

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

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## Abstract

Nitrous oxide (N<sub>2</sub>O) production pathways in a single stage, continuously fed partial nitrification-anammox reactor were investigated using online isotopic analysis of offgas N<sub>2</sub>O with quantum cascade laser absorption spectroscopy (QCLAS). N<sub>2</sub>O emissions increased when reactor operating conditions were not optimal, for example, high dissolved oxygen concentration. SP measurements indicated that the increase in N<sub>2</sub>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<sub>2</sub>O monitoring is an ideal method to detect imbalances in reactor operation and regulate aeration, to ensure optimal reactor conditions and minimise N<sub>2</sub>O emissions.

Under normal operating conditions, the N<sub>2</sub>O isotopic site preference (SP) was much higher than expected - up to 40‰ - which could not be explained within the current understanding of N<sub>2</sub>O production pathways. Various targeted experiments were conducted to investigate the characteristics of N<sub>2</sub>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<sub>2</sub>O reduction, ii) unknown inorganic or anammox-associated N<sub>2</sub>O production pathway, iii) previous underestimation of SP fractionation during N<sub>2</sub>O production from NH<sub>2</sub>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<sub>2</sub>O production pathways in PN-anammox systems and definitively distinguish between these hypotheses.

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*Keywords:* nitrous oxide, isotopic composition, laser spectroscopy, nitrifier  
denitrification, process control

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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 ( $\text{NO}_2^-$ ) and ammonium ( $\text{NH}_4^+$ ) to form  $\text{N}_2$ , 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 nitrification (PN), where  $\sim 50\%$  of  $\text{NH}_4^+$  is first oxidised to  $\text{NO}_2^-$  by ammonia oxidizing bacteria (AOBs) under oxic conditions, coupled to the anoxic anammox reaction to produce  $\text{N}_2$ . As an organic carbon source is not required, PN-anammox is particularly suited for the treatment of high  $\text{NH}_4^+$ , 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  $0.2 \text{ mg L}^{-1}$  ( $6.25 \text{ } \mu\text{M}$ ) [23] and potentially also by high  $\text{NO}_2^-$  concentrations (eg.  $>600 \text{ mg L}^{-1}$  or  $10 \text{ mM}$ ) [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  $\text{NO}_2^-$  has the potential to serve as control parameter to monitor activity. However, as no

suitable electrode is available for the online measurement of  $\text{NO}_2^-$ , online measurements of  $\text{N}_2\text{O}$  mixing ratio<sup>1</sup> have been suggested to act as a proxy for  $\text{NO}_2^-$ , thus presenting an ideal method to monitor and control reactor dynamics [70].

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

$\text{N}_2\text{O}$  production by AOBs can be envisaged as a ‘leaky pipeline’ [13] because  $\text{N}_2\text{O}$  is an unwanted by-product during the goal reaction, oxidation of  $\text{NH}_4^+$  to  $\text{NO}_2^-$ . Two major pathways are implicated in the emission of  $\text{N}_2\text{O}$  by AOBs: Nitrifier denitrification, and  $\text{NH}_2\text{OH}$  oxidation (see Table 1). When the overall turnover of  $\text{NH}_4^+$  by AOBs increases, the ‘leaking’ of  $\text{N}_2\text{O}$  also increases - thus  $\text{N}_2\text{O}$  emissions can roughly indicate AOB activity. Biotic and abiotic  $\text{N}_2\text{O}$  production by hydroxylamine ( $\text{NH}_2\text{OH}$ ) oxidation can be minimised by keeping the concentration of  $\text{NH}_4^+$  relatively low (eg. by intermittent dosing of wastewater); previous studies suggest that even at high  $\text{NH}_4^+$  concentrations this pathway contributes less than 20-30% of total  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  [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  $\text{N}_2\text{O}$  [36]. This is much lower than the percent of ammonium released as  $\text{N}_2\text{O}$  by AOBs

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<sup>1</sup>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  $\text{N}_2\text{O}$  production. Online  $\text{N}_2\text{O}$  measurements can therefore act as a proxy for  $\text{NO}_2^-$  concentration, which is an indicator for the relative activity of AOBs compared to anammox.

A major challenge for process regulation via  $\text{N}_2\text{O}$  measurements is the activity of NOBs, which reduce  $\text{N}_2\text{O}$  emissions from nitrifier denitrification by consuming  $\text{NO}_2^-$ . Monitoring the net  $\text{NO}_3^-$  production to  $\text{NH}_4^+$  consumption ratio (hereafter  $r(\text{NO}_3^-)/r(\text{NH}_4^+)$ ) - which should be approximately 10-11% due to  $\text{NO}_3^-$  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  $\text{N}_2\text{O}$  during the stepwise reduction of  $\text{NO}_3^-$  to  $\text{N}_2$ , however HET activity is minimal in PN-anammox systems due to low organic carbon concentrations [24, 49].

$\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  production pathways is lacking, which is necessary both to mitigate emissions and to effectively use online  $\text{N}_2\text{O}$  measurements for process control.  $\text{N}_2\text{O}$  isotopic composition is particularly useful to quantify  $\text{N}_2\text{O}$  production via different pathways (Table 1). The most abundant four isotopocules of  $\text{N}_2\text{O}$  are  $^{14}\text{N}^{14}\text{N}^{16}\text{O}$ ,  $^{14}\text{N}^{14}\text{N}^{18}\text{O}$ ,  $^{14}\text{N}^{15}\text{N}^{16}\text{O}$  ( $\alpha$ ) and  $^{15}\text{N}^{14}\text{N}^{16}\text{O}$  ( $\beta$ ). The bulk  $^{15}\text{N}$  isotopic composition refers to the average  $\delta^{15}\text{N}$  at both positions:

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

The  $\text{N}_2\text{O}$  ‘site preference’ (SP) refers to the difference in  $^{15}\text{N}$  isotopic composition of the

central ( $\alpha$ ) position N compared to the terminal ( $\beta$ ) position N:

$$\text{SP} = \delta^{15}\text{N}^{\alpha} - \delta^{15}\text{N}^{\beta} \quad (2)$$

Site preference of  $\text{N}_2\text{O}$ , unlike  $\delta^{15}\text{N}^{\text{bulk}}$ , is independent of the substrate isotopic composition, and it is therefore a robust tool to differentiate between  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  by anammox [36], are still undetermined. In addition, reduction of  $\text{N}_2\text{O}$  by HET increases the site preference of the remaining  $\text{N}_2\text{O}$ , which can complicate the partitioning of different production pathways. To overcome these difficulties, combination of site preference data with simultaneous measurements of  $\delta^{18}\text{O}$  and  $\delta^{15}\text{N}^{\text{bulk}}$  can help to constrain the effect of  $\text{N}_2\text{O}$  reduction by HET [46, 63]. Recent developments in spectroscopy [66, 40, 17] allow online monitoring of  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  production and consumption pathways shown in Table 1.

The aim of this study is to investigate the major pathways leading to  $\text{N}_2\text{O}$  production in a continuously fed PN-anammox reactor, and thereby facilitate the use of online  $\text{N}_2\text{O}$  measurements to control process stability. A threshold limit for the net production of  $\text{N}_2\text{O}$  per  $\text{NH}_4^+$  consumed in the reactor (hereafter  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$ ) is used to automatically control the reactor aeration rate and maintain balance between AOB and anammox activity. We report the first online measurements of  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  production under different conditions. These results will be used to improve our understanding of  $\text{N}_2\text{O}$  production pathways during PN-anammox treatment, and to

investigate the possible production of  $\text{N}_2\text{O}$  by anammox bacteria.

## 2. Materials and methods

### 2.1. Pilot-scale partial nitrification-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  $\text{NH}_4^+$  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.  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$  and  $\text{N}_2\text{O}$ ) are always reported as net rates.

