Isotopic evidence for nitrous oxide production pathways in a partial nitritation-anammox reactor

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Abstract

Nitrous oxide (N\textsubscript{2}O) production pathways in a single stage, continuously fed partial nitritation-anammox reactor were investigated using online isotopic analysis of offgas N\textsubscript{2}O with quantum cascade laser absorption spectroscopy (QCLAS). N\textsubscript{2}O emissions increased when reactor operating conditions were not optimal, for example, high dissolved oxygen concentration. SP measurements indicated that the increase in N\textsubscript{2}O was due to enhanced nitrifier denitrification, generally related to nitrite build-up in the reactor. The results of this study confirm that process control via online N\textsubscript{2}O monitoring is an ideal method to detect imbalances in reactor operation and regulate aeration, to ensure optimal reactor conditions and minimise N\textsubscript{2}O emissions.

Under normal operating conditions, the N\textsubscript{2}O isotopic site preference (SP) was much higher than expected - up to 40\% - which could not be explained within the current understanding of N\textsubscript{2}O production pathways. Various targeted experiments were conducted to investigate the characteristics of N\textsubscript{2}O formation in the reactor. The high SP measurements during both normal operating and experimental conditions could potentially be explained by a number of hypotheses: i) unexpectedly strong heterotrophic N\textsubscript{2}O reduction, ii) unknown inorganic or anammox-associated N\textsubscript{2}O production pathway, iii) previous underestimation of SP fractionation during N\textsubscript{2}O production from NH\textsubscript{3}OH, or strong variations in SP from this pathway depending on reactor conditions. The second hypothesis - an unknown or incompletely characterised production pathway - was most consistent with results, however the other possibilities cannot be discounted. Further experiments are needed to fully resolve N\textsubscript{2}O production pathways in PN-anammox systems and definitively distinguish between these hypotheses.

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1. Introduction

In recent years, interest in wastewater treatment with anaerobic ammonium oxidizers (anammox) has increased, resulting in significant technological developments [64, 24, 23, 20, 12, 16], as the process offers the potential for reductions of >50% in energy consumption compared to traditional nitrification-denitrification, without decreasing nitrogen removal efficiency [54]. The anammox reaction, which involves conversion of nitrite (NO$_2^-$) and ammonium (NH$_4^+$) to form N$_2$, plays an important role in a diverse range of artificial, marine and terrestrial environments [42, 60, 34, 33, 65]. Wastewater treatment with anammox involves partial nitritation (PN), where ∼50% of NH$_4^+$ is first oxidised to NO$_2^-$ by ammonia oxidizing bacteria (AOBs) under oxic conditions, coupled to the anoxic anammox reaction to produce N$_2$. As an organic carbon source is not required, PN-anammox is particularly suited for the treatment of high NH$_4^+$, low carbon wastes, such as digester liquor from conventional wastewater treatment.

For process stability in single-reactor PN-anammox systems, it is critical to balance AOB and anammox activity while minimising growth of nitrite-oxidising bacteria (NOBs). This is achieved by controlling the air supply rate to be the rate-limiting factor for AOB activity and therefore for the entire process, thus avoiding nitrite accumulation [23]. Anammox bacteria are inhibited by dissolved oxygen (DO) concentrations higher than 0.2 mg L$^{-1}$ (6.25 µM) [23] and potentially also by high NO$_2^-$ concentrations (eg. >600 mg L$^{-1}$ or 10 mM) [10]. If AOB activity is reduced, DO can increase as less oxygen is consumed in the reactor, inhibiting anammox and interrupting operation. Thus the maximal sustainable treatment rate depends on the sludge activity, which - according to experience from long-term full-scale operations - may not be assumed constant. Online monitoring of NO$_2^-$ has the potential to serve as control parameter to monitor activity. However, as no
suitable electrode is available for the online measurement of NO$_2^-$, online measurements of N$_2$O mixing ratio$^1$ have been suggested to act as a proxy for NO$_2^-$, thus presenting an ideal method to monitor and control reactor dynamics [70].

Wastewater treatment is an important, growing source of N$_2$O, through a number of pathways summarised in Table 1. The wastewater sector currently contributes $\sim$6% of N$_2$O emissions globally, and best estimates predict that emissions from the sector will increase by $>$25% between 1990 and 2020 [4]. N$_2$O is a potent greenhouse gas with a global warming potential 298 times higher than CO$_2$ (100-yr) [14] and the most important ozone depleting substance currently released [9, 50]. Online N$_2$O monitoring for process stability results in control and reduction of N$_2$O emissions, thus providing an additional benefit in terms of greenhouse gas (GHG) release.

N$_2$O production by AOBs can be envisaged as a ‘leaky pipeline’ [13] because N$_2$O is an unwanted by-product during the goal reaction, oxidation of NH$_4^+$ to NO$_2^-$. Two major pathways are implicated in the emission of N$_2$O by AOBs: Nitrifier denitrification, and NH$_2$OH oxidation (see Table 1). When the overall turnover of NH$_4^+$ by AOBs increases, the ‘leaking’ of N$_2$O also increases - thus N$_2$O emissions can roughly indicate AOB activity. Biotic and abiotic N$_2$O production by hydroxylamine (NH$_2$OH) oxidation can be minimised by keeping the concentration of NH$_4^+$ relatively low (eg. by intermittent dosing of wastewater); previous studies suggest that even at high NH$_4^+$ concentrations this pathway contributes less than 20-30% of total N$_2$O emissions in PN-anammox reactors [43, 21]. In contrast to AOBs, it was generally agreed until recently that anammox bacteria do not produce significant amounts of N$_2$O [26, 44]; results from Lotti et al. (2014) suggest however that up to 0.2% of nitrogen removed by anammox may in fact be released as N$_2$O [36]. This is much lower than the percent of ammonium released as N$_2$O by AOBs.

$^1$Mixing ratio is the ratio of the component of interest to the total of all other constituents in a mixture. In this paper, mixing ratio always refers to the molar mixing ratio, ie. ppm = parts per million = moles of X per million moles of the total mixture.
during imbalances in the reactor, thus in a PN-anammox reactor, nitrifier denitrification is expected to dominate N$_2$O production. Online N$_2$O measurements can therefore act as a proxy for NO$_2^-$ concentration, which is an indicator for the relative activity of AOBs compared to anammox.

A major challenge for process regulation via N$_2$O measurements is the activity of NOBs, which reduce N$_2$O emissions from nitrifier denitrification by consuming NO$_2^-$. Monitoring the net NO$_3^-$ production to NH$_4^+$ consumption ratio (hereafter $r$(NO$_3^-$)/$r$(NH$_4^+$)) - which should be approximately 10-11% due to NO$_3^-$ production by anammox when NOBs are not active [55, 6] - to detect NOB activity is therefore important for process control. In addition, NOB activity should be minimised, eg. with regular washout [23]. Heterotrophic denitrifiers (HET) also release N$_2$O during the stepwise reduction of NO$_3^-$ to N$_2$, however HET activity is minimal in PN-anammox systems due to low organic carbon concentrations [24, 49].

