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Interactive comment on "Continuous measurements of nitrous oxide isotopomers during incubation experiments" *by* Malte Winther et al.

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[bg, manuscript]copernicus

[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 have taken the addressed issues into account and adjusted the manuscript accordingly. We are grateful and appreciate the two referees for their comments which we believe led

to an improved version of the manuscript.

One major point that both reviewers raised is the analyzers dependence on the presence of oxygen. We indeed forgot to make this correction which however has no effect on the isotopic fractionation we find. The correction is now included.

In the editing process we found a small mistake in the calculations of the combined standard deviation of our standard gasses. This has been corrected in the revised version of the manuscript.

Thank you for the consideration,

[Response to referee comment # 1]

General comments:
The manuscript of Malte Winther and co-authors with the title "Continuous measurements of nitrous oxide isotopomers during incubation experiments" presents measurements of N_2O isotopic composition ($\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$) with a prototype Picarro CRDS analyser. Research on GHG isotopologues is very active and the manuscript is therefore timely and of high interest for readers of Biogeosciences and potential future users of this technique. The wording is colloquial and should be strongly improved. I have a number of suggestions for technical corrections the authors have to consider for improving the consistency and readability of the manuscript.
The manuscript gives details on "prototype" applications of the novel technique on N_2O produced by two bacterial strains. I have strong concerns, regarding the interpretation of results using a simplified modelling approach, which in the end leads to results for isotope enrichment factors (ϵ_{SP}), which are in contrast to existing literature! I would strongly recommend to either reassess the data analysis + interpretation or focus more on performance tests of the developed analyser and mention the limitations of the ap-

plied approach.
Moreover, the manuscript gives the impression that this is the first time that N_2O isotopomers were analysed continuously by mid-infrared spectroscopy. The author ignore previously published using a similar CRDS analyser (D. V. Erler et al., Limnol. Oceanogr. Methods 13, 391–401 (2015)) and several years earlier with mid-infrared absorption spectroscopy QCLAS (see specific comments below related to page 2 Line 20). The respective citations have to be included in the manuscript before publication!
In summary, I suggest publication in Biogeosciences after careful revisions.

All above comments are detailed below and we respond to them under specific comments.

Specific comments:
Page 1 Line 3 -5: I would suggest to rephrase this sentence possibly to: "In the linear N=N=O molecule 15N substitution is possible in two distinct positions, central and terminal. The respective molecules, 14N15NO and 15N14NO, are called isotopomers."

We rephrased the sentence as suggested.

Page 1 Line 5: The sentence is colloquial and should be changed to something like: "... that N_2O produced by nitrifying or denitrifying microbes exhibits a different relative abundance of both isotopomers."

We agree and have rephrased the sentence in the revised version. Now we write:"It has been demonstrated that N_2O produced by nitrifying or denitrifying microbes exhibits a different relative abundance of both isotopomers."

Page 1 Line 6: Please define the term "site preference".

In the revised version, we write: "Therefore, measurements of the site preference (difference in the abundance of the two isotopomers) in N_2O can be used to..."

Page 1 Line 7: What is the meaning of the term "in the order of days" – would it not be feasible to perform analysis for weeks or even months – please comment?

It is certainly feasible to perform continuous measurements over a longer period of time. The analyzer is not the limit it is rather the leak rate of the setup. What we meant here was that our experiments took place over a couple of days. We removed the statement as it leads to a misunderstanding as we can see from the reviewers comment.

Page 1 Line 10: The term "position dependent measurement" might be changed to" analysis of N_2O isotopomers" or similar.

As suggested we changed the sentence to: "The continuous analysis of N_2O isotopomers reveal the....".

Page 1 Line 16: The limitations of the applied data analysis, in particular to disentangle N_2O production and N_2O reduction should be mentioned. In addition, the discrepancy of the enrichment factor ϵ_{SP} for N_2O reduction with existing literature should be mentioned or the number deleted from the abstract.

The limitations of our analyses are discussed in detail throughout the manuscript. We

believe that our results are robust and that a focus on the limitations in the abstract is not appropriate, though a sentence has been added to the abstract: “The slightly increased isotopic fractionation during reduction is believed to be due to diffusive isotopic fractionation and kinetic isotope effect.”

