions, denitrification can proceed within anoxic microsites/microggregates (Daiger et al., 2007). After the biological treatment to eliminate fixed N, the wastewater enters the secondary clarifiers. Two activated sludge lanes share one secondary clarifier, respectively, and therefore receive the same return sludge (Fig. 1). The biological treatment is operated with a fixed total-solids retention time (SRT) of 13 days. Excess activated sludge is treated in an anaerobic digestion process (not shown in Fig. 1), delivering ammonium-rich reject water to the biological treatment. Increasing the ammonium load in the inflow, reject water is dosed into the primary clarifier to make sure that the N load is equally distributed among the lanes. Typically, reject water from sludge treatment is added overnight from 11 pm to 7 am in batches, every 30 min.

2.2. Continuous N2O monitoring

Continuous N2O emission monitoring was done using the flux chamber approach, as described in Gruber et al., (2020). A part of the monitoring results (November 2019 – December 2020) has already been presented by Gruber et al. (2021). Flux chambers were installed in Zone 1, 2 and 3 according to Fig. 1. Additionally, 1.5-meter-long columns, called anox tubes (Fig. S 1), were installed in Zone 1 of selected lanes (1.1, 2.1, 2.2, 3.2) to sample N2O from the mixed liquor during non-aerated operation by gas stripping with a blower. This technique provides qualitative information on temporal fluctuations of dissolved N2O concentrations for Zone 1. N2O concentrations from the anox tubes are not quantitative, since the efficiency of the stripping process can only be roughly quantified (Fig. S 2). However, anox tubes provide a temporal trend of dissolved N2O concentrations, relevant for interpretation of N2O production/consumption processes. A small share of the off-gas from the chambers and anox tubes was diverted to a central N2O measuring unit, consisting of an automated valve system, preceding a dehumidifier and a non-dispersive infra-red sensor (X-stream, Emerson, St. Louis MO, USA). The N2O monitoring system was installed in October 2019, and since then is running continuously.
2.3. Campaigns with isotope measurements

In 2019, 2020, and 2021 three intensive sampling campaigns supported by N₂O isotopic measurements were performed on two selected lanes (2.1 and 2.2, Table 1). Campaigns were conducted on days with rather dry weather conditions on the day of sampling, since rain weather reduces emissions substantially (Gruber et al., 2020). Gaseous and aqueous samples of specific zones were collected for isotopic analyses and concentrations measurements during the experiments. Details on the experiments are given in Table 1.

2.4. Collection of gaseous and aqueous samples and isotopic analyses

Gas samples for N₂O isotopocule analyses were collected from the sampling lines of the N₂O monitoring device, and the sample gas was extracted with a membrane pump (model PM25032-022, KNF Neuberger AG, Switzerland). Gas samples were integrated over 15 to 20 min to ensure representativeness, dehumidified by permeation drying and stored in 40 L aluminum coated gas bags (model GSB-P/44, Wohlgroth AG, Switzerland) until analysis at the Laboratory for Air Pollution / Environmental Sciences, University of Basel, Switzerland.

Nitrogen and oxygen isotope analyses were conducted at the Department of Environmental System Sciences, ETH Zurich. The average 15N composition of N₂O is referred to as δ¹⁵Nbulk(N₂O) (δ¹⁵Nbulk(N₂O) = (δ¹⁵N + δ¹⁵N°)/2 and the difference between δ¹⁵N° and δ¹⁵N° is termed the site preference (SP ≡ δ¹⁵N° − δ¹⁵N°).

For the analysis of δ¹⁵N and δ¹⁸O in the dissolved N species (NO₃⁻, NO₂⁻, NH₄⁺), mixed liquor samples from the wastewater reactors were filtered with 0.45 and 0.2 µm single-use membrane filters, and stored refrigerated until further processing (Magyar et al., 2021). Nitrogen and oxygen isotope analyses of NO₃⁻, NO₂⁻, and NH₄⁺ were conducted at the Department of Environmental Sciences, University of Basel, Switzerland.

δX‰ = \frac{(R_{sample} - R_{standard})}{R_{standard}} \tag{1}

where X refers to the rare isotopocule, i.e. ¹⁵N and ¹⁸O for dissolved nitrogen species as well as water and ¹⁴N¹⁵N²H₁⁶O (abbreviated as ¹⁸O), ¹¹N¹⁵N²O (¹⁵N²O) and ¹⁵N¹⁴N¹⁶O (¹⁵N¹⁶O) for N₂O, and Rsample and Rstandard are the ratios of the abundance of the least and the most abundant isotopic species in the sample and the standard, respectively. The international scales for nitrogen and oxygen isotope ratios are atmospheric N₂ (AIR-N₂) and Vienna Standard Mean Ocean Water (VSMOW) (Mohn et al., 2016; Toyoda and Yoshida, 1999).

