Response to Editor

Dear Professor Kuzyakov,

Thank you very much for considering our manuscript for publication in Biogeosciences after minor revision. The manuscript has been now corrected according to the comments of three reviewers. Below please find the detailed outline of changes made in the manuscript according to particular comments and our revised manuscript with tracked changes.

Sincerely yours,
Dominika Lewicka-Szczebak

Anonymous Referee #2

The authors' changes in the manuscript are in red fond.

The title of this paper should be specified to show that they studied denitrification "in soils" because there are many publications with respect to isotope fractionation during N2O production in soils, waters, pure culture of microbes.

The title has been specified to soil denitrification: 'Oxygen isotope fractionation during N_2O production by soil denitrification'.

I found an error in their model-based discussion on the branching isotope effect, and consider the error might be critical as shown below. In summary, I consider that this paper might be acceptable for publication in Biogeoscience after correction for the error and improvement of some minor points below.

This 'error' is not really an error, but an assumption that can be well justified. More discussion on this issue has been added (P20, L5-10).

P17018, L17 The authors used "Delta" series mass spec, for which I think linearity problem has been previously reported for NO+ fragment analysis. I suggest to add correction procedure/method if they applied.

This information has been added (P10, L17-20).

P17026, L23 "19.1+-0.5 (Table 1)" Does this mean average and 1sd of 12 data presented in Table 1?

This has been clarified (P18, L5) .

P17026, L26 "It can be noted . . ." I cannot follow this because Figure 3 is complicated. It seems this figure shows more data than those presented in Table 1. For example, I thought Exp. 1.1a was conducted with nitrate with high d18O from Table 1, but blue open triangle in Figure 3 suggests this experiment was also carried out with low-d18O nitrate.

To be consistent, we have deleted the samples with synthetic nitrate in Exp1.2 from Table 1, since the O-exchange could not be precisely determined there and they are not further used for modelling. Moreover, we have added an appendix with a summary of all the treatments and way of their presentation in tables and figures. We have better explained the selection of different treatments for tables and graphs in the Section 2.1.1 and appendix.

P17028, eqs. (7) and (8) It seems the authors assume that epsilons for NIR- and NOR-mediated O exchange processes are identical. But I think it is not trivial because chemical species that exchange O atom with water are different between the two processes. Rationale or speculation should be added.

We have discussed this uncertainty in the manuscript (P21, L1-6).

P17029 , L1 "We have neglected the possible fractionation associated with the NAR reduction, . . ." I disagree with this statement. The authors write this was investigated in Rohe et al. (2014a), but I could not find any experimental evidence in the cited paper. I found a quotation from Casciotti et al. (2007) in the caption of Table 4 in Rohe et al. But Casciotti et al. (2007) describes that "branching isotope effect between nitrate and nitrite is 25-30 permil". Please explain why the authors considered the branching isotope effect is significant in nitrite-NO reduction step, not the nitrate-nitrite step. If nitrate nitrite step is more important regarding the branching isotope effect as Casciotti et al. showed, delta-n in equation (11) should be d18O of nitrite, not nitrate, and the authors' model calculation results presented in Table 4 would change especially for Exp. 2.

This statement has been better clarified in the manuscript (P20, L3-10 and P4, L16-19).

3. Technical corrections
P17032, L25 and 27 "intra-molecular effect". This should be "inter-molecular effect"?
This has been corrected (P24, L10,12).
P17042, second column of Table 2. The unit of production rate should be consistent with those appear in Table 1 and text: microgram/kg/h.
This has been corrected (Table 2, P34).
P17044, caption of Table 4. Number or position of bracket(s) are awkward in the first sentence.
This has been corrected (Table 4, P37).
P17045, Figure 1 "epsilon-n"s are better noted as "epsilon-NAR, -Nir, -NOR" to be consistent with text.
This has been corrected (Fig.1).

Response to Anonymous Referee #3
The authors' changes in the manuscript are in red fond.

General comments:
1. Title
The results of this paper mainly indicate that the isotopic signatures of $\delta^{18}O$, especially the values of $\delta^{18}O(N_2O/H_2O)$, could be used as indicators for differentiation of the N2O production processes by denitrification, hence, the title "The mechanism of oxygen isotope fractionation during N2O production by denitrification" did not reflect the main results in this paper and should be corrected accordingly.
We have changed the title to: 'Oxygen isotope fractionation during N_2O production by soil denitrification'.

2. Abstract
Because the results and conclusions in this paper were not focused and well demonstrated, I recommend the authors rewrite this part. In p. 17010. Line 17-24: these sentences indicates that the results found bacterial denitrification and fungal denitrification had different oxygen isotope exchange and leaded to different values of $\delta^{18}O(N2O/H2O)$, however, as my understanding of the results, the results showed different oxygen isotope exchange between a static and a dynamic incubation experiments at first. With the results of 15N site preference, the authors demonstrated that the different oxygen isotope exchange between a static and a dynamic incubation experiments was probably due to the fungal denitrification processes.
This has been reformulated (P2, L3-9)

3. Introduction
Many corrections should be made in this part. The authors should focus the scientific questions which need to be solved and introduce the research progresses for these questions. The hypothesis based on the previous researches should be summarized and outlined at the last part of the introduction, furthermore, the research methods and objects should be introduced in detail for a good understanding of this research. Several scientific questions were provided and introduced in this part: (1) How the isotope oxygen exchange with soil water during denitrification responses to different abiotic factors such as temperature and soil moisture? (2) Do the different NOR mechanisms for fungi and bacteria have effects on the value of $\delta^{18}O$? The authors also made hypothesis according to these questions, however, the hypothesis was not well demonstrated in the results and discussion of this paper.
We made numerous corrections to introduction (P3 – P6)

4. Methods
The experiment set-ups was not written with a clear and detailed description.

In p. 17015. Line 23-25: The two sentences were related to the results and should be put in the results part. Furthermore, which data in the results has been published in the previous paper? The authors need to highlight it with reference in the results.
The precise information on this has been added in the caption of Table 1 (P31).

In the descriptions of Experiment 1 and Experiment 2, the authors did not provide the detailed information about the treatments, the replicate number or the number of incubation jars in each treatment, and this information should be added to the method for a clear understanding of the experiment set-ups.
This is quite complex information, how we combined treatments and incubations. I have prepared an extra table showing all the experimental treatments, including soil moisture, applied nitrate and water, addition of acetylene, soil type and temperature. This table is added to the manuscript as an extra appendix.
Additionally, we have also better clarify these issues in the Section 2.1.1.

In p. 17016. Line 14-15: Could the selected jars be considered as one treatment, and the non-selected jars be considered as another treatment?
'C_2H_2 treatment' has been added (P8, L14). The respective treatments has been also indicated in Table 1. Moreover, an appendix with detailed treatments description has been added for better clarification of treatments strategy.

In p. 17016. Line 22-25: how the N2O mole fraction f(N2O) was estimated by addition of 15N-labelled NaNO3? If this method has been described in the previous papers, it is better to add the papers as references to make a clear description of the experiment design.
Short clarification with reference has been added to the manuscript (P8, L19-20)

In p. 17017. Line 27-28: the sentence "f(N2O) was determined based on the direct measurement of N2O and N2 fluxes" should be followed after the sentence "The fluxes of N2O and N2 were analyzed immediately (see Sect. 2.2)" in Line 24-25.
This sentence has been moved (P9, L15,17-18).

In p. 17020. Line 19-20: I could not understand this sentence "For both presented methods it is assumed that no further O isotope exchange between N2O and H2O occurs". Could the authors rewrite this sentence to make it be understood ?
This sentence has been rewritten (P11, L25-26).

In p. 17021. Line 1-7: I suggest that this description of the parallel incubations for isotope exchange investigation could be inserted and fused into the contents of the experiment set-ups in p. 17015-17018. The authors should make a comprehensive introduction of the experiment design for the following analysis in the method. In addition, the authors said the parallel incubations to determine the isotope exchange were carried out in Exp 1 (p. 17021. Line 2). Did this method also carried out in Exp 2 for the isotope exchange determination? The authors did not show this content in the method.
The description of parallel incubations has been moved to description of experimental set-up (P7, L26-30).

In p. 17022. Line 16-17: the experiment design for the inhabitation of N2O reduction in Exp 1 were not clearly written in the part of experiment set-ups, and the sentences here could fused into the experiment set-ups. Which treatments were carried out with distinct water or nitrate isotopic signatures, and which treatments were added with acetylene for the inhabitation of N2O reduction? The authors should clarified and identified these experimental treatments in the description of the experiment design. The same corrections should be made for the contents in p. 17022. Line 2-6.
We have indicated the C_2H_2 inhibited treatments in Table 1 and Appendix.

