

We would like to thank the referee for the effort and time she/he put in to review our manuscript. We are grateful for her/his valuable comments and will make every attempt to carefully address these comments that will improve the quality of the revised manuscript. Hereafter, the points raised by the referee are written in black, whereas our responses are shown in blue. Citation of our corrections that will take place in the revised version of the manuscript are highlighted in grey.

General comments

Humbert et al. report emissions and production/consumption processes of N₂O in a nitrifying biofilm reactor which simulates a part of a biological waste water treatment system. Although several similar studies have been published, knowledge of key factors that should be controlled to mitigate N₂O emission is still insufficient because there are various type of biological waste water treatment and because processes related to N₂O depend on many factors.

Major findings of this paper are that N₂O is mainly produced by nitrifier-denitrification in a nitrifying biofilm reactor and that temperature control is more important than oxygen concentration or ammonia concentration. They may be worth publishing in Biogeosciences if the authors add implications of their research not only for a specific waste water treatment system but for other systems including natural water or soils.

Although the purpose and conclusion are clearly described, I found several flaws in the manuscript. First, a couple of related studies (Tumendelger et al., 2014; 2016) are not cited and compared with the findings of this study. Second, presentation of results (tables and figures in main text and supplement) is not well organized and is confusing. For example, Table S1 seems to show all the experimental conditions but corresponding results are not shown and figures seem to show only a part of the results. Third, a part of interpretation of isotopic data is not appropriate or based on assumptions that are not clearly explained. Fourth, several sentences are not readable or clear.

In summary, I consider this paper may be acceptable after careful revisions with respect to concerns above and below.

In the revised manuscript, we attempted to add implications of our research for a diversity of systems by adding sentences that underline these perspectives at some strategical parts of the manuscript (i.e. the ends of introduction, and discussion sections).

Our responses to the specific comments of the referee are listed below and allow to address the four concerns lastly raised by the referee to our knowledge.

Specific comments

L39 Add Tumendelger et al., 2014 and 2016.

We agree that the results presented in Tumendelger et al., 2014 and 2016 fall perfectly within the scope of our study. Therefore, we added the references suggested by the referee and removed Tallec et al. 2006 from this reference list.

Temperature, electron donor and acceptor concentrations have been identified to control the N₂O emission from WRRFs (Bollon et al., 2016; Kampschreur et al., 2009; Tumendelger et al., 2014, 2016; Wunderlin et al., 2012).

L58–60 I think this statement is vague because equilibrium process is involved in biotic process (e.g., O-exchange between nitrate and water during nitrification and denitrification) and kinetic fractionation also occur in abiotic processes (e.g., diffusion in air or water).

We reworded this sentence.

The isotopic fractionation results from the difference in equilibrium constant or reaction rate observed between the heavier and lighter isotopes in both abiotic and biotic processes.

L73 This statement is misleading because many of previous studies cited elsewhere in this manuscript did use combination of isotope data of N₂O to analyze production/consumption processes.

We agree and reworded this sentence.

Nitrogen and oxygen isotope ratios of N₂O have lower potential for N₂O source identification as compared to ¹⁵N-SP. However, we believe that the use of both isotope approaches should strengthen the conclusions from ¹⁵N-SP and reveal additional isotope effects (Fig. 1).

L93–94 I think a schematic of the reactor helps readers to understand the experiment and how monitoring of environmental parameters and sample collection were conducted. What is “continuous down-flow counter current mode”?

As suggested by the referee, we added a schematic of the reactor as new Figure S1 in the supplementary material.

A lab-scale reactor with a working volume of 9.9 L (colonized Biostyrene® beads and interstitial volume) and a headspace of 1.4 L was operated in continuous down-flow counter-current mode for seven weeks (Fig. S1).

L102 Here it can be read that the authors made 24 experiments, but in Table S1 total 26 conditions are shown. But in Table S1, the first line in the list of oxygenation tests and the second one for NH₄⁺ concentration tests seem the exactly same condition, and the same for the second of oxygenation tests and the last of NH₄ concentration tests. Are these pairs from actually a single experiment? Please explain in footnotes. Also, there is no “n.a.” entry in Table S1 in spite of footnote.

Twenty-four experiments were performed. Two of them tested effects of both oxygenation and ammonium concentration. As suggested by the referee, we added explanations in the text and in the footnote of Table S1.

The influence of environmental conditions on the ammonium oxidation rates and the N₂O emissions from various combinations of oxygenation levels, temperatures and ammonium concentrations were tested in twenty-four experiments (Tables 1). Note that two of them were used twice; as oxygenation and concentration tests.

Note that two experiments tested both oxygenation and ammonium concentration.

L106 If the numbers in Table S1 are correct, NH₄ concentration should be “20.3” –21.1 mg N/L and temperature should be “19.0 to 19.8” C. Please check the data carefully.

We are grateful to the referee for raising this mistake. We rechecked our data and changed therefore the text in consistency with Table S1 which was correct.

The oxygenation tests consisted of mixing compressed air and pure nitrogen gas to reach 0 to 21 % O₂ in the gas mixture (Fig. S1a). They were performed at five substrate concentrations and at a temperature between 19.2 and 20.6 °C. The temperature tests consisted of cooling the feeding solution directly in the feeding tank (22.3 to 13.5 °C), with an inflow ammonium concentration of 20.3–21.1 mg NH₄⁺-N L⁻¹. At temperatures ranging from 19 to 19.8 °C, the ammonium concentration tests

consisted of an increase (6.2, 28.6 and 62.1 mg NH₄⁺-N L⁻¹) and a decrease (56.1, 42.9, 42.7 and 20.2 mg NH₄⁺-N L⁻¹) in the NH₄⁺ concentrations in the feeding solution.

L108 How did the authors determine that the optimal oxygen concentration is 21%? Table 1. This table is just showing reduced information presented in Table S1 and is not helpful. I suggest to use Table S1 here.

We reworded this sentence and added an explanation about the meaning of optimal oxygenation level for the next of the manuscript. Further, as suggested by the referee, Table S1 was used instead of Table 1 in the main text.