Identify N₂O production processes (3.3)

2.4.1. N₂O isotope measurement (gas phase)

N₂O sample gas concentrations were determined with a non-dispersive infrared spectrometer (X-stream, Emerson, St. Louis MO, USA). Thereafter, sample gases were diluted to ambient N₂O concentrations (approx. 330 ppb) with high-purity synthetic air using mass flow controllers (Vogtlin Instruments GmbH, Switzerland), and the

Table 1

| Campaign | Weather conditions | Date (start-end) | Experiment | Sampling of gas and liquid phase for Isotope analysis in zones | Research focus (results section) |
|----------|--------------------|-----------------|------------|----------------------------------------------------------------|----------------------------------|
| 1        | Short and light rain before and after the experiment | 28.11.2019 (09:00–12:00) | Lane 2.1, Zone 1: aerated | Lane 2.1: 1 per Zone 1–3<br>Lane 2.2: 1 per Zone 1–3<br>– 6 samples | Impact of process control (Zone 1 aeration) on N₂O emissions and processes (3.4) |
|          |                    |                 | Lane 2.2, Zone 1: not aerated |                                             |                                  |
| 2        | Dry weather         | 08.12.2020 (13:00–15:00) | Lane 2.1, Zone 1: not aerated | Lane 2.1: 1 per Zone 1–3<br>Lane 2.2: 1 per Zone 1–3<br>– 6 samples | Identify N₂O production processes under standard operation (3.2) |
|          |                    |                 | Lane 2.2, Zone 1: not aerated |                                             |                                  |
| 3        | Dry weather         | 24.02.2021 (6:00–15:30) | Lane 2.1, Zone 1: not aerated | Lane 2.1: Temporal profile, 5 samples in Zone 1–2<br>Lane 2.2: 10 samples | Impact of daily COD and N inflow variation on N₂O production processes (3.3) |
|          |                    |                 | Lane 2.2, Zone 1: aerated |                                             |                                  |
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2.4.4. Isotope analysis in dissolved N species

The N and O isotopic abundances in NO₃⁻ were determined using the azide method, where NO₃⁻ is chemically converted to gaseous N₂O at low pH (4 to 4.5) (Magyar et al., 2021; Mcllvyn and Altabet, 2005). For the conversion, a sample volume equivalent to 40 or 10 nmol of NO₃⁻ (depending on the concentration in the sample) was added to 3 ml of nitrite-free seawater in a 20 ml headspace vial, and crimp-sealed. The precollection (TREX), as described in Ibraim et al. (2018). All quantum cascade laser absorption spectroscopy (QCLAS), preceded by samples were analysed in triplicate and standard deviations for repeated two-point delta calibration approach was implemented (CG1: 36.12 ± 0.32 ‰; CG2: 18.21 ± 0.12 ‰), and instrumental drift, as well as differences in N₂O concentration corrected (Harris et al., 2020).

2.4.3. H₂O isotope measurement

In experiment 3, aqueous samples were analyzed for δ¹⁸O-H₂O using the high-temperature carbon reduction method. For that purpose, a high-temperature elemental analyzer (TC/EA; Finnigan MAT, Germany) was coupled to a Delta²²XP isotope ratio mass spectrometer via a ConFlo III interface (Finnigan MAT, Germany; Werner et al., 1999).

The TC/EA was additionally equipped with a custom-made Nafion-trap followed by a 4-port valve (Werner, 2003) between the carbon reduction tube and the GC column. The set-up of the carbon reduction tube follows the “MPI-BGC method” described by Gehre et al. (2004). Water was injected automatically with a GC PAL autosampler (CTC, Switzerland) equipped with a 10 µl gas-tight syringe. Preparation for injection of 0.5 µl of water was made with three washing cycles (3 µl) and five pull-ups. All results were normalized to VSMOW and SLAP, assigning consensus values of 0 and 55.5 ‰ for δ¹⁸O and 0 and 428 ‰ for δD to VSMOW and SLAP reference waters, respectively (Coplen, 1988).

2.5. Analyses of reactive N-species

Concentrations of cations (NH₄⁺ -N) and anions (NO₂⁻ -N, NO₃⁻ -N) were analyzed using flow injection analysis (i.e., Faastar flow injection 5000 analyzer, Denmark) and anion chromatography (Methrom 881 compact IC, Switzerland), respectively.