Measurements were made under both ‘baseline’ and experimental conditions. Baseline conditions were defined as when  $\text{NO}_2^-$  concentration was low ( $<1 \text{ mg-N L}^{-1}$ ) and no compounds were added to perturb the system (see Table 2 and Figure S2 for all cycle designations). During baseline experiments,  $\text{N}_2\text{O}$  mixing ratios in the offgas were used as a proxy for  $\text{NO}_2^-$  concentrations in the reactor in order to infer the relative activity of AOBs vs anammox and regulate reactor aeration accordingly. Minimum ( $180\text{-}200 \text{ L h}^{-1}$ ) and maximum ( $400\text{-}750 \text{ L h}^{-1}$ ) ranges for aeration rates were set, between which aeration could automatically vary: When  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$  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  $\text{NH}_4^+$ , whereby  $\text{NH}_4^+$  concentration was set to  $\sim 10\text{-}20 \text{ mg-N L}^{-1}$  or  $100 \text{ mg-N L}^{-1} \text{ h}^{-1}$  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 N<sub>2</sub>O measurements for process control by improving our understanding of how the offgas N<sub>2</sub>O mixing ratio responds to various ‘extreme’ situations. The following short-term experiments were performed:

- increase of the aeration rate,
- external addition of NO<sub>2</sub><sup>-</sup> under both normal and N<sub>2</sub> aeration, and
- external addition of NH<sub>2</sub>OH

The goal was to favor different N<sub>2</sub>O formation pathways, and to investigate the isotopic end-member signature of the ‘unknown’ pathway. Isotopic measurements were carried out to gain insight into N<sub>2</sub>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 N<sub>2</sub>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 N<sub>2</sub>O, CO<sub>2</sub>, CH<sub>4</sub>, CO and NO with one minute resolution. The FT-IR measurement of N<sub>2</sub>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 CO<sub>2</sub> and CO [32].

The isotopic composition of offgas N<sub>2</sub>O ( $\delta^{15}\text{N}^{\alpha}$ ,  $\delta^{15}\text{N}^{\text{beta}}$ ,  $\delta^{15}\text{N}^{\text{bulk}}$  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  $\delta^{18}\text{O}$  and Air- $\text{N}_2$  for  $\delta^{15}\text{N}$  as described in the supplementary information, Section S1.3. Compatibility of  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  mixing ratios measured in the three instruments, and all data was corrected to the time scale of the SBR process monitor. Agreement between  $\text{N}_2\text{O}$  mixing ratios from the three measurements was very good ( $\pm 5\text{-}10\%$ ), although peaks in  $\text{N}_2\text{O}$  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 $\text{N}_2\text{O}$ production rate

##### 3.1.1. Baseline conditions with low $\text{NH}_4^+$ 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 preceeding 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  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$  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  $\text{N}_2\text{O}$  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 $\text{NH}_4^+$ concentration

Automatic regulation during baseline cycles with elevated  $\text{NH}_4^+$  (setpoint 100 mg-N  $\text{L}^{-1}$   $\text{h}^{-1}$ ) was tested, to investigate  $\text{N}_2\text{O}$  production with higher  $\text{NH}_4^+$  consumption rates. At high  $\text{NH}_4^+$  concentrations,  $\text{N}_2\text{O}$  emissions can be expected to increase [69, 48]. This was confirmed with the results of this study. When the  $\text{NH}_4^+$  setpoint was 100 mg-N  $\text{L}^{-1}$  (cycle  $n$ ), the initial  $\text{NH}_4^+$  consumption rate was much higher than under baseline conditions; 30 mg-N  $\text{L}^{-1}$   $\text{h}^{-1}$  compared to  $<10$  mg-N  $\text{L}^{-1}$   $\text{h}^{-1}$  with low  $\text{NH}_4^+$  load (Figure 1). In the initial phase of cycle  $n$  (up to 4 hours after onset) both the net  $\text{N}_2\text{O}$  production rate (0.3 mg-N  $\text{L}^{-1}$   $\text{h}^{-1}$ , average over complete cycle 0.08 mg-N  $\text{L}^{-1}$   $\text{h}^{-1}$ ) and the  $\text{NH}_4^+$  consumption rate peaked, although the  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$  ratio was not especially high at this point. Around 5 hours after the onset of cycle  $n$ ,  $\text{N}_2\text{O}$  emissions and the  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$  drastically increased, thus the aeration rate was automatically reduced and consequently  $\text{NH}_4^+$  consumption rate decreased. Throughout the rest of the cycle,  $\text{N}_2\text{O}$  emissions varied cyclically in response to the automatic regulation of the aeration rate. We did not observe an increase in either  $\text{NO}_2^-$  concentration or net  $\text{NO}_3^-$  production rate during high  $\text{NH}_4^+$  experiments ( $\text{NO}_3^-$  production level was  $10.4 \pm 2.4\%$  of  $\text{NH}_4^+$  consumption; similar to low  $\text{NH}_4^+$  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  $\text{N}_2\text{O}$  measurement was able to control the process to achieve approximately the desired ratio of  $\text{N}_2\text{O}$  production to  $\text{NH}_4^+$  consumption (with minimal  $\text{NO}_3^-$  production) even at high  $\text{NH}_4^+$  concentration (cycle  $n$ ), the system was in a much more dynamic state than with a low  $\text{NH}_4^+$  concentration (Figure 1). This resulted in much larger variations in aeration rate and  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$  than under low  $\text{NH}_4^+$  conditions (cycles  $j$  and  $k$ ). Consistent results have been observed in previous studies, which also reported peak  $\text{N}_2\text{O}$  emissions when  $\text{NH}_4^+$  concentrations were high, attributed to enhanced

nitriifier denitrification [21, 51, 35]. The  $\text{NH}_4^+$  consumption peak at the beginning of cycle  $n$  is most likely due to enhanced AOB activity, however the current understanding of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  consumption and  $\text{N}_2\text{O}$  production pathways does not allow us to interpret features of both the baseline and high  $\text{NH}_4^+$  measurements based on  $\text{N}_2\text{O}$  mixing ratio and process parameters alone. Therefore, in the following section the isotopic composition of  $\text{N}_2\text{O}$  emitted from the reactor under baseline conditions (both low and high  $\text{NH}_4^+$ ) will be discussed, to understand which pathway(s) contribute the majority of  $\text{N}_2\text{O}$  emissions.

### *3.2. Isotopic composition of $\text{N}_2\text{O}$ produced under baseline conditions (low and high $\text{NH}_4^+$ concentrations)*

Baseline experiments with low  $\text{NH}_4^+$  represent optimal operating conditions, therefore the isotopic measurements made during these experiments reveal the  $\text{N}_2\text{O}$  production pathway(s) that will be most important in a stable reactor (Section 3.1.1). The mean SP during baseline experiments with low  $\text{NH}_4^+$  was  $34.3 \pm 2.8\text{‰}$  (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  $\text{NH}_4$  and  $\text{NO}_2^-$  [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  $\text{NH}_4^+$  concentrations under baseline conditions (cycle  $n$ ; Section 3.1.2), SP was  $28.3 \pm 1.9\text{‰}$ ; this is significantly lower than with low  $\text{NH}_4^+$  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  $\text{NH}_2\text{OH}$  pathway is expected

at higher  $\text{NH}_4^+$  concentrations, resulting in SP values closer to 33‰ [57, 69]. However, the SP values for  $\text{N}_2\text{O}$  produced during both low and high ammonium concentrations under baseline conditions were close to previously measured values for  $\text{N}_2\text{O}$  from  $\text{NH}_2\text{OH}$  oxidation. A low contribution of  $\text{NH}_2\text{OH}$  oxidation to  $\text{N}_2\text{O}$  production is supported by the observed lowest SP values when the  $\text{NH}_4^+$  consumption rate ( $r(\text{NH}_4^+)$ ) was highest at the beginning of cycle  $n$  ( $24.5 \pm 0.5\%$ ;  $p < 0.001$ ).

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

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

- i) significant reduction of  $\text{N}_2\text{O}$  by HET causing increased SP in the residual  $\text{N}_2\text{O}$ ,
- ii) a significant contribution from an unknown  $\text{N}_2\text{O}$  production pathway with high SP, and/or
- iii) previous measurements of SP from the  $\text{NH}_2\text{OH}$  oxidation pathway are too low, either due to influence from  $\text{N}_2\text{O}$  produced via denitrification during experiments, or an incomplete coverage of all process conditions and thus of the total range of fractionation factors.

272 The potential role of heterotrophic N<sub>2</sub>O reduction (hypothesis i) during baseline con-  
 273 ditions was considered based on the relationship between net N<sub>2</sub>O production rate, SP  
 274 and  $\delta^{18}\text{O}$  under low  $\text{NH}_4^+$  baseline conditions (Figure 2; [46, 63]). Figure 2 displays a  
 275 considerable increase in both  $\delta^{18}\text{O}$  and SP with decreasing N<sub>2</sub>O net N<sub>2</sub>O production. For  
 276 the maximum net N<sub>2</sub>O production rate ( $0.086 \text{ mg-N L}^{-1} \text{ h}^{-1}$ ), N<sub>2</sub>O with a  $\text{SP} = 30.2\text{‰}$   
 277 and  $\delta^{18}\text{O} = 44.1\text{‰}$  was observed; this was assumed to be the data least affected by het-  
 278 erotrophic N<sub>2</sub>O reduction to N<sub>2</sub>. Assuming a constant N<sub>2</sub>O source process or mixture  
 279 of processes (ie. constant isotopic source signature) and fractionation factors for N<sub>2</sub>O  
 280 reduction ( $\epsilon_{18}$  and  $\epsilon_{\text{SP}}$  of  $-12.6 \pm 5.4$  and  $-5.6 \pm 1.9\text{‰}$  respectively; see Table S2), the  
 281 isotopic composition of N<sub>2</sub>O that would be emitted after partial heterotrophic reduction  
 282 can be estimated. In Figure 2 the range of  $\delta^{18}\text{O}$  versus SP values expected after par-  
 283 tial N<sub>2</sub>O reduction is given together with the data obtained from the low  $\text{NH}_4^+$  baseline  
 284 measurements.