Atmospheric oxygenation level (i.e. 21 % O₂ in the gas mixture) was imposed for both tests (Figs. S1b and c). This gas mixture using compressed air with 21 % O₂ was considered hereafter as optimal as compared to the oxygen-depleted atmosphere used during the oxygenation tests. Noticeably, the atmospheric oxygenation level is the condition that represents the most optimal conditions of oxygenation applied in nitrification BAF of domestic WRRF.

L131 This sentence seems to explain the calibration of dN and dO for ammonium, nitrite, and nitrate. In the case of N₂O, dO cannot be calibrated using nitrate standards because there is a kinetic fractionation during N-O bond rupture in nitrate reduction to N₂O. SP is also not determinable using the standards listed here. Please explain more.

We clarified and reorganized this paragraph.

These methods consist in the conversion of the substrate (ammonium or nitrite or nitrate) into dissolved N₂O. The δ¹⁵N and δ¹⁸O for ammonium, nitrite, and nitrate were hence determined from a calibration curve created with a combination of nitrate or ammonium standards that have undergone the same chemical conversion as the samples (USGS-32, δ¹⁵N-NO₃⁻ = 180 ‰, δ¹⁸O-NO₃⁻ = 25.7 ‰; USGS-34, δ¹⁵N-NO₃⁻ = -1.8 ‰, δ¹⁸O-NO₃⁻ = -27.9 ‰ and USGS-35 δ¹⁵N-NO₃⁻ = 2.7 ‰, δ¹⁸O-NO₃⁻ = 57.5 ‰; or IAEA-N1, δ¹⁵N-NH₄⁺ = 0.4 ‰, IAEA-305A, δ¹⁵N-NH₄⁺ = 39.8 ‰, USGS-25, δ¹⁵N-NH₄⁺ = -30.4 ‰). The quality of calibration was controlled with additional international standards (IAEA-NO-3, δ¹⁵N-NO₃⁻ = 4.7 ‰, δ¹⁸O-NO₃⁻ = 25.6 ‰; or IAEA-N2, δ¹⁵N-NH₄⁺ = 20.3 ‰). Basically, an analytical sequence was comprised of triplicate standards for calibration and quality controls and duplicate samples. The average of the analytical replicates was then used for calibration, quality control and as result.

Since no international standards were available for N₂O isotopes, these were determined the same day as nitrate or ammonium standard analysis insuring correct functioning of the method and analysis. In addition to this, the internal N₂O standards were previously calibrated by exchange with the laboratory of Naohiro Yoshida and Sakae Toyoda at the Tokyo Institute of Technology. All isotope measurements were determined using an isotope ratio mass spectrometer (IRMS, DeltaVplus; Thermo Scientific) in continuous-flow with a purge and trap system coupled with a Finnigan GasBench II system (Thermo Scientific). The precision was 0.8 ‰, 1.5 ‰ and 2.5 ‰ for δ¹⁵N, δ¹⁸O, and ¹⁵N-SP, respectively.

L140 Confirm the unit. If concentration is multiplied by flow rate, dimension should be mass per time (e.g., mg N /min).

The unit was corrected.

The ammonium oxidation rate (AOR) was estimated in each experiment for time ≥ 1 HRT from the difference between influent and effluent NH₄⁺ concentrations multiplied by the liquid flow rate (kg NH₄⁺-N d⁻¹).

L147 Consider more appropriate title of the section, for example, “Estimation of the range of nitrogen isotope ratio in N₂O produced by each biological process”.

We clarified the title as suggested by the referee.

2.5 Estimation of ranges of nitrogen isotope ratio in biologically produced N₂O

L157–171 It is strange that these sentences describe how to estimate dN values of output NH₄⁺ and NO_x⁻, because in Fig. S2 concentrations and isotope ratios of N compounds in inflow and outflow are shown as “measured” parameters. If these are really measured, I think it is worth calculating isotope enrichment factors (epsilons) in the studied system and comparing with previous studies.

The isotope ratios of N compounds in inflow and outflow were measured only for the three experiments shown in Fig.S2. Although these experiments allow calculating isotope enrichment factors (epsilons), these epsilons unlikely describe the whole range of operating conditions tested in our study. Therefore, we think that using isotope ratio estimates from the literature is more appropriate than values based on a limited number of experiments. We added sentences throughout the manuscript (in 2.5 and 3.1 sections) to better explain this and hence to improve its clarity. In addition, detailed data related to Figure S2 were added as new Table S1.

The isotope effect of reaction step can be determined from the isotope composition of substrates or products. Although being performed on a few tests here, the obtained value can only be applied to a limited number of environmental conditions. The use of estimates from the literature seems therefore suitable.

The δ¹⁵N of inflow ammonium, nitrite and nitrate were -3 ± 0.1 ‰ (n = 3), -15 ± 0.1 ‰ (n = 2), 6.9 ± 0.3 ‰ (n = 3) respectively during ammonium concentration experiments (Fig. S2). The δ¹⁵N of outflowing NH₄⁺ and NO_x⁻ were estimated from Eqs. (1-4) with f = 0 or 1, ε_{ao} = -30 ‰, the highest [NH₄⁺]_{in} (62.1 mg N L⁻¹) and the lowest [NO_x⁻]_{in} (1.4 mg N L⁻¹). They ranged from -3 to 27 ‰ and from -32 to 7 ‰, respectively, which encompasses a few isotope compositions measured in the outflow during ammonium concentration tests (Fig. S2 and Table S1).

L171 It seems that produced NO_x⁻ (=NO_xout – NO_xin) is assumed to be derived from reacted ammonium. Then “f” in eq (4) should be “1-f”.

