Response to the referees

Dear Kees Jan van Groenigen,

In the following document we comment on and explain how we address the issues and comments raised by the two referees. We found the comments of the referees very useful in highlighting important points, missing in the original manuscript. We will take the raised issues into account and adjust the manuscript accordingly. We are grateful and appreciate the two referees for their comments which we believe leads to an improved version of the manuscript.

Thank you for the consideration,

Response to referee comment # 1

General comments:

The study by Winther et al. presents continuous measurements of nitrous oxide isotopomers at concentration levels close to ambient and intends to determine isotope effects for two different bacterial organisms. Especially the endeavour to bring such measurements towards ambient concentration levels is valuable for the scientific community. In this context, this paper is of interest for the broad audience Biogeosciences attracts. There are some flaws, such as 1. The initial NO_3^- isotopic composition had to be estimated. 2. There is no nitrate balance provided, which would be helpful with regard to constraining f. 3. Concentration of other products, such as NO have not been accounted for. However, the manuscript provides some interesting calculation approaches, and the experiments involving P. chlororaphis are straightforward. All in all, I recommend acceptance for publication after addressing the below comments.

Specific comments:

See some more detailed comments below.

Title ok

Highlights Not given

Abstract P1, L10: Please specify the sentence “the continuous analysis of . . .”. The meaning is unclear without knowing the manuscript.

The sentence has been changed to “the continuous measurements of …”.

Please change reveal to reveals.

The spelling error has been corrected.

Introduction P2, L19: please change to positions, instead of position.

The spelling error has been corrected.

The objectives and the added value of another experiment on fractionation factors could be more to the point.

We added a phrase at the end of the introduction. “Isotope effects during denitrification are diverse and species dependent (e.g. Denk et al., 2017). Our study demonstrates a new way to determine fractionation factors from continuous measurements of N_2O.”

Materials and Methods P4, L12 and following: I suggest changing “before” to “upstream of”.
Agreed and changed

In addition, the text does not comply with Figure1. From Figure 1, it seems like a Nafion and a Magnesium perchlorate / Ascarite trap was used. The Nafion reduces the water vapor to a certain dewpoint (please specity) and the magnesium perchlorate $Mg(ClO_4)_2$! removes the remaining water chemically. The Ascarite (this is not $Mg(ClO_4)_2$, but sodium hydroxide coated silica!!) removes the CO2!. This section needs to be corrected.
We agree that the description is incorrect and changed accordingly to: “Before the analyzer, a Nafion unit and an Ascarite trap is installed. The Nafion unit removes the bulk of H_2O vapor. Remaining water vapor is removed chemically by magnesium perchlorate ($Mg(ClO_4)_2$) in front and after the Ascarite (NaOH) section of the trap.”

P6, L1: the subsection head number is 2.4.1, but there is no 2.4.2, which does not make sense. I suggest numbering the subsection head to 2.5
Head number has been changed.

P7, L20: please change results to result
The spelling error has been corrected.

L7,P19/20: with regard to the reference to the supplementary, I suggest changing “unreacted” to “reacted” in P4,L14 of the supplementary (“. . .can therefore be calculated as the sum of the immediate product calculated for all reacted fractions of the substrate”), as the accumulated product is the result of the reacted fractions of the substrate.
We agree that the formulation is confusing and simplify to: “…be calculated as the sum of the respective immediate products.”

P8, (8): The numerator terms are quite clear, the denominator term is not required to my understanding. Please clarify.
We verified and the mass balance is correct.

P8, L26: Please change to net production.
We agree and corrections has been made accordingly.

P8, L30: This assumption is not in agreement with your expectations given on P3, L16-20. There it is assumed that $\delta^{15}N^{\alpha}$ becomes enriched (I agree with this assumption). In general, all N_2O isotopic species should become enriched in a situation in which only reduction occurs, as N_2O is the substrate in this case, and a normal isotope effect occurs. However, this is not the case in Figures 6A/B. Please comment.
We see the origin of the confusion. The outcome of our analysis for *P. fluorescens* is indeed conflicting with the expectation given on P3, L16-20. This is obvious in Figure 6A/B and discussed

later in the manuscript. On P8, L30 we only define the start of reduction for determining the fractionation coefficient. The bracket stating "(assumption based on reduction…)" is misleading and we removed it.