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

299 [31], thus other evidence for the extent of HET activity should be examined.

300 HET they would need to reduce  $>70\%$  of  $\text{N}_2\text{O}$  to achieve the highest SP values ob-  
301 served in this study<sup>2</sup>. However, HET activity is expected to be low as the  $\text{NH}_4^+$  to COD  
302 ratio is low ( $\sim 1:1$ , compared to  $1:8$ - $1:10$  for typical municipal wastewater; see Table S1  
303 for full details of influent water quality) [24, 49, 62]; in addition, the supernatant liquor  
304 used in this study has already passed an aerobic (sludge formation) and anaerobic (sludge  
305 digestion) stage, thus the COD remaining is quite refractory to microbial degradation.  
306 High HET activity, to reduce  $>70\%$  of  $\text{N}_2\text{O}$ , should be evident through reduced  $\text{NO}_3^-$   
307 concentration and decreased ratio of  $\text{NO}_3^-$  production to  $\text{NH}_4^+$  consumption. There was,  
308 however, no correlation between SP and  $r(\text{NO}_3^-)/r(\text{NH}_4^+)$  - in fact, the highest site prefer-  
309 ences were simultaneous with some of the highest  $\text{NO}_3^-$  production rates (data not shown).  
310 Although Ishii et al. [21] observed that HET activity was strong enough for HET  $\text{N}_2\text{O}$   
311 production to be the major  $\text{N}_2\text{O}$  source in a heterotrophic PN reactor,  $\text{N}_2\text{O}$  reduction in  
312 their study was only strong enough to bring SP to a maximum of  $14\%$ .  
313 In summary, the results during baseline conditions are not consistent with strong  
314 heterotrophic  $\text{N}_2\text{O}$  reduction causing the observed high SP, thus the other potential ex-  
315 planations for the data should be considered. The currently accepted fractionation factors  
316 for the  $\text{N}_2\text{O}$  production pathways shown in Table 1 are not able to explain the measured  
317 isotopic composition under baseline conditions. It is possible that the fractionation factor  
318 for  $\text{N}_2\text{O}$  production from  $\text{NH}_2\text{OH}$  oxidation has been strongly underestimated in previous  
319 studies, due to inorganic  $\text{N}_2\text{O}$  production occurring simultaneously to reduce the isotopic  
320 composition of the final measured  $\text{N}_2\text{O}$  in these studies - or the fractionation factors  
321 for  $\text{N}_2\text{O}$  reduction in Table S2 could be an incomplete assessment of the ‘true’ range  
322 of  $\epsilon_{18}/\epsilon_{\text{SP}}$ . Alternatively, an unknown microbial or inorganic  $\text{N}_2\text{O}$  production pathway  
323 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  $\text{N}_2\text{O}$  production pathways so that the various potential hypotheses could be examined with specific experiments, the parameters affecting  $\text{N}_2\text{O}$  production and isotopic composition under low  $\text{NH}_4^+$  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,  $\delta^{18}\text{O}$  and  $\delta^{15}\text{N}^{\text{bulk}}$ , which correlate inversely with net  $\text{N}_2\text{O}$  production ( $\text{N}_2\text{O}$  mixing ratio, net  $\text{N}_2\text{O}$  production rate ( $r\text{N}_2\text{O}$ ) and  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$ ) and  $DO$ . This suggests that during baseline conditions,  $DO$  is the most important factor controlling partitioning of  $\text{N}_2\text{O}$  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,  $\text{NO}_3^-$  ( $r(\text{NO}_3^-)$  and  $\text{NO}_3^-/\text{NH}_4^+$ ) is unimportant in PC1, which is consistent with a minor role of HETs and NOBs under baseline conditions.  $\text{NH}_4^+$  concentration is also unimportant in determining isotopic composition under baseline conditions, which suggests that  $\text{N}_2\text{O}$  production via  $\text{NH}_2\text{OH}$  oxidation is minor in this reactor as observed in previous studies, because this pathway is expected to be sensitive to the availability of  $\text{NH}_4^+$  [69, 21, 43, 48].

Overall, the measurements taken during baseline conditions are most consistent with hypothesis ii) above: A previously-unidentified  $\text{N}_2\text{O}$  production pathway is occurring in

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<sup>2</sup>Considering 100% of the initial released  $\text{N}_2\text{O}$  was derived from  $\text{NH}_2\text{OH}$  oxidation with  $\text{SP} = 33\text{‰}$ ,  $\epsilon_{\text{SP}} = -5.6$  (Table S2), and a maximum measured SP of  $39.7\text{‰}$ , and using the equation  $\delta = \delta_0 + \epsilon_{\text{SP}} \ln f$  [38, 46],  $f$  is equal to 0.3 thus 70% of the  $\text{N}_2\text{O}$  must be reduced. This represents a minimum level of reduction, as it is highly unlikely 100% of the initial  $\text{N}_2\text{O}$  was from  $\text{NH}_2\text{OH}$  oxidation, thus  $\delta_0$  is likely  $<33\text{‰}$ .

the PN-anammox reactor. The PCA results suggest that heterotrophic  $\text{N}_2\text{O}$  reduction and  $\text{NH}_2\text{OH}$  oxidation by AOBs are unimportant for the  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  emissions from pure anammox culture [36]. The ‘unknown pathway’ would have  $\text{SP} > 40\text{‰}$  to account for our observations. This is the first indication for a possible high SP from  $\text{N}_2\text{O}$  production in anammox systems, because in previous studies SP has only been measured in a PN reactor with high  $\text{NH}_4^+$  concentrations and very high  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$ , where  $\text{N}_2\text{O}$  production is dominated by HET denitrification [21]. The ‘unknown pathway’ appears to be most important when  $\text{N}_2\text{O}$  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  $\text{NH}_2\text{OH}$  oxidation may reach far higher values than observed in previous studies, and the extent of this pathway may not depend on the  $\text{NH}_4^+$  concentrations, in contrast to results from previous studies [57, 69]. The isotopic measurements following various perturbations 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 $\text{N}_2\text{O}$ production pathways

#### 3.3.1. $\text{N}_2\text{O}$ production with high aeration

The goal of this experiment was to favor  $\text{N}_2\text{O}$  production by AOBs - via the nitrifier denitrification pathway and/or  $\text{NH}_2\text{OH}$  oxidation (end-member SPs of  $\approx 0\text{‰}$  and  $\approx 33\text{‰}$  respectively; Table 1) - by reversibly inhibiting anammox and HET activity through elevated dissolved oxygen concentrations. The PCA of the baseline data revealed that  $\text{DO}$  was the most important parameter controlling  $\text{N}_2\text{O}$  production pathways and isotopic composition (Figure S5), although changes in  $\text{DO}$  during baseline conditions were small ( $0.5 - 54.4 \mu\text{g L}^{-1}$ ;  $0.02 - 1.7 \mu\text{M}$ ). The aeration rate was manually increased from 250-750

373 L h<sup>-1</sup> during baseline conditions to 1500-3000 L h<sup>-1</sup>, with normal NH<sub>4</sub><sup>+</sup> setpoint (20-30  
 374 mg-N L<sup>-1</sup>) in cycle *s* and the start of cycle *aa* (*aa*(1)) and higher NH<sub>4</sub><sup>+</sup> setpoints (50-  
 375 60 mg-N L<sup>-1</sup>) later in cycle *aa* (*aa*(2)), as shown in Figure 3. High aeration increased  
 376 *DO* to >0.2 mg L<sup>-1</sup> (>6.25 μM), reversibly inhibiting anammox, which are inhibited by  
 377 relatively low dissolved oxygen concentrations in suspended biomass systems [6, 23]. It is  
 378 possible some anammox activity remained in anoxic microsites, however this is assumed  
 379 to be very low as evidenced by rapid NO<sub>2</sub><sup>-</sup> build-up. High *DO* can also potentially lower  
 380 HET activity, particularly nitrous oxide reductase activity [11, 3, 37], although previous  
 381 studies suggest that nitrous oxide reductase can be active at *DO* up to 5 mg L<sup>-1</sup> (156  
 382 μM) [28].