We thank the referee for raising this mistake. We corrected Eq. (4) and modified subsequent information (text and figures) related to Eq. (4) throughout the manuscript.

$$\delta^{15}\text{N-NO}_x^-, \text{int} \approx \frac{(\delta^{15}\text{N-NO}_x^-, \text{in} \times ([\text{NO}_3^-]_{\text{in}} + [\text{NO}_2^-]_{\text{in}}) + \delta^{15}\text{N-NO}_x^-, \text{p} \times (1-f) \times [\text{NH}_4^+]_{\text{in}})}{([\text{NO}_3^-]_{\text{in}} + [\text{NO}_2^-]_{\text{in}} + (1-f) \times [\text{NH}_4^+]_{\text{in}})}, \quad (4)$$

L172–176 These statements are not correct in a strict sense and are misleading. In a closed system, approximation of isotope effect using difference in delta values is valid only when isotope ratio in substrate can be treated as constant as described in Denk et al. (2017). In an open system, it is true that isotope effect can be expressed as the difference in delta values between product and residual substrate that exit from the system (Fry, 2006). But d15Ns in eq (5) can be read as isotope ratio in substrate before reaction (input) and hence is not applicable to open system. Equation (5) can be derived from an equation similar to eq (2) when f=1, but the authors do not state the assumption that f=1 is appropriate in this study. In fact, the value of f decreases as low as 0.2 (Fig. S1).

We revised this paragraph considering directly the open system used in this study, and introducing in details the assumptions made and additional calculations that allow describing our system more appropriately than before. Further, we specified that the notations used here are reversed as

compared to those defined in Fry, 2006 while being similar to those presented in Denk et al. 2017. Finally, we renamed $\text{NH}_4^+_{,\text{out}}$ remaining in the reactor after nitrification as $\text{NH}_4^+_{,\text{res}}$; i.e. residual substrate, for better clarity. The NO_x^- pool resulting from mixing between the NO_x^- produced by ammonium oxidation and inflow NO_x^- was renamed as intermediate pool ($\text{NO}_x^-_{,\text{int}}$).

The nitrifying reactor used in this study can be described as an open-system continuously supplied by an infinite substrate pool with constant isotopic composition. A small amount of the infinite substrate pool is transformed into a product pool or a residual substrate pool when flowing through the system. The equations describing the input, output and processes considered here are presented in Fig. 2 after Fry (2006). Note that the definitions of f and Δ are inverse to the cited literature and that Δ_1 and Δ_4 are null because no fractionation alter the residual substrate exiting the reaction (Fry, 2006). The balance between input and output of each reactional step allows to propose equations for calculation of the nitrogen isotope ratio of compounds in the inflow and outflow of the system (Denk et al., 2017; Fry, 2006). These equations can be simplified under the assumption that limited amount of N compounds are transformed into N_2O ; i.e. f_2 close to 0 and f_3 close to 1. Therefore, the N isotope ratios of the residual substrate pool can be approximated from Eq. (1).

$$\delta^{15}\text{N-NH}_4^+_{,\text{res}} \approx \delta^{15}\text{N-NH}_4^+_{,\text{in}} - \Delta_2(1 - f_1), \quad (1)$$

Where f_1 is the remaining substrate fraction leaving the reactor (i.e. remaining fraction of ammonium), ranging from 0 to 1 (0-100 in %), and Δ_2 is the N isotope enrichment factor associated with ammonium oxidation. In their review, Denk et al. (2017) reported a mean value of -29.6 ± 4.9 ‰ for Δ_2 . Therefore, $\delta^{15}\text{N}$ is higher for residual than the initial substrate pool ($\delta^{15}\text{N-NH}_4^+_{,\text{in}} < \delta^{15}\text{N-NH}_4^+_{,\text{res}}$; where 'in' and 'res' represent inflow and outflow, respectively). Consequently, the pool of product is depleted in heavier isotope (i.e. nitrite and nitrate hereafter defined as NO_x^- pool; $\delta^{15}\text{N-NO}_x^-_{,\text{in}} > \delta^{15}\text{N-NO}_x^-_{,\text{int}}$). It is estimated from Eq. (2):

$$\delta^{15}\text{N-NO}_x^-_{,\text{p}} \approx \delta^{15}\text{N-NH}_4^+_{,\text{in}} + \Delta_2 f_1, \quad (2)$$

Where $\delta^{15}\text{N-NO}_x^-_{,\text{p}}$ is the nitrogen isotope ratio of the product pool produced by nitrification. The nitrogen isotope ratio of the overall intermediate NO_x^- exiting this process results of mixing between initial and produced NO_x^- pools ($\delta^{15}\text{N-NO}_x^-_{,\text{int}}$) and can be estimated from Eqs. (3) and (4):

$$\delta^{15}\text{N-NO}_x^-_{,\text{in}} = \frac{(\delta^{15}\text{N-NO}_2^-_{,\text{in}} \times [\text{NO}_2^-]_{\text{in}} + \delta^{15}\text{N-NO}_3^-_{,\text{in}} \times [\text{NO}_3^-]_{\text{in}})}{([\text{NO}_3^-]_{\text{in}} + [\text{NO}_2^-]_{\text{in}})}, \quad (3)$$

$$\delta^{15}\text{N-NO}_x^-_{,\text{int}} \approx \frac{(\delta^{15}\text{N-NO}_x^-_{,\text{in}} \times ([\text{NO}_3^-]_{\text{in}} + [\text{NO}_2^-]_{\text{in}}) + \delta^{15}\text{N-NO}_x^-_{,\text{p}} \times (1-f_1) \times [\text{NH}_4^+]_{\text{in}})}{([\text{NO}_3^-]_{\text{in}} + [\text{NO}_2^-]_{\text{in}} + (1-f_1) \times [\text{NH}_4^+]_{\text{in}})}, \quad (4)$$

Note that $\delta^{15}\text{N-NO}_x^-_{,\text{out}}$ equals $\delta^{15}\text{N-NO}_x^-_{,\text{int}}$ when f_3 is close to 1 which means that nitrifier-denitrification and heterotrophic denitrification are negligible. Finally, two options must be considered to approximate the nitrogen isotope ratio of N_2O that exits the reactor. On the one hand, $\delta^{15}\text{N-N}_2\text{O}$ can be estimated from Eq. (5), when the hydroxylamine oxidation is the producing process of N_2O :

$$\delta^{15}\text{N-N}_2\text{O} \approx \delta^{15}\text{N-NH}_4^+_{,\text{in}} - \Delta_2(1 - f_1) + \Delta_3, \quad (5)$$