Page 2 Line 17: The positions in the N_2O molecule are named α and β but not the isotopomers, please correct.

We changed the sentence to: ”The position in the N_2O molecule are named $^{15}N^{\alpha}$ and $^{15}N^{\beta}$ or short α and β for $^{14}N^{15}N^{16}O$ and $^{15}N^{14}N^{16}O$, respectively”.

Page 2 Line 20: “. . . spectral regions . . .”

The sentence has been changed to: ”... the two isotopomers providing spectral regions where absorptions of the two isotopomers do not overlap.”

Page 2 Line 20: Mid-infrared spectroscopy and exactly the same spectral region (around 2188 cm-1) was already applied earlier for continuous analysis of N_2O isotopologues. The authors have to cite the respective publications: H. Wächter et al. Optics Express 16 (12), 9239-9244 (2008), J. Heil et al. Geochimica et Cosmochimica Acta 139, 72–82 (2014), J. R. Köster et al. Rapid Commun. Mass Spectrom. 27, 216–222 (2013), J. Mohn et al. Atmos. Meas. Tech., 5, 1601–1609 (2012).

We agree and have added the references as requested.

Page 2 Line 25: The international isotope ratio scale is AIR-N2 and the standard is

atmospheric nitrogen.

We agree and the sentence has been changed accordingly.

Page 3 Lines 0 – 17: Please add a few sentences on the effect of N_2O reduction on the N_2O isotopic composition.

In the revised version the following has been added: ”The cleavage of the covalent N=O bond of N_2O leading to N_2 and H_2O is the result of N_2O reduction during bacterial denitrification. According to kinetic isotope theory, the cleavage of N_2O is expected to have an increased fractionation effect on $^{15}N^{\beta}$, due to the weaker $N-O$ bond, diffusion into the cell, and enzymatic reduction. N_2O reduction during bacterial denitrification is therefore expected to lead to an increase in SP. (Popp et al., 2002; Tilsner et al., 2003; Wrage et al., 2004)”

Page 3 Line 20: “determined”

The spelling error has been corrected.

Page 3 Line 25: Please rephrase the term “14N absorption feature”.

We agree that the term ^{14}N in N_2O has not been defined. We added the definition further up in the manuscript when it is mentioned for the first time.

Page 3 Line 26 – 28: Please give information for which “time interval” the precision values are given.

In the revised version we write: "The typical precision of the instrument over 10 minutes averaging is < 0.3 ppb for the N_2O mixing ratio and < 0.4 for each of the delta values of the isotopomers for concentrations in the range of 200 ppb – 2000 ppb."

Page 4 Line 0 ff: Was there any provision to avoid under- or overpressure in the setup?

The system is operated at ambient pressure. We have added a sentence to clarify this: "The resulting overpressure in the incubator is released prior to switching back to the closed loop position."

Page 4 Line 7: Flushing the system with N2 most probably has changed the O2/N2 ratio in the setup, which in turn would have affected the analysis of N_2O isotopologues – please comment? The statement given on page 6 Line 7 – 8 is not sufficient as the gas matrix (O2/N2 ratio), i.e. differences in pressure broadening, is known to affect the of the analysed spectral lines (e.g. D. V. Erler et al., Limnol. Oceanogr. Methods 13, 391–401 (2015)).

We would like to thank both referee #1 and referee #2 for bringing up this important point. The N_2/O_2 ratio has indeed a significant effect on measurements of the isotopomers of N_2O, when using the Picarro G5101-i analyzer. This oxygen dependence effect, as first presented by Erler et al. (2015), has a linear dependence on the isotopomers.
In our incubation experiments no O_2 is available in the incubation system. However, our instrument has been calibrated with N_2O in N_2/O_2 at atmospheric ratio. This fact got lost in our analyses and all data needs to be corrected for a constant offset. However, since the offset is constant it has no effect on the isotope enrichment factors.

We have performed experiments similar to the once presented by Erler et al. (2015). Our experiments (although a different approach) show the same linear dependence on O_2 concentration for the isotopomers. In the revised version all data is presented with the correction for the addressed effect. In the process of incorporating the O_2 dependence a small fault in the original data analysis script was noticed and corrected. This has lead to a small change in the isotopic fractionation values presented in tables 2, 3, and 4, providing adjustments which are within the order of the reported deviations. We stress that the O_2 dependence has no effect on the results of our study, namely the isotope enrichment factors.
We will include the experiments concerning the oxygen dependence of the analyzer in a supplement to the publication.