383 In the absence of significant anammox activity, NO<sub>2</sub><sup>-</sup> accumulated up to 50 mg-N L<sup>-1</sup>  
 384 during the peak *DO* period in cycle *s* (NO<sub>2</sub><sup>-</sup> was not measured during cycle *aa*). In paral-  
 385 lel, the NH<sub>4</sub><sup>+</sup> consumption rate and the net N<sub>2</sub>O and NO production rates increased, and  
 386 the SP and δ<sup>18</sup>O strongly decreased (grey shading in Figure 3). SP values close to 0‰  
 387 indicate that N<sub>2</sub>O production is primarily via nitrifier denitrification or heterotrophic  
 388 denitrification, consistent with the increase in NO production [11, 2, 48]. However, a  
 389 strong increase in N<sub>2</sub>O production via heterotrophic denitrification is not consistent with  
 390 increased NH<sub>4</sub><sup>+</sup> consumption rate, as heterotrophs do not consume NH<sub>4</sub><sup>+</sup>. Therefore, the  
 391 low SP and the increase in NH<sub>4</sub><sup>+</sup> consumption rate together indicate that nitrifier den-  
 392 itrification by AOBs is responsible for the large spikes in N<sub>2</sub>O production under high  
 393 aeration.

394 The minimum SP value reached at the peak *DO* in each high aeration period (val-  
 395 ues shown in Figure 3) correlates with the NH<sub>4</sub><sup>+</sup> setpoint during the increased aeration.  
 396 This can be explained by NH<sub>2</sub>OH oxidation, which is expected to be enhanced at high  
 397 NH<sub>4</sub><sup>+</sup> concentration in the presence of abundant *DO* [69, 43]. However, the low SP values  
 398 under these conditions show that NH<sub>2</sub>OH oxidation makes a small contribution to N<sub>2</sub>O  
 399 production - although its importance does in fact increase with increasing concentration



of  $\text{NH}_4^+$ . Even at  $\text{NH}_4^+ = 50 \text{ mg-N L}^{-1}$  ( $DO > 1 \text{ mg L}^{-1}$  ( $31 \mu\text{M}$ ), cycle *aa*(2)), SP is 1.8‰ (Figure 3), thus  $\text{NH}_2\text{OH}$  oxidation contributes  $<8\%$  of  $\text{N}_2\text{O}$  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.

$\text{N}_2\text{O}$  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  $\text{NO}_3^-$  concentrations showed NOBs were active and consuming some  $\text{NO}_2^-$ . Although it is unlikely that during normal reactor operation (aeration rate  $250\text{--}750 \text{ L h}^{-1}$ ) the aeration rate would suddenly increase to  $3000 \text{ L h}^{-1}$ , 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  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$  remains a robust signal for process control (Figure 3), even though  $\text{N}_2\text{O}$  production shifted from the unknown pathway into a nitrifier denitrification-dominated regime. Aeration rate would be reduced in response to online measurements of  $\text{N}_2\text{O}$  mixing ratio to successfully return the reactor to stable operation.

### 3.3.2. $\text{NO}_2^-$ addition under aerobic conditions (normal aeration)

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

The net  $\text{N}_2\text{O}$  production rate and  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$  increased, and the SP decreased

by between 7‰ (cycle *f*, second peak) and 21.5‰ (cycle *h*) each time  $\text{NO}_2^-$  was added<sup>3</sup>. The  $\delta^{18}\text{O}$  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  $\delta^{18}\text{O}$  with no affect on SP. The difference between  $\delta^{18}\text{O}$  and SP varies between -7.8 and 20‰ with the smallest differences during  $\text{NO}_2^-$  addition, which suggests a shift between two dominant  $\text{N}_2\text{O}$  production pathways, with little influence from confounding factors such as the dependence of fractionation factors on process conditions [5, 72]. During cycle *o*, the  $\text{NH}_4^+$  setpoint was raised to 100 mg-N L<sup>-1</sup> to simultaneously enhance  $\text{N}_2\text{O}$  production via nitrifier denitrification and  $\text{NH}_2\text{OH}$  oxidation [69, 43]. The observed SP change was in the same range as for all other  $\text{NO}_2^-$  addition cycles shown in Figure 4, therefore it appears that  $\text{NH}_2\text{OH}$  oxidation plays a minor role in  $\text{N}_2\text{O}$  production, in agreement with the results presented in the previous subsections (Sections 3.2 and 3.3.1). The baseline SP before  $\text{NO}_2^-$  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  $\text{NO}_2^-$  addition rate (data not shown): Increasing  $\text{NO}_2^-$  addition rate results in a lower SP minimum. This shows that, although  $\text{NO}_2^-$  did not build up substantially in the bulk phase of the reactor, heterotrophic or nitrifier denitrification was enhanced following  $\text{NO}_2^-$  addition. This suggests a transition between two pathways, in agreement with the strong correlation between  $\delta^{18}\text{O}$  and SP. The high SP pathway could either be unknown, or  $\text{NH}_2\text{OH}$  oxidation with a previously unseen high SP fractionation; the former is more likely as the SP change at high  $\text{NH}_4^+$  concentrations discussed in the previous paragraph suggests  $\text{NH}_2\text{OH}$

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<sup>3</sup>SP decrease = SP during  $\text{NO}_2^-$  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  $\text{NO}_2^-$  addition is that  $\text{NO}_2^-$  inhibits HET activity [11, 3], resulting in less reduction of  $\text{N}_2\text{O}$  by HET and consequently lower SP. However, the highest observed  $\text{NO}_2^-$  concentration was  $<3 \text{ mg-N L}^{-1}$  (see Figure 4) while previous studies have observed HET activity at  $\text{NO}_2^-$  concentrations up to  $50 \text{ mg-N L}^{-1}$  [37, 1, 19].

### 3.3.3. $\text{NO}_2^-$ addition under anoxic conditions ( $\text{N}_2$ purging)

In cycles *r*, *z*, and *bb*  $\text{NO}_2^-$  was added to the reactor (Figure 5) and the gas flow was switched to  $\text{N}_2$ , thus  $\text{N}_2\text{O}$  production via nitrifier denitrification was promoted by excess  $\text{NO}_2^-$  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  $\text{NH}_4^+$  consumption shows that anammox remained active with  $\text{N}_2$  aeration because  $\text{NO}_2^-$  was continuously added.

In cycles *r* and *z*  $\text{NO}_2^-$  addition began under aerobic conditions and SP dropped by 9 and 15‰ respectively, showing that nitrifier denitrification was enhanced by  $\text{NO}_2^-$  addition as discussed in the previous subsection (Section 3.3.2;  $\text{NO}_2^-$  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  $\text{NO}_2^-$  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  $\text{N}_2$ , however, SP increased immediately by  $\sim 9\text{‰}$  as  $\text{N}_2\text{O}$  production by AOB ceased [73]. In cycle *bb*,  $\text{NO}_2^-$  addition and  $\text{N}_2$  aeration were started simultaneously and SP increased significantly by

476 7‰ ( $p < 0.001$ ) to reach the highest value measured over the whole experimental period,  
477 +45.9‰.

478 The simultaneous decrease in  $\delta^{15}\text{N}^{\text{bulk}}$  following  $\text{NO}_2^-$  addition shows consumption  
479 of the isotopically-light  $\text{NO}_2^-$  substrate (supplementary material Section S1.2); however  
480 unlike SP,  $\delta^{15}\text{N}^{\text{bulk}}$  remains lower with  $\text{N}_2$  aeration, particularly in cycle *bb*, reflecting  
481 the low  $\delta^{15}\text{N}$  of the added salt relative to the supernatant liquor and showing that the  
482 added  $\text{NO}_2^-$  substrate is being used to produce  $\text{N}_2\text{O}$  - even though reactor conditions ( $\text{N}_2$   
483 aeration; no  $\text{O}_2$ ) suggest nitrifier denitrification will not be occurring.  $\text{N}_2\text{O}$  production  
484 by HET also involves  $\text{NO}_2^-$  reduction [35] and agrees with the low  $\delta^{15}\text{N}^{\text{bulk}}$  discrimination  
485 between the added  $\text{NO}_2^-$  and the observed  $\text{N}_2\text{O}$  [45]; however the  $\text{N}_2\text{O}$  produced by HET  
486 has low SP ( $\sim 0\%$  [57, 70]). This pathway can therefore not explain the results, unless  
487  $> 90\%$  of the  $\text{N}_2\text{O}$  is subsequently reduced to  $\text{N}_2$  by HET (using Rayleigh fractionation  
488 equations as described in Section 3.2). This is not consistent with low COD in the system,  
489 or with the relatively high  $\text{N}_2\text{O}$  emissions, particularly during cycle *bb*. Furthermore,  $\text{NO}_3^-$   
490 production is close to the 11% expected from anammox and therefore not consistent with  
491 strong HET activity (Figure 5).