In addition to the influence of the nitrogen isotope composition of the substrate, the $\delta^{15}\text{N-N}_2\text{O}$ depends therefore on the difference between the isotope effects related to the oxidation of NH_4^+ to NO_2^- and the oxidation of NH_2OH to N_2O for complete nitrification ($f_1=0$), while depending only on the latter for limited nitrification ($f_1=1$). On the other hand, $\delta^{15}\text{N-N}_2\text{O}$ can be estimated from Eq. (6), when the nitrite reduction is the producing process of N_2O :

$$\delta^{15}\text{N-N}_2\text{O} \approx \delta^{15}\text{N-NO}_x^-_{,\text{int}}(1 - f_1)^{-1} + \Delta_5, \quad (6)$$

In addition to the influence of the nitrogen isotope composition of the substrate, when negligible amounts of N₂O are produced by nitrite reduction during nitrifier-denitrification or heterotrophic denitrification its nitrogen isotope ratio depends on isotope effect related to this process (Δ_5).

L182–183 Is “the ratio between ammonium oxidation rate and influent ammonium concentrations” different from “nitrification efficiency” (=oxidation rate/NH₄ feeding rate) defined in L140–141? It is odd that ratio of parameters with different dimension is additionally introduced.

We thank the referee for raising this mistake. We reworded the sentence consistently with parameters previously introduced. We are speaking about nitrification efficiency here.

Over the range of tested conditions, the ratio between ammonium oxidation rate and influent ammonium load ranged from 10 to 82 %, never exceeding 40 % for suboptimal nitrifying conditions imposed during oxygenation and temperature tests.

L184–186 I cannot understand what this sentence means. Please rephrase.

We rephrased the sentence.

The ammonium concentration, oxygenation level and temperature affected the ammonium oxidation rates, as well N₂O emission rates and factors.

L191–193 Here, the possible range of dN of outflowing NH₄⁺ and NO_x⁻ is shown, but the dN for each timing is plotted as a single value in Fig. S2. How were these individual values calculated with what assumption?

The standard error plotted here depending on the number of samples after one hydraulic retention time. We added precision in the method section 2.4.

The measurements related to liquid or gas samples were average by experiment; i.e. average of data obtained from the samples taken after one hydraulic retention time.

L195–200 In multi-step reaction (in this case, two step reaction of NH₃->NH₂OH->N₂O is considered), overall isotope effect does not necessarily equal to sum of the isotope effect of each step, but depends on substrate availability and ratio of backward to forward flows in the middle step of the reaction (Rees 1973). Please add basis of the authors’s assumption.

Please refer to the previous answer regarding the estimates of $\delta^{15}\text{N-N}_2\text{O}$ ranges.

L203–204 I believe this is incorrect. The authors did not “observe” the net nitrogen isotope effect for each pathway, but they used literature values.

We rephrased the sentence.

A higher net nitrogen isotope effect for nitrite reduction than hydroxylamine oxidation pathway was estimated for N₂O production.

L224–227 I would like to see the mass balance of N. Judging from Table S2 (which shows results only from NH₄⁺ concentration tests though), increase in NO_x⁻ is always lower than decrease in NH₄⁺. Is apparent nitrogen loss explainable by gaseous emission of N₂O and NO, or was there significant nitrogen assimilation by the biofilm?

We agree that the presentation of N mass balance would have improved our discussion. However, due to technical problems, NO₃⁻ analysis were lacking for some oxygenation tests and for the temperature tests, whereas NH₂OH, N₂ production, as well as N mineralization and assimilation in the biofilm were

not quantified. Therefore, the amount of N_2O emitted alone does not explain the apparent nitrogen loss. No significant amounts of NO were detected during the tests (< 1 ppm measured in the outlet gas), and the accumulation of NH_2OH is unlikely. Heterotrophic denitrification, i.e. the reduction of NO_x^- to N_2 , most likely adds up the N mass balance. However, N_2 measurements (in the gas mixture comprising of at least 79 % N_2) were not been feasible and thus not carried out. We added precisions about this in the manuscript.

Ammonium concentrations decreased from 20.2-37.3 to 11.4-31.1 mg N L^{-1} ; with 45 to 89 % of the inflow ammonium remaining in the outflow during the oxygenation tests (Fig. S1d). When measured, the cumulated concentrations of NO_2^- and NO_3^- ($[NO_x^-]$) increased from 2.4-4.1 to 4.7-11 mg N L^{-1} between inflow and outflow and were composed by at least 74 and 82 % of NO_3^- , respectively. The mass balance between N compounds that enter and exit the reactor evidenced a default of up to 5 mg N and impacted each test. No significant amount of NO was detected during any tests (data not shown) whereas neither NH_2OH , N_2 , nor N mineralization/assimilation in the biofilm were quantified. The accumulation of such amounts of NH_2OH is unlikely. Heterotrophic denitrification, i.e. the reduction of NO_x^- and more particularly of N_2O to N_2 , may explain the incomplete N mass balance. However, the measurement of small N_2 variations in the gas mixture that exiting the reactor and comprising at least 79 % N_2 was not measured.

L228–231 I cannot agree that ammonium oxidation rates were “low and stable” for 0–10.5% O_2 because two high values were observed at 5% (Fig. 3a). It is not clear whether the authors excluded the two data (because of large error bars?) or not. I understand that these rates are calculated from influent and effluent NH_4^+ concentrations measured over time as presented in Fig. S2 (again, this figure only shows results from NH_4^+ concentration tests though), but cannot understand why the error bar (“standard deviation”) for the two data is significantly large. Please explain it as well as detailed procedure for calculating “average and standard deviation” (e.g., how many measured data were used for averaging?).

We improved clarity of this sentence and precise according to the referee’s comment that the average presented here was calculated from all data point in the considered interval (i.e. 0-10.5 % O_2 in the gas mixture).