N$_2$O can be the most important greenhouse gas released from wastewater treatment and in addition, shows great potential as an online parameter to monitor process conditions in PN-anammox systems. However, a thorough understanding of N$_2$O production pathways is lacking, which is necessary both to mitigate emissions and to effectively use online N$_2$O measurements for process control. N$_2$O isotopic composition is particularly useful to quantify N$_2$O production via different pathways (Table 1). The most abundant four isotopocules of N$_2$O are $^{14}$N$^{14}$N$^{16}$O, $^{14}$N$^{14}$N$^{18}$O, $^{14}$N$^{15}$N$^{16}$O ($\alpha$) and $^{15}$N$^{14}$N$^{16}$O ($\beta$). The bulk $^{15}$N isotopic composition refers to the average $\delta^{15}$N at both positions:

$$\delta^{15}$N$^{\text{bulk}} = \frac{\delta^{15}$N$^{\alpha} + \delta^{15}$N$^{\beta}}{2} \quad (1)$$

The N$_2$O ‘site preference’ (SP) refers to the difference in $^{15}$N isotopic composition of the
central (α) position N compared to the terminal (β) position N:

\[ SP = \delta^{15}N^\alpha - \delta^{15}N^\beta \]  

Site preference of N₂O, unlike \( \delta^{15}N^{\text{bulk}} \), is independent of the substrate isotopic composition, and it is therefore a robust tool to differentiate between N₂O production pathways. While isotopic signatures are known for some pathways, as shown in Table 1 [57, 47], characteristic SP values of other pathways, for example the possible production of N₂O by anammox [36], are still undetermined. In addition, reduction of N₂O by HET increases the site preference of the remaining N₂O, which can complicate the partitioning of different production pathways. To overcome these difficulties, combination of site preference data with simultaneous measurements of \( \delta^{18}O \) and \( \delta^{15}N^{\text{bulk}} \) can help to constrain the effect of N₂O reduction by HET [46, 63]. Recent developments in spectroscopy [66, 40, 17] allow online monitoring of N₂O isotopic composition including site preference, which can be combined with online measurements of specific reactor operating conditions in a well-mixed experimental system, to investigate N₂O production and consumption pathways shown in Table 1.

The aim of this study is to investigate the major pathways leading to N₂O production in a continuously fed PN-anammox reactor, and thereby facilitate the use of online N₂O measurements to control process stability. A threshold limit for the net production of N₂O per NH₄⁺ consumed in the reactor (hereafter \( \frac{r(N_2O)}{r(NH_4^+)} \)) is used to automatically control the reactor aeration rate and maintain balance between AOB and anammox activity. We report the first online measurements of N₂O offgas isotopic composition during wastewater treatment, made possible by developments in laser spectroscopy in recent years [66, 40, 17]. A series of experiments were carried out for targeted investigation of N₂O production under different conditions. These results will be used to improve our understanding of N₂O production pathways during PN-anammox treatment, and to
investigate the possible production of N$_2$O by anammox bacteria.

2. Materials and methods

2.1. Pilot-scale partial nitritation-anammox reactor

The measurements made in this study were carried out using a pilot-scale sequencing batch reactor, which is described in detail in the supplementary information (Section S1.1, Figure S1). The reactor had a maximum fill level of 400 L and was operated in ‘intermittent feeding’ mode, which involves semi-continuous addition of supernatant liquor to maintain NH$_4^+$ concentration at the setpoint over an entire reactor cycle. The one-month measurement period included 32 fill cycles of 9-33 hours, with an average length of 21 hours, designated a-ff as shown in Figure S2. These cycle names will be used throughout the paper to discuss the different experiments. Throughout the paper, production and consumption of nitrogen species (eg. NH$_4^+$, NO$_2^-$, NO$_3^-$ and N$_2$O) are always reported as net rates.

Measurements were made under both ‘baseline’ and experimental conditions. Baseline conditions were defined as when NO$_2^-$ concentration was low (<1 mg-N L$^{-1}$) and no compounds were added to perturb the system (see Table 2 and Figure S2 for all cycle designations). During baseline experiments, N$_2$O mixing ratios in the offgas were used as a proxy for NO$_2^-$ concentrations in the reactor in order to infer the relative activity of AOBs vs anammox and regulate reactor aeration accordingly. Minimum (180-200 L h$^{-1}$) and maximum (400-750 L h$^{-1}$) ranges for aeration rates were set, between which aeration could automatically vary: When $r$(N$_2$O)/$r$(NH$_4^+$) reached a defined maximum (eg. 0.5 or 1%), the aeration flow was reduced to decrease AOB activity relative to anammox activity (Figure 1). Baseline conditions were considered with both low and high NH$_4^+$, whereby NH$_4^+$ concentration was set to $\sim$10-20 mg-N L$^{-1}$ or 100 mg-N L$^{-1}$ h$^{-1}$ respectively by continuous feeding of digester liquid.
The reactor was also run under a range of different conditions designed to represent boundary settings for normal operation, in order to test the suitability of online \( \text{N}_2\text{O} \) measurements for process control by improving our understanding of how the offgas \( \text{N}_2\text{O} \) mixing ratio responds to various ‘extreme’ situations. The following short-term experiments were performed:

- increase of the aeration rate,
- external addition of \( \text{NO}_2^- \) under both normal and \( \text{N}_2 \) aeration, and
- external addition of \( \text{NH}_2\text{OH} \)

The goal was to favor different \( \text{N}_2\text{O} \) formation pathways, and to investigate the isotopic end-member signature of the ‘unknown’ pathway. Isotopic measurements were carried out to gain insight into \( \text{N}_2\text{O} \) production pathways. The experiments are summarised in Table 2 and an overview of the measurements is given in Figure S2. The results are presented in Section 3.3.

2.2. Online spectroscopic analysis of trace gas emissions and \( \text{N}_2\text{O} \) isotopic composition

Isotopic measurements were carried out with a Quantum Cascade Laser Absorption Spectrometer (QCLAS; Aerodyne Research, Inc.) [66, 39, 40] using the set up shown in Figure S1 and described in detail in Section S1.3. In brief, 50 sccm of offgas from the SBR is pulled through an FT-IR (CX 400, Gasmet Technologies Inc.) which measures the mixing ratios of \( \text{N}_2\text{O}, \text{CO}_2, \text{CH}_4, \text{CO} \) and \( \text{NO} \) with one minute resolution. The FT-IR measurement of \( \text{N}_2\text{O} \) mixing ratio is used to dynamically dilute the offgas to a mixing ratio of 45 ppm, which is then measured in the QCLAS at a pressure of 21 hPa following chemical removal of \( \text{CO}_2 \) and \( \text{CO} \) [32].

The isotopic composition of offgas \( \text{N}_2\text{O} \) (\( \delta^{15}\text{N}^\alpha, \delta^{15}\text{N}^{\beta}, \delta^{15}\text{N}^{\text{bulk}} \) and \( \delta^{18}\text{O} \)) is measured continuously at one second time resolution over \( \approx 30 \) minute periods (see Figure S3). Measurements are corrected and calibrated to the international isotopic standard
scales, V-SMOW (Vienna Standard Mean Ocean Water) for $\delta^{18}$O and Air-N$_2$ for $\delta^{15}$N as described in the supplementary information, Section S1.3. Compatibility of N$_2$O isotopomer analysis by QCLAS with isotope ratio mass spectrometry (IRMS) laboratories was recently demonstrated in an interlaboratory comparison campaign [41]. Lags in the temporal response of SBR process monitor, FTIR and QCLAS measurements were determined by comparing the N$_2$O mixing ratios measured in the three instruments, and all data was corrected to the time scale of the SBR process monitor. Agreement between N$_2$O mixing ratios from the three measurements was very good (±5-10%), although peaks in N$_2$O mixing ratios analysed by QCLAS tended to be slightly dampened due to mixing of process gas in the laser cell.