5. Results and Discussion
Many problems existed in the presentation of the results.
In p. 17024. Line 3-12: the paragraph had an introduction of calculation method for δ 18O(N2O/H2O) and δ 018O(N2O/H2O) , hence, this part belongs to the method and should be migrated to the method part in the paper.
The description of calculation method has been moved to method section as requested (P14, L15-21).

In addition, the results only included the estimated values related to Table 1 and 2, without the contents related to other tables and figures. The authors should tell the results according to the tables and figures presented in the paper, and tell the story completely and fluently.

To avoid the problematic distinction into results and discussion, we have combined both parts into a common results & discussion section (from P15).

Table 1 and 2 showed the results of Exp 1 and 2, however, the contents in the tables were not well organized and structured. The treatments, such as reduction inhibited or non-inhibited, soil adding with heavy or light water, with natural Chile saltpeter or synthetic NaNO3, should be noted in the tables. Only one target moisture level (80% WFPS) and three target moisture level (50%, 65% and 80% WFPS) were set in the Exp 1.1 and 1.2, while one target moisture level (70% WFPS) was set in Exp 2. In the table 1 and 2, the moisture levels with small differences in the same moisture treatment could be uniformed with the target moisture levels (50%, 65%, 70%, or 80% WFPS). In p. 17015.
Treatments have been clarified in Table 1, and additional detailed outline of treatments has been added as Table A1 (appendix). I have uniformed the moisture levels for exp1, as they were very consistent, however due to large differences it could not be done for exp2.

In p. 17024. Line 14 and Line 22: the values of $\delta^{18}O(N2O/H2O)$ were not shown in the tables, should the $\delta^{18}O(N2O/H2O)$ here be rewritten to $\delta^{18}O(N2O/H2O)$?
Yes, thank you, this mistake will be corrected (P15, L15; L23)).

Many contents in Discussion were about the results in the tables and figures, and should be classified into the results part. I recommend the authors reorganize the contents in Discussion. The authors mainly discussed the results based on the analyzed data, and I recommend the authors use other previous researches to demonstrate these conclusions.
We have combined results and discussion section.

In p. 17027. Line 25-26: the authors said that the different values of x between the static and dynamic incubations may be due to activity of different microorganism groups, but I could not understand this conclusion based on the presented data and other information provided in the paper.
This sentence has been deleted (P19, L2-4). In Section 3.7 we present a justification for this assumption.

In p. 17028. Line 1-5: the authors said that the correlation between x and $\delta^{18}O(N2O/H2O)$ seems to differ for different soil types, and try to explain this conclusion by deciphering the theoretical model of the denitrification. However, the results of the theoretical model indicates that majority of isotope exchange associated mainly with nitrite reduction, and how did it explain the differences correlation between x and $\delta^{18}O(N2O/H2O)$ for different soil types?
An explanation has been added to the discussion (P23, L22 –L27).

Response to Anonymous Referee #4
The authors' changes in the manuscript are in red fond.

General Comments
Based on my understanding (and also the reading of Casciotti et al., (2007)), the exchange of O atoms between nitrite and water occurs as the result of the chemical dissociation of nitrous acid and is enhanced depending on their respective equilibrium concentrations. With a pKa value of _3.4, a lower pH accelerates the exchange of oxygen atoms because the pool size of nitrous acid increases, increasing the rates of forward/reverse equilibrium reactions between nitrite and nitrous acid (during which O atoms are lost/gained). This pH influence is well-known in other oxy-anion systems as well (sulfate, carbonate and phosphate, etc.). Given this important control on oxygen isotope equilibrium dynamics, I am surprised that the authors have not reported pH values for their soil incubations and solutions. Can more consideration be included about the pH of these soils and the porewaters – and their possible role in the oxygen isotope dynamics?
Information about soil pH values has been added (P7, L12; P8, L28).

The authors report traditional 'delta' and 'epsilon' values in units of 'permil,' yet in all of the equations the authors use 'un-normalized' delta and epsilon values. Perhaps this is simply style issue – but I feel that it can lead to confusion. For example – in the text when the branching isotope effect is estimated as '17‰´-- this is not the numerical value that is used in the equations throughout. At a minimum, some clarity might be

provided by stating how the values should be converted (e.g., not multiplied by 1000). For example, on P 18, L 16 – here the epsilon values which were reported on P 15, L2 as "18.2‰´' and "17.1‰´' are being reported as equal to 0.0181 and 0.0172. While I understand the desire to somewhat simplify the equations – there appears to be some inconsistency – which I think would be very confusing to the casual reader.
The information about permil unit has been added (P10, L21).
And the mistake in fraction values has been corrected (P20, L21).

P 4 L5-10: In general I think it would be good to be clearer about how epsilon-n here is calculated (e.g., I think it would be useful to see this mathematically expressed).
It is just a sum of both intra and intermolecular effect. I do not think introducing an extra equation in introduction would be needed, as it would be just epsilon-n = intramolecular effect + intermolecular effect, associated with each reduction step. We have clarified this better in the text (P4, L13).

P 5 L 11-13: This sentence seems out of place.
I think the use of the word "Dynamic" incubations seems a little misleading – perhaps consider using 'steady-state' or 'open-system' or 'flow-through' experiments instead? I think of 'dynamic' as indicating an important changing parameter – whereas here conditions are held constant (with the exception of the temperature and perhaps soil moisture).
Good idea, thanks for this suggestion, this has been changed to 'flow-through' experiments.

Does one need to account for the non-random 17O in the calculations of Site Preference?
Or does the low abundance of 17O not impact the accuracy?
The ^{17}O correction changed Site Preference values of up to 0.4 permil. It has been applied for Exp2 data, where the measured $\Delta^{17}O$ in N_2O is pronounced - up to 5.3 permil. For Exp1 the measured $\Delta^{17}O$ in N_2O is very low, hence the correction was not needed. The corrected values have been shown in the manuscript.

P 20, L 25: "Out of plausible range of values" – please include reference to your line of reasoning here (e.g., based on what?).
The references providing plausible range of values (Casciotti et al., 2007; Rohe et al., 2014a) has been added (P23, L9-10).

P21 L 10-15: Something is not clear about these statements. I understand how the effect observed by Casciotti (2007) represents only the 'intra-molecular' effect – (e.g., the O abstraction) – and that Casciotti (2007) refers to this as the branching effect. Here the authors then refer to this as being the 'maximal possible branching effect' – which also makes sense. Then, referring to the work by Rohe et al (2014), since the NO3- pool is not completely consumed – both the 'inter-' and 'intra-molecular effects' should be observed. But I fail to understand the next statement about the values of e-NIR of 10‰ and eNAR assumed to be 0‰ – and how this supports their observations. Please clarify.
These are values indicated by the model applied in that study. The lower values obtained indicate that the maximal branching effect was partially compensated by intermolecular effect, which results in lower values for net branching effect. This has been better clarified in the revised manuscript (P24, L4-5).

Specific Comments
The title could be a little more specific (e.g., referring to soils).
Title has been corrected: 'Oxygen isotope fractionation during N_2O production by soil denitrification'
P 17 L 11: 'Oxygen fractionation' = not clear whether you are referring here to molecular O2, O in water or O in N-bearing species.
We have clarified in the text that this fractionation is related to the entire process of investigated N_2O formation (P19, L11).
P21 L 17 and L19: I think this should be 'inter-molecular effects.'
Yes, this has been corrected (P24, L10,L12).

~~The mechanism of oxygen~~Oxygen isotope fractionation during N_2O production by soil denitrification

D. Lewicka-Szczebak[1], J. Dyckmans[3], J. Kaiser[2], A. Marca[2], J. Augustin[4], R. Well[1]

[1]~~]~~[~~{~~]Thünen Institute of Climate-Smart Agriculture, Federal Research Institute for Rural Areas, Forestry and Fisheries, Bundesallee 50, ~~D~~ 38116 Braunschweig, Germany~~}~~

[2]~~]~~[~~{~~]Centre for Ocean and Atmospheric Sciences, School of Environmental Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom~~}~~

[3]~~]~~[~~{~~]Centre for Stable Isotope Research and Analysis, University of Göttingen, Büsgenweg 2, 37077 Göttingen, Germany~~}~~

[4]~~]~~[~~{~~]Leibniz Centre for Agricultural Landscape Research, Eberswalder Straße 84, 15374 Müncheberg, Germany~~}~~

Correspondence to: D. Lewicka-Szczebak (dominika.lewicka@ti.bund.de)

Abstract

The isotopic composition of soil-derived N_2O can help differentiate between N_2O production pathways and estimate the fraction of N_2O reduced to N_2. Until now, $\delta^{18}O$ of N_2O has been rarely used in the interpretation of N_2O isotopic signatures because of the rather complex oxygen isotope fractionations during N_2O production by denitrification. The latter process involves nitrate reduction mediated through the following three enzymes: nitrate reductase (NAR), nitrite reductase (NIR) and nitric oxide reductase (NOR). Each step removes one oxygen atom as water (H_2O), which gives rise to a branching isotope effect. Moreover, denitrification intermediates may partially or fully exchange oxygen isotopes with ambient water, which is associated with an exchange isotope effect. The main objective of this study was to decipher the mechanism of oxygen isotope fractionation during N_2O production by soil denitrification and, in particular, to investigate the relationship between the extent of oxygen isotope exchange with soil water and the $\delta^{18}O$ values of the produced N_2O.