492 Both microbial and inorganic production of  $\text{N}_2\text{O}$  from  $\text{NH}_2\text{OH}$  have been observed to  
493 produce SP values of 29-36‰ [61, 57, 15, 45, 69, 18, 71]; SP of inorganic  $\text{N}_2\text{O}$  production  
494 via  $\text{NO}_2^-$  reduction by  $\text{Fe}^{2+}$  has not been measured and can therefore not be assessed.  
495 A recent study by Yang et al. [72] considering fungal nitric oxide reductase (P450nor)  
496 suggests that SP may not always be a conservative tracer of reaction pathways, in contrast  
497 to pure culture studies on fungal denitrification by Sutka et al. [56] and more recently  
498 Rohe et al. [52], which show highly constant SP values for a particular species, with some  
499 variation between species. Yang et al. [72] suggest that the variation in SP (15-29‰)  
500 observed in their study was due to non-steady state conditions regarding NO supply and  
501 consumption and cell growth conditions; however non-steady state conditions resulted in  
502 lower, not higher, SP in their study. It is possible that high SP in the present experiments

is due to  $\text{N}_2\text{O}$  production from  $\text{NH}_2\text{OH}$  oxidation when conditions are far from steady state; another possibility is that previous attempts to measure the SP of the  $\text{NH}_2\text{OH}$  pathway were contaminated by low SP- $\text{N}_2\text{O}$  from inorganic or microbial  $\text{N}_2\text{O}$  production by denitrification. In the latter case, the present study would need to have significantly less  $\text{N}_2\text{O}$  from denitrification than all previous studies, to make the ‘true’ high SP of the  $\text{NH}_2\text{OH}$  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  $\text{NH}_2\text{OH}$  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  $\text{NO}_2^-$  addition under  $\text{N}_2$  aeration therefore may suggest, in agreement with the other experimental results as discussed in the previous sections, that an anoxic pathway is producing  $\text{N}_2\text{O}$  with high SP ( $\geq 45\%$ ), enhanced by  $\text{NO}_2^-$  addition and anoxic conditions - and therefore possibly associated with anammox activity. The unknown pathway may have a low level of isotopic discrimination for  $\delta^{15}\text{N}^{\text{bulk}}$ , given the close agreement between the  $\delta^{15}\text{N}$  of added  $\text{NO}_2^-$  and  $\delta^{15}\text{N}^{\text{bulk}}$  of  $\text{N}_2\text{O}$  in cycles *z* and *bb* - however  $\delta^{15}\text{N}$  discrimination can be highly variable, and in addition it is unclear if all of the N in  $\text{N}_2\text{O}$  derives from  $\text{NO}_2^-$ .

#### 3.3.4. Addition of $\text{NH}_2\text{OH}$

In cycle *y*,  $\text{NH}_2\text{OH}$  was continuously added at a rate of 20-80 mg-N/min for a total of 204 minutes (total addition = 9.3 g-N  $\approx$  23 mg-N  $\text{L}^{-1}$ ) to promote  $\text{N}_2\text{O}$  production via  $\text{NH}_2\text{OH}$  oxidation (results shown in supplementary information, Figure S6). The  $\text{N}_2\text{O}$  production rate increased twofold when  $\text{NH}_2\text{OH}$  was added. The SP of emitted  $\text{N}_2\text{O}$  also increased significantly, from  $34.4 \pm 1.1\%$  to  $41.3 \pm 1.8\%$  ( $\Delta\text{SP} = 6.9 \pm 2.1$ ;  $p < 0.001$ ). The final SP of  $41.3\%$  is  $5\%$  higher than the highest values reported in the literature for  $\text{NH}_2\text{OH}$  oxidation [15, 57] (see Table 1).

As in the previous experiments, we can consider several possibilities for these high values: i) significant  $\text{N}_2\text{O}$  reduction by HET, ii) an unknown inorganic or microbial  $\text{N}_2\text{O}$  production pathway with a much higher end-member SP, where  $\text{NH}_2\text{OH}$  is somehow involved either directly, or indirectly via the formation of intermediates, or iii) previous measurements of  $\text{NH}_2\text{OH}$  characteristic SP are too low due to ‘contaminant’  $\text{N}_2\text{O}$  from denitrification, or SP of  $\text{N}_2\text{O}$  from  $\text{NH}_2\text{OH}$  is higher when growth conditions are not at steady state [72]. HET reduction would need to remove >50-70% of  $\text{N}_2\text{O}$  to achieve the observed SP of 41‰ [46, 22, 62] produced following  $\text{NH}_2\text{OH}$  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  $\text{N}_2\text{O}$  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 ( $\epsilon_{\text{SP}} = -2.9$  to  $-8.2$ ‰, see Table S2) and are therefore considered robust. Such high levels of reduction are also inconsistent with the observed increase in  $\text{N}_2\text{O}$  production rate following  $\text{NH}_2\text{OH}$  addition. SP of  $\text{N}_2\text{O}$  produced from abiotic reduction of  $\text{NO}_2^-$  by  $\text{Fe}^{2+}$  [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  $\text{N}_2\text{O}$  produced from the inorganic oxidation of  $\text{NH}_2\text{OH}$  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  $\text{N}_2\text{O}$  production via  $\text{NH}_2\text{OH}$  oxidation are unlikely given the literature but cannot be discounted. An unknown microbial pathway with high SP involving  $\text{NH}_2\text{OH}$  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 $\text{N}_2\text{O}$ production

The online isotopic measurements of  $\text{N}_2\text{O}$  produced in a combined PN-anammox reactor provided a rich dataset that revealed unexpected results regarding  $\text{N}_2\text{O}$  production

555 pathways. The results of this study showed that nitrifier denitrification is responsible  
 556 for  $\text{N}_2\text{O}$  emission peaks at high  $\text{NH}_4^+$  concentrations, when AOB and anammox activities  
 557 are not optimally balanced (eg. when the aeration rate or the  $\text{DO}$  is too high; see Sec-  
 558 tion 3.3.1). However, consistently high SP observed during normal operation (Figures 2,  
 559 S1 and S4) show that either  $\text{N}_2\text{O}$  production also occurs via an unknown pathway with  
 560 high SP, HET activity and thus  $\text{N}_2\text{O}$  reduction is much stronger than expected, or frac-  
 561 tionation factors reported in previous studies (Table 1) cover an incomplete range.  $\text{N}_2\text{O}$   
 562 emissions of this type, which cannot be explained within our current understanding - low  
 563  $\text{N}_2\text{O}$  production rate and high SP - accounted for  $>75\%$  of the total  $\text{N}_2\text{O}$  production<sup>4</sup> over  
 564 the entire experimental period, and close to 100% of emissions during normal operating  
 565 conditions. A number of experimental perturbations were applied to the system to test  
 566 these hypotheses.

567 The results of these experiments are most consistent with the hypothesis that high  
 568 SP is due to an unknown  $\text{N}_2\text{O}$  production pathway (Table 2) - although the other pro-  
 569 posed hypotheses cannot be dismissed. HET reduction would need to consume  $>90\%$  of  
 570  $\text{N}_2\text{O}$  to achieve the highest SP value of  $45.9\text{‰}$  observed during the entire experimental  
 571 period (Section 3.3.3), and  $>70\%$  of  $\text{N}_2\text{O}$  to reach the maximum ‘baseline’ SP value of  
 572  $39.7\text{‰}$  (Section 3.2), calculated using Rayleigh fractionation as described in Section 3.2  
 573 [38]. This level of reduction is unlikely due to low COD concentrations and observed N  
 574 transformation rates - eg. high  $\text{N}_2\text{O}$  production rates, and  $\sim 10\%$   $\text{NO}_3^-$  production relative  
 575 to  $\text{NH}_4^+$  consumption - however it cannot be entirely ruled out without isotopic tracer ex-  
 576 periments. O isotope exchange could explain why the slope of SP to  $\delta^{18}\text{O}$  does not agree  
 577 with  $\text{N}_2\text{O}$  reduction during baseline experiments (Figure 2) as discussed in Section 3.2,  
 578 but an important role of heterotrophic  $\text{N}_2\text{O}$  reduction is generally not consistent with our  
 579 other experimental results, particularly during  $\text{NO}_2^-$  addition (Sections 3.3.2 and 3.3.3).