3. Results and discussion

3.1. Process control using online monitoring of net N$_2$O production rate

3.1.1. Baseline conditions with low NH$_4^+$ concentration

Achieving a balance between AOB and anammox activity while maintaining low NOB activity is one of the key challenges in PN-anammox reactor systems [23, 70]. Effective process control is therefore a critical factor preceding the adoption of single-reactor PN-anammox systems in full scale wastewater treatment operations [23]. In the combined PN-anammox system used in this study, the $r$(N$_2$O)/$r$(NH$_4^+$) was used as a trigger to set the aeration rate and thus control the relative activities of aerobic AOBs and anaerobic anammox bacteria, as suggested by Wunderlin et al. [70]. Figure 1 illustrates that reactor operation was stable over two full 24-hour baseline cycles (j and k). This confirms that online measurement of N$_2$O mixing ratio in reactor offgas can be effectively used to control the aeration rate, and thus microbial activities, of a single-stage PN-anammox system under specific baseline conditions.
3.1.2. Baseline conditions with high NH$_4^+$ concentration

Automatic regulation during baseline cycles with elevated NH$_4^+$ (setpoint 100 mg-N L$^{-1}$ h$^{-1}$) was tested, to investigate N$_2$O production with higher NH$_4^+$ consumption rates. At high NH$_4^+$ concentrations, N$_2$O emissions can be expected to increase [69, 48]. This was confirmed with the results of this study. When the NH$_4^+$ setpoint was 100 mg-N L$^{-1}$ (cycle $n$), the initial NH$_4^+$ consumption rate was much higher than under baseline conditions; 30 mg-N L$^{-1}$ h$^{-1}$ compared to $<$10 mg-N L$^{-1}$ h$^{-1}$ with low NH$_4^+$ load (Figure 1). In the initial phase of cycle $n$ (up to 4 hours after onset) both the net N$_2$O production rate (0.3 mg-N L$^{-1}$ h$^{-1}$, average over complete cycle 0.08 mg-N L$^{-1}$ h$^{-1}$) and the NH$_4^+$ consumption rate peaked, although the $r$(N$_2$O)/$r$(NH$_4^+$) ratio was not especially high at this point. Around 5 hours after the onset of cycle $n$, N$_2$O emissions and the $r$(N$_2$O)/$r$(NH$_4^+$) drastically increased, thus the aeration rate was automatically reduced and consequently NH$_4^+$ consumption rate decreased. Throughout the rest of the cycle, N$_2$O emissions varied cyclically in response to the automatic regulation of the aeration rate. We did not observe an increase in either NO$_2^-$ concentration or net NO$_3^-$ production rate during high NH$_4^+$ experiments (NO$_3^-$ production level was 10.4±2.4% of NH$_4^+$ consumption; similar to low NH$_4^+$ baseline data at p<0.05), showing that the anammox population was strong, while the NOB population in the reactor was weak and the NOB washout and aeration control were effective.

Although the online N$_2$O measurement was able to control the process to achieve approximately the desired ratio of N$_2$O production to NH$_4^+$ consumption (with minimal NO$_3^-$ production) even at high NH$_4^+$ concentration (cycle $n$), the system was in a much more dynamic state than with a low NH$_4^+$ concentration (Figure 1). This resulted in much larger variations in aeration rate and $r$(N$_2$O)/$r$(NH$_4^+$) than under low NH$_4^+$ conditions (cycles $j$ and $k$). Consistent results have been observed in previous studies, which also reported peak N$_2$O emissions when NH$_4^+$ concentrations were high, attributed to enhanced
nitrifier denitrification [21, 51, 35]. The NH$_4^+$ consumption peak at the beginning of cycle $n$ is most likely due to enhanced AOB activity, however the current understanding of NH$_4^+$ and NO$_3^-$ consumption and N$_2$O production pathways does not allow us to interpret features of both the baseline and high NH$_4^+$ measurements based on N$_2$O mixing ratio and process parameters alone. Therefore, in the following section the isotopic composition of N$_2$O emitted from the reactor under baseline conditions (both low and high NH$_4^+$) will be discussed, to understand which pathway(s) contribute the majority of N$_2$O emissions.

3.2. Isotopic composition of N$_2$O produced under baseline conditions (low and high NH$_4^+$ concentrations)

Baseline experiments with low NH$_4^+$ represent optimal operating conditions, therefore the isotopic measurements made during these experiments reveal the N$_2$O production pathway(s) that will be most important in a stable reactor (Section 3.1.1). The mean SP during baseline experiments with low NH$_4^+$ was 34.3±2.8‰ (Figure 2). This is significantly higher than SP values reported for conventional treatment systems, where measured SP was between 4.5 and 23‰ [49, 62], and compared to between 4.9 and 14.1‰ for a batch-fed heterotrophic PN reactor with high concentrations of NH$_4$ and NO$_3^-$ [21]. All the measured SP values under baseline conditions in the present study were larger than 28‰, which is much higher than previously measured values (Figure 2). In 36% of measurements, the SP was even larger than 36‰, which is the highest currently measured value for any known production pathway [15] (Table 1) - although it is possible that previous studies do not encompass the full range of process conditions and thus of isotopic fractionation factors that are possible for known reaction pathways.

At high NH$_4^+$ concentrations under baseline conditions (cycle $n$; Section 3.1.2), SP was 28.3±1.9‰; this is significantly lower than with low NH$_4^+$ concentrations (p<0.001) but still higher than reported previous studies. This finding is quite surprising, since based on the current understanding, a higher contribution from the NH$_2$OH pathway is expected
at higher NH$_4^+$ concentrations, resulting in SP values closer to 33‰ [57, 69]. However, the SP values for N$_2$O produced during both low and high ammonium concentrations under baseline conditions were close to previously measured values for N$_2$O from NH$_2$OH oxidation. A low contribution of NH$_2$OH oxidation to N$_2$O production is supported by the observed lowest SP values when the NH$_4^+$ consumption rate ($r$(NH$_4^+$)) was highest at the beginning of cycle n (24.5±0.5‰; p<0.001).

Over the entire course of the experiments (all experiments, including baseline), higher concentrations of NO$_2^-$ resulted in enhanced N$_2$O emissions with low SP (Figure S4), showing enhanced nitrifier denitrification consistent. SP of 4.9-14.1‰ was measured in a batch-fed reactor with NH$_4^+$$>$200 mg-N L$^{-1}$ and elevated DO where N$_2$O emissions were 5.6% of NH$_4^+$ turnover [21]. Our results show that higher NH$_4^+$ and DO result in lower SP and more N$_2$O, thus extrapolating to the conditions in [21] suggests that our measurements are consistent with the results presented by [21]. Higher NH$_4^+$ concentrations and conversion rates therefore seem to support N$_2$O formation via the nitrifier denitrification pathway.

In summary, isotopic data under baseline conditions (with both low and high NH$_4^+$ concentrations) showed surprisingly high SP values, which are difficult to explain based on the current understanding of N$_2$O formation mechanisms. The following hypotheses may offer an explanation for our findings:

i) significant reduction of N$_2$O by HET causing increased SP in the residual N$_2$O,

ii) a significant contribution from an unknown N$_2$O production pathway with high SP, and/or

iii) previous measurements of SP from the NH$_2$OH oxidation pathway are too low, either due to influence from N$_2$O produced via denitrification during experiments, or an incomplete coverage of all process conditions and thus of the total range of fractionation factors.
The potential role of heterotrophic N\(_2\)O reduction (hypothesis i) during baseline conditions was considered based on the relationship between net N\(_2\)O production rate, SP and \(\delta^{18}O\) under low NH\(_4^+\) baseline conditions (Figure 2; [46, 63]). Figure 2 displays a considerable increase in both \(\delta^{18}O\) and SP with decreasing N\(_2\)O net N\(_2\)O production. For the maximum net N\(_2\)O production rate (0.086 mg-N L\(^{-1}\) h\(^{-1}\)), N\(_2\)O with a SP = 30.2‰ and \(\delta^{18}O = 44.1\%\) was observed; this was assumed to be the data least affected by heterotrophic N\(_2\)O reduction to N\(_2\). Assuming a constant N\(_2\)O source process or mixture of processes (ie. constant isotopic source signature) and fractionation factors for N\(_2\)O reduction (\(\epsilon_{18}\) and \(\epsilon_{SP}\) of \(-12.6 \pm 5.4\) and \(-5.6 \pm 1.9\%)\) respectively; see Table S2), the isotopic composition of N\(_2\)O that would be emitted after partial heterotrophic reduction can be estimated. In Figure 2 the range of \(\delta^{18}O\) versus SP values expected after partial N\(_2\)O reduction is given together with the data obtained from the low NH\(_4^+\) baseline measurements.