The oxygenation level had contrasting effects on ammonium oxidation rates, and N_2O emission rates and factors (Figs. 3a-c). Between an oxygenation of 0 to 10.5 % O_2 in the gas mixture, no clear trend in ammonium oxidation rates was observed although being rather low (1.1 ± 0.5 mg NH_4^+-N min^{-1}). In the same oxygenation levels interval, the N_2O emission rates and factors increased from $0.35 \cdot 10^{-3}$ to $1.6 \cdot 10^{-3}$ mg N min^{-1} and from 0.05 to 0.16 %, respectively.

Further, the standard error plotted here depending on the number of gas sampled after one hydraulic retention time. We added precision in the method section 2.4.

The measurements related to liquid or gas samples were average by experiment; i.e. average of data obtained from the samples taken after one hydraulic retention time.

L234–235 I see 8 data points in Fig. 3d (also 3e and 3f), but Table S1 indicates total 13 data were obtained for oxygenation tests. Does this mean isotopic measurements were not conducted for all samples?

The referee is correct. The isotopic measurements were not conducted for all samples. However, the concentration data were all presented to capture the whole dynamic constrained by environmental

conditions tested. We added sentences in the method section 2.2 and in the caption of Fig. 6 for improved clarity.

Note that gas sampling was lacking for 5 of 13 oxygenation tests.

Note that the isotopic measurements of gas samples taken at inflow ammonium concentration of 42.7 and 42.9 mg N L⁻¹ were averaged.

L235–236 I cannot see “similar marked change” in d15N at 16.8% O₂ and 21% O₂. The two data points for each O₂ condition depart each other, and when average is taken, there would be no significant difference.

We understand the concern of the referee, however, it should be noticed that ammonium concentration is also modified between 16.8 and 21 % O₂. This additional effect of ammonium mitigates the effect of oxygenation alone that is intended to be shown here. Therefore, the average isotope ratios at 21 % O₂ should be compared to the data solely measured for 23.8 NH₄⁺-N L⁻¹ at 16.8 % O₂. We specified it more clearly in the manuscript.

A similar marked change in nitrogen and oxygen isotope ratios of N₂O (decrease and increase, respectively) was observed when oxygenation increased from 16.8 to 21 % O₂ (Figs. 3e and f). Note that to observe the latter variations the effect of ammonium concentration was not included. One way to do so is to compare the isotope composition average at 21% O₂ with the isotope composition measured for 23.8 NH₄⁺-N L⁻¹ at 16.8 % O₂.

L238–239 Relatively higher SP value was observed not only at 4.2% O₂ but also at 20% O₂. But do the authors consider N₂O reduction occurs at 4.2% O₂ just because SP is larger than the range estimated for N₂O produced NO₂⁻ reduction? It seems to me that the two high SP data is not significantly different considering the large error bars for 20% O₂, and that the upper end of estimated range might be underestimate (see Fig. 6 in Denk et al.).

We agree with the referee. We did suggest that N₂O reduction to N₂ likely occurs for oxygen-depleted air due to the anti-correlation observed between the oxygen-depletion and ¹⁵N-SP for oxygenation levels between 4.2 and 16.8 % O₂, not due to ¹⁵N-SP at 4.2 % O₂ higher than the range of ¹⁵N-SP proposed for the N₂O derived from nitrite reduction.

L244–245 Alternative explanations can be made for the decrease in N₂O emission. For example, change in branching ratio between NO₂⁻, N₂O, and NO production during NH₂OH oxidation might reduce N₂O emission. Are there any other evidences for N₂O reduction? I agree that N₂O reduction might occur when oxygen concentration is really 0%, but as shown in Fig. S1, measured DO is ca. 1.5 mg/L and this enabled NH₄ oxidation. It is unlikely that NH₄ oxidation and N₂O reduction occur at the same time unless there are specific anoxic sites in the system.

Our point here is to suggest the likely occurrence of anoxic microsites within the reactor biomass, due to heterogeneous and varying distribution of air circulation. Currently, two evidences are presented: (i) the increasing ¹⁵N-SP with the decreasing oxygenation levels; and (ii) the decrease in N₂O emission. This could explain the co-occurrence of NH₄⁺ oxidation and N₂O reduction. Further, the DO presented in Fig. S1 is unfortunately an undetailed picture of what happened within the reactor and more particularly within the colonized media. We were not able to measure the DO within the media and DO was therefore measured at the top of the reactor, where it is likely higher than within the colonized media. This is attested by a few measurements performed in the outflow of the reactor during concentration tests. We observed DO 2.8-3.9 mg O₂ L⁻¹ lower in the outflow than at the top of the

reactor. We added a reference and an explanation describing the likely co-occurrence of ammonium oxidation and N_2O reduction.

This is also consistent with a possible onset of anoxic microsites within the reactor biomass more likely at 4.2 than 16.8 % O_2 . The dissolved oxygen (DO) concentration never decreased below ca. 1.5 mg O_2 L^{-1} in the bulk solution at the top of the reactor (Fig. S1). However, DO decreases from the bulk reactor solution toward the deeper layers of biofilm due to the activity of ammonium oxidizers (Sabba et al., 2018). This is further exacerbated by heterogeneous and varying distribution of air circulation within the static bed. Therefore, oxygen depletion can be assumed within the biofilm.

L249 What does “independence of samples” mean?

L249–251 I cannot follow these sentences. Please rephrase and describe why the different trends of d15N and d18O can be explained with reaction rates in more detail.

Independence of samples means that the samples are not temporally linked to each other. The N_2O sampled at 4.2 % O_2 is not a residual fraction of N_2O produced at 16.8 % O_2 . We clarified these sentences.

The independence of samples taken during the oxygenation tests can explain this. The N_2O sampled at 4.2 % O_2 is not a residual fraction of N_2O produced at 16.8 % O_2 that would have undergone a partial reduction. The oxygenation level can alter the isotope fractionation factors through the control of reaction rates, as evidenced for the reduction of N_2O to N_2 by Vieten et al. (2007). These authors reported lower reaction rates and increased isotope fractionation factors with increasing oxygenation levels. In our case, a similar phenomenon might have influenced both oxidative and reductive processes leading to the production of N_2O and occurring before its ultimate reduction to N_2 . However, knowledge, that the controls such as the oxygenation level have on the net isotope effect related to a sequence of non-exclusive oxidative and reductive processes is still lacking and require further investigations.