580 Highly varying SP fractionation for the  $\text{NH}_2\text{OH}$  pathway is unlikely given the relatively  
 581 tight range of  $29\text{--}36.3\text{‰}$  measured in numerous previous studies considering a variety of

pathways, including inorganic, pure culture, and mixed culture  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  with high SP ( $\geq 45\%$  - the maximum SP observed, during  $\text{NO}_2^-$  addition under anoxic conditions) and appears to be associated with anammox metabolism directly or indirectly as an associated inorganic pathway, possibly involving  $\text{NH}_2\text{OH}$ . Further experimentation is needed to confirm the existence of this pathway and gain a definitive understanding of  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  monitoring is an ideal method for process control in a combined, continuously-fed PN-anammox reactor, extending the current set of available online parameters (eg. pH, conductivity,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , aeration rate). During stable operation  $\text{N}_2\text{O}$  emissions are within the range required for anammox systems to have a positive energy balance compared to traditional nitrification-denitrification wastewater treatment processes. When  $\text{DO}$  or  $\text{NO}_2^-$  increase due to eg. changes in AOB or anammox activity,  $\text{N}_2\text{O}$  emissions via nitrifier denitrification are enhanced; thus aeration can be decreased in response to high measured offgas  $\text{N}_2\text{O}$  mixing ratio to keep the reactor in a stable state while simultaneously minimizing GHG emissions.

## 4. Conclusions

This study uses online measurements of  $\text{N}_2\text{O}$  concentration and isotopic composition to understand  $\text{N}_2\text{O}$  production pathways under a variety of conditions, and assess the use of  $\text{N}_2\text{O}$  measurements for process control in a single-stage PN-anammox reactor. The main conclusions of this study are:

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<sup>4</sup>Estimated as  $\text{N}_2\text{O}$  production rate  $\times$  time where  $\text{SP} > 33\%$ , compared to production rate  $\times$  time where  $\text{SP} < 33\%$ .



- Online measurements of  $\text{N}_2\text{O}$  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  $\text{N}_2\text{O}$  emissions are relatively low during optimal operation indicates that our current understanding of  $\text{N}_2\text{O}$  production and isotopic fractionation is incomplete, and may suggest the existence of an unknown  $\text{N}_2\text{O}$  production pathway associated with anammox metabolism.
- When  $\text{NH}_4^+$  or  $\text{DO}$  are elevated in the reactor,  $\text{N}_2\text{O}$  production by nitrifier denitrification is enhanced.  $\text{N}_2\text{O}$  production via  $\text{NH}_2\text{OH}$  oxidation is relatively unimportant in the reactor under all conditions.

## Acknowledgments

We thank Thomas Kuhn, Moritz Lehmann and Caitlin Frame from the University of Basel for measuring the  $\delta^{15}\text{N}$  of  $\text{NaNO}_2$  and  $\text{NH}_2\text{OHCl}$  salts and Beat Beyeler from WWTP Zürich Werdhölzli for providing the supernatant liquor. Instrumental developments at Empa were funded by the State Secretariat for Education and Research (SER) within COST Action ES0806 and the Swiss National Science Foundation (SNSF). Eliza Harris was supported by the European Union’s Seventh Framework Programme for research, technological development and demonstration under grant agreement no. 2010-267161.

The supplementary information file contains Section S1: Materials and Methods, Figures S1-S6 and Table S1-S2.

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## Figures and tables

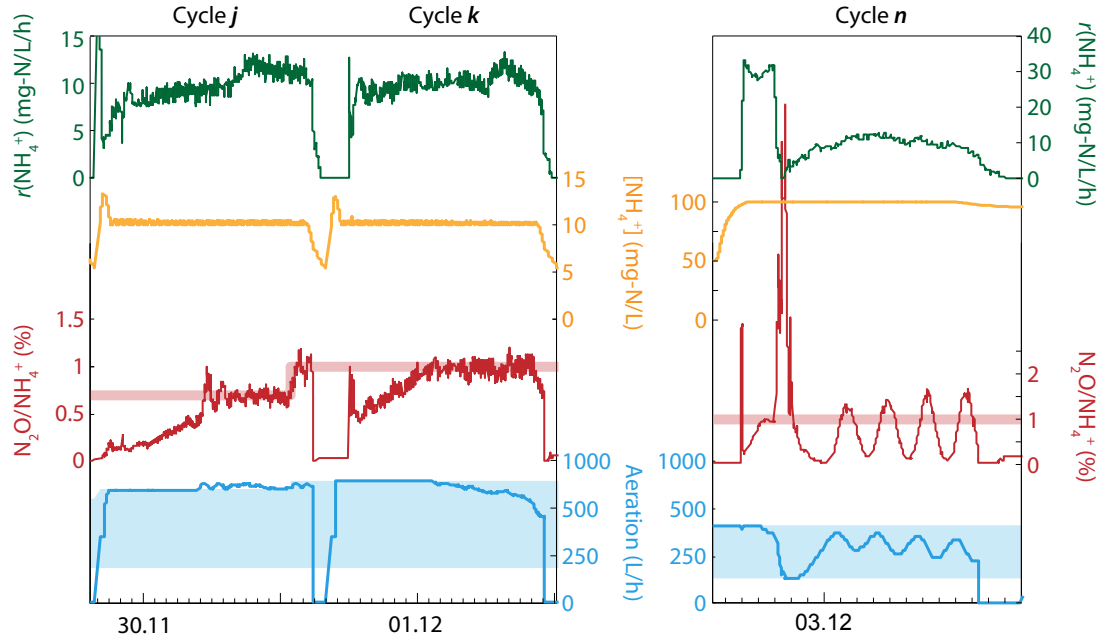


Figure 1: Automatic regulation of aeration flow rate based on online measurement of  $\text{N}_2\text{O}$  production divided by  $\text{NH}_4^+$  consumption rate  $r(\text{NH}_4^+)$  under baseline conditions with low  $\text{NH}_4^+$  (left) and high  $\text{NH}_4^+$  (right) concentration. The setpoint 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.

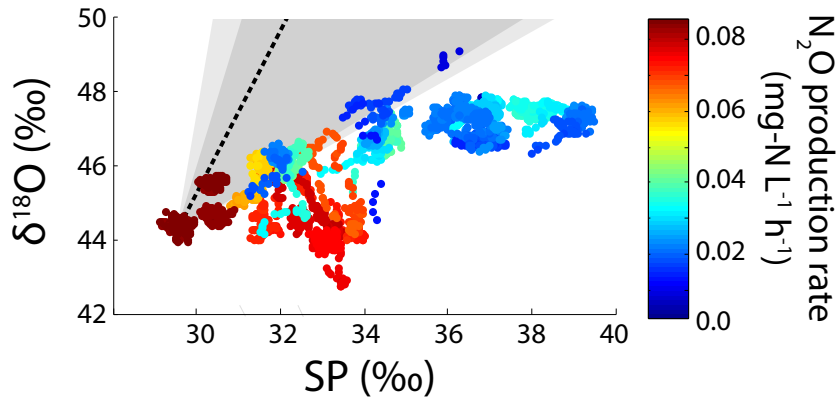


Figure 2: Relationship between SP and  $\delta^{18}\text{O}$  during baseline experiments with low  $\text{NH}_4^+$ , shown as points coloured by  $\text{N}_2\text{O}$  production rate. The expected relationship using the mean  $\epsilon_{18}/\epsilon_{\text{SP}}$  from previous studies (see Table S2) is shown as a dashed line and the dark grey shading shows the  $1\sigma$  error; the pale grey shading shows the maximum and minimum slopes from previous studies. These estimates assume that deviation from the measured SP and  $\delta^{18}\text{O}$  (30.2 and 44.1‰ respectively) at an  $\text{N}_2\text{O}$  production rate of  $0.09 \text{ mg-N L}^{-1} \text{ h}^{-1}$  is solely due to  $\text{N}_2\text{O}$  reduction. Slope from Well et al. [67] added to determination of mean slope in revised version; see Table S2.