Page 5 Line 1: If the two diluted gases are new standard gases they might be named different than the original ones.

The two new standard gasses have an identical isotopic composition as the original pure standards. To make that clear we kept the original name but added CIC in front.

Page 5 Line 3 – 14: The two statements "measured according to an international standard reference" and "relative to atmospheric air" is contradictory as the primary anchor of the international scale is atmospheric N2 and not N_2O – please correct. If measurements were anchored to atmospheric N_2O please give the values which were adopted for $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ of atmospheric N_2O by different laboratories.

We agree that the statements were incorrect, and this has been corrected. In the revised version the sentence reads: "All measurements were performed relative to our standard gasses anchored to atmospheric N_2. Our position dependent $\delta^{15}N$

measurements are reported relative to atmospheric N_2."

Page 5 Line 3 – 14: The spread of results observed by different laboratories for the same calibration gas is considerable. This should be mentioned in the text with reference to a recent inter-laboratory campaign, which showed similar results (Mohn et al. Rapid Commun. Mass Spectrom. 28, 1995–2007 (2014)).

We thank the referee for pointing out this publication. The section in question has been adjusted accordingly and is now: "The standard deviation (Table 1) that we see from our measurements are similar to those presented by Mohn et al. (2014)."

In addition it should be mentioned that Tokyo Institute of Technology is supposed to be the only laboratory in the group to anchor their measurements to the AIR-N2 scale through NH4NO3 thermal decomposition.

ll of the laboratories anchor their measurements to AIR-N2.

Page 6 Section 2.5: All acronyms should be defined in the text.

All acronyms are defined in the revised version.

Page 6 Line 14: Rs is supposed to be the isotope ratio of the substrate at time t.

Correct, the wording has been changed accordingly.

Page 6 Line 15: For "ε" the wording "enrichment factor" is usually applied (e.g. Well et al. JOURNAL OF GEOPHYSICAL RESEARCH, VOL. 114, G02020 (2009)) – please correct throughout the text.

We thank the referee for bringing this issue up. Throughout the manuscript we have changed "isotope enrichment" with "isotopic fractionation" according to Coplen, (2011).

Page 6 Line 16: "We did not measure . . ."

Corrected.

Page 6 Line 17: The statement "has to be identical" might be too strong and should be replaced by "can be used to estimate".

We disagree: When all KNO_3 has reacted to N_2O the initial isotopic composition of KNO_3 and the final isotopic composition in N_2O are identical by definition. However, due to the experimental uncertainty we do agree that the measurement gives only an estimate of the initial composition. We reworded to make this clear.
"By definition when all KNO_3 has reacted to N_2O, its isotopic composition is identical to the initial composition of KNO_3. The final isotopic values of N_2O for P. chlororaphis can therefore be used to estimate the initial isotopic composition of KNO_3."

Page 6 Line 19: ". . . the bulk 15N/14N isotope ratio . . ."

Corrected

Page 6 Line 20: "... for the accumulated product Rbulk p, acc is:"

Corrected.

Page & Formula 4: It should be "Rs,0" instead of "R0".

Corrected.

Page 7 Formula 8: Some of the acronyms are not defined and for "φ" "ÏŢα" might be correct.

The missing acronyms are defined in the revised version.

Page 7 Formula 9 + 10: It is hard to follow the argumentation as some of the acronyms are not explained in the text. Please add the definition and give more details on the derivation of the formula and the involved literature.

The missing acronyms are defined in the revised version.
Equations 9 and 10 are original (we are not aware that Rayleigh fractionation for isotopomers has been formulated elsewhere). We changed the formulation to make that clear.

Page 7 Line 7: The assumption that the ratio of reduction and production rate is constant is highly questionably based on past experimental evidence (e.g. Lewicka et al., Rapid Commun. Mass Spectrom. 29, 269–282 (2015)) – please comment?