It can be seen that the isotopic changes observed in relation to the N\(_2\)O production rates are not consistent with N\(_2\)O reduction being responsible for high SP at low N\(_2\)O mixing ratio, considering previous measurements of isotopic fractionation. Nearly all the measurement points are outside of the 1\(\sigma\) range for N\(_2\)O reduction from previous studies (Figure 2). Correspondingly, the ratio of \(\epsilon_{18}:\epsilon_{SP}\) in this study is smaller than 1, which has only been observed in one study looking at reduction in soils [31] (see Table S2). A potential explanation for this is oxygen isotope exchange during the formation of N\(_2\)O, as observed in a number of studies [7, 30, 29, 67]; although a ‘normal’ slope of 1.1-1.2 was seen by [67] following extensive O isotope exchange during N\(_2\)O formation, followed by extensive reduction of N\(_2\)O by denitrifiers. The anomalously low \(\epsilon_{18}:\epsilon_{SP}\) ratio of 0.69 observed by [31] for clayey soils was attributed to \(\delta^{18}O\) fractionation during N\(_2\)O diffusion through water, while SP was not fractionated. However, even this slope is much steeper than the \(\epsilon_{18}:\epsilon_{SP}\) relationship observed in the present study. It is possible that diffusion effects on \(\delta^{18}O\) were particularly strong in the present study, resulting in a very low \(\epsilon_{18}:\epsilon_{SP}\)
[31], thus other evidence for the extent of HET activity should be examined.

HET they would need to reduce >70% of N₂O to achieve the highest SP values observed in this study². However, HET activity is expected to be low as the NH₄⁺ to COD ratio is low (≈1:1, compared to 1:8-1:10 for typical municipal wastewater; see Table S1 for full details of influent water quality) [24, 49, 62]; in addition, the supernatant liquor used in this study has already passed an aerobic (sludge formation) and anaerobic (sludge digestion) stage, thus the COD remaining is quite refractory to microbial degradation.

High HET activity, to reduce >70% of N₂O, should be evident through reduced NO₃⁻ concentration and decreased ratio of NO₃⁻ production to NH₄⁺ consumption. There was, however, no correlation between SP and \( r(NO_3^-)/r(NH_4^+) \) - in fact, the highest site preferences were simultaneous with some of the highest NO₃⁻ production rates (data not shown).

Although Ishii et al. [21] observed that HET activity was strong enough for HET N₂O production to be the major N₂O source in a heterotrophic PN reactor, N₂O reduction in their study was only strong enough to bring SP to a maximum of 14%.

In summary, the results during baseline conditions are not consistent with strong heterotrophic N₂O reduction causing the observed high SP, thus the other potential explanations for the data should be considered. The currently accepted fractionation factors for the N₂O production pathways shown in Table 1 are not able to explain the measured isotopic composition under baseline conditions. It is possible that the fractionation factor for N₂O production from NH₂OH oxidation has been strongly underestimated in previous studies, due to inorganic N₂O production occurring simultaneously to reduce the isotopic composition of the final measured N₂O in these studies - or the fractionation factors for N₂O reduction in Table S2 could be an incomplete assessment of the ‘true’ range of \( \epsilon_{18}/\epsilon_{SP} \). Alternatively, an unknown microbial or inorganic N₂O production pathway with high SP could be occurring. Various targeted experiments will be discussed in the
following sections to distinguish between these and other possible explanations.

3.2.1. Principal components analysis

To identify the most important factors regulating N₂O production pathways so that the various potential hypotheses could be examined with specific experiments, the parameters affecting N₂O production and isotopic composition under low NH₄⁺ baseline conditions were examined with principal components analysis (PCA, described in Section S1.4). Simple regression between SP and potential controlling factors such as pH or T could not provide useful results due to the complexity of the data set and strong intercorrelations between the many parameters monitored. Nine PCs were identified, with PC1 accounting for 43% of the variability (Figure S5). PC1 shows a strong relationship between SP, δ¹⁸O and δ¹⁵Nbulk, which correlate inversely with net N₂O production (N₂O mixing ratio, net N₂O production rate (rN₂O) and r(N₂O)/r(NH₄⁺)) and DO. This suggests that during baseline conditions, DO is the most important factor controlling partitioning of N₂O production between nitrifier denitrification (high production at high DO with low SP) and other production pathway(s) (characterised by low production at low DO with high SP). In contrast, NO⁻₃ (r(NO⁻₃) and NO⁻₃/NH₄⁺) is unimportant in PC1, which is consistent with a minor role of HETs and NOBs under baseline conditions. NH₄⁺ concentration is also unimportant in determining isotopic composition under baseline conditions, which suggests that N₂O production via NH₂OH oxidation is minor in this reactor as observed in previous studies, because this pathway is expected to be sensitive to the availability of NH₄⁺ [69, 21, 43, 48].

Overall, the measurements taken during baseline conditions are most consistent with hypothesis ii) above: A previously-unidentified N₂O production pathway is occurring in

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²Considering 100% of the initial released N₂O was derived from NH₂OH oxidation with SP = 33‰, εSP = -5.6 (Table S2), and a maximum measured SP of 39.7‰, and using the equation δ = δ₀ + εSP ln f [38, 46], f is equal to 0.3 thus 70% of the N₂O must be reduced. This represents a minimum level of reduction, as it is highly unlikely 100% of the initial N₂O was from NH₂OH oxidation, thus δ₀ is likely <33‰.
the PN-anammox reactor. The PCA results suggest that heterotrophic N₂O reduction and NH₂OH oxidation by AOBs are unimportant for the N₂O budget, making hypotheses i) and iii) less likely explanations. An unknown pathway, inorganic or associated with anammox, may be consistent with a recent study showing low-level N₂O emissions from pure anammox culture [36]. The ‘unknown pathway’ would have SP>40‰ to account for our observations. This is the first indication for a possible high SP from N₂O production in anammox systems, because in previous studies SP has only been measured in a PN reactor with high NH₄⁺ concentrations and very high r(N₂O)/r(NH₄⁺), where N₂O production is dominated by HET denitrification [21]. The ‘unknown pathway’ appears to be most important when N₂O emissions are relatively low; however, overall this can be the most important emission pathway from a reactor operating under optimal conditions. Alternatively, it is possible that the SP from NH₂OH oxidation may reach far higher values than observed in previous studies, and the extent of this pathway may not depend on the NH₄⁺ concentrations, in contrast to results from previous studies [57, 69]. The isotopic measurements following various pertubations as shown in Table 2 will be discussed in the following subsections to distinguish between the proposed explanations.