P9, L5: the fractionation factor during reduction is varied between 1 and 2, this means only not-normal isotope effects are allowed for the reduction of N_2O. A recent review on isotope effects in the N cycle, Denk et al. 2017 in Soil Biol. Biochem. (The nitrogen cycle: A review of isotope effects and isotope modeling approaches), shows that the literature has reported that N_2O reduction is associated with a normal isotope effect for $\delta^{15}N^{bulk}$. Please comment why this limitation was necessary.
We apologize, this is a typo. The reduction fractionation factor was varied between 0 and 2 which includes all possible isotope effects. Corrected.

Results Discussion P11, L2: The statement that the production rate is 10 times higher for P. Chlororaphis is ambiguous, since a net rate is compared to a "gross" rate (assuming direct conversion of NO_3^- to N_2O). Please add this to your interpretation that reaction rate cannot account for the difference in isotopic fractionation alone.
The reviewer is correct. We changed "production" to "net production"

P11, L15-17: This is pertinent information. Thank you. I only suggest to change the numbering from 1) and 2) for the results of the DNA comparisons to i) and ii). I was a little confused with the (1)-(4) numbering.
We agree and have changed accordingly.

Response to referee comment # 2

The manuscript of Malte Winther et al. describes the real-time analysis of site-specific N_2O isotopic composition from two denitrifying bacterial strains with a novel Picarro CRDS analyser. A setup for a closed-loop experiment was designed and applied in a number of prototype experiments. A correction function was developed for the spectrometer raw data and a modified Rayleigh model applied to derive fractionation factors.
The manuscript is an important contribution to research on N_2O isotopes and therefore of interest for a number of readers of Biogeosciences. The presented interpretation of singular incubation experiments might be questionable; at least given the "surprising" results, e.g. for ε_{SP} of N_2O reduction. But the manuscript should still be accepted after a number of minor revisions as detailed below:

Page 1 Line 8: The main application of the instrument might be for biogeochemical applications, e.g. soil sciences, at enhanced concentrations and not for atmospheric chemistry.
We agree that the manuscript would be good in soil sciences as well.

Page 1 Line 10 – 11: The expression "… reveal the transient pattern" is incomplete.
Changed to: "The continuous analysis of N_2O isotopomers reveals the transient isotope exchange between KNO_3, N_2O, and N_2."

Page 1 Line 15 – 17: The explanation for the SP isotopic fractionation for N_2O reduction above zero, "diffusive isotopic fractionation and a difference in active enzymes during production of N_2O", is not convincing.
We remove the statement (see also our comment to Page 12 Line 26)

Page 2 Line 18 – 19: Please rephrase the sentence "The position in the N_2O molecule are named …" to "N_2O molecules with ^{15}N substitution in the central or terminal position are named $^{15}N^{\alpha}$ for $^{14}N^{15}N^{16}O$ or $^{15}N^{\beta}$ for $^{15}N^{14}N^{16}O$, respectively."
The suggested correction is incorrect. Since it is the positions of the N atom which defines the name, and not the molecule. Changed to: "The positions in the N_2O molecule have been named N^{α} and N^{β} or short α and β (Yoshida and Toyoda, 2000). N_2O molecules with ^{15}N substitution in the central or terminal position are named $^{15}N^{\alpha}$ for $^{14}N^{15}N^{16}O$ or $^{15}N^{\beta}$ for $^{15}N^{14}N^{16}O$, respectively."

Page 2 Line 25: Please rephrase the expression to "… to enable continuous and selective measurements of the isotopomer abundances."
Correction has been applied.

Page 3 Line 4 – 6: The sentences "The primary anthropogenic sources of N_2O are organic and inorganic N fertilizers used for agriculture. The natural sources are primarily nitrification and denitrification in terrestrial and aquatic ecosystems." are misleading as the biotic (and abiotic) source processes for anthropogenic and natural N_2O emissions are similar, but anthropogenic emissions are enhanced due to fertilizer application. Please rephrase the sentences.
The sentences has been corrected. Now it reads "The primary anthropogenic increase in N_2O emission originate from organic and inorganic N fertilizers used for agriculture. The natural sources are primarily nitrification and denitrification in terrestrial and aquatic ecosystems."

Page 3 Line 17 – 18: The expression "… , the cleavage of N_2O is expected to have an increased fractionation effect on $^{15}N^{\alpha}$, due to …" might be rephrased to "the cleavage of N_2O is expected to fractionate in favor of the $^{15}N^{\alpha}$ molecule, due to …".
We adapt the suggestion and changed accordingly.