3. Results and discussion

3.1. N₂O emissions at the Hofen WWTP

The average N₂O emissions of lanes 2.1 and 2.2 at the Hofen WWTP were 0.8 kg N₂O-N/d during the monitoring campaign (Table 2). The resulting emission factor (0.2% of the total nitrogen load) is low compared to other WWTPs with full-year nitrification and denitrification (median of: 0.4%) (Gruber et al., 2021). Emissions from both lanes displayed similar temporal patterns, with high emissions in winter, and lower emissions during the summer season (Fig. 2). However, the emission pattern is not reproducible in different years. By far the highest N₂O emissions were observed over several weeks starting in January 2021. The emission peak occurred in parallel with increased NO₃⁻ concentrations in the effluent of the WWTP, which is known to enhance N₂O emissions via both nD and hD pathways and has been linked to emission peak phases in other WWTPs (Gruber et al., 2021b, Ren et al., 2019, Kuokkanen et al., 2021).

In fact, all lanes were fully aerated during the peak emission phase to increase NO₂⁻ oxidation capacities of the biological treatment, which in turn favours N₂O stripping and strongly lowers NO₂⁻ as well as N₂O reduction capacities during denitrification. Consequently, during full aeration of Zone 1, emissions in all zones of both lanes increase. However, the major share of the emissions occurs in Zone 2 (Fig. 2), where likely most of the nitrogen turnover happens in case of full aeration of a lane.

The detrimental effect of aeration of Zone 1 (in terms of N₂O production) compared to anoxic operation was also shown in Campaigns 1 and 3, where the first zone of lane 2.1 or 2.2 were aerated (Table 2). Similarly, in April 2020 only Zone 1 of lane 2.1 was aerated, which led to substantially higher net N₂O emissions as compared to lane 2.2 (Figs. 2, and 5).

Table 2

| Phase                          | Emissions lane 2.1 (kg N₂O−N/d) | Emissions lane 2.2 (kg N₂O−N/d) |
|-------------------------------|---------------------------------|---------------------------------|
| Average (Nov 2019-Mar 2021)   | 0.8 (standard operation, variable) | 0.8 (standard operation, variable) |
| Peak phase (Jan 2021)         | 3.6 (aerated)                   | 4.4 (aerated)                   |
| Campaign 1                    | 1.9 (aerated)                   | 0.4 (anoxic)                    |
| Campaign 2                    | 0.1 (anoxic)                    | 0.3 (anoxic)                    |
| Campaign 3                    | 0.7 (anoxic)                    | 1.7 (aerated)                   |
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Fig. 2. N₂O emissions of individual zones of lanes 2.1 and 2.2 (panel (a)) and effluent NO₂⁻, NO₃⁻ and NH₄⁺ concentrations of all lanes (panel (b)) at Hofen WWTP. Blue lines indicate the day of the three intensive sampling campaigns and numbers in brackets refer to the campaign number.

3.2. Identification of N₂O production pathways using dual isotope mapping approaches

The isotope sampling campaigns at the Hofen WWTP were conducted during different seasons and day times, and under either oxic or anoxic operation of Zone 1 (Tables 1 and 2). The mean SP value for N₂O emitted from oxygen-replete zones in all three experiment was -1.7 ± 2.7 ‰, which is somewhat lower than results (4.5 ‰) from a previous full-scale WWTP study (Toyoda et al., 2011) and literature results for N₂O from Ni, which yields consistently higher SP values (+32.0 to +38.7 ‰). However, values are fully in the range of isotopic signatures measured for nD and hD at a lab-scale WWTP (Wunderlin et al., 2013), as well as in pure culture studies (hD: -7.5 to -13.6 ‰, nD: -13.6 to +1.9 ‰) (summarized in Denk et al. (2017), Ostrom and Ostrom (2017), Yu et al. (2020)). In contrast, N₂O liberated from Zone 1 under anoxic operation, using the anox tube, displayed significantly higher SP values of 12.3 ± 2.2 ‰.

To evaluate the N₂O production pathways during the experiments in more detail, we applied the dual isotope mapping approach, where SP values are plotted against either Δδ₁⁵Nbulk(N₂O, substrate) or Δδ¹⁸O(N₂O, H₂O) and compared to the isotope signatures known from literature for a given process (Yu et al., 2020). In this approach, the Δδ¹⁵Nbulk(N₂O) values are corrected for δ¹⁵N of possible N substrates (NH₄⁺, NO₂⁻, NO₃⁻), with Δδ¹⁵Nbulk(N₂O, substrate) = Δδ¹⁵Nbulk(N₂O) - δ¹⁵Nsubstrate, while δ¹⁸O(N₂O) is compared to δ¹⁸O(H₂O), with Δδ¹⁸O(N₂O, H₂O) = δ¹⁸O(N₂O) - δ¹⁸O(H₂O) (Fig. 3). Wunderlin et al., (2013) followed this approach relating SP to Δδ¹⁵Nbulk(N₂O) values to verify process conditions that are most conducive to distinct production pathways (e.g., hD, nD, Ni) during batch experiments in a laboratory-scale reactor with activated sludge. Since no elevated SP was observed in the aerated zones, no significant contribution of Ni to N₂O production was anticipated. Moreover, Δδ¹⁵N(N₂O, NH₄⁺) values, which considers ammonium as a possible substrate, did not co-vary with the SP values towards Ni source endmember signatures (Fig. S.3).