L259–261 Although NH_4^+ oxidation rate has linear relation to NH_4^+ concentration (Fig. 4b), the remaining NH_4^+ fraction does not (Fig. S1f). It seems to increase nonlinearly. Please discuss why this happened. In addition, I cannot see that remaining fraction of NH_4 or NH_4^+ oxidation rate is “negatively correlated to temperature” in Fig. S1e or Fig. 4a. It seems almost constant irrespective of temperature. Is stated correlation really significant? Please show p values.

We think that the discussion about the difference between ammonium oxidation rate and remaining ammonium fraction in relationship with the ammonium concentration (i.e. linear vs. nonlinear) is out of the scope of our study. However, the nonlinear increase that describes the relationship between the remaining ammonium fraction and the ammonium concentration, and highlighted here by the referee is mainly due to the data recorded at the lowest ammonium concentration (6.2 mg N L^{-1}). The low remaining ammonium fraction measured at this inflow NH_4^+ concentration can be explained by oxygen limitation being lower at 6.2 mg N L^{-1} than at higher ammonium concentration. Indeed, the decrease in DO toward the deeper layers of biofilm due to the activity of ammonium oxidizers is likely less important at 6.2 mg N L^{-1} than at higher ammonium concentration. A way to check this would have been to measure the isotope composition of N_2O emitted at 6.2 mg N L^{-1} inflow [NH_4^+]; i.e. the ^{15}N -SP is expected much higher at 6.2 mg N L^{-1} due to increase contribution of hydroxylamine oxidation pathway in N_2O production. Unfortunately, the N_2O emission was very low at this inflow ammonium concentration and N_2O isotope composition was not analyzed.

Further, the negative correlation between the remaining ammonium fraction and temperature exists and is significant with $p < 0.05$ (please see statement about the p values used in our study lines 144-145 in the method section). In addition, please note that the y-axis scale of Fig. S4e is the same as Fig. S4d and f. We applied the same y-axis scale to be able to compare with each other the effects of the tested conditions had on the remaining ammonium fraction. Consequently, the variations are less visible in response to temperature than ammonium concentration. However, the negative correlation does exist.

L264–267 Although I think there is no significant relationship between NH_4^+ oxidation rate or remaining fraction and temperature, the authors argue that NH_4^+ remaining fraction is negatively correlated with temperature whereas NH_4^+ oxidation rate is positively correlated with temperature. Please explain why this apparently contradict trend was observed.

It is not surprising that NH_4^+ remaining fraction was negatively correlated to NH_4^+ oxidation rate in the temperature tests. At constant inflow ammonium concentration and hydraulic retention time, the increase in the ammonium oxidation rate decreases the amount of residual ammonium that exits the reactor. Consequently, the ammonium remaining fraction decreases.

L267–268 Temperature effect (if any) might be explained with enzymatic activity, but I think NH_4^+ concentration effect can be explained with kinetics of enzymatic reaction like Michaelis-Menten kinetics.

We agree with the referee and clarified the text.

An increase in temperature and inflow ammonium concentrations both positively influenced the rates of NH_4^+ oxidation and N_2O emissions and the emission factor (Fig. 4). The NH_4^+ oxidation rate linearly increased from 1.3 to 1.5 $\text{mg NH}_4^+\text{-N min}^{-1}$ with temperature ($r = 0.89$; Fig. 4a) and increased from 0.97 to 3.49 $\text{mg NH}_4^+\text{-N min}^{-1}$ with a tenfold increase in the inflow ammonium concentration ($r = 0.82$; Fig. 4b). These positive correlations are well known in the temperature range investigated here and are likely due to enhanced enzymatic activity and Michaelis-Menten kinetics, respectively (Groeneweg et al., 1994; Kim et al., 2008; Raimonet et al., 2017).

L274 Based on which parameter do the authors find “stronger” effect of temperature and NH_4^+ concentration on the N_2O emission rate than on NH_4^+ oxidation”? For example, slopes in Fig. 4b and 4d look similar.

The emission factor is calculated as the ratio between N_2O emission and NH_4^+ oxidation rates. Therefore, the N_2O emission factor enables to identify when the N_2O emission is more responsive than the NH_4^+ oxidation to the ammonium inflow. Furthermore, note that the slopes in Figs. 4b and d cannot be compared with each other, as the y-axis scales are different.

L278–281 Please explain in detail why the authors consider nitrite oxidation (to nitrate) is less important than nitrite reduction (to N_2O) in this case.

Given the mass balance of the N compounds, the oxidation of nitrite to nitrate remains the main pathway consuming nitrite. However, an increasing N_2O emission factor results from increase in ammonium oxidation rate driven by temperature or NH_4^+ concentration. Therefore, the increase in reduction of NO_2^- to N_2O seems to be a bit more pronounced than the increase in oxidation of nitrite to nitrate. We added precisions in the manuscript.

Both experiments suggest that the increase in N_2O emissions results from the increasing production of N_2O by hydroxylamine oxidation or nitrite reduction in combination with a slow rate of or absence of

N₂O reduction to N₂. Furthermore, no nitrite accumulation was observed with increasing ammonium oxidation rate and N₂O emission factor (Fig. S1i). Therefore, if N₂O emission results mainly from the nitrite reduction pathway, this suggests that the nitrite reduction pathway is more responsive to the increasing ammonium oxidation rate than the nitrite oxidation pathway; the latter remaining the main pathway of nitrite consumption.

L284–285 In Tumendelger et al. (2014, 2016), larger SP values were reported under aerobic condition.

We added references to works performed by Tumendelger et al.

This is consistent with previous findings based on the ¹⁵N-SP of N₂O emitted from aerobic activated sludge (Toyoda et al., 2011; Tumendelger et al., 2016; Wunderlin et al., 2013). Authors reported ¹⁵N-SP as high as 10 ‰, which can suggest more oxygen limitation favorable to the contribution of the nitrite reduction to N₂O production in the nitrifying reactor studied here. The hydroxylamine oxidation can even be the main N₂O producing pathway, as evidenced by Tumendelger et al. (2014) in some aerated tank.