~~We performed several~~In our soil incubation experiments~~. For the first time,~~ $\Delta^{17}O$ isotope tracing was applied for the first time to simultaneously determine the extent of oxygen isotope exchange and any associated oxygen isotope effect. We found ~~bacterial denitrification to be~~that N_2O formation in static anoxic incubation experiments was typically associated with ~~almost complete~~ oxygen isotope exchange close to 100 % and a stable difference ~~in $\delta^{18}O$~~ between the $^{18}O/^{16}O$ ratio of soil water and the ~~produced~~ N_2O product of $\delta^{18}O(N_2O/H_2O)$ = (17.5±1.2) ‰. However, ~~some experimental setups yielded~~flow-through experiments gave lower oxygen isotope exchange ~~as low as~~down to 56 % and a higher $\delta^{18}O(N_2O/H_2O)$ of up to 37 ‰. The extent of isotope exchange and $\delta^{18}O(N_2O/H_2O)$ showed a ~~very~~ significant correlation (R^2 = 0.70, p < 0.00001). We hypothesise that this observation was due to the contribution of N_2O from another production process, most probably fungal denitrification.

An oxygen isotope fractionation model was used to test various scenarios with different magnitudes of branching isotope effects at different steps in the reduction process. The results suggest that during denitrification ~~the,~~ isotope exchange occurs prior to ~~the~~ isotope branching and that ~~the mechanism of~~ this exchange is mostly associated with the enzymatic nitrite reduction mediated by NIR. For bacterial denitrification, the branching isotope effect can be surprisingly low, about (0.0±0.9) ‰; in contrast to fungal denitrification where higher values of up to 30 ‰ have been reported previously. This suggests that $\delta^{18}O$ might be used as a tracer for differentiation between bacterial and fungal denitrification, due to their different magnitudes of branching isotope effects.

1. Introduction

Our ability to mitigate soil N_2O emissions is limited due to poor understanding of the complex interplay between N_2O production pathways in soil environments. In order to develop effective fertilizing strategies and reduce the loss of nitrogen through microbial consumption as well as related adverse environmental impacts~~,~~ (IPCC, 2013; Ravishankara et al., 2009), it is very important to fill the existing knowledge gaps. Isotopocule analyses of N_2O, including $\delta^{18}O$, average $\delta^{15}N$ ($\delta^{15}N^{av}$) and ^{15}N site preference within the linear N_2O molecule ($\delta^{15}N^{sp}$) have been used for several years to help differentiate between N_2O production pathways (Opdyke et al., 2009; Perez et al., 2006; Sutka et al., 2006; Toyoda et al., 2005; Well et al., 2008), the various microbes involved (Rohe et al., 2014a; Sutka et al., 2008; Sutka et al., 2003) and to estimate the ~~magnitude~~fraction of N_2O ~~reduction~~reduced to N_2 (Ostrom et al., 2007; Park et al., 2011; Toyoda et al., 2011; Well and Flessa, 2009). However, the usefulness of these analyses would be enhanced further if the isotope fractionation mechanisms were better understood. In particular, we need to ~~know~~recognize the isotope ~~fractionations~~effects associated with nitrate and N_2O reduction to quantify the ~~fraction of~~entire gaseous nitrogen losses as N_2O ~~reduced to~~and N_2 based on the N_2O isotopic signatures (Lewicka-Szczebak et al., 2015; Lewicka-Szczebak et al., 2014). This would be most effective if either of the isotopic signatures ($\delta^{18}O$, $\delta^{15}N^{av}$ or $\delta^{15}N^{sp}$) were stable or predictable for N_2O produced by each of the relevant N_2O forming processes (*e.g.* heterotrophic bacterial denitrification, fungal denitrification, nitrifier denitrification and nitrification). We hypothesize that this could be the case for $\delta^{18}O$, ~~which was the focus of this study~~and this study aims to increase the understanding of the factors controlling $\delta^{18}O$ during N_2O production in soils. .

$\delta^{18}O$ ~~of~~ (N_2O) has been rarely applied in the interpretation of N_2O isotopic signatures because of the rather complex oxygen isotope fractionations during N_2O production by denitrification (Kool et al., 2007)~~. It~~. Denitrification is a stepwise process of nitrate reduction mediated by three enzymes: nitrate reductase (NAR), nitrite reductase (NIR) and nitric oxide reductase (NOR) (Fig. 1). $\delta^{18}O(N_2O)$ is controlled by the origin of the oxygen atom in the N_2O molecule (nitrate, nitrite, soil water or molecular O_2) and by the isotope fractionation during nitrate reduction or during oxygen isotope exchange with soil water.

[Fig. 1]

~~N_2O production during denitrification is a stepwise process of nitrate reduction mediated by the following three enzymes: nitrate reductase (NAR), nitrite reductase (NIR) and nitric oxide reductase (NOR) (Kool et al., 2007) as presented in the simplified scheme in Fig. 1.~~ During each reduction step, one oxygen atom is detached and removed as water (H_2O), which is associated with branching isotope effects (Casciotti et al., 2007; Snider et al., 2013). Conceptually, these can be regarded as a combination of two isotope fractionations with opposite effects on the $\delta^{18}O$ signature of the reduction product: (i) intermolecular fractionation due to preferential reduction of ^{18}O-depleted molecules, which results in ^{18}O-enriched residual substrate and ^{18}O-depleted product, and (ii) intramolecular fractionation due to preferential ^{16}O abstraction, which results in ^{18}O-enriched nitrogen-bearing reduction products and ^{18}O-depleted H_2O as side product. Since intermolecular fractionation causes ^{18}O depletion of the reduction product and intramolecular fractionation causes ^{18}O enrichment, the net branching effect (ε_n~~))~~, as the sum of both, can theoretically vary between negative and positive values. However, pure cultures studies show that ε_n is mostly positive, *i.e.* between 25 and 30 ‰ for bacterial denitrification (Casciotti et al., 2007) and between 10 and 30 ‰ for fungal denitrification (Rohe et al., 2014a). Importantly, the intra- and intermolecular isotope effects can only manifest together during incomplete substrate consumption (Rohe et al., 2014a). In case of complete substrate conversion, the net branching effect reflects the intramolecular effect only (Casciotti et al., 2007).

Moreover, denitrification intermediates may partially or fully exchange oxygen isotopes with ambient water (Kool et al., 2009). The isotopic signature of the incorporated O-atom depends on the isotopic signature of ambient water and the isotope fractionation associated with this exchange. Under typical soil conditions, *i.e.* pH close to neutral and moderate temperatures, abiotic isotope exchange between nitrate and water is negligibly slow. In extremely acid conditions (pH < 0), the equilibrium effect is $\varepsilon(NO_3^-/H_2O)$ = 23 ‰ (Böhlke et al., 2003). Casciotti et al. (2007) showed that for nitrite the abiotic exchange can ~~also take place~~occur at neutral pH, but for achieving an isotopic equilibrium over 8 months are needed. The observed isotope equilibrium effect between nitrite and water is $\varepsilon(NO_2^-/H_2O)$ = 14 ‰ at 21 ºC. Nothing is known yet about the possible abiotic exchange between NO and ambient water.