3.3. Targeted experiments with continuous isotopic measurements to understand N₂O production pathways

3.3.1. N₂O production with high aeration

The goal of this experiment was to favor N₂O production by AOBs - via the nitrifier denitrification pathway and/or NH₂OH oxidation (end-member SPs of ≈ 0‰ and ≈ 33‰ respectively; Table 1) - by reversibly inhibiting anammox and HET activity through elevated dissolved oxygen concentrations. The PCA of the baseline data revealed that DO was the most important parameter controlling N₂O production pathways and isotopic composition (Figure S5), although changes in DO during baseline conditions were small (0.5 - 54.4 µg L⁻¹; 0.02 - 1.7 µM). The aeration rate was manually increased from 250-750
L h\(^{-1}\) during baseline conditions to 1500-3000 L h\(^{-1}\), with normal NH\(_4^+\) setpoint (20-30 mg-N L\(^{-1}\)) in cycle s and the start of cycle aa (aa(1)) and higher NH\(_4^+\) setpoints (50-60 mg-N L\(^{-1}\)) later in cycle aa (aa(2)), as shown in Figure 3. High aeration increased DO to >0.2 mg L\(^{-1}\) (>6.25 µM), reversibly inhibiting anammox, which are inhibited by relatively low dissolved oxygen concentrations in suspended biomass systems [6, 23]. It is possible some anammox activity remained in anoxic microsites, however this is assumed to be very low as evidenced by rapid NO\(_2^-\) build-up. High DO can also potentially lower HET activity, particularly nitrous oxide reductase activity [11, 3, 37], although previous studies suggest that nitrous oxide reductase can be active at DO up to 5 mg L\(^{-1}\) (156 µM) [28].

In the absence of significant anammox activity, NO\(_2^-\) accumulated up to 50 mg-N L\(^{-1}\) during the peak DO period in cycle s (NO\(_2^-\) was not measured during cycle aa). In parallel, the NH\(_4^+\) consumption rate and the net N\(_2\)O and NO production rates increased, and the SP and δ\(^{18}\)O strongly decreased (grey shading in Figure 3). SP values close to 0‰ indicate that N\(_2\)O production is primarily via nitrifier denitrification or heterotrophic denitrification, consistent with the increase in NO production [11, 2, 48]. However, a strong increase in N\(_2\)O production via heterotrophic denitrification is not consistent with increased NH\(_4^+\) consumption rate, as heterotrophs do not consume NH\(_4^+\). Therefore, the low SP and the increase in NH\(_4^+\) consumption rate together indicate that nitrifier denitrification by AOBs is responsible for the large spikes in N\(_2\)O production under high aeration.

The minimum SP value reached at the peak DO in each high aeration period (values shown in Figure 3) correlates with the NH\(_4^+\) setpoint during the increased aeration. This can be explained by NH\(_2\)OH oxidation, which is expected to be enhanced at high NH\(_4^+\) concentration in the presence of abundant DO [69, 43]. However, the low SP values under these conditions show that NH\(_2\)OH oxidation makes a small contribution to N\(_2\)O production - although its importance does in fact increases with increasing concentration.
of NH$_4^+$. Even at NH$_4^+ = 50$ mg-N L$^{-1}$ ($DO > 1$ mg L$^{-1}$ (31 µM), cycle $aa(2)$), SP is 1.8‰ (Figure 3), thus NH$_2$OH oxidation contributes <8% of N$_2$O production [57]. This suggests that this pathway will also be relatively unimportant throughout the experimental period, consistent with baseline results (Section 3.2) and previous laboratory [21, 69] and modelling [43] studies.

N$_2$O production rate and offgas mixing ratio were observed to increase as DO was raised in the high aeration experiments, due to a shift in production pathways. The increase was strong even though elevated NO$_3^-$ concentrations showed NOBs were active and consuming some NO$_2^-$. Although it is unlikely that during normal reactor operation (aeration rate 250-750 L$^{-1}$) the aeration rate would suddenly increase to 3000 L h$^{-1}$, high DO could occur due to, for example, a sudden decrease in AOB activity and therefore in oxygen consumption [23]. These experiments show that the $r$(N$_2$O)/$r$(NH$_4^+$) remains a robust signal for process control (Figure 3), even though N$_2$O production shifted from the unknown pathway into a nitrifier denitrification-dominated regime. Aeration rate would be reduced in response to online measurements of N$_2$O mixing ratio to successfully return the reactor to stable operation.

3.3.2. NO$_2^-$ addition under aerobic conditions (normal aeration)

NO$_2^-$ was added to the reactor in several experiments to enhance N$_2$O production via nitrifier denitrification, as shown in Figure 4. Despite significant addition of NO$_2^-$, the concentration of NO$_2^-$ did not increase in the reactor, showing anammox and/or nitrifier denitrification activity was high. In all cases, the $\delta^{15}$N$^{\text{bulk}}$ changed significantly to approach the $\delta^{15}$N of the added NaNO$_2$ salt (grey dashed line in Figure 4), showing the additional NO$_2^-$ was consumed and contributed to N$_2$O emissions. The fractionation of $\delta^{15}$N during N$_2$O production cannot be inferred as almost all NO$_2^-$ is consistently consumed, thus the effective fractionation is very small.

The net N$_2$O production rate and $r$(N$_2$O)/$r$(NH$_4^+$) increased, and the SP decreased
by between 7% (cycle ƒ, second peak) and 21.5% (cycle h) each time NO$_2^-$ was added$^3$. The $\delta^{18}$O and SP values show a strong correlation ($R^2 = 0.72$), which suggests minimal influence from O-isotope exchange, which would be expected to alter $\delta^{18}$O with no affect on SP. The difference between $\delta^{18}$O and SP varies between -7.8 and 20% with the smallest differences during NO$_2^-$ addition, which suggests a shift between two dominant N$_2$O production pathways, with little influence from confounding factors such as the dependence of fractionation factors on process conditions [5, 72]. During cycle o, the NH$_4^+$ setpoint was raised to 100 mg-N L$^{-1}$ to simultaneously enhance N$_2$O production via nitrifier denitrification and NH$_2$OH oxidation [69, 43]. The observed SP change was in the same range as for all other NO$_2^-$ addition cycles shown in Figure 4, therefore it appears that NH$_2$OH oxidation plays a minor role in N$_2$O production, in agreement with the results presented in the previous subsections (Sections 3.2 and 3.3.1). The baseline SP before NO$_2^-$ addition (black dashed line in Figure 4) was lower in cycles m and o than in cycles f - i, which may indicate that AOBs were relatively more active compared to anammox in these cycles, perhaps due to favourable growth conditions - however in the absence of specific microbial activity measurements, the SP differences cannot be definitively explained.

The SP decrease in each case is significantly correlated with the NO$_2^-$ addition rate (data not shown): Increasing NO$_2^-$ addition rate results in a lower SP minimum. This shows that, although NO$_2^-$ did not build up substantially in the bulk phase of the reactor, heterotrophic or nitrifier denitrification was enhanced following NO$_2^-$ addition. This suggests a transition between two pathways, in agreement with the strong correlation between $\delta^{18}$O and SP. The high SP pathway could either be unknown, or NH$_2$OH oxidation with a previously unseen high SP fractionation; the former is more likely as the SP change at high NH$_4^+$ concentrations discussed in the previous paragraph suggests NH$_2$OH oxidation.

$^3$SP decrease = SP during NO$_2^-$ addition (minima in Figure 4) - SP before/after addition (dashed line in Figure 4)
oxidation is unimportant overall. An alternative explanation for decreasing SP following NO\textsubscript{2}\textsuperscript{-} addition is that NO\textsubscript{2}\textsuperscript{-} inhibits HET activity [11, 3], resulting in less reduction of N\textsubscript{2}O by HET and consequently lower SP. However, the highest observed NO\textsubscript{2}\textsuperscript{-} concentration was <3 mg-N L\textsuperscript{-}1 (see Figure 4) while previous studies have observed HET activity at NO\textsubscript{2}\textsuperscript{-} concentrations up to 50 mg-N L\textsuperscript{-}1 [37, 1, 19].