L285–286 Ambiguous sentence. Do the authors intend to argue that SP value increases with temperature (13.5°C < T < 19.5°C) and that it also increases with NH₄⁺ concentration when T is set around 19°C? Please rephrase. I cannot agree with the latter statement because SP value obtained at 42.8 mg N/L is lower than SP at 28.6 (Fig. 5a).

We rephrased the sentence for improved clarity.

Furthermore, the ¹⁵N-SP increased with temperature between 13.5 and 19.8 °C. Our data suggest that temperature mainly controlled the change in N₂O producing pathways within this temperature range (Fig. 5a). The temperature control seems to mitigate here the effect that ammonium concentration can have on the N₂O producing pathways evidenced elsewhere. Wunderlin et al. (2012, 2013) observed an increase in ¹⁵N-SP from -1.2 to 1.1 ‰ when inflow [NH₄⁺] increased from 9 to 15 mg N L⁻¹. They also observed 3-6 ‰ decreases in ¹⁵N-SP over the course of ammonium oxidation experiments and suggested that NH₂OH oxidation contribution to N₂O production increased when conditions of NH₄⁺ excess, low NO₂⁻ concentrations and high nitrogen oxidation rate occur simultaneously. Our findings are consistent with the observation of Groeneweg et al. (1994) showing that temperature rather than ammonium concentration influenced the ammonium oxidation rate.

Figure 5 caption. As I pointed out at L228–231 above, it is not clear how the authors made data reduction based on primary data. How “average and standard deviation” were calculated? How did the authors ensure “the steady-state conditions”?

Please see our previous answers. Furthermore, we rephrased the sentence.

Average and standard deviation (error bars) are calculated for the samples taken after one hydraulic retention time.

L315–317 Does “stable NO₂⁻” mean that NO₂⁻ concentration was constant over time or that it did not depend on NH₄⁺ concentration? If the authors intend to mean the latter, I cannot agree with them because three data points at 20% O₂ in Fig. S1g show a large variation.

We mean that the NO₂⁻ concentration did not depend on oxygenation levels. We modified this sentence.

The oxygenation, temperature and ammonium concentration tests revealed a strong control of nitrite oxidizing activity and the contribution of the nitrite reduction pathway to N₂O production. No

relationship was observed between NO_2^- concentrations and oxygenation. In addition to this, higher ^{15}N -SP at 21 % compared to the 10.5-16.8 % O_2 was observed while temperature remained below 20 °C (Figs. S1g and 3d). This is most likely due to higher nitrite oxidation than nitrite reduction rates in response to increasing oxygenation levels to 21 % O_2 , which is consistent with the nitrite oxidation step sensitivity to oxygen limitation (Pollice et al., 2002; Tanaka and Dunn, 1982).

L323–324 Sect. 3.2 -> Sect. 3.3? There, the authors wrote temperature range as 13.5–19.8°C, and did not describe “exponentially increase”. It seems to me that SP increases with temperature linearly.

We agree with the referee. We rephrased the sentence.

During the temperature and ammonium concentration tests, the contribution of the hydroxylamine oxidation pathway to N_2O emissions increased with temperature between 13.5 and 19.8 °C (Sect. 3.3) and decreased in favor of the nitrite reduction pathway when temperature exceeded 20 °C (Fig. 5a).

Technical corrections

L44 a large “extent”

This change was made.

L67 enriched in “ ^{15}N at” central position

This change was made.

L81 Add question mark at the end of the sentence.

This change was made.

L103–105 Awkward sentence. Consider other expression than “consisted of”.

We modified our sentences.

The oxygenation tests were carried out by mixing compressed air and pure nitrogen gas to reach 0 to 21 % O_2 in the gas mixture (Fig. S1a). The tests were performed at five substrate concentrations and at a temperature between 19.2 and 20.6 °C. The temperature tests were carried out by cooling the feeding solution directly in the feeding tank (22.3 to 13.5 °C), with an inflow ammonium concentration of 19.9-21.1 mg $\text{NH}_4^+\text{-N L}^{-1}$. The ammonium concentration tests were run at an increase (6.2, 28.6 and 62.1 mg $\text{NH}_4^+\text{-N L}^{-1}$) and a decrease (56.1, 42.9, 42.7 and 20.2 mg $\text{NH}_4^+\text{-N L}^{-1}$) in the NH_4^+ concentrations in the feeding solution, at temperatures ranging from 18.8 to 19.9 °C. An optimal oxygenation level was imposed for both tests (Figs. S1b and c).

L230 and elsewhere. Insert “x” between significant (i.e. 0.35) and exponent (i.e. 10⁻³)

This change was made.

Figure 3 legend. Open circle represents NH_4 concentration of 25.1, not 25.3 if Table S1 is correct.

This change was made.

L238–239 Rephrase the subject part (“The ^{15}N -SP ... levels”) of this sentence. A higher amount of N_2O “was” reduced to N_2 .

We rephrased this sentence.

An additional suggestion can be made from the ^{15}N -SP dynamics between and variations within the oxygenation levels. They suggest a higher amount of N_2O was reduced to N_2 at 4.2 than 16.8 % O_2 .

L273 correlation between ... and the ammonium oxidation rates (delete "to")

This change was made.

L333 a larger "extent"

This change was made.