The isotope exchange between denitrification intermediates and ambient water is most probably accelerated by enzymatic catalysis, since numerous ^{18}O tracer studies documented nearly complete O isotope exchange (Kool et al., 2009; Rohe et al., 2014b; Snider et al.,

2013) within short incubation times like a few hours. Hence, it can be assumed that at least one enzymatic step must be responsible for exchange of O isotopes with soil water (Rohe et al., 2014a; Snider et al., 2013)~~. Consequently, the final δ^{18}O of produced N_2O may vary over a wide range, depending on the extent of isotope exchange with soil water associated with particular enzyme (Rohe et al., 2014a).~~

~~Pure culture studies indicated large differences between various denitrifying microbes. The~~. In pure culture studies the extent of oxygen isotope exchange ranged from 4 to 100 % for bacterial denitrification (Kool et al., 2007) and from 11 to 100 % for fungal denitrification (Rohe et al., 2014b). In contrast, unsaturated soil incubation experiments, with a natural whole microbial community, showed consistently high magnitudes of ~~O~~oxygen isotope exchange between 85 and 99 % (Kool et al., 2009; Lewicka-Szczebak et al., 2014; Snider et al., 2013). If the high extent of isotope exchange was characteristic of soil denitrification processes, we would expect quite stable δ^{18}O values of the produced N_2O during denitrification~~, provided that these values are not influenced by N_2O reduction~~.

It is difficult to quantitatively link isotope exchange and apparent isotope effects, because using the ^{18}O tracer technique to quantify isotope exchange prevents simultaneous study of isotope oxygen fractionation. However, two studies that conducted parallel ^{18}O traced and natural abundance experiments allowed ~~the authors to propose the first~~formulating general oxygen isotope fractionation models (Rohe et al., 2014a; Snider et al., 2013). These models showed that the magnitude of overall isotope fractionation depends not only on the ~~overall~~ extent of oxygen isotope exchange but also on the enzymatic reduction step ~~when it occurs~~associated with this exchange (Fig. 1). It was found that the oxygen isotope exchange is predominantly associated with NIR for fungal denitrification (Rohe et al., 2014a). Fungi and bacteria are characterized by different NOR mechanisms (Schmidt et al., 2004; Stein and Yung, 2003), ~~which result~~resulting in distinct $\delta^{15}N^{sp}$ values for bacterial and fungal denitrification. It ~~can be assumed~~is possible that these ~~differences in~~different NOR mechanisms also influence δ^{18}O~~, but this hypothesis has not been tested yet~~.

~~[Fig. 1]~~

In the present study, we used ^{17}O as tracer to determine the extent of O isotope exchange, in order to separate isotope exchange and apparent isotope effects. We applied a nitrate fertilizer of natural atmospheric deposition origin with high ^{17}O excess, as a result of non-random oxygen isotope distribution. Then we measured ^{17}O excess of the produced N_2O and, based on the observed loss of ^{17}O excess, calculated the extent of isotope exchange with water. Simultaneously, we could measure the $^{18}O/^{16}O$ fractionation in the same incubation vessels, since the ^{17}O tracing method has no impact on $\delta^{18}O$. This is the first time that such an approach has been used ~~and to~~. To validate this method, we applied an alternative approach~~.~~ ~~Namely~~, namely, soil water with distinct $\delta^{18}O$ values within the range of natural abundance isotopic signatures was applied to quantify isotope exchange (Snider et al., 2009).

The latter method has also been applied in a recent soil incubation study (Lewicka-Szczebak et al., 2014) and indicated almost complete oxygen isotope exchange with soil water associated with a stable isotope ratio difference between soil water and produced N_2O of $\delta^{18}O(N_2O/H_2O)$ = (19.0±0.7) ‰. However, the results of other experiments presented in the same study (Lewicka-Szczebak et al., 2014) indicated much higher $\delta^{18}O(N_2O/H_2O)$ values of up to 42 ‰. The higher values may be due to a lower extent of oxygen isotope exchange, but no data were available ~~for~~regarding the extent of exchange for those samples. ~~Interestingly, a tight correlation was found between $\delta^{18}O(N_2O/H_2O)$ and soil moisture (Lewicka-Szczebak et al., 2014), suggesting that the extent of isotope exchange may be influenced by soil moisture.~~ In the present study, ~~this hypothesis has been tested with~~we investigated possible controlling factors for oxygen isotope exchange by applying various experimental ~~results of soil incubations with three different~~treatments differing in soil moisture ~~levels.~~and temperature.

~~The isotope fractionation associated with oxygen isotope exchange is expected to be temperature-dependent, but this assumption has never been tested. Hence, in this study we used incubations at two different temperatures to check the temperature dependence.~~

The combination of various experimental approaches allowed us to further improve the $\delta^{18}O$ fractionation model proposed by Snider et al. (2013) and Rohe et al. (2014a), to decipher the mechanism of oxygen isotope fractionation during N_2O production by denitrification and to determine the associated isotope effects. We investigated the variability of isotope exchange with soil water and of the $\delta^{18}O$ values of produced N_2O under varying conditions as well as the relation between these quantities. Ultimately, our aim was to check to what level of accuracy $\delta^{18}O$ can be predicted based on the known controlling factors.

Additionally, the ^{17}O analyses of N_2O produced by denitrification gave us the opportunity to ~~check~~test the hypothesis of soil denitrification contributing to the non-random distribution of oxygen isotopes (^{17}O excess, or $\Delta^{17}O$) in atmospheric N_2O (Kaiser et al., 2004; Michalski et al., 2003).

2. Methods

2.1. Experimental set-ups

2.1.1. Experiment 1 (Exp 1) – static anoxic incubation

The static incubations were performed under an anoxic atmosphere (N_2) in closed, gas-tight vessels where denitrification products accumulated in the headspace. Two arable soil types were used: a *Luvisol* with loamy sand texture and *Haplic Luvisol* with silt loam texture ~~(same as~~ with pH (in ~~previous study, where more~~0.01 M $CaCl_2$) of 5.7 and 7.4, respectively. More details on soil properties can be found ~~(Lewicka-Szczebak et al., 2014)). The~~in Lewicka-Szczebak et al. (2014). For the first part of these incubations (Exp 1.1) ~~was performed for both soils at~~ two different ~~temperatures~~temperature treatments were applied (8 and 22 ºC) ~~but with~~and only one moisture ~~level~~treatment of 80 % WFPS (water filled pore space). The results of $\delta^{18}O(N_2O)$ analyses for these samples have already been published (Lewicka-Szczebak et al., 2014). Here we expand these data with $\Delta^{17}O(N_2O)$ analyses. The second part of the static incubations (Exp 1.2) was performed for the same two soils ~~but for~~with three different moisture ~~levels~~treatments of 50, 65 and 80 % WFPS ~~(target, for actual values see Table 1)~~ at one temperature (22 ºC). Details on the treatments are presented as supplementary information in Table A1.

This experimental approach is described in detail in Lewicka-Szczebak et al. (2014). In short, the soil was air dried and sieved at 2 mm mesh size. Afterwards, the soil was rewetted to obtain the target WFPS and fertilised with 50 (Exp 1.1) or 10 (Exp 1.2) mg N equivalents (as $NaNO_3$) per kg soil. Various nitrate and water treatments were applied (Table A1). The soils were rewetted using two waters with distinct isotopic signatures: *heavy water* ($\delta^{18}O$ = -1.5 ‰) and *light water* ($\delta^{18}O$ = -14.8 ‰) and fertilized with two different nitrate fertilizers: natural *Chile saltpeter* ($NaNO_3$, Chili Borium Plus, Prills-Natural origin, supplied by Yara, Dülmen, Germany, $\delta^{18}O$ = 56 ‰) and *synthetic* $NaNO_3$ (Sigma Aldrich, Taufkirchen, Germany, $\delta^{18}O$ =

27 ‰). The soils were thoroughly mixed to obtain a homogenous distribution of water and fertilizer and an equivalent of 100 g of dry soil was repacked into each incubation jar at bulk densities of 1.3 g cm^{-3} for the silt loam soil and 1.6 g cm^{-3} for the loamy sand soil. The 0.8 dm^3 Weck jars (J. WECK GmbH u. Co. KG, Wehr, Germany) were used with airtight rubber seals and with two three-way valves installed in their glass cover to enable sampling and flushing. The jars were flushed with N_2 at approximately 500 cm^3 min^{-1} (STP: 273.15 K, 100 kPa) for 10 min to create anoxic conditions. Immediately after flushing, acetylene (C_2H_2) was added to inhibit N_2O reduction in selected jars~~,~~ (C_2H_2 inhibited treatment), by replacing 80 cm^3 of N_2 with C_2H_2, which resulted in 10 kPa C_2H_2 in the headspace. Each treatment (Table 1A) had three replicates. The soils were incubated for approximately 25 hours and three to four samples were collected at 4 to 12 hour-intervals by transferring 30 cm^3 of headspace gases into two pre-evacuated 12 cm^3 Exetainer vials (Labco Limited, Ceredigion, UK). The excess 3 cm^3 of headspace gas in each vial ensured that no ambient air entered the vials. The removed sample volume was immediately replaced by pure N_2 gas.