3.3.3. NO\textsubscript{2}\textsuperscript{-} addition under anoxic conditions (N\textsubscript{2} purging)

In cycles r, z, and bb NO\textsubscript{2}\textsuperscript{-} was added to the reactor (Figure 5) and the gas flow was switched to N\textsubscript{2}, thus N\textsubscript{2}O production via nitrifier denitrification was promoted by excess NO\textsubscript{2} until all AOB activity was stopped by anoxic conditions. Cessation of NO emissions shows both AOB and HET activity was very low due to a lack of suitable electron donor compounds, consistent with the initially low HET activity expected given the low organic carbon concentrations in the supernatant liquor [73, 37]. Continuing NH\textsubscript{4}\textsuperscript{+} consumption shows that anammox remained active with N\textsubscript{2} aeration because NO\textsubscript{2}\textsuperscript{-} was continuously added.

In cycles r and z NO\textsubscript{2}\textsuperscript{-} addition began under aerobic conditions and SP dropped by 9 and 15‰ respectively, showing that nitrifier denitrification was enhanced by NO\textsubscript{2}\textsuperscript{-} addition as discussed in the previous subsection (Section 3.3.2; NO\textsubscript{2}\textsuperscript{-} under aerobic conditions) - this is consistent with nitrifier denitrification being active at much lower organic C concentrations than heterotrophic denitrification [68]. There is a strong correlation between \(\delta^{18}\text{O}\) and SP \((R^2 = 0.53)\), and the difference between \(\delta^{18}\text{O}\) and SP \((0.2\) to \(16\%\)) is lowest when NO\textsubscript{2} is added before anoxia is introduced. This is also in agreement with the results of the previous subsection and again suggests a shift between two dominant pathways with little influence from dependence of fractionation factors on varying conditions or O-isotope exchange [5, 72]. When the air flow was switched to N\textsubscript{2}, however, SP increased immediately by \(\sim 9\%\) as N\textsubscript{2}O production by AOB ceased [73]. In cycle bb, NO\textsubscript{2} addition and N\textsubscript{2} aeration were started simultaneously and SP increased significantly by
7‰ (p<0.001) to reach the highest value measured over the whole experimental period, +45.9‰.

The simultaneous decrease in δ\textsuperscript{15}N\textsubscript{bulk} following NO\textsubscript{2} addition shows consumption of the isotopically-light NO\textsubscript{2} substrate (supplementary material Section S1.2); however unlike SP, δ\textsuperscript{15}N\textsubscript{bulk} remains lower with N\textsubscript{2} aeration, particularly in cycle bb, reflecting the low δ\textsuperscript{15}N of the added salt relative to the supernatant liquor and showing that the added NO\textsubscript{2} substrate is being used to produce N\textsubscript{2}O - even though reactor conditions (N\textsubscript{2} aeration; no O\textsubscript{2}) suggest nitrifier denitrification will not be occurring. N\textsubscript{2}O production by HET also involves NO\textsubscript{2} reduction \cite{35} and agrees with the low δ\textsuperscript{15}N\textsubscript{bulk} discrimination between the added NO\textsubscript{2} and the observed N\textsubscript{2}O \cite{45}; however the N\textsubscript{2}O produced by HET has low SP (\textasciitilde0‰ \cite{57, 70}). This pathway can therefore not explain the results, unless >90% of the N\textsubscript{2}O is subsequently reduced to N\textsubscript{2} by HET (using Rayleigh fractionation equations as described in Section 3.2). This is not consistent with low COD in the system, or with the relatively high N\textsubscript{2}O emissions, particularly during cycle bb. Furthermore, NO\textsubscript{3}\textsuperscript{−} production is close to the 11% expected from anammox and therefore not consistent with strong HET activity (Figure 5).

Both microbial and inorganic production of N\textsubscript{2}O from NH\textsubscript{2}OH have been observed to produce SP values of 29-36‰ \cite{61, 57, 15, 45, 69, 18, 71}; SP of inorganic N\textsubscript{2}O production via NO\textsubscript{2} reduction by Fe\textsuperscript{2+} has not been measured and can therefore not be assessed. A recent study by Yang et al. \cite{72} considering fungal nitric oxide reductase (P450nor) suggests that SP may not always be a conservative tracer of reaction pathways, in contrast to pure culture studies on fungal denitrification by Sutka et al. \cite{56} and more recently Rohe et al. \cite{52}, which show highly constant SP values for a particular species, with some variation between species. Yang et al. \cite{72} suggest that the variation in SP (15-29‰) observed in their study was due to non-steady state conditions regarding NO supply and consumption and cell growth conditions; however non-steady state conditions resulted in lower, not higher, SP in their study. It is possible that high SP in the present experiments
is due to N₂O production from NH₂OH oxidation when conditions are far from steady state; another possibility is that previous attempts to measure the SP of the NH₂OH pathway were contaminated by low SP-N₂O from inorganic or microbial N₂O production by denitrification. In the latter case, the present study would need to have significantly less N₂O from denitrification than all previous studies, to make the ‘true’ high SP of the NH₂OH pathway visible, which is unlikely given that this study uses a mixed culture and real supernatant liquor rather than a well-constrained pure culture and growth medium. Additionally, results in Sections 3.2, 3.3.1 and 3.3.2 suggest the NH₂OH pathway is not important in this system, consistent with previous results [43, 21]. However, these two explanations cannot be definitively discounted based on the available evidence.

The experiments testing NO⁻₂ addition under N₂ aeration therefore may suggest, in agreement with the other experimental results as discussed in the previous sections, that an anoxic pathway is producing N₂O with high SP (≥45‰), enhanced by NO⁻₂ addition and anoxic conditions - and therefore possibly associated with anammox activity. The unknown pathway may have a low level of isotopic discrimination for δ¹⁵Nbulk, given the close agreement between the δ¹⁵N of added NO⁻₂ and δ¹⁵Nbulk of N₂O in cycles z and bb - however δ¹⁵N discrimination can be highly variable, and in addition it is unclear if all of the N in N₂O derives from NO⁻₂.

### 3.3.4. Addition of NH₂OH

In cycle y, NH₂OH was continuously added at a rate of 20-80 mg-N/min for a total of 204 minutes (total addition = 9.3 g-N ≈ 23 mg-N L⁻¹) to promote N₂O production via NH₂OH oxidation (results shown in supplementary information, Figure S6). The N₂O production rate increased twofold when NH₂OH was added. The SP of emitted N₂O also increased significantly, from 34.4±1.1‰ to 41.3±1.8‰ (ΔSP = 6.9±2.1; p<0.001). The final SP of 41.3‰ is 5‰ higher than the highest values reported in the literature for NH₂OH oxidation [15, 57] (see Table 1).
As in the previous experiments, we can consider several possibilities for these high values: i) significant N\textsubscript{2}O reduction by HET, ii) an unknown inorganic or microbial N\textsubscript{2}O production pathway with a much higher end-member SP, where NH\textsubscript{2}OH is somehow involved either directly, or indirectly via the formation of intermediates, or iii) previous measurements of NH\textsubscript{2}OH characteristic SP are too low due to ‘contaminant’ N\textsubscript{2}O from denitrification, or SP of N\textsubscript{2}O from NH\textsubscript{2}OH is higher when growth conditions are not at steady state [72]. HET reduction would need to remove >50-70% of N\textsubscript{2}O to achieve the observed SP of 41‰ [46, 22, 62] produced following NH\textsubscript{2}OH addition, calculated using Rayleigh fractionation as described in Section 3.2 [38], which is very unlikely given the low organic carbon content. The SP fractionation during N\textsubscript{2}O reduction may vary depending on conditions such as substrate availability; however the above simulations are based on enrichment factors from four different studies under widely varying environmental conditions ($\epsilon_{SP} = -2.9$ to -8.2‰, see Table S2) and are therefore considered robust. Such high levels of reduction are also inconsistent with the observed increase in N\textsubscript{2}O production rate following NH\textsubscript{2}OH addition. SP of N\textsubscript{2}O produced from abiotic reduction of NO\textsubscript{2} by Fe\textsuperscript{2+} [25] has been measured by only one study, and found to be in the range of -13.3 to +22.6‰ [53]; thus this pathway is unlikely to explain our observations. SP of N\textsubscript{2}O produced from the inorganic oxidation of NH\textsubscript{2}OH is in a very narrow range of 34-35‰, with little sensitivity to process conditions [18]. As discussed in the previous subsection, incorrect or varying SP values for microbial N\textsubscript{2}O production via NH\textsubscript{2}OH oxidation are unlikely given the literature but cannot be discounted. An unknown microbial pathway with high SP involving NH\textsubscript{2}OH may be the best explanation for the results, although no previous study has shown clear evidence of such a pathway [27, 36].