Page 3 Line 17 – 19: The statement that "diffusion into the cell (Tilsner et al., 2003) and enzymatic reduction (Wrage et al., 2004)" might be deleted.
These introductory statements line out what ideas have been presented to explain changes in SP. We would like to keep them.

Page 4 Line 8: The phrase "by placing the sample delivery system … in a closed loop" might be rephrased to "by a closed-loop gas flow through the …".
We adapt the suggestion and changed accordingly.

Page 4 Line 13: $Mg(ClO_4)_2$ is the chemical formula for Magnesiumperchlorate used for drying the measuring gas, but not for Ascarite used for removing CO_2. The scheme in Figure 1 shows the correct setup of the trap.
We agree and the correction has been made accordingly. Also see comments to referee #1.

Page 4 Line 22 – 29: Please re-write this section, as the same information, that a concentration dependent correction for delta values is needed is given several times.
We removed duplicate statements from the section and write now: "Isotopomer measurements made

with the G5101i-CIC have a N_2O concentration dependence and need to be corrected. There is a 1/concentration dependence, caused by small offsets in the measurement of the $^{14}N^{15}N^{16}O$ and $^{15}N^{14}N^{16}O$ peaks. These offsets are caused by baseline ripple created by optical cavity etalons. An etalon is an optical effect in which a beam of light undergoes multiple reflections between two reflecting surfaces, and whose resulting optical transmission or reflection is periodic in wavelength. The ripples are not always constant in phase, which means that the ripples can shift spectrally, which can cause the offset to drift over time. Because baseline ripple effects become more dominant as N_2O concentration decreases, the offset is largest at low concentrations."

Page 5 Line 5: The section on the O_2 correction (now Page 6 Line 18 – 23) could better be placed here.

The O_2 correction is related to the calibration gases we use for the specific experiments introduced on page 6. Therefore we prefer to leave it where it is. Moving the section to page 5 would lead to repeating the arguments on page 6.

Page 5 Section calibration gases (Table 1): Please check whether there is a mistake in the mean values in Table 1, e.g. the mean of 1.34, 1.08, 2.62 is not 1.32.

The mean values in Table 1 is not the mean of the three numbers, but rather the combined mean values, which depends on the number of measurements performed. That is the reason for the difference. That we use the combined mean values has been clarified.

Page 7 Line 6: The statement to give $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ values for KNO_3 is wrong or at least a misunderstanding.

We agree that the way we phrased is misleading and reformulated to clarify the link between $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$ and the isotopic composition of KNO_3: "The initial isotopic composition of KNO_3 calculates as the average of the end values for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ to -3.08 ‰ ± 1.05 (identical to the $\delta^{15}N^{bulk}$ value)."

Page 12 Line 3: The statement that differences in net production rates affect ε_{SP} seems questionable.

We agree and also write earlier in the manuscript that net production differences account for less than 10% of the effect. We now write: "We hypothesize that the slight difference in ε_{SP} originates predominantly from 4) fractionation associated with nitrous oxide reductase in *P. fluorescens*."

Page 12 Line 26: The statement that higher ε_{SP} values as reported in literature could be rationalized by diffusive isotope fractionation seems questionable, as diffusion is generally assumed to not affect the N_2O SP.

We agree that diffusive isotope fractionation is mass dependent and therefore has no effect on SP. Rereading the section we realize that the reduction part of Figure 7 is not fully described and would therefore like to slightly adjust the phrasing. As to the statement in question it is misplaced and we remove it. The paragraph now reads: "A number of studies have investigated N_2O reduction from denitrification in soils (e.g. (Well and Flessa, 2009b; Köster et al., 2013a; Lewicka-Szczebak et al., 2014, 2015)). The results are only partly in accord with our findings for specific bacteria strains. While our results for ε_{bulk} are within the range of their findings, they find consistently negative ε_{SP} values while our results are generally positive. The only study on pure bacteria we know of is from Ostrom et al. (2007) for two bacteria strains different from ours namely *P. stutzeri* and *P. denitrificans*. They found ε_{SP} values between -6.8 ‰ and -5 ‰. At this point we have no explanation for the discrepancy but can find no artifact in our incubation setup."

Page 13 Line 2: The term “isotope depletion” is incomplete, it should be mentioned which isotopic species is depleted.

We agree and have adapted the formulation. Now we write ”… find a bulk isotope depletion.”