As far as we understand Lewicka et al. were able to separate production and reduction steps and observe different rates. It seems that both production and reduction rates were quite constant throughout their experiment, which seems to favor of our assumption. However, we did not find a statement about the ratio of production versus reduction. Also their experiment is not directly comparable to ours as they work with a bacterial community not with individual bacteria.
We clearly state that a constant ratio between reduction and production rate is an assumption. We agree that this may be different but from our experiments we are not able to tell. A constant ratio is the most conservative assumption we can make.

Page 7 Line 21 – 22: This sentence might be wrong, as the "net production rate" is negative after the point of maximum N_2O concentration (Figure 3) – please clarify?

Rephrasing to: "For P. fluorescens the section of production is defined as being from the start of the measurements until the net production (net emission) rate turns negative."

Page 7 Line 30: The term "CDC" is given here for the first time, please define.

Changed to: " The models are fitted using both the concentration dependent corrected (CDC) data and..."

Page 8 Line 4: "Éč" was defined as ratio between reduction and production rate (page 7 Line 6 – 7) and is named reduction correction parameter here – please unify.

We agree on the issue and have changed the wording accordingly. " N_2O depends

on the ratio between reduction rate and production rate, from here referred to as the reduction correction parameter (γ)."

Page 8 Line 27: The statement, that N_2O production is absent when both $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ decrease is without proof – please comment?

That production is absent is not intended as a statement, but rather an assumption for the further analysis. We emphasize this by writing: "We defined the start of the section where P. fluorescens is only reducing N_2O to the point where both $\delta^{15}N^{\alpha}$ and $\delta^{15}N^{\beta}$ start decreasing (assumption based on reduction of $\delta^{15}N^{\alpha}$, $\delta^{15}N^{\beta}$, δ15Nbulk, and concentration)." – on page 7 line 27

Page 8 Line 24: Please delete “the”.

Corrected.

Page 8 Line 27: The wording “the bulk” is colloquial, please correct.

Corrected.

Page 9 Section 4 Discussion: Some statements in the discussion are in contrast to existing literature. Therefore, the authors should carefully check the interpretation of their results in relation to existing literature – details are given below.

See response to detailed comments below.

Page 9 Line 13: To clarify “nutrient” might be exchanged by “substrate”.

Corrected.

Page 9 Line 18 – 19: The wording “the isotopomers are depleted” is colloquial as the term isotopomer relates to the molecules 15N14NO and 14N15NO.

The sentence is changed to: ”We therefore find that the isotope enrichment is significantly increased for the isotopomers produced by *P. fluorescens* than for those produced by *P. chlororaphis*.”

Page 9 Line 19: The wording “the Rayleigh” is colloquial please change.

The sentence is corrected to: ”... since the Rayleigh model is calculated as product-to-substrate fractionation.”

Page 9 Line 21: What are “measurements of P. chlororaphis”?

The sentence has been changed to: ”This conclusion is based on measurements of two denitrifiers (*P. chlororaphis* (ATCC 43928) and *P. aureofaciens* (ATCC 13985))....”

Page 9 Line 23: The statement “that the conclusion applies to all denitrifying bacteria” is to strong based on the presented measurements and might be deleted.

We agree that the statement is too aggressive and it has therefore been deleted.

Please also discuss results in relation to relevant work by other authors: e.g. Sakae Toyoda et al. Soil Biology & Biochemistry 37 1535–1545 (2005).

Thank you for bringing this study to our attention. We added: "Toyoda et al. (2005) present contrasting results for ϵ_{SP} of *P. fluorescens* (ATCC 13525) of 23.3 . The results may, however, not be comparable to ours as Toyoda et al., suspect an abiological reaction within the incubation flask to be responsible for N_2O production in the incubation experiment."

Page 9 Line 24: The observed difference in the enrichment factor ϵ_{bulk} could also be explained by just differences in the reaction rate as already demonstrated by A. Mariotti et al. Can. J. Soil Sci. 62: 221-241 (1982).

We thank the referee for pointing out this matter. We modified the section accordingly: "The observed difference in the isotopic fractionation during production of N_2O could originate from 1) a difference in the production rate (Mariotti et al., 1982), or 2) a difference in the nitric oxide reductase enzymes. 1) The production rates in our experiments (Table 2 and 4) show an isotopic fractionation dependent on the production rate similar to the one observed by Mariotti et al. (1982). Our experiments show a production rate 10 times higher for *P. chlororaphis* than for *P. fluorescens*, which cf. Mariotti et al. (1982) would account for only approximately a 10 offset. We therefor believe that a change in production rate does not account for the 37.1 difference in isotopic fractionation."