Additional treatments with addition of ^{15}N-labelled $NaNO_3$ (98 % ^{15}N isotopic purity) were used to control the efficiency of acetylene inhibition and to determine the N_2O mole fraction $f(N_2O) = c(N_2O)/[c(N_2)+c(N_2O)]$ (c: volumetric concentration) in non-inhibited treatments. This method allows determination of the N_2 concentration originating from the ^{15}N labelled pool and hence the N_2O mole fraction (Lewicka-Szczebak et al., 2013).

2.1.2. Experiment 2 (Exp 2) – ~~dynamic~~flow-through incubation under He atmosphere

The ~~dynamic~~flow-through incubations were performed using a special gas-tight incubation system allowing for incubation under N_2-free atmosphere to enable direct quantification of soil N_2 fluxes (Butterbach-Bahl et al., 2002; Scholefield et al., 1997). This system has been described in detail by Eickenscheidt et al. (2014). Four different soils were incubated: two arable soils, same as in Exp 1 (loamy sand and silt loam) and two grassland soils: an organic soil classified as *Histic Gleysol* and a sandy soil classified as *Plaggic Anthrosol*~~.~~, with pH (in 0.01 M $CaCl_2$) of 5.9 and 5.3, respectively. All soils were incubated at the target moisture level of 80 % WFPS and the two most active soils (organic and silt loam soil) were additionally incubated at the lower moisture level of 70 % WFPS (target values, for actual values see Table 2).

The soils were air dried and sieved at 4 mm mesh size. Afterwards, the soil was rewetted to obtain 70 % WFPS and fertilised with 50 mg N equivalents (as $NaNO_3$) per kg soil~~.~~ with natural fertilizer *Chile saltpetre.* The soils were thoroughly mixed to obtain a homogenous distribution of water and fertilizer and 250 cm^3 of wet soil was repacked into each incubation vessel at bulk densities of 1.4 g cm^{-3} for the silt loam soil, 1.6 g cm^{-3} for the loamy sand soil, 1.5 g cm^{-3} for the sandy soil, and 0.4 g cm^{-3} for the organic soil. Afterwards, the water deficit to the target WFPS was added on the top of the soil ~~if needed~~for 80% WFPS treatments. Each treatment had three replicates. The incubation vessels were cooled to 2 ºC and repeatedly evacuated (to 4.7 kPa) and flushed with He to reduce the N_2 background and afterwards flushed with a continuous flow of 20 % O_2 in helium (He/O_2) mixture at 15 cm^3 min^{-1} (STP) for at least 60 hours. When a stable and low N_2 background (below 10 µmol mol^{-1}) was reached, temperature was increased to 22 ºC. During the incubation the headspace was constantly flushed with He/O_2 mixture (first 3 days; Part 1) and then with He (last 2 days; Part 2) at a flow rate of approximately 15 cm^3 min^{-1} (STP). The fluxes of N_2O and N_2 were analyzed immediately (see Sect. ~~2.2).~~2.2) and $f(N_2O)$ was determined. Samples for N_2O isotopocule analyses were collected by connecting the sampling vials in line with the exhaust gas of each incubation vessels and exchanging them at least twice a day. ~~$f(N_2O)$ was determined based on the direct measurement of N_2O and N_2 fluxes.~~ The results presented in this study originate from the anoxic Part 2 of the incubation, since the N_2O fluxes during the Part 1 were too low for $\Delta^{17}O$ analyses. The results for two samples taken approximately 8 and 24 h after switch to anoxic conditions are shown.

2.2. Gas chromatographic analyses

In Exp 1 the samples for gas concentration analyses were collected in Exetainer vials (Labco Limited, Ceredigion, UK) and were analysed using an Agilent 7890A gas chromatograph (GC) (Agilent Technologies, Santa Clara, CA, USA) equipped with an electron capture detector (ECD). Measurement repeatability as given by the relative standard deviation (1σ) of four standard gas mixtures was typically 1.5 %.

In Exp 2, online trace gas concentration analysis of N_2 was performed with a micro-GC (Agilent Technologies, 3000 Micro GC), equipped with a thermal conductivity detector (TCD) and N_2O was measured with a GC (Shimadzu, Duisburg, Germany, GC–14B) equipped with ECD detector. The measurement repeatability (1σ) was better than 0.02 µmol mol^{-1} for N_2O and 0.2 µmol mol^{-1} for N_2.

2.3. Isotopic analyses

2.3.1. Isotopocules of N_2O

Gas samples were analyzed using a Delta V isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to automatic preparation system: Precon + Trace GC Isolink (Thermo Scientific, Bremen, Germany) where N_2O was preconcentrated, separated and purified. In the mass spectrometer, N_2O isotopocule signatures were determined by measuring *m/z* 44, 45, and 46 of intact N_2O^+ ions as well as *m/z* 30 and 31 of NO^+ fragments ions. This allows the determination of average $\delta^{15}N^{av}$, $\delta^{15}N^{\alpha}$ ($\delta^{15}N$ of the central N position of the N_2O molecule), and $\delta^{18}O$ (Toyoda and Yoshida, 1999). $\delta^{15}N^{\beta}$ ($\delta^{15}N$ of the peripheral N position of the N_2O molecule) is calculated using $\delta^{15}N^{av} = (\delta^{15}N^{\alpha} + \delta^{15}N^{\beta}) / 2$. The ^{15}N site preference ($\delta^{15}N^{sp}$) is defined as $\delta^{15}N^{sp} = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$. The scrambling factor and ^{17}O-correction were taken into account (Kaiser and Röckmann, 2008; Röckmann et al., 2003). Pure N_2O (Westfalen, Münster, Germany) was used as internal reference gas and was analyzed in the laboratory of the Tokyo Institute of Technology using calibration procedures reported previously (Toyoda and Yoshida, 1999; Westley et al., 2007). Moreover, the comparison materials from an intercalibration study (S1, S2) were used to perform a two-point calibration (Mohn et al., 2014). For correction of non-linear effect due to variable sample amount five different standard gas mole fractions (0.3, 1, 5, 10, 20 µmol mol^{-1}) were analyzed in each sample run. Samples with similar N_2O mole fractions were run together with at least two standard gases with similar mole fractions.

All isotopic signatures are expressed as relative deviation (in ‰) from the $^{15}N/^{14}N$, $^{17}O/^{16}O$ and $^{18}O/^{16}O$ ratios of the reference materials (*i.e.*, atmospheric N_2 and Vienna Standard Mean Ocean Water (VSMOW), respectively). The measurement repeatability (1σ) of the internal standard (filled into vials and measured in the same way as the samples) for measurements of $\delta^{15}N^{av}$, $\delta^{18}O$ and $\delta^{15}N^{sp}$ was typically 0.1, 0.1, and 0.5 ‰, respectively.

2.3.2. $\delta^{18}O$ of NO_3^-

Soil nitrate was extracted in 0.01 M aqueous $CaCl_2$ solution (weight ratio soil:solution 1:10) by shaking at room temperature for one hour. $\delta^{18}O$ of nitrate in the soil solution was determined using the bacterial denitrification method (Casciotti et al., 2002). The

measurement repeatability (1σ) of the international standards (USGS34, USGS35, IAEA-NO-3) was typically 0.5 ‰ for $\delta^{18}O$.

2.3.3. $\Delta^{17}O$ excess in N_2O and NO_3^-

N_2O samples collected from soil incubation and N_2O produced from soil NO_3^- by the bacterial denitrifier method ~~was~~were analysed for $\Delta^{17}O$ using the thermal decomposition method (Kaiser et al., 2007) with a gold oven (Exp 1.1b,c and 1.2a,b) and with a gold-wire oven (Exp 1.1a and 2) (Dyckmans et al., 2015) . The ^{17}O excess, $\Delta^{17}O$, is defined as (Kaiser et al., 2007):

$$\Delta^{17}O = \frac{1+\delta^{17}O}{(1+\delta^{18}O)^{0.5279}} - 1 \qquad (1)$$

The measurement repeatability (1σ) of the international standards (USGS34, USGS35) was typically 0.5 ‰ for $\Delta^{17}O$.

2.3.4. *Soil water analyses*

Soil water was extracted with the method described by Königer et al. (2011) and $\delta^{18}O$ of water samples (with respect to VSMOW) was measured using cavity ringdown spectrometer Picarro L1115-*i* (Picarro Inc., Santa Clara, USA). The measurement repeatability (1σ) of the internal standards (three calibrated waters with known $\delta^{18}O$: -19.67 ‰, -8.60 ‰, +1.37 ‰) was below 0.1 ‰. The overall error associated with the soil water extraction method determined as standard deviation (1σ) of the 5 samples replicates was below 0.5 ‰.