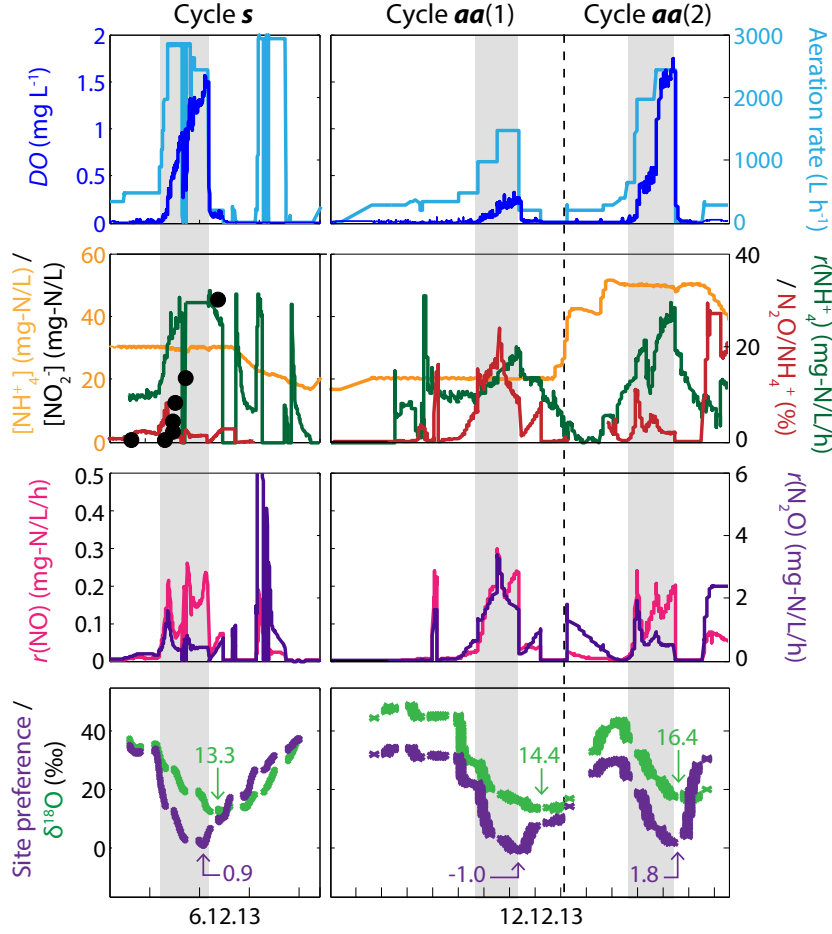


Figure 3: High aeration experiments with normal  $\text{NH}_4^+$  setpoint during cycles *s* and *aa*(1) and with high  $\text{NH}_4^+$  setpoint during cycle *aa*(2). Cycles *aa*(1) and *aa*(2) are divided with a dashed line. The top panel shows the increase in aeration (light blue) and the resultant increase in *DO* (dark blue); periods where aeration and *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.



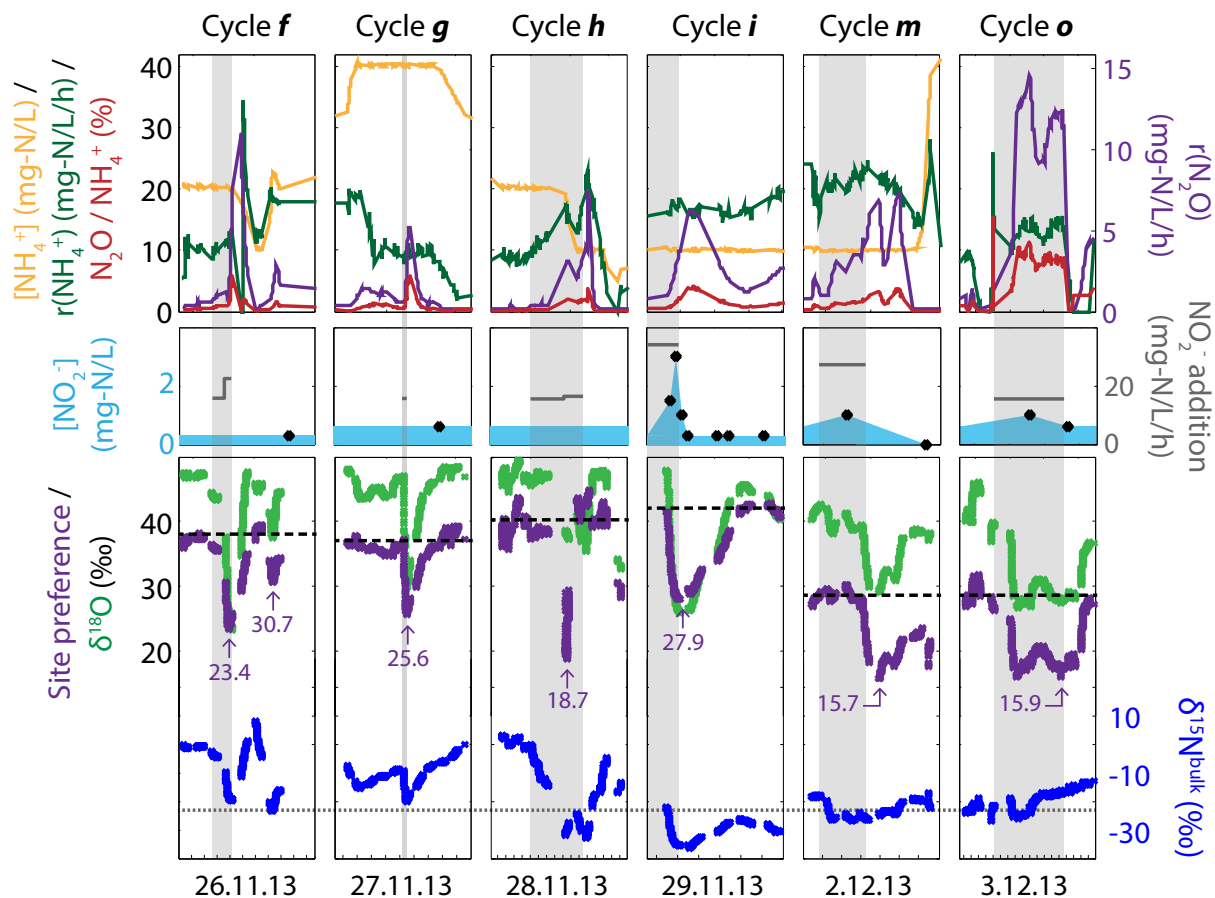


Figure 4: Addition of  $\text{NO}_2^-$  (as  $\text{NaNO}_2$ ) during six cycles with normal aeration.  $\text{NO}_2^-$  was continuously added to the reactor during the periods indicated with grey shading. The top panel shows the  $\text{NH}_4^+$  consumption rate ( $r(\text{NH}_4^+)$ ; green), the  $\text{N}_2\text{O}$  production rate ( $r(\text{N}_2\text{O})$ ; purple),  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$  (%) (red), and the  $\text{NH}_4^+$  concentration (yellow). In cycle o the  $\text{NH}_4^+$  setpoint was 100  $\text{mg-N L}^{-1}$  which is off-scale in the figure. In the second panel, the  $\text{NO}_2^-$  addition rate is shown as a thick grey line and the  $\text{NO}_2^-$  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  $\text{N}_2\text{O}$ . The minimum SP reached after each  $\text{NO}_2^-$  addition is indicated and the mean SP before and after addition for each cycle is shown with a black dashed line. The  $\delta^{15}\text{N}$  of the added  $\text{NaNO}_2$  salt is shown with a grey dotted line in the bottom panel. X-axis tickmarks are spaced 1 hour apart.