Page 10 Line 9: R. Well et al. Rapid Commun. Mass Spectrom. 2008; 22: 2621–2628 published ϵ_{SP} values for diffusion, which are in contrast to the authors speculation,

please add the reference and comment?

We thank the referee for the comment, and we have changed the sentence: "As this is a diffusion driven process it is mass-dependent and a slight effect on SP is expected. This is also in line with the kinetic isotope effect theory which suggest a small offset towards higher ϵ_{α} and therefore higher ϵ_{SP} (Popp et al., 2002; Tilsner et al., 2003; Wrage et al., 2004; Well and Flessa, 2008)."

Page 10 Line 24 – 25: The authors state that "for N_2O reduction their results ϵ_{bulk} and ϵ_{SP}) are in line with earlier studies". However, all previous studies consistently show negative ϵ_{SP} values for N_2O reduction, e.g. D. Lewicka Geochimica et Cosmochimica Acta 134 55–73 (2014); D. Lewicka et al. Rapid Commun. Mass Spectrom. 29, 269–282 (2015), R. Well et al. Rapid Commun. Mass Spectrom. 23: 2996–3002 (2009); J. R. Köster et al. Rapid Commun. Mass Spectrom., 27, 2363–2373 (2013). Please add references and comment!

We agree that the lines/section was incomplete and in need of further comments.
"A number of studies have investigated N_2O originating 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. I.e. they find consistently negative isotopic fractionation for SP while we find slightly positive values on average. Ostrom et al. (2007) investigated bacterial reduction of N_2O using P. stutzeri and P. denitrificans, and the SP resulting from this bacterial reduction of N_2O was between -6.8 and -5 . However, they note that while for high net production (predominantly production of N_2O) SP is negative while with reduced net production that goes with an increasing rate of reduction the SP value increases, in line with our finding. This is also in agreement with kinetic isotope theory presented e.g. (Popp et al., 2002; Tilsner et al., 2003; Wrage et al., 2004)."

Figure 3: I assume the "N_2O production" is "net N_2O production" – please clarify and change here and elsewhere in the text?

Corrected.

Figure 4A, 4B, 5A and 5B: The wording in the legends is "poor" and should be rephrase to something like $\delta^{15}N^{\alpha}$ as a function of N_2O concentration . . .The blue curve is the instantaneous signal of the CRDS analyser, the black curve the five minutes running average. The blue arrow indicates ..." Please add information on the bacterial strains involved in Figure 4 and 5.

The figure legends has been corrected to: "$\delta^{15}N^{\alpha}$ as a function of N_2O concentration as produced by P. fluorescens and the modeled Rayleigh type distillation. High resolution CRDS data (blue line) and five minutes running average (black line). The red and magenta curve is the modeled apparent Rayleigh type distillation curve for the production and reduction of N_2O, respectively. The blue arrow indicates the direction of time during production of N_2O whereas the green arrow indicates the direction of time during reduction of N_2O."

Table 3: The enrichment factors given for N_2O reduction are in contrast to all existing literature. Therefore the limitations of the applied data analysis should be mentioned.

We believe that the response to this comment is covered by our response to comment to Page 10 Line 24 – 25:

[Response to referee comment # 2]

The study by Winther et al. presents continuous measurements of nitrous oxide isotopomers to determine isotope effects for two different bacterial organisms. N_2O isotopomers have been identified as a promising tool for the identification of the different processes generating N_2O. For this reason, such measurements are valuable for the scientific community. Especially spectroscopic measurements have been shown to be very well suited for the determination of the isotopomers (Mohn et al., 2014, Rapid Comm. Mass Spec.), owing to the selectivity of this method. In this context, this paper is also from a methodologic perspective of interest for the broad audience Biogeosciences attracts. However, I want to raise some general points:

1. The paper unfortunately stops short of providing technical details on the instrument used and the performance during the incubation experiments. For potential users, information on the stability of the measurements over a deployment period of weeks to months would be interesting. Further, the stability of the concentration dependence and the calibration coefficients is of interest for readers interested in spectroscopic methods. In my opinion the authors should elaborate on the named points a little more than stating "P4, L29: Over the course of the experiments, no further instrumental drift was observed". For example, what is the duration of the course of the experiments?