3.4. Implications for our understanding of N\textsubscript{2}O production

The online isotopic measurements of N\textsubscript{2}O produced in a combined PN-anammox re-
actor provided a rich dataset that revealed unexpected results regarding N\textsubscript{2}O production
pathways. The results of this study showed that nitrifier denitrification is responsible for N₂O emission peaks at high NH₄⁺ concentrations, when AOB and anammox activities are not optimally balanced (e.g. when the aeration rate or the DO is too high; see Section 3.3.1). However, consistently high SP observed during normal operation (Figures 2, S1 and S4) show that either N₂O production also occurs via an unknown pathway with high SP, HET activity and thus N₂O reduction is much stronger than expected, or fractionation factors reported in previous studies (Table 1) cover an incomplete range. N₂O emissions of this type, which cannot be explained within our current understanding - low N₂O production rate and high SP - accounted for >75% of the total N₂O production⁴ over the entire experimental period, and close to 100% of emissions during normal operating conditions. A number of experimental pertubations were applied to the system to test these hypotheses.

The results of these experiments are most consistent with the hypothesis that high SP is due to an unknown N₂O production pathway (Table 2) - although the other proposed hypotheses cannot be dismissed. HET reduction would need to consume >90% of N₂O to achieve the highest SP value of 45.9‰ observed during the entire experimental period (Section 3.3.3), and >70% of N₂O to reach the maximum ‘baseline’ SP value of 39.7‰ (Section 3.2), calculated using Rayleigh fractionation as described in Section 3.2 [38]. This level of reduction is unlikely due to low COD concentrations and observed N transformation rates - e.g. high N₂O production rates, and ∼10% NO₃⁻ production relative to NH₄⁺ consumption - however it cannot be entirely ruled out without isotopic tracer experiments. O isotope exchange could explain why the slope of SP to δ¹⁸O does not agree with N₂O reduction during baseline experiments (Figure 2) as discussed in Section 3.2, but an important role of heterotrophic N₂O reduction is generally not consistent with our other experimental results, particularly during NO₂⁻ addition (Sections 3.3.2 and 3.3.3).

Highly varying SP fractionation for the NH₂OH pathway is unlikely given the relatively tight range of 29-36.3‰ measured in numerous previous studies considering a variety of
pathways, including inorganic, pure culture, and mixed culture N₂O production ([57, 15]; [62] and [45] and references therein; [69, 18, 71]). If an unknown pathway is occurring in the reactor, it would be characterised by production of N₂O with high SP (≥45‰ - the maximum SP observed, during NO₂⁻ addition under anoxic conditions) and appears to be associated with anammox metabolism directly or indirectly as an associated inorganic pathway, possibly involving NH₂OH. Further experimentation is needed to confirm the existence of this pathway and gain a definitive understanding of N₂O production in PN-anammox systems. The new questions regarding the nitrogen cycle in a PN-anammox reactor raised by the results of this study are shown schematically in Figure 6.

The results of this study show that online N₂O monitoring is an ideal method for process control in a combined, continuously-fed PN-anammox reactor, extending the current set of available online parameters (e.g. pH, conductivity, NH₄⁺, NO₃⁻, aeration rate). During stable operation N₂O emissions are within the range required for anammox systems to have a positive energy balance compared to traditional nitrification-denitrification wastewater treatment processes. When DO or NO₂⁻ increase due to e.g. changes in AOB or anammox activity, N₂O emissions via nitrifier denitrification are enhanced; thus aeration can be decreased in response to high measured offgas N₂O mixing ratio to keep the reactor in a stable state while simultaneously minimizing GHG emissions.

4. Conclusions

This study uses online measurements of N₂O concentration and isotopic composition to understand N₂O production pathways under a variety of conditions, and assess the use of N₂O measurements for process control in a single-stage PN-anammox reactor. The main conclusions of this study are:

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4Estimated as N₂O production rate × time where SP>33‰, compared to production rate × time where SP<33‰.
• Online measurements of N₂O offgas mixing ratio are an ideal method to balance
activity of AOBs and anammox, to maintain process stability in a single-stage PN-
anammox reactor.

• High isotopic SP when N₂O emissions are relatively low during optimal operation
indicates that our current understanding of N₂O production and isotopic fraction-
atation is incomplete, and may suggest the existence of an unknown N₂O production
pathway associated with anammox metabolism.

• When NH₄⁺ or DO are elevated in the reactor, N₂O production by nitrifier denitrifi-
cation is enhanced. N₂O production via NH₂OH oxidation is relatively unimportant
in the reactor under all conditions.