Alternatively, Lewicka-Szczebak et al. (2016) showed that a dual isotope mapping approach with SP versus Δδ¹⁸O(N₂O, H₂O) is especially suitable to elucidate mixing of N₂O produced by hD or Ni and partial N₂O reduction by denitrification. N₂O produced by Ni typically bears oxygen isotope values of δ¹⁸O(H₂O) ~ 25 ‰, inherited from atmospheric O₂ (Frame and Casciotti, 2010). For N₂O produced from hD or nD, the parameter Δδ¹⁸O(N₂O, H₂O) offers additional insights over δ¹⁸O alone, as discussed below.

The SP values of N₂O emitted under aerated conditions indicate nD or hD as main N₂O production pathway. The relationship of SP with Δδ¹⁸O(N₂O, H₂O) (Fig. 3a) displays a considerable decrease in both SP and Δδ¹⁸O(N₂O, H₂O) during the change from anoxic (Zone 1) to oxic (Zone 2) conditions. This corresponds to a decline in partial N₂O reduction for Zone 2, in relation to Zone 1, as reduction of N₂O to N₂ by hD increases SP of the residual N₂O pool, since the ¹⁵N-O bond is more stable than ¹⁸N-O (summarized in Denk et al. (2017), Ostrom and Ostrom (2017), Yu et al. (2020)). Additional support for the concurrent reduction of nitrite and N₂O through hD comes from the concomitant increase in δ¹⁸O(NO₂⁻) and δ¹⁸N(N₂O) shown in Fig. 3b.

Interpreting the Δδ¹⁸O(N₂O, H₂O) signatures of N₂O emitted in the aerobic zone (i.e., in parallel with low SP values) requires a more nuanced interpretation, but yields additional information. The Δδ¹⁸O (N₂O, H₂O) value is controlled by both equilibrium isotope effects during O-exchange of precursors with water and branching isotope effects during O-abstraction (Casciotti et al., 2007; Casiotti et al., 2010; Kool et al., 2007). Both effects depend strongly on the bacterial community that performs denitrification, and can differ substantially among systems (Kool et al., 2007; Martin and Casciotti, 2016). The observed δ¹⁸O (NO₂⁻) is consistent with complete exchange between NO₂⁻ and water for samples in the aerated zone; the measured δ¹⁸O(H₂O) plus the equilibrium fractionation of 13‰ at 15 to 20 °C yields a composition of ~3‰ (Buchwald and Casciotti, 2013) (Fig. 3b). Complete exchange can be associated with nitrite produced in nitrification (Buchwald et al., 2012; Casiotti et al., 2010), but can also be mediated by the iron-containing nitrite reductase NirS, which is present in many heterotrophic denitrifiers (Casciotti et al., 2007; Casiotti et al., 2002; Kool...
et al., 2007). Then, the final $\Delta^{18}O(N_2O, H_2O)$ of $N_2O$ is determined by the branching kinetic isotope effects associated with nitrite reduction to NO, followed by NO reduction to $N_2$ (Casciotti et al., 2007; Casciotti et al., 2002; Martin and Casciotti, 2016; Rohe et al., 2017). The conversion of NO$_2^-$ to $N_2$ by the nitrite reductase and nitric oxide reductase enzymes then imparts a branching kinetic isotope effect (Casciotti et al., 2007; Casciotti et al., 2002). The identity of the nitrite reductase enzyme (NirK, NirS) controls the size of this branching isotope effect, and thus $\delta^{18}O(N_2O, H_2O)$. $N_2O$ production from nitrite that has an equilibrium value of $\delta^{18}O(NO_2^-, H_2O)$ by bacteria with NirS is associated with a larger oxygen isotope effect and so that $N_2O$ will display values for $\Delta^{18}O(N_2O, H_2O)$ of $28 \pm 6$‰ (Martin and Casciotti, 2016). Various hD species are known to have either NirK or NirS, but only NirK has been found in nD (Kozlowski et al., 2016; Nikaido, 2003; Zumft, 1997; Wei et al., 2015). Therefore, $N_2O$ associated with nD and hD exhibits overlapping ranges for $\Delta^{18}O$ ($N_2O, H_2O$), but values greater than 30% are likely to be associated with hD. The only pure-culture constraint on $\Delta^{18}O$ ($N_2O, H_2O$) for $N_2O$ generated by nD, with a value of $22 \pm 6$‰ (Frame and Casciotti, 2010), falls at the low end of the above-mentioned range, and, thus, consistent with the expectation from the enzyme-based framework provided.