Following the recommendation of the reviewer we now give more details about the technical side of the experiments.

We have edited the last paragraph in section 2.2. The concentration dependence experiments presented consist of a total of seven similar experiments now shown together in figure 2.

We further added the following paragraph to section 2.4: "Continuous measurements

of the bacterial production of N_2O from P. chlororaphis were performed for approximately 500 minutes for each replica. All five replicas were measured within one week, starting at the same hour of the day and after equally long cultivation prior to the measurements. The bacterial evolution, of N_2O production and reduction, from P. fluorescens was continuously measured for 1000 minutes on average. The seven replicas were measured in three one week measuring campaigns over the course of half a year, but always with an equally long cultivation prior to measurements."

2. The headspace was flushed with pure N2 and, thus, the composition of N2 and O2 was not constant. At the same time, the calibration gases were provided in synthetic air. The composition of the analysed gas is crucial for adequate calibration, and the authors need to show the influence of changing N2/O2 ratio.

The reviewer is right and the manuscript has been corrected for this error (see our comments to referee # 1: Page 4 Line 7).

3. The determination of isotope effects for the bacterial organisms is based on the Rayleigh approach. However, for P. fluorescens the determination of the isotope effect during production was considered. In this consideration, a parameter, gamma, is introduced reflecting the ratio between reduction rate and production rate. In the next section, the authors state that they used an iterative procedure to fit their experimental data to the derived Rayleigh model, but the sensitivity of the resulting isotope effect is not mentioned at all throughout the manuscript. Also the authors state that it is assume that the ratio between consumption and production is constant. In my opinion, a sensitivity analysis of the model towards non-constant gamma should be provided.

That production to reduction is constant is clearly stated as an assumption. It is

possible that this ratio deviates over time but there is no way in knowing how in our experiment. In our view, without further knowledge on how the ratio could have changed, a sensitivity analysis is not appropriate. See also our answer to Referee #1 comment for P7, L7.

4. It is surprising that for SP, the measurements seem to differ quite a bit from published values, though the isotope effect associated with SP, and especially for N_2O reduction, is in general considered to be the most reproducible. I suggest discussing this in more detail.

We thank the referee for bringing up this issue. The answer to this is written in the response to referee # 1 comment for Page 10 Line 24 – 25

5. The manuscript uses informal language at many points, not always provides adequate references (e.g., section 2.5) and also the figure captions sometimes lack basic information such as which bacterial organism is the subject of the figure (see below). The introduction is currently rather a sequence of statements, so that I suggest rearranging it to a concise introduction. See some more detailed comments below.

Title
Ok

Abstract
P1, L1: "feed-back loop": global warming is not necessarily enforcing global N_2O emissions per se. Please explain the feed-back loop mentioned.

We deleted the questioned statement since it is not the core topic of the manuscript.

P1, L3/4 sounds odd. I suggest “A rare 15N atom can substitute the abundant 14N atom either at the central or terminal position in the linear N=N=O molecule.”

We rephrased: ”In the linear N=N=O molecule ^{15}N substitution is possible in two distinct positions, central and terminal. The respective molecules, $^{14}N^{15}N^{16}O$ and $^{15}N^{14}N^{16}O$, are called isotopomers.”

P1, L6: Please also define site preference

We now write: ”Therefore, measurements of the site preference (difference between the isotopomers) in N_2O can be used to...”

Introduction
The introduction draws an arch from isotopomers to climate change. This needs to be specified as currently the capabilities of quantitative source partitioning still need to be proven.

See comment further down (P2, L13/14).

P2,L5: point (1) sounds odd. I suggest “(1) enhanced radiative forcing with N_2O being the third most important GHG”. Further, e.g. is followed by a comma. Please change all e.g. to e.g.,

The sentence has been changed to: ” N_2O being the third most important greenhouse

gas, e.g., N_2O has the third highest contribution to the radiative forcing of the naturally occurring greenhouse gasses (Hartmann et al., 2013),...”

P2,L9: Please make clear that there was a positive correlation.