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The supplementary information file contains Section S1: Materials and Methods, Fig-
ures S1-S6 and Table S1-S2.
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**Figures and tables**
Figure 1: Automatic regulation of aeration flow rate based on online measurement of N₂O production divided by NH₄⁺ consumption rate $r(\text{NH}_4^+)$ under baseline conditions with low NH₄⁺ (left) and high NH₄⁺ (right) concentration. The setpoint for $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$ (%) is shown in pale red and the measured value in dark red. The set range for aeration is shown in pale blue and the actual aeration flow is shown in darker blue. Tick mark spacing on the x-axis is one hour.
Figure 2: Relationship between SP and δ¹⁸O during baseline experiments with low NH₄⁺, shown as points coloured by N₂O production rate. The expected relationship using the mean ε₁⁸/εSP from previous studies (see Table S2) is shown as a dashed line and the dark grey shading shows the 1σ error; the pale grey shading shows the maximum and minimum slopes from previous studies. These estimates assume that deviation from the measured SP and δ¹⁸O (30.2 and 44.1‰ respectively) at an N₂O production rate of 0.09 mg-N L⁻¹ h⁻¹ is solely due to N₂O reduction. Slope from Well et al. [67] added to determination of mean slope in revised version; see Table S2.
Figure 3: High aeration experiments with normal \( \text{NH}_4^+ \) setpoint during cycles \( s \) and \( aa(1) \) and with high \( \text{NH}_4^+ \) setpoint during cycle \( aa(2) \). Cycles \( aa(1) \) and \( aa(2) \) are divided with a dashed line. The top panel shows the increase in aeration (light blue) and the resultant increase in \( \text{DO} \) (dark blue); periods where aeration and \( \text{DO} \) were elevated are highlighted with grey shading. The \( \text{NH}_4^+ \) concentration (orange) and consumption rate (dark green), \( r(\text{N}_2\text{O})/r(\text{NH}_4^+) \) (red), and \( \text{NO}_2^- \) concentration (black circles) are shown in the second panel. The third panel shows the \( \text{N}_2\text{O} \) and \( \text{NO} \) production rates in purple and pink respectively. The \( \delta^{18}\text{O} \) (green) and SP (purple) of \( \text{N}_2\text{O} \) is shown in the bottom panel, with the minimum values indicated for each experiment. X-axis tickmarks are spaced 1 hour apart.
Figure 4: Addition of NO$_2^-$ (as NaNO$_2$) during six cycles with normal aeration. NO$_2^-$ was continuously added to the reactor during the periods indicated with grey shading. The top panel shows the NH$_4^+$ consumption rate ($r$(NH$_4^+$); green), the N$_2$O production rate ($r$(N$_2$O); purple), $r$(N$_2$O)/$r$(NH$_4^+$) (%; red), and the NH$_4^+$ concentration (yellow). In cycle o the NH$_4^+$ setpoint was 100 mg-N L$^{-1}$ which is off-scale in the figure. In the second panel, the NO$_2^-$ addition rate is shown as a thick grey line and the NO$_2^-$ concentration is shown in blue (offline measurements) with the individual measurement points are indicated in black. The bottom panel shows the isotopic composition of offgas N$_2$O. The minimum SP reached after each NO$_2^-$ addition is indicated and the mean SP before and after addition for each cycle is shown with a black dashed line. The $\delta^{15}$N of the added NaNO$_2$ salt is shown with a grey dotted line in the bottom panel. X-axis tickmarks are spaced 1 hour apart.
Figure 5: Addition of NO\textsubscript{2}\textsuperscript{−} (as NaNO\textsubscript{2}) during three cycles with N\textsubscript{2} purge gas. NO\textsubscript{2}\textsuperscript{−} was continuously added to the reactor during the periods indicated with grey shading, and N\textsubscript{2} was used for aeration during the periods shaded blue, as shown in the legend. The top panel shows the NH\textsubscript{4}\textsuperscript{+} consumption rate ($r$(NH\textsubscript{4}\textsuperscript{+}); green), the NH\textsubscript{4}\textsuperscript{+} concentration (yellow) and NO\textsubscript{3}\textsuperscript{−}:NH\textsubscript{4}\textsuperscript{+} (dark blue). The second panel shows the net N\textsubscript{2}O production rate ($r$(N\textsubscript{2}O); purple) and $r$(N\textsubscript{2}O)/$r$(NH\textsubscript{4}\textsuperscript{+}) (%; red), and the NO\textsubscript{2}\textsuperscript{−} addition rate (grey). The isotopic composition of offgas N\textsubscript{2}O is presented in the bottom panel; the $\delta^{15}$N of the added NaNO\textsubscript{2} salt is shown with a grey dotted line. X-axis tickmarks are spaced 1 hour apart.
Figure 6: The nitrogen cycle in a PN-anammox reactor. Pathways attributed to ammonia-oxidizing bacteria (AOBs) are shown in yellow, including N₂O production via NH₂OH oxidation (SP ∼ 33‰) and via nitrifier denitrification (SP = 0‰). Anaerobic NH₄⁺ oxidizer (anammox) pathways are shown in blue. Other reactions that may be occurring to a minor degree in the system are shown in grey: reduction by heterotrophic denitrifiers (HET), NO₃⁻ production by NO₂⁻ oxidizing bacteria (NOB), and chemical oxidation of NH₂OH with SP ∼ 33‰. The four most likely explanations for the high SP values observed throughout this study are shown in red: i) unexpectedly strong heterotrophic N₂O reduction, ii) unknown inorganic or anammox-associated N₂O production pathway, or iii) the SP end-member for NH₂OH has been underestimated in previous studies.
Table 1: Known N₂O production and consumption pathways that may be occurring in a partial nitritation-anammox reactor, and the site preference and ¹⁵N fractionation factors associated with the pathways. AOB = NH₄⁺ oxidizing bacteria, HET = heterotrophic denitrifiers.

<table>
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<tr>
<th>Pathway</th>
<th>Population</th>
<th>Reaction</th>
<th>Site preference (‰)</th>
<th>Δδ¹⁵N (‰)</th>
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<td>NH₂OH oxidation</td>
<td>AOB</td>
<td>NH₂OH → NO → N₂O [8]</td>
<td>30 to 36 [69]</td>
<td>-37 to -17 [58, 59]</td>
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<tr>
<td>Nitrifier denitrification</td>
<td>AOB</td>
<td>NO₂⁻ → NO → N₂O [8]</td>
<td>-10 to 0 [57, 69]</td>
<td>-68 to -30 [58, 59, 69]</td>
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<td>NO₅⁻/NO₂⁻ reduction</td>
<td>HET</td>
<td>NO₅⁻ → NO₂⁻ → N₂O</td>
<td>-5 to 0 [61, 63, 69]</td>
<td>-39 to -12 [61]</td>
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<tr>
<td>N₂O reduction</td>
<td>HET</td>
<td>N₂O → N₂</td>
<td>-16.4 to -2.9⁵</td>
<td>-39 to -12 [61]</td>
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<td>Abiotic production</td>
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<td>Various [61, 18]</td>
<td>29.5 to 35 [61, 18]</td>
<td>-27 to 1.4 [18]</td>
</tr>
</tbody>
</table>

⁵Reduction favours N₂O with low site preference as the ¹⁴N-O bond is more easily broken than the ¹⁵N-O bond, thus the site preference of the remaining N₂O pool is increased. The values -2.9 to -16.4‰ refer to ε(SP), the site preference fractionation factor for reduction (see Table S2 for references).
Table 2: Overview of experiments conducted to understand N₂O production pathways during wastewater treatment in a PN-Anammox reactor. ‘Action’ refers to the experimental perturbation made over baseline conditions. Baseline conditions are detailed in Section 2.1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cycle(s)</th>
<th>Action</th>
<th>Observation</th>
<th>Potential explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline: Low NH₄⁺</td>
<td>a-e, j-l, t-w, cc-ce</td>
<td>No perturbation; NH₄⁺ setpoint 10-20 mg-N L⁻¹</td>
<td>SP higher than expected</td>
<td>Unknown (anammox) N₂O production pathway, rather than significant HET reduction of N₂O</td>
</tr>
<tr>
<td>Baseline: High NH₄⁺</td>
<td>g, n, o, p, q</td>
<td>No perturbation; NH₄⁺ setpoint 100 mg-N L⁻¹</td>
<td>SP decreased compared to low NH₄⁺</td>
<td>Enhanced N₂O production via nitrifier denitrification</td>
</tr>
<tr>
<td>High aeration</td>
<td>s, aa</td>
<td>Aeration rate increased to 1500-3000 L h⁻¹, thus DO &gt;0.2 mg L⁻¹</td>
<td>Increased N₂O production, SP reduced to ~0‰</td>
<td>Enhanced nitrifier denitrification with anammox and possibly HET inhibited; NH₂OH oxidation unimportant</td>
</tr>
<tr>
<td>NO₂⁻ addition (oxic)</td>
<td>f-i, m, o</td>
<td>NO₂⁻ added at 5-36 mg NO₂⁻-N L h⁻¹</td>
<td>SP decrease (7-21‰), correlated with NO₂⁻ addition rate</td>
<td>Enhanced nitrifier denitrification - NH₂OH oxidation unimportant</td>
</tr>
<tr>
<td>NO₂⁻ addition (anoxic)</td>
<td>r, z, bb</td>
<td>NO₂⁻ added at 11-27 mg NO₂⁻-N L h⁻¹ and aeration switched to N₂</td>
<td>SP remains high or increases (&gt;46‰)</td>
<td>Nitrifier denitrification inhibited, anammox activity and unknown N₂O production pathway enhanced</td>
</tr>
<tr>
<td>NH₂OH addition</td>
<td>y</td>
<td>NH₂OH added at 20-80 mg NH₂OH-N min⁻¹</td>
<td>SP increased to ~41‰</td>
<td>Unknown (anammox?) N₂O production pathway involves NH₂OH</td>
</tr>
</tbody>
</table>