2.4. Determination of the extent of isotope exchange

The extent of isotope exchange (x) was determined with two independent methods described below. In Exp 1 both approaches were applied simultaneously on the same soil samples, which allowed quantifying the oxygen isotope exchange with two different methods independently. This enabled the validation of the ^{17}O excess method, which was used here for the first time for quantification of isotope exchange. Afterwards this validated method was applied in the following Exp 2. For both presented methods it is assumed that after N_2O is formed, no further ~~O~~oxygen isotope exchange ~~between N_2O and~~with H_2O occurs.

2.4.1. $\delta^{18}O$ method

This method determines the isotope exchange based on the relative difference between $\delta^{18}O$ of produced N_2O and its potential precursors: soil water and soil nitrate (Snider et al., 2009). To make this method applicable, parallel incubations with distinct water and/or nitrate isotopic signatures must be carried out. ~~In Exp 1 this was achieved by rewetting the soils with two different waters of distinct isotopic signatures: *heavy water* ($\delta^{18}O$ = -1.5 ‰) and *light water* ($\delta^{18}O$ = -14.8 ‰) and by adding two different nitrate fertilizers: natural *Chile saltpeter* ($NaNO_3$, Chili Borium Plus, Prills Natural origin, supplied by Yara, Dülmen, Germany, $\delta^{18}O$ = 56 ‰) and *synthetic* $NaNO_3$ (Sigma Aldrich, Taufkirchen, Germany, $\delta^{18}O$ = 27 ‰).~~

Therefore, treatments with different water and nitrate isotopic signatures were applied in Exp. 1 (Table 1, Table A1). The calculation is based on two end member mixing model (water (δ_w) and nitrate (δ_n); δ stands for $\delta^{18}O(N_2O)$) taking into account the isotope fractionation associated with O atom incorporation into N_2O from each end member (ε_w - fractionation associated with oxygen isotope exchange with water, ε_n - fractionation associated with branching effect during nitrate reduction). This is expressed as:

$$1+\delta = x(1+\delta_w)(1+\varepsilon_w)+(1-x)(1+\delta_n)(1+\varepsilon_n) \quad (2)$$

which can be rearranged to:

$$\frac{\delta-\delta_n}{1+\delta_n} = x(1+\varepsilon_w)\frac{\delta_w-\delta_n}{1+\delta_n} + x\varepsilon_w + (1-x)\varepsilon_n \quad (3)$$

where:

$\frac{\delta-\delta_n}{1+\delta_n} = \delta^{18}O(N_2O/NO_3^-) =$ dependent variable of the linear regression

$\frac{\delta_w-\delta_n}{1+\delta_n} = \delta^{18}O(H_2O/NO_3^-) =$ independent variable of the linear regression

$x(1+\varepsilon_w) =$ slope of the linear regression ≅ the magnitude of isotope exchange (x)

$x\varepsilon_w + (1-x)\varepsilon_n =$ intercept of the linear regression ≅ total fractionation (ε)

Hence, from the linear correlation between $\delta^{18}O(N_2O/NO_3^-)$ and $\delta^{18}O(H_2O/NO_3^-)$ we can ~~read~~ approximate x (the deviation from the exact value may be up to 0.02, for ε_w < 20 ‰) and the total fractionation ε comprised of both ε_w and ε_n.

2.4.2. $\Delta^{17}O$ method

This method determines the isotope exchange based on the comparison of $\Delta^{17}O$ in soil nitrate and produced N_2O. It requires the application of nitrate characterised by high $\Delta^{17}O$. ~~In Exps 1 and 2~~Therefore, soils were amended with natural $NaNO_3$ *Chile saltpeter* showing high $\Delta^{17}O$ (ca. 20 ‰) and ~~with synthetic $NaNO_3$ showing slight negative $\Delta^{17}O$ (ca. -5 ‰) and~~ the $\Delta^{17}O$ of the N_2O product was measured. $\Delta^{17}O$ of soil water was assumed to be 0 ‰.

The magnitude of oxygen isotope exchange (x) was calculated as:

$$x = 1 - \frac{\Delta^{17}O(N_2O)}{\Delta^{17}O(NO_3^-)} \quad x = 1 - \frac{\Delta^{17}O(N_2O)}{\Delta^{17}O(NO_3^-)} \qquad (4)$$

The error due to the use of the power-law definition of $\Delta^{17}O$ in combination with a linear mixing relationship (Eq. (4)) causes a negligible relative bias of <1 % for x.

2.5. Correction for N_2O reduction

Since $\delta^{18}O(N_2O)$ values of emitted N_2O are strongly affected by partial N_2O reduction, the measured isotope values can only be informative for the mechanism of N_2O production if the reduction is inhibited or the isotope effects associated with reduction are taken into account. ~~In~~ Exp 1~~.2 N_2O reduction was completely~~ , where we applied both C_2H_2-inhibited~~, whereas in Exp 1.1 we had~~ as well as uninhibited treatments ~~with and without inhibition. Exp 1.1 thus~~(Table 1), allows us to check the validity of our correction methods as it directly yields the impact of N_2O reduction on the measured $\delta^{18}O(N_2O)$ values. In Exp 2, reduction was not inhibited and the mathematical correction described below was applied.

The correction was made using the Rayleigh fractionation equation (Mariotti et al., 1981):

$$\frac{1+\delta_S}{1+\delta_{S0}} = f^{\varepsilon} \qquad (5)$$

where: δ_S – isotopic signature of the remaining substrate, here: measured $\delta^{18}O$ of the final, partially reduced, N_2O, δ_{S0} – initial isotopic signature of the substrate, here: $\delta^{18}O$ of the produced N_2O unaffected by the reduction ($\delta_0{}^{18}O$); to be calculated; f – remaining unreacted fraction, here: the N_2O mole fraction $f(N_2O)$; directly measured; ε – isotope effect between product and substrate, here: $\varepsilon(N_2/N_2O)$, the isotope effect associated with N_2O reduction,

taken from the literature (Lewicka-Szczebak et al., 2014). As it has been shown that the experimental approach largely influences O isotope effect during reduction (Lewicka-Szczebak et al., 2015; Lewicka-Szczebak et al., 2014), we used different $\varepsilon^{18}O(N_2/N_2O)$ values for static and ~~dynamic conditions.~~flow-through incubations. For the static Exp. 1 a mean $\varepsilon^{18}O(N_2/N_2O)$ value of -17.4 ‰ is used, based on one common experiment between the study of Lewicka-Szczebak et al. (2014) (Experiment 1) and this study (Exp 1.1). For the ~~dynamic~~flow-through Exp 2 we accept the $\varepsilon^{18}O(N_2/N_2O)$ value of -12 ‰ recently determined for ~~a dynamic~~similar flow-through experiments under He/O_2 atmosphere (Lewicka-Szczebak et al., 2015). For the correction of $\delta^{15}N^{sp}$ values one common $\varepsilon^{15}N^{sp}(N_2/N_2O)$ value of -5 ‰ was used, since it was shown that this value is applicable for all experimental setups (Lewicka-Szczebak et al., 2014). The error due to the simplified use of $\varepsilon^{15}N^{sp}$ for the Rayleigh model (Eq. (5)) instead of separate calculations with $\varepsilon^{15}N^{\alpha}$ and $\varepsilon^{15}N^{\beta}$, causes a negligible bias of the calculated $\delta_0{}^{15}N^{sp}$ values of <0.15 ‰ for the presented dataset.

2.6. N_2O isotopic signatures related to water

Relative isotope ratio differences between N_2O and soil water, $\delta^{18}O(N_2O/H_2O)$, were calculated as the difference between the measured $\delta^{18}O$ of produced N_2O and of soil water:

$$\delta^{18}O(N_2O/H_2O) = \frac{\delta^{18}O(N_2O) - \delta^{18}O(H_2O)}{1+\delta^{18}O(H_2O)} \quad (6)$$

In samples where N_2O reduction occurred $\delta^{18}O(N_2O/H_2O)$ values were corrected as described above (Sect. 2.5) and for statistical analyses and modelling exercises the reduction-corrected values were used ($\delta_0{}^{18}O(N_2O/H_2O)$).

~~2.6.~~2.7. Statistical methods

For results comparisons, ANOVA variance analysis was used with the significance level α of 0.05. The uncertainty values provided for the measured parameters represent the standard deviation (1σ) of the replicates. The propagated uncertainty was calculated using Gauss' error propagation equation taking into account standard deviations of all individual parameters.