Sentence changed to: ”Ice core records show that N_2O concentrations positively correlate with northern hemispheric temperature variations, e.g., during the last glacial-interglacial termination as well as over the rapid climate variations occurring during the glacial period, known as Dansgaard-Oeschger events (D-O events) (Schilt et al., 2010).”

P2,L13/14: Please elaborate more in how isotopomers can help understanding climate change. The radiative forcing is almost exclusively due to 14N14N16O and, as said before, atmospheric N_2O concentration is correlated.

Added: Isotopomers of N_2O provide information on the sources (Pérez et al., 2000; Perez et al., 2001; Park et al., 2011) i.e. whether N_2O originates predominantly from nitrification or denitrification processes. As the conditions/ circumstances leading to emissions from the two processes differ both for the marine and terrestrial sources measuring isotopomers will potentially improve our understanding on the climate conditions leading early release of N_2O over some rapid climate change.

P2,L24/25: I suggest starting the d-value description form the generic d15Nsample: “The isotopic composition of a sample is usually reported as d-value which represents the deviation of the elemental isotope ration R in the sample from a reference material. Delta values can be calculated for bulk N_2O as well as for d15Na and d15N beta. ” Please refer to AIR-N2 as standard material.

We agree and changed the introductory sentence accordingly. The reference to N2 of atmospheric air being the standard has been added.

P2,L27: Park et al is not suited for the definition of SP. It was introduced in 1999 by Brenninkmeijer and Röckmann (as indicated in the text) and by Toyoda and Yoshida, Annal. Chem 71, 4711-4718. I think these two publications deserve the credit for SP.

We removed Park et al., 2011 from the sentence.

P3, L2: It sounds like denitrifying bacteria exclusively produces N_2, which is not the case. Please rephrase.

We rephrased to: "Denitrification is a stepwise biological reduction process in which denitrifying bacteria ultimately produce nitrogen (N_2)"

P3, L14-17: Is it a method application or investigation of different bacteria? The latter is not really new.

While the reviewer is correct that bacteria and also the specific bacteria in our study have been investigated before, it is the first time (to our knowledge) those bacteria have been analyzed continuously allowing not only to determine the fractionation but (in the case of *P. fluorescens*) also distinguish between production and reduction. In that way it is a new method applied to a selection of bacteria. Also contradicting results indicate that further experiments are still needed to obtain a more complete understanding of the N_2O part of the nitrogen cycle.

Materials and Methods
P3, L22: Please change spectrometry to spectroscopy. The paper reports on a spectroscopic method.

Changed.

P5, L30: The incubation chamber's headspace was flushed with pure N2 to create anaerobic conditions. However, the calibration gases are N_2O in synthetic air. For spectroscopy, the gas matrix is of importance as it affects the line shape of the investigated gas. Please show the influence of increasing N2 content on determined isotope ratios at constant N_2O concentration. It is fundamental to address gas matrix in such an experiment.

We would like to thank the referee for bringing up this important point. A similar comment was received from referee # 1. Please see our response and corresponding changes to "Page 4 Line 7". We stress that the issue has been fixed and that this has no effect on the results of our study, namely the isotope enrichment factors.

P6, L7-8: Though this statement addresses the concern raised above, but actually it is not only O2 spectral lines that affect the measurements, but also the collision partner itself. For this reason, the authors need to show empirical evidence here that the effect of a changed gas matrix did not change the determined isotope ratio (as described above).

See comment above.

P6, L12: Equation 3 is given with Rs and Rs,0, whereas L12 refers to “reactant”. The “s” in Rs,0 stands for substrate. In my opinion, it is easier to follow if the abbreviation would be the same as the word used. Thus, I suggest using “substrate instead of reactant”

The sentence has been changed to: ”... the isotope ratio of the substrate as: .... where $R_{s,0}$ is the initial isotope ratio of the substrate, R_s is the isotope ratio of the substrate at time *t*.”

P6, L22: The term isotope enrichment for the bulk is a little confusing here. For normal isotope effects, the accumulated product is depleted compared to the initial substrate. In this context, the section head 2.5 “Analysis of isotope enrichment” is also not ideal. As far as I can see the whole section aims at the determination of the isotope effect. I suggest using “Determination of isotope effects” as section head and introduce epsilon as isotope effect, which is from my point of view the agreed term (this also refers to line 15/16).