Fig. 3. Isotopic signatures of $N_2O$ liberated from aerated (blue symbols) and anoxic (red symbols) zones of the WWTP Hofen, normalized for the substrate isotopic composition ($H_2O$, NO$_2^-$, NO$_3^-$) for the three campaigns that included isotopic measurements. Dual-isotope plots for SP and $\Delta^{18}O(N_2O, H_2O)$ (panel a), $\Delta^{15}N(N_2O, NO_2^-$) (panel c), and $\Delta^{15}N(N_2O, NO_3^-$) (panel d) are provided. $\delta^{18}O(N_2O)$ vs. $\delta^{18}O(NO_2^-)$ values are displayed in panel (b). Gray lines in panel (b) represent the expected $\delta^{18}O$ values for NO$_2^-$ in equilibrium with water and the measured $\delta^{18}O$ of H$_2O$. Colored areas in panels a, c, and d indicate expected isotopic signatures for $N_2O$ production pathways (Ni = hydroxylamine oxidation, nD = nitrifier denitrification, hD = heterotrophic denitrification) according to Yu et al. (2020). The expected change in isotopic composition during partial reduction of $N_2O$ to $N_2$ is indicated by black "reduction lines". For panels (a) and (c), all data points fall on one line, while for panel (c) data points of individual days present individual reduction lines for Campaigns 1 and 3. Numbers next to data points of Campaign 3 (squares) indicate the sampling sequence (t1: 6 – 7 am, t2: 8 – 9 am, t3 = 10 – 11 am, t4 = 1 – 2 pm, t5 = 2:30 – 3:30 pm).
Orschler et al. (2021) that although hD can theoretically involve both NirK or NirS, in activated sludge systems, it is predominantly performed by NirS. $\Delta^{15}$O(N$_2$O, H$_2$O) values from the aerated zones are about 10‰ higher than those reported by Lewicka-Szczebak et al. (2016) of 16.7 to 23.3‰. The observed discrepancy may be explained by the fact that the underlying values reported by Lewicka-Szczebak et al. (2016) were derived from soil systems that likely differ significantly in terms of the active microbial communities and expressed enzymes, as compared to wastewater systems (Wu et al., 2019).

The prevalence of anaerobic hD under oxic conditions can easily be rationalized by anoxic microsites in sludge flocs even in aerated zones (Sexstone et al., 1985; Daigger et al., 2007). Nevertheless, given the variability seen in $\Delta^{15}$O(N$_2$O, H$_2$O), we cannot exclude a variable contribution from hD under certain conditions, which could be what drives differences between aerobic samples in Fig. 3a. Slightly lower SP and lower $\Delta^{15}$O(N$_2$O, H$_2$O) values may be due to an increased contribution of nD. Alternatively, the higher values may also be caused by a partial reduction of N$_2$O also during aerobic phases, assuming that the organic substrate is not fully consumed in Zone 1 and leaks into Zone 2. Furthermore, N$_2$O with a high SP and $\Delta^{15}$O(N$_2$O, H$_2$O) might be transported, and mixed in, from Zone 1, as discussed in Section 3.3 in more detail.

Plotting SP values relative to $\Delta^{15}$N(N$_2$O, NO$_2^-$) indicates a higher variability among the three intensive sampling campaigns (Fig. 3d). Co-variations in SP and $\Delta^{15}$N(N$_2$O, NO$_2^-$) values between N$_2$O from aerated and anoxic zones during individual campaigns were driven by the partial N$_2$O reduction, indicated by the reduction line. Differences in $\Delta^{15}$N(N$_2$O, NO$_3^-$) between experiments, e.g., 31.6‰ (Campaigns 1 and 2) versus 41.1‰ (Campaign 3), were possibly caused by concentration-dependent variations (affecting cell-specific rates) in the isotope effects associated with denitrification (Kritee et al., 2012). More precisely, the higher NO$_3^-$ concentrations during experiment 3 (10–18 mg NO$_3^-$-N/L) compared to experiment 1 and 2 (0–7 mg NO$_3^-$-N/L) may manifest in substantially higher isotope effects. The increased nitrate concentrations were due to the full aeration of all zones over multiple weeks before experiment 3. The operation led to reduced denitrification activity and NO$_3^-$ accumulation in the biological treatment.