3. Results & Discussion

3.1. Exp 1

In Table 1 the results are presented as average values from three replicated incubation vessels with respective standard deviation. Soil nitrate and water were analysed at the beginning of the experiment from the prepared homogenised soils, hence no standard deviation but the standard analytical uncertainty is given. Relative isotope ratio differences between N_2O and soil water, $\delta^{18}O(N_2O/H_2O)$, were calculated as the difference between the measured $\delta^{18}O$ in produced N_2O and soil water:

$$\delta^{18}O(N_2O/H_2O) = \frac{\delta^{18}O(N_2O) - \delta^{18}O(H_2O)}{1+\delta^{18}O(H_2O)} \quad (6)$$

In samples where N_2O reduction occurred these values were corrected as described above (Sect. 2.5) and for statistical analyses and modelling exercises the reduction-corrected values were used ($\delta_0^{18}O(N_2O/H_2O)$).

For different temperature treatments, x (determined by the $\Delta^{17}O$ method) was not significantly different (p = 0.19) but $\delta^{18}O$ $\delta_0{}^{18}O(N_2O/H_2O)$ was slightly higher (p = 0.009) for 8 ºC ((19.5±0.3) ‰) than for 22 ºC ((18.6±0.3) ‰) treatment. No significant differences were observed between the two analysed soil types or between various soil moisture levels.

When comparing Exp 1.1 and 1.2, x did not show any significant differences, but the $\delta_0{}^{18}O(N_2O/H_2O)$ values were significantly different (p < 0.001) with higher values for Exp 1.1 ((19.1 ± 0.5) ‰) than for Exp 1.2 ((16.9 ± 0.8) ‰). It should be noted that the $\delta^{18}O$ values of soil nitrate were much lower in Exp 1.2 (from -2.0 to 6.5 ‰) when compared to Exp 1.1 (from 31.8 to 42.6 ‰) which might have affected the observed differences in $\delta^{18}O$ $\delta_0{}^{18}O(N_2O/H_2O)$.

[Table 1]

3.2. Exp 2

Moreover, forIn Table 2 the results are presented as average values from three replicate incubation vessels with respective standard deviation. The extent of oxygen isotope exchange (x) ranges from 55 to 85 % and is lower and much more variable when compared to Exps 1.1 and 1.2. $\delta_0{}^{18}O(N_2O/H_2O)$ varies between 18.6 and 36.9 ‰, which is significantly higher when compared to the values determined in Exp 1.

[Table 2]

4. Discussion

4.1. Determination of oxygen isotope exchange

For Exp 1 the $\delta^{18}O$ method was applied to estimate x and ε from the relationship between $\delta^{18}O(N_2O/NO_3)$ and $\delta^{18}O(H_2O/NO_3)$ as described in 2.4.1.

[Fig. 2]

According to this method, from the linear regression one can decipher x (slope) and ε (intercept) (Snider et al., 2009). The correlation is excellent (R^2 from 0.989 to 0.997) which indicates that the x and ε are very stable for all the treatments (Fig. 2). The x is about 1 (complete exchange) and ε varies from 17.1 (Exp 1.2) to 18.2 ‰ (Exp 1.1). When compared to the results presented in Table 1, we see slightly higher isotope exchange with $\delta^{18}O$ method when compared to $\Delta^{17}O$ method. This may be partially due to the fact that the slope in $\delta^{18}O$ method (Fig. 2) is actually slightly higher than x (from Eq. (3): $x(1+\varepsilon_w)$). But theThe difference between the two experiments is mostly within the error of each method, so far the results are consistent. The $\Delta^{17}O$ method is more useful, since it allows for individual determinations of x, whereas the correlation obtained from the $\delta^{18}O$ method is based on all data, hence provides a mean result for x and ε for a whole experiment.

Importantly, we found that the $\delta^{18}O$ method is not applicable forto samples with uninhibited N_2O reduction, if $\delta^{18}O(N_2O)$ values are not corrected for N_2O reduction. The treatment with uninhibited reduction of Exp 1.1 was tested and provided very different results, *i.e.* largely

overestimated x (1.5) and ε (44.8) (red dashed fit line, Fig.2). Hence, for proper determination of these factors the results from treatments with inhibited N_2O reduction were used (solid black fit line, Fig.2). However, the $\delta^{18}O$ values after mathematical correction for N_2O reduction (red '+' points, Fig.2) fitted very well to the correlation found for inhibited samples. Hence, the reduction corrected values ($\delta_0{}^{18}O(N_2O)$) should rather be used when applying this method in experiments with uninhibited N_2O reduction. Moreover, in both static experiments we used the C_2H_2 inhibition technique, and our results indicate almost complete exchange of oxygen isotopes with soil water, which indicates ~~clearly~~ that the isotope exchange process is not inhibited by C_2H_2 addition.

3.2. Exp 2

In Table 2 the results are presented as average values from three replicate incubation vessels with respective standard deviation. The extent of oxygen isotope exchange (x) ranges from 55 to 85 % and is lower and much more variable when compared to Exp 1. $\delta_0{}^{18}O(N_2O/H_2O)$ varies between 18.6 and 36.9 ‰, which is significantly higher when compared to the values determined in Exp 1.

[Table 2]

~~4.2.~~3.3. Oxygen isotope effects at nearly complete isotope exchange

In case of very high, almost complete, isotope exchange with soil water (Exp 1), the relative isotope ratio difference between N_2O and H_2O ($\delta_0{}^{18}O(N_2O/H_2O)$) is quite stable and ranges from 15.6 to 19.8 ‰ (Table 1). In contrast, the relative isotope ratio difference between N_2O and NO_3^- ($\delta_0{}^{18}O(N_2O/NO_3^-)$) shows large variations from -36.1 to 18.0 ‰ (Fig. 3).

[Fig. 3]

ε determined in Fig.2 represents theoretically the total oxygen isotope fractionation (from Eq. (3): $x\varepsilon_w + (1–x)\varepsilon_n$), but in case of the nearly whole isotope exchange (x = 1) ε equals ε_w and ε_w

= (δ_{N2O} - δ_w)/(δ_w+1) = $\delta^{18}O(N_2O/H_2O)$, hence both - the intercept in Fig. 2 and $\delta^{18}O(N_2O/H_2O)$ in Fig. 3 should provide rough estimates for ε_w. However, for x<1 $\delta^{18}O(N_2O/H_2O)$ depends also on δ_n and ε_n and the intercept (Fig.2) includes ε_n. Both these values indicate a slight difference between both experiments, for Exp 1.1 ε of (18.2±0.6) (intercept, Fig.2) and $\delta^{18}O(N_2O/H_2O)$ of (19.1±0.5) (mean±SD, Table 1) are higher than for Exp 1.2, (17.1±0.3) and (16.7±0.8), respectively. This slight difference is most probably due to *x* slightly lower than 1, as indicated by $\Delta^{17}O$ method and additional impact of δ_n and ε_n. It can be noted that $\delta_0{}^{18}O(N_2O/H_2O)$ slightly increases with higher $\delta^{18}O$ values of nitrate (Fig. 3), *i.e.* the difference of about 40 ‰ in $\delta^{18}O$ of applied NO_3^- results in about 2 ‰ change in $\delta_0{}^{18}O(N_2O/H_2O)$. Hence, only about 5 % of the difference in nitrate isotopic signature is reflected in the produced N_2O, suggesting that an equivalent percentage of $O(N_2O)$ originated from NO_3^-. This is very consistent with the determined extent of isotope exchange with soil water, which was (95.6±2.6) % (Table 1).

Taken together, the data indicates that the $\delta^{18}O(N_2O)$ values are clearly influenced by the $\delta^{18}O$ of soil water, whereas $\delta^{18}O$ of soil nitrates has only very little influence. Hence, the O isotope fractionation during N_2O production by denitrification should be considered in relation to soil water, rather than soil nitrates.