We thank the referee for bringing this issue up. Throughout the manuscript we have changed “isotope enrichment” with “isotopic fractionation” according to Coplen, (2011). Furthermore we have changed the section title to: “Determination of isotopic fractionation”.

P7, L2: in equation 8, the correction term is not specified, however the isotope ratio is. I assume, the correction term needs a subscript identifier as well, e.g., a or b.

Correct and corrected.

In addition, the section beginning P6, L 25 needs references.

We are not sure what the reviewer means. If he is refereeing to the application of Rayleigh fractionation to isotopomers, we derived those equations ourselves. We changed the sentence to make that clear (see also comment to referee # 1- Page 7 Formula 9 + 10)

P7, L3-10: Also this section needs references and at least the terms in eq. 9 and 10 should be explained properly. Rp,r is most likely the isotope ratio of remaining N_2O after reduction? The manuscript is cumbersome toe read if this (basic) information needs to be deduced by the reader.

Equations 9 and 10 are original (we are not aware that Rayleigh fractionation for isotopomers has been formulated elsewhere). We changed the formulation to make that clear.

P7, L17: In my opinion its odd to have a quite extensive section 2.5 followed by a sole subsection 2.5.1. This type of structure does not make sense to me and I suggest the following structure: 2.5 Determination of the isotope effects, 2.51. Modifications to the Rayleigh model, 2.5.2 Fitting procedure.

We agree and the suggested structure is applied.

P7, L 30: I am not aware that CDC has been introduced so far. What does it mean?

Sentence changed to: "The models are fitted using both the concentration dependent corrected (CDC) data ..."

Whole 2.5.1: The second section is somewhat repetitive as the R2 value is mentioned twice as optimization criterion and, thus is too verbose. Please make this section more concise and provide information on what "is iteratively found" means exactly (P7, L23).

Corrected.
"... is iteratively found (calculations of the accumulated product (eq. 8 and eq. 10) are calculated with all possible combinations of 1) the unreacted fraction at the start and at the end, 2) reduction correction parameter, 3) fractionation factor during production, 4) fractionation factor during reduction) ..."

Results
P8, L7ff. The evolution of N_2O concentration (please add the word concentration in line 7) during the experiments is described for both organisms, though there are sub-sections for the respective organisms following. From this perspective, the introduction in section 3 is odd. Though it may be controversial, I suggest starting the subsection 3.1 directly following section 3 and moving the description of N_2O concentration to the respective subsection.

We added "concentration" after N_2O in the introductory sentence. We believe it makes sense to keep four lines of general introduction at the beginning of the section and would like to keep it the way it is.

P8, L14: The reference to Fig 4a and 4B is ok, but the caption of fig. 4 does not indicate which organism the figure refers to. Please add this information.

We have changed the captions to: " $\delta^{15}N^{\alpha}$ as a function of N_2O concentration as produced by P. chlororaphis and the modeled Rayleigh type distillation. High resolution CRDS data (blue line) and five minutes running average (black line). The red line shows the modeled apparent Rayleigh type distillation for the production of N_2O. The blue arrow indicates the direction of time during production of N_2O."

P8, L15: Please avoid terms like "in fig : : : we plot : : : ". This occurs throughout the manuscript, sounds informal and could easily be replaced by Figure xxx shows : : : .

Corrected.

P8, L17-18: The match was relatively high to what? The correlation coefficient is R, the coefficient of determination is R2. Please consider.

Reworded to: " The average coefficient of determination (R^2)..."

P8, L24: please also here add the organism to caption of fig. 5 and remove "the" in front of $\delta^{15}N^{\alpha}$. The results section does not contain any information on the parameter gamma, representing the share of reduction and production. Please give details.

Corrected – similar to the caption of fig. 4.

Discussion

The discussion lacks information on the share of reduction and production of N_2O during the onset of the experiment involving P. fluorescens. What is the uncertainty of this parameter on derived isotope effects?

We agree that this information has been missing. The share of reduction and production is the result of the iterative calculations leading to the best coefficient of determination between the data and the Rayleigh model. The only assumption we make is that the ratio is constant as clearly stated throughout the manuscript. We added the missing information in Table 2 for all the experiments providing also information on the uncertainty of the parameter.