Interestingly, $\Delta^{15}$N(N$_2$O, NO$_2^-$) was more consistent than $\Delta^{15}$N(N$_2$O, NO$_3^-$) between campaigns, i.e., isotope effects seemed less strongly affected by N substrate concentrations (Fig. 3c). Therefore, isotopic signatures for samples from aerated and anoxic compartments cluster significantly closer to the predicted reduction line (Fig. 3c). The observed correlation of delta values for individual campaigns hence supports the notion that the isotopic composition of NO$_3^-$, NO$_2^-$ and N$_2$O are mostly controlled by the sequential reduction of NO$_3^-$ to N$_2$ during complete denitrification.

In summary, the isotopic composition of N$_2$O, NO$_2^-$, and NO$_3^-$ consistently point towards a high contribution of hD to N$_2$O production during aeration on all days. hD may be of variable relevance, yet Ni can be excluded as a significant contributor. hD was previously shown to be more sensitive than the $\Delta^{15}$N(N$_2$O, substrate) approaches. However, combining both approaches as shown here, has the benefit of being able to additionally validate interpretations, and to provide independent process information to assess the full complexity of concurrent N$_2$O formation and reduction.

3.3. Diurnal variation in N$_2$O emissions and production pathways

The main focus of the third campaign was to investigate the effect of the diurnal patterns in N loading (controlled by reject water dosage) and COD substrate inflow on N$_2$O emissions and variations in N$_2$O reduction. For this, we analysed the isotopic signatures of N$_2$O and nitrogenous substrates in Zone 1 and 2 for five different time points during one day at lane 21 (Fig. 4). N$_2$O emissions exhibited a clear diurnal pattern, with a peak at 9 am, right before the reject water dosage was stopped (Fig. 4a). N$_2$O concentration changes in the anoxic zone, measured with the anox tube, were consistent with changes in the N$_2$O flux from Zone 2 and 3. While NH$_4^+$ concentrations also exhibited a clear diurnal variation pattern, NO$_3^-$ concentrations were relatively stable throughout the study period (Fig. 4c, Figs. S.4 and S.5, (SI)). NO$_3^-$ was highest in Zone 1 and gradually decreased in Zone 2 and 3, respectively (Fig. 5a, (SI)).

The diurnal trend of the N$_2$O site preference in Zone 1 indicates a decreasing importance of N$_2$O reduction from 7 am to 9 am (sampling points 1 and 2), also shown in the dual isotope mapping approach, e.g., for SP vs. $\Delta^{15}$O(N$_2$O, H$_2$O) (Fig. 3a). After 10 am, SP and $\Delta^{15}$O(N$_2$O, H$_2$O) values for N$_2$O from Zone 1 increased along the predicted reduction line, which suggests a return to an increasing relevance of N$_2$O reduction for samples 3 to 5. NO$_3^-$ concentrations remain stable in Zone 1 (Fig. 4c) despite an increase of N$_2$O inflow from the return sludge (Fig. 5a, (SI)), confirming that heterotrophic nitrate reduction (hD) was very active after 9 am. We suggest two main causes for the strong daily variation in N$_2$O emissions and N removal.

First, the dosage of reject water and the morning peak in N inflow, typically seen in WWTPs, led to a NH$_4^+$ concentration increase (Fig. 4c, t1 – t2), while the N$_2$O reduction capacity of the WWTP was lower due to the increased supply of NO$_3^-$ . Second, and more importantly, the availability of organic substrate typically exhibits daily fluctuations. Therefore, despite high NH$_4^+$ loads from 10 am to 2 pm (t3 – t4), high availability of organic substrate led to increased nitrogen removal and, in turn, increased fractional N$_2$O reduction rates. Notably, COD concentrations were not measured during the campaign, but are expected to correlate with the inflow rate to the wastewater treatment plant, which exhibits reproducible daily variation (Fig. S.7, (SI)).

The N$_2$O SP in Zone 2 is at its maximum between 6 and 9 am, probably due to transport of N$_2$O produced in Zone 1, where both N$_2$O production and reduction were high during this part of the diurnal cycle, as described above (Fig. 4b). This would imply that N$_2$O emissions from Zone 2, before and during the peak phase, i.e., the end of the reject water dosage, comprise a substantial contribution of N$_2$O from Zone 1. hD as the main source of this N$_2$O is supported by the high $\Delta^{15}$O(N$_2$O, H$_2$O) values (36.2 ± 2.3‰). Alternatively, high SP values in Zone 2 before 9 am can be explained by partial N$_2$O reduction, but this is unlikely given COD limitation during reject water dosage. Moreover, transport of N$_2$O produced in an anoxic zone to an aerobic zone has been reported earlier for other WWTPs (Mampaey et al., 2016). After 10 am, the difference in SP values between Zone 1 and 2 was increasing again, indicating that N$_2$O transport and mixing was less important.