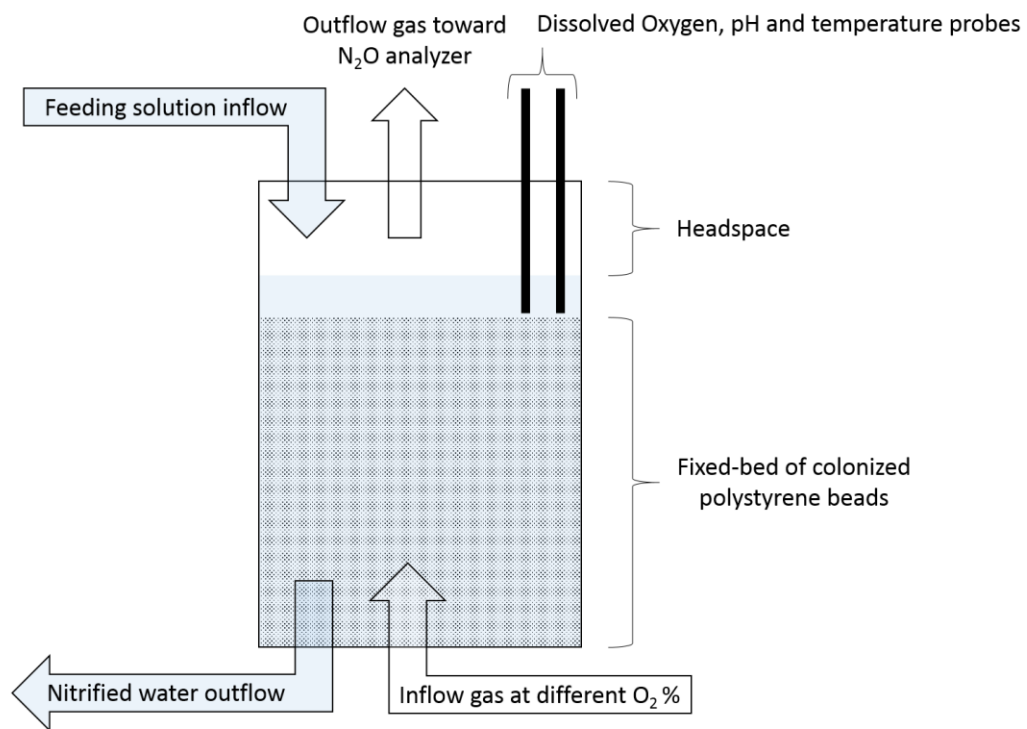
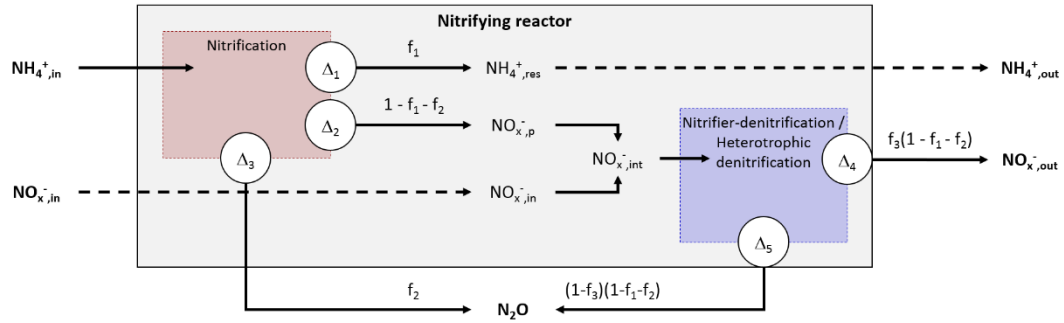


Figure S1. Schematic of the nitrifying reactor used in this study. Note that solution was down-flowing, while air was up-flowing.



Equations:

Nitrification:

$$\delta^{15}\text{N}-\text{NH}_4^+_{,\text{res}} \approx \delta^{15}\text{N}-\text{NH}_4^+_{,\text{in}} - \Delta_2(1 - f_1) + (\Delta_3 - \Delta_2)f_2$$

$$\delta^{15}\text{N}-\text{NO}_x^-_{,\text{p}} \approx \delta^{15}\text{N}-\text{NH}_4^+_{,\text{in}} + \Delta_2f_1 + (\Delta_3 - \Delta_2)f_2$$

$$\delta^{15}\text{N}-\text{NO}_x^-_{,\text{int}} \approx \frac{(\delta^{15}\text{N}-\text{NO}_x^-_{,\text{in}} \times ([\text{NO}_3^-]_{\text{in}} + [\text{NO}_2^-]_{\text{in}}) + \delta^{15}\text{N}-\text{NO}_x^-_{,\text{p}} \times (1 - f_1 - f_2) \times [\text{NH}_4^+]_{\text{in}})}{([\text{NO}_3^-]_{\text{in}} + [\text{NO}_2^-]_{\text{in}} + (1 - f_1 - f_2) \times [\text{NH}_4^+]_{\text{in}})}$$

$$\delta^{15}\text{N}-\text{N}_2\text{O} \approx \delta^{15}\text{N}-\text{NH}_4^+_{,\text{in}} - \Delta_2(1 - f_1 + f_2) + \Delta_3(1 + f_2)$$

**Nitrifier-denitrification /
Heterotrophic denitrification:**

$$\delta^{15}\text{N}-\text{NO}_x^-_{,\text{out}} \approx \delta^{15}\text{N}-\text{NO}_x^-_{,\text{int}}(1 - f_1 - f_2)^{-1} - \Delta_5(1 - f_3)$$

$$\delta^{15}\text{N}-\text{N}_2\text{O} \approx \delta^{15}\text{N}-\text{NO}_x^-_{,\text{int}}(1 - f_1 - f_2)^{-1} + f_3\Delta_5$$

Figure 2. Diagram and equations of the nitrifying reactor adapted from Fry (2006). It is considered as a sequence of two reactor boxes. (i) The nitrification of inflow ammonium ($\text{NH}_4^+_{,\text{in}}$) to a pool of nitrite and nitrate ($\text{NO}_x^-_{,\text{p}}$), residual ammonium ($\text{NH}_4^+_{,\text{res}}$), and nitrous oxide (N_2O) through the hydroxylamine oxidation pathway. (ii) The subsequent reduction of intermediate $\text{NO}_x^-_{,\text{int}}$; mixing of inflow $\text{NO}_x^-_{,\text{in}}$ and formed $\text{NO}_x^-_{,\text{p}}$ to nitrous oxide (N_2O) through the nitrite reduction pathway, and residual NO_x^- that exits the reactor ($\text{NO}_x^-_{,\text{out}}$). Note that residual substrates and formed product exit the reactor without further isotope fractionation. See text for details.