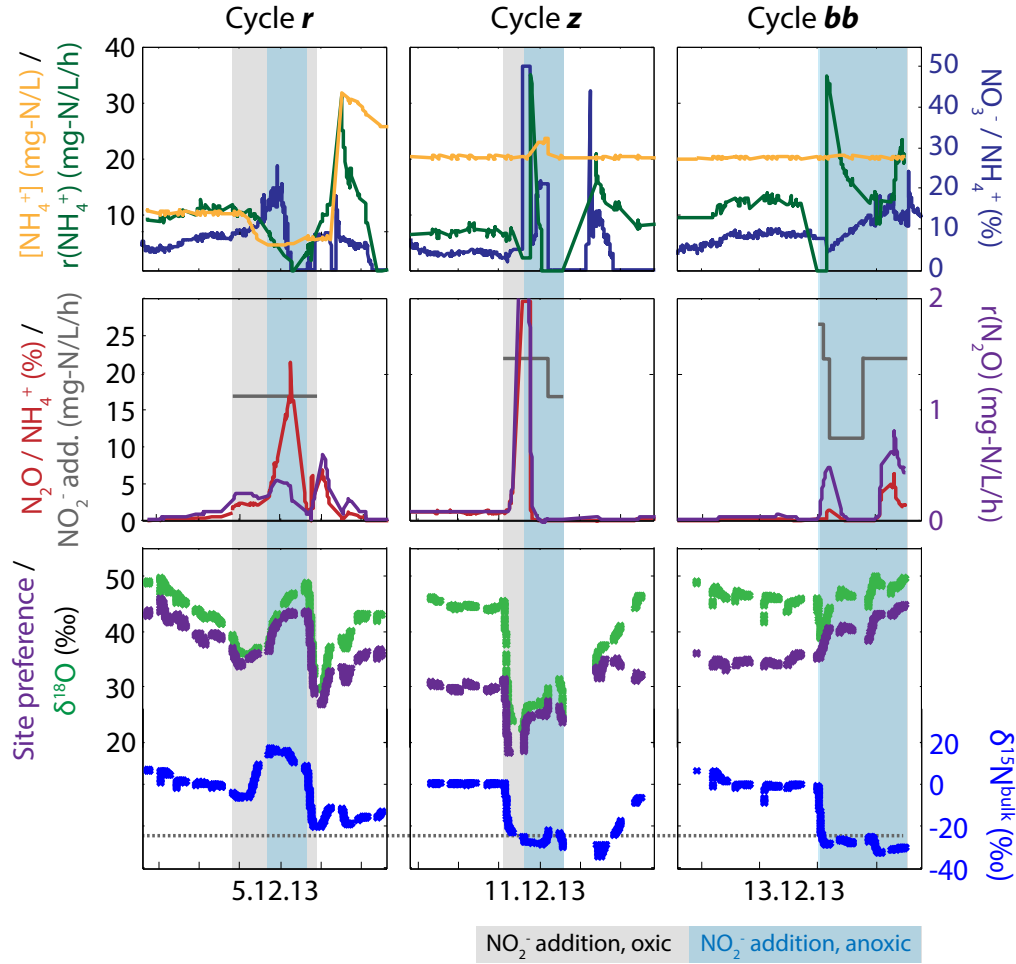


Figure 5: Addition of  $\text{NO}_2^-$  (as  $\text{NaNO}_2$ ) during three cycles with  $\text{N}_2$  purge gas.  $\text{NO}_2^-$  was continuously added to the reactor during the periods indicated with grey shading, and  $\text{N}_2$  was used for aeration during the periods shaded blue, as shown in the legend. The top panel shows the  $\text{NH}_4^+$  consumption rate ( $r(\text{NH}_4^+)$ ; green), the  $\text{NH}_4^+$  concentration ( $[\text{NH}_4^+]$ ; yellow) and  $\text{NO}_3^-/\text{NH}_4^+$  (dark blue). The second panel shows the net  $\text{N}_2\text{O}$  production rate ( $r(\text{N}_2\text{O})$ ; purple) and  $r(\text{N}_2\text{O})/r(\text{NH}_4^+)$  (%; red), and the  $\text{NO}_2^-$  addition rate (grey). The isotopic composition of offgas  $\text{N}_2\text{O}$  is presented in the bottom panel; the  $\delta^{15}\text{N}$  of the added  $\text{NaNO}_2$  salt is shown with a grey dotted line. X-axis tickmarks are spaced 1 hour apart.

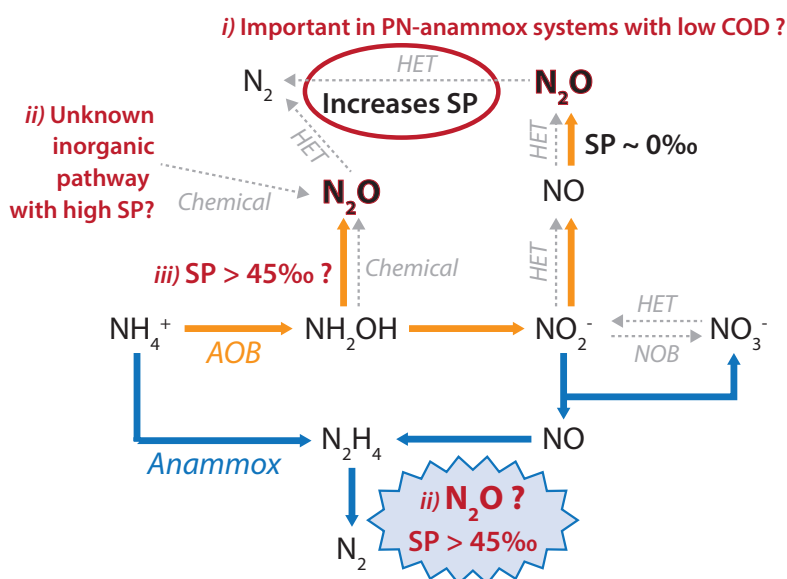


Figure 6: The nitrogen cycle in a PN-anammox reactor. Pathways attributed to ammonia-oxidizing bacteria (AOBs) are shown in yellow, including  $\text{N}_2\text{O}$  production via  $\text{NH}_2\text{OH}$  oxidation ( $\text{SP} \sim 33\text{‰}$ ) and via nitrifier denitrification ( $\text{SP} = 0\text{‰}$ ). Anaerobic  $\text{NH}_4^+$  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),  $\text{NO}_3^-$  production by  $\text{NO}_2^-$  oxidizing bacteria (NOB), and chemical oxidation of  $\text{NH}_2\text{OH}$  with  $\text{SP} \sim 33\text{‰}$ . The four most likely explanations for the high SP values observed throughout this study are shown in red: i) unexpectedly strong heterotrophic  $\text{N}_2\text{O}$  reduction, ii) unknown inorganic or anammox-associated  $\text{N}_2\text{O}$  production pathway, or iii) the SP end-member for  $\text{NH}_2\text{OH}$  has been underestimated in previous studies.

Table 1: Known  $\text{N}_2\text{O}$  production and consumption pathways that may be occurring in a partial nitrification-anammox reactor, and the site preference and  $^{15}\text{N}$  fractionation factors associated with the pathways. AOB =  $\text{NH}_4^+$  oxidizing bacteria, HET = heterotrophic denitrifiers.

Pathway	Population	Reaction	Site preference (‰)	$\Delta\delta^{15}\text{N}$ (‰)
$\text{NH}_2\text{OH}$ oxidation	AOB	$\text{NH}_2\text{OH} \rightarrow \text{NO} \rightarrow \text{N}_2\text{O}$ [8]	30 to 36 [69]	-37 to -17 [58, 59]
Nitrifier denitrification	AOB	$\text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O}$ [8]	-10 to 0 [57, 69]	-68 to -30 [58, 59, 69]
$\text{NO}_3^-/\text{NO}_2^-$ reduction	HET	$\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{N}_2\text{O}$	-5 to 0 [61, 63, 69]	-39 to -12 [61]
$\text{N}_2\text{O}$ reduction	HET	$\text{N}_2\text{O} \rightarrow \text{N}_2$	-16.4 to -2.9 <sup>5</sup>	-39 to -12 [61]
Abiotic production	-	Various [61, 18]	29.5 to 35 [61, 18]	-27 to 1.4 [18]

<sup>5</sup>Reduction favours  $\text{N}_2\text{O}$  with low site preference as the  $^{14}\text{N}$ -O bond is more easily broken than the  $^{15}\text{N}$ -O bond, thus the site preference of the remaining  $\text{N}_2\text{O}$  pool is increased. The values -2.9 to -16.4‰ refer to  $\epsilon(\text{SP})$ , the site preference fractionation factor for reduction (see Table S2 for references).

Table 2: Overview of experiments conducted to understand  $N_2O$  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.

Experiment	Cycle(s)	Action	Observation	Potential explanation
Baseline: Low $NH_4^+$	<i>a-e, j-l, t-w, cc-ee</i>	No perturbation; $NH_4^+$ setpoint 10-20 mg-N $L^{-1}$	SP higher than expected	Unknown (anammox) $N_2O$ production pathway, rather than significant HET reduction of $N_2O$
Baseline: High $NH_4^+$	<i>g, n, o, p, q</i>	No perturbation; $NH_4^+$ setpoint 100 mg-N $L^{-1}$	SP decreased compared to low $NH_4^+$	Enhanced $N_2O$ production via nitrifier denitrification
High aeration	<i>s, aa</i>	Aeration rate increased to 1500-3000 $L h^{-1}$ , thus $DO > 0.2$ mg $L^{-1}$	Increased $N_2O$ production, SP reduced to $\sim 0\%$	Enhanced nitrifier denitrification with anammox and possibly HET inhibited; $NH_2OH$ oxidation unimportant
$NO_2^-$ addition (oxic)	<i>f-i, m, o</i>	$NO_2^-$ added at 5-36 mg $NO_2-N L h^{-1}$	SP decrease (7-21%), correlated with $NO_2^-$ addition rate	Enhanced nitrifier denitrification - $NH_2OH$ oxidation unimportant
$NO_2^-$ addition (anoxic)	<i>r, z, bb</i>	$NO_2^-$ added at 11-27 mg $NO_2-N L h^{-1}$ and aeration switched to $N_2$	SP remains high or increases ( $> 46\%$ )	Nitrifier denitrification inhibited, anammox activity and unknown $N_2O$ production pathway enhanced
$NH_2OH$ addition	<i>y</i>	$NH_2OH$ added at 20-80 mg $NH_2OH-N min^{-1}$	SP increased to $\sim 41\%$	Unknown (anammox?) $N_2O$ production pathway involves $NH_2OH$