In addition, the contribution of hD to N$_2$O formation might have increased after 10 am in Zone 2, which could further explain the lower SP and $\Delta^{15}$O(N$_2$O, H$_2$O) here. Nevertheless, we believe that hD also contributed a major part to the emissions in the aerobic zones between 11 am and 4 pm, given the still-high $\Delta^{15}$O(N$_2$O, H$_2$O) values.

3.4. N$_2$O emissions depend on process operation

The seasonal dynamics in N$_2$O emissions indicate that phases when the air consumption in Zone 3 exceeded a defined threshold, and thus when Zone 1 was aerated, were generally characterized by high net N$_2$O production (Fig. 2). To better understand the effect of aerobic conditions in the first zone on overall N$_2$O formation, we compared the isotopic signatures of N$_2$O produced along a fully aerated lane (2.1) and a lane under standard operation, i.e., with anoxic conditions in the Zone 1 (2.2)
The episodes of reject-water dosage in the morning had a high impact on the emissions (i.e., high N$_2$O emissions in Campaign 3), but N$_2$O emissions were even higher from the fully aerated lane (Table 2). The difference between lanes was primarily driven by emissions in Zones 1 and 2, while emissions in the third zone were comparable (Fig. 5a).

The explanation for increased N$_2$O emissions from the fully aerated lane 2.1 can be assessed when comparing isotopic signatures of the N$_2$O released from Zone 1 of both lanes (Fig. 5b, Campaign 1). The N$_2$O isotopic signature measured in the Zone 1 of lane 2.2, with conventional operation, i.e., Zone 1 mostly anoxic, indicates a substantial reduction of N$_2$O. In contrast, for lane 2.1, with Zone 1 aerated, the share of N$_2$O reduction was substantially lower. The proportion of N$_2$O reduction can be estimated quantitatively by the expression $\Delta SP = \varepsilon SP \times \ln f$ (Jinuntuya-Nortman et al., 2008; Lewicka-Szczebak et al., 2017; Mariotti et al., 1981), with $\varepsilon$ being the enrichment factor (-8.2 to -2.9 ‰ (Yu et al., 2020)), and f the fraction of unreacted N$_2$O. The isotopic enrichment factor between product P and substrate S is defined as $\varepsilon X_P/S = \alpha X_P/S - 1 = \delta X_P / \delta X_S - 1$, where $\alpha$ is the isotopic fractionation factor. Applying this approach yields an estimate of 92% of N$_2$O (84 to 99% using max and min fractionation factors) reduced for the anoxic Zone 1 of lane 2.2, while only 68% (56 to 90% using max and min fractionation factors) is reduced in the aerated Zone 1 of lane 2.1 (assuming that the SP values for N$_2$O from Zone 2 are representative for the N$_2$O production process).

As during Campaign 3, N$_2$O production was very likely driven by hD, given the increased $\Delta^{18}O$ (N$_2$O, H$_2$O) values (35.2 ± 0.6 ‰) in the aerobic zones.

Campaigns 1 and 3 revealed that organic carbon availability, aeration of Zone 1, and reject-water N dosage are the most important modulators of N$_2$O emissions during standard operation at the Hofen WWTP, and at a given time of the year. Notably, emissions were lowest in Campaign 2 (Table 2), with anoxic conditions in Zone 1 of both lanes, without reject-water dosage and sampling times in the afternoon, where increased organic substrate concentrations are expected. While it seems relatively clear that aerobic conditions in Zone 1 and low organic substrate availability both lead to higher emissions by impairing a more efficient N$_2$O reduction, the mechanism behind the increased production of N$_2$O caused by elevated reject-water dosage (which leads to an increase in NH$_4^+$ concentrations) is not fully understood (Gruber et al., 2020). Most plausibly, elevated N$_2$O emissions are directly linked to the high NH$_4^+$ concentrations (following substrate- vs- intermediate product systematics). Alternatively, it is possible that the composition of the
reject water is somehow unfavorable for heterotrophic denitrifiers and nitrifiers. Further research is needed to unravel underlying mechanisms, e.g., by comparing the effects of dosages of reject-water NH$_4^+$ versus (NH$_4$)$_2$SO$_4$ solution in activated sludge. Nevertheless, our results already yield important information regarding efficient strategies to reduce N$_2$O emissions during normal operation at the Hofen WWTP. The guiding principle for the mitigation of N$_2$O emissions is to maximize N$_2$O reduction by avoiding aeration of Zone 1, and dosing reject-water primarily during periods with high organic carbon load, e.g., in the afternoon. The adaptation of the feeding strategy to optimize organic carbon utilization towards most efficient N$_2$O reduction has been successfully applied in side-stream treatment (Peng et al., 2017). However, changing reject-water dosage operation strategies should be critically evaluated, as the effects of the NH$_4^+$ loading are multifaceted. That is, besides potential impacts of the NH$_4^+$ dosage on net N$_2$O emissions, other constraints need to be considered. For example, increased NH$_4^+$ peak concentrations can lead to NH$_4^+$ breakthrough, and load equilibration in the diurnal pattern is beneficial for the nitrification performance (Meyer and Wilderer 2004). We propose to apply conventional activated sludge modeling and full-scale testing, combined with extensive process monitoring, to optimize reject-water dosage in terms of effluent quality and maximized reduction capacities for N$_2$O mitigation (Henze et al., 2000).

Isotopic technologies were successfully applied to analyze the contribution of N$_2$O production pathways at the Hofen WWTP, and provided mechanistic understanding to support mitigation strategies. Still, long-term monitoring of the isotopic composition of N$_2$O and other nitrogen species is needed in future studies to evaluate the consistency and robustness of the approach. A major advantage to characterize contributions of N$_2$O reduction and production pathways at the Hofen WWTP involved the cascaded lanes, with clearly defined redox conditions in each zone. We expect that the application in flow-through, non-compartmented activated sludge systems can be more challenging due to increased mixing over a whole lane, leading to a higher exchange of the nitrogen pools. Furthermore, continuous long-term monitoring is important for the extrapolation and interpretation of the data and the characterization of the seasonal emission peaks. The lion’s share of the total annual N$_2$O emissions can be attributed to the January peak emission period (Fig. 2; 50% of the total emissions) in association with elevated NO$_2^-$ concentration levels. Seasonally impaired NO$_2^-$ oxidation in WWTPs, leading to NO$_2^-$ accumulation, has been linked to low abundances of nitrite oxidizing bacteria (NOB) and drastic changes in the whole activated sludge microbial community (Gruber et al., 2021). However, the NOB loss observed by Gruber et al. (2021) at the Uster WWTP led to NO$_2^-$ accumulation over a periods of 1–2 months, and it is unclear whether similar process were also responsible for the accumulation of nitrate over a few weeks at the Hofen WWTP.

4. Summary and conclusions

- Measurements of relative $^{15}$N and $^{18}$O abundances in nitrogen-bearing molecules were successfully applied to characterize dynamics of N$_2$O formation pathways under normal operation in a full-scale activated sludge WWTP. N$_2$O was mainly produced by heterotrophic denitrification, while nitrifier denitrification appeared to be less important and of rather variable influence; NH$_3$OH oxidation was negligible. 
- Seasonal emission peaks occurred during winter when NO$_2^-$ accumulates, and when the biological treatment is operated at full aeration, but NOB activity is still impaired. 
- Based on N$_2$O isotopic measurements, N$_2$O reduction was identified under anoxic conditions, and to lesser extent also underoxic conditions, when it is restricted to anoxic micro-niches. Fractional N$_2$O reduction was most pronounced under organic-substrate-replete conditions, while N$_2$O accumulation in the anoxic zone was primarily observed when organic substrate was limiting. Hence, the daily variation of organic substrate has a strong impact on the reduction of N$_2$O, and in turn, diurnal N$_2$O emission fluctuations. 
- The dosage of reject-water and full aeration of the biological treatment significantly increased N$_2$O emissions, since N$_2$O reduction was strongly impeded. Hence, an efficient mitigation strategy towards optimized N$_2$O reduction may involve shifting reject-water dosage to periods with high organic substrate availability, as well as avoiding full aeration of the biological treatment. 
- Coupling isotopic technologies with continuous long-term monitoring of N$_2$O emissions is a powerful tool for qualitative N$_2$O pathway identification and the development of N$_2$O mitigation strategies in full-scale WWTPs. However, clearly defined conditions in a reactor system are required to interpret the data.
Declaration of Competing Interest

The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The study and WG were financially supported by the Swiss Federal Office for the Environment (FOEN), the Swiss National Foundation for Scientific Research, the canton of Bern (AWA), the canton of Basel-Landschaft (AIB), the canton of Thurgau (ARU), the canton of Zurich (AWEL), TBF + Partner AG Consulting Engineers, Holinger AG, Hunziker Betatech AG, Alpha Wassertechnik AG, arabern WWTP, REAL Entsorgung St. Gallen and Uster WWTP. P.M.M., J.M., A.J. and M.F.L. received funding from the SNF Synergia project, CRSII1709876. We acknowledge Thomas Kuhn for the help in isotopic measurements, Hans-Ulrich Bauer and Daniel Gahler for providing access to the Hofen WWTP and assisting during sampling. We would also like to thank Sylvia Richter and Karin Rottemann for analytical support and Tobias Bührer for assisting during sampling.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wra.2020.101303.

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