4.3.3.4. Oxygen isotope effects at variable isotope exchange

In contrast to the above presented results, Section 3.3, *x* was more variable for the dynamicflow-through incubation (Exp. 2), *x* was more variable) and also significantly lower. In general, the lower *x* was associated with higher $\delta_0{}^{18}O(N_2O/H_2O)$ values. In Fig. 4 we can compare results from static incubations (red symbols) with the dynamicflow-through incubations (black symbols). This comparison clearly shows that the pattern of isotope exchange and the associated oxygen fractionation differs significantly between both experimental approaches. The essential difference in Exp 2 was the use of a flow-through system and ofwith an oxic atmosphere at the beginning of the incubation (though results presented originate from the anoxic phase). This resulted in lower production rates for N_2O when comparing the respective soil (Table 1 and 2), *e.g.*, 80 μg kg^{-1} h^{-1} (mass of N as sum of N_2O and N_2 per mass of dry soil) for the silt loam soil at 80 % WFPS in Exp 2.3 but 261 μg kg^{-1} h^{-1} in Exp 1.1c. This may suggest an impact of N_2O production rate on extent of isotope exchange. However, for static experimentsanoxic incubations the effect of production rate

was not observed, *e.g.* between 1.1a and 1.1b (Table 1), where we have different production rates but similar x and $\delta_0{}^{18}O(N_2O/H_2O)$. ~~Hence, we rather suppose that the trend observed here may be due to activity of different microorganism groups, which have been activated by oxic atmosphere in Exp 2 and are characterised by lower x and higher $\delta_0{}^{18}O(N_2O/H_2O)$.~~

[Fig. 4]

Interestingly, the correlation between x and $\delta_0{}^{18}O(N_2O/H_2O)$ seems to differ for different soil types. Very clearly both sandy soils represent distinct and weaker correlation when compared to silt loam and organic soil. Most probably this is due to different oxygen fractionation pattern during N_2O formation in both soils, which we try to ~~decipher~~elucidate in the theoretical model presented below.

3.5 The mechanism of oxygen isotope fractionation – a fractionation model

To better understand the mechanism of oxygen isotope fractionation and the relation between the apparent isotope effect and the extent of isotope exchange we applied a simulation calculation where the total isotope effect was calculated from the theoretical isotope fractionation associated with two enzymatic reduction steps: NIR and NOR. This model was based on the calculations presented by Rohe et al. (2014a) for pure fungal cultures, where this approach has been described in detail. The model assumes that $\delta^{18}O(N_2O)$ is determined by two isotope fractionation processes associated (i) with the branching isotope effect (ε_n) and (ii) with the isotope effect due to isotope exchange with soil water (ε_w), both possible at NIR or NOR. This can be expressed by the following isotope mass balance equations:

$$1+\delta = x_{NOR}(1+\delta_w)(1+\varepsilon_w)+(1-x_{NOR})(1+\delta_{NO})(1+\varepsilon_{NOR}) \quad (7)$$

$$1+\delta_{NO} = x_{NIR}(1+\delta_w)(1+\varepsilon_w)+(1-x_{NIR})(1+\delta_n)(1+\varepsilon_{NIR}) \quad (8)$$

where:

$$1-x = (1-x_{NIR})(1-x_{NOR}) \quad (9)$$

$$1+\varepsilon_n = (1+\varepsilon_{NIR})(1+\varepsilon_{NOR}) \quad (10)$$

After substitution and transformation, this gives

$$\frac{\delta-\delta_w}{1+\delta_w} = (1-x)(1+\varepsilon_n)\frac{\delta_n-\delta_w}{1+\delta_w} + (x-x_{NOR})\varepsilon_{NOR}(1+\varepsilon_w) + x\varepsilon_w + (1-x)\varepsilon_n \quad (11)$$

We ~~have~~ neglected the possible fractionation associated with the NAR reduction, *i.e.* $\delta(NO_2^-) = \delta(NO_3^-) = \delta_n$ in Eq. (11). This enzymatic step was investigated by Rohe et al. (2014a), and appeared to have ~~very minor~~no significant impact on the total oxygen fractionation, *i.e.* ~~this step~~the branching fractionation for nitrate treatments was ~~relevant only for one fungus species~~in no case higher than for nitrite treatment. This indicates that the oxygen fractionation between nitrate and nitrite is low due to cancellation of the intramolecular effect of about 30 ‰ (Casciotti et al. 2007) by the intermolecular effect when the nitrate pool is not completely consumed. Hence, we only focused here on differentiating between NIR and NOR enzymatic reduction steps, which are most likely the enzymatic reactions crucial for determining final N_2O isotopic values (Kool et al., 2007).

There are ~~a lot of~~many unknown factors in the Eq. (11); first of all, isotopic fractionation factors ε_n and ε_w. We have compiled the results of both methods applied for Exp 1 data: □^{18}O method and Δ^{17}O method to estimate these factors. Using δ^{18}O method ε was determined from the intercept in Fig. 2 and this value represents total fractionation: $\varepsilon = x\,\varepsilon_w + (1-x)\,\varepsilon_n$ (see Sect. 2.4.1). Using the Δ^{17}O method ~~the,~~ individual x ~~was~~values were calculated for each sample. We have also measured $\delta^{18}O(N_2O/H_2O)$ and $\delta^{18}O(NO_3^-/H_2O)$ for each sample, hence from the transformed Eq. (3):

$$\frac{\delta-\delta_w}{1+\delta_w} = (1-x)(1+\varepsilon_n)\frac{\delta_n-\delta_w}{1+\delta_w} + x\varepsilon_w + (1-x)\varepsilon_n \quad (12)$$

and knowing that $x\,\varepsilon_w + (1-x)\,\varepsilon_n$ = 0.~~0181~~0182 for Exp 1.1 and $x\,\varepsilon_w + (1-x)\,\varepsilon_n$ = 0.~~0172~~0171 for Exp 1.2 (Fig. 2) we have calculated ε_w and ε_n for each sample. Table 3 summarises the results:

[Table 3]

The determination of ε_w is very precise, with no significant difference between Exp 1.1 and 1.2 (p~~=~~ = 0.868). The value obtained (17.5±0.7) ‰ is within the range of the previous values

determined for chemical exchange $\varepsilon(NO_2^-/H_2O)$ = 14 ‰ and $\varepsilon(NO_3^-/H_2O)$ = 23 ‰ (Böhlke et al., 2003; Casciotti et al., 2007). So far there are no data for the isotope effect of chemical exchange $\varepsilon(NO/H_2O)$. ~~The~~ Therefore, we assumed equal ε_w values for isotope exchange associated with NIR and NOR, similarly to previous studies (Rohe et al., 2014a; Snider et al., 2012). Hence, the ε_w value determined here is a hypothetical mean value of enzymatically mediated isotope exchange associated with NIR ($\varepsilon_w(NO_2^-/H_2O)$) and NOR ($\varepsilon_w(NO/H_2O)$).

ε_n is also quite stable with a weak (p~~=~~ = 0.006) and very small (below 1 ‰) difference between Exp 1.1 and 1.2. The ε_n values found are very low and vary around 0, from -1.9 to 2.1 ‰. This is much lower ~~compared to~~than in previous studies, which reported ε_n from 10 to 30 ‰ (Casciotti et al., 2007; Rohe et al., 2014a).

We checked how well these calculated values fit for the individual samples of both experiments. We started with the simplest Scenario 0, where we assume the values determined in Table 3 for ε_w and ε_n and calculate the $\delta^{18}O(N_2O)$ with Eq. (11), which is then compared with the measured $\delta^{18}O(N_2O)$ and the difference between measured and calculated $\delta^{18}O(N_2O)$ value (D) is determined (Table 4). Since the mean value of 0 was assumed for ε_n in this scenario, the isotope exchange can be associated either with NIR or NOR without any effect on the final $\delta^{18}O(N_2O)$, because ~~the~~ Eq. (11) is simplified to:

$$\frac{\delta-\delta_w}{1+\delta_w} = (1-x)\frac{\delta_n-\delta_w}{1+\delta_w} + x\varepsilon_w \qquad (13)$$

This scenario works quite well for Exp 1 data with the maximal D of 1.4 ‰. However, for Exp 2 data we obtain significant overestimation of the calculated $\delta^{18}O(N_2O)$ values for sandy soils (Exp 2.1 and 2.2) up to 6.1 ‰ and underestimation for two other soils, reaching up to 12.2 ‰ for organic soil (Exp 2.5). Why the model developed based on Exp 1 data do not work for Exp 2 data? We expect that the ε_w value should be quite stable for all the samples. It was observed in the study by Casciotti et al. (2007) that $\varepsilon(NO_2^-/H_2O)$ values varied in a very narrow range. Also in our study in Fig. 2 we obtained very good correlation with stable slope which suggests that the ε_w value must be very stable and almost identical for all the samples. It can be supposed that rather ε_n values can be more variable, but due to nearly complete isotope exchange in Exp 1 these potential variations cannot be reflected in $\delta^{18}O(N_2O)$ values. Also, the ~~previous~~ study by Rohe et al. (2014a) indicated possibly wide variations of ε_n from 10 to 30 ‰.

[Table 4]

Therefore, for the next scenarios (Scenario 1, 2 and 3 - Table 4) we assumed stable ε_w value of 17.5 ‰, as determined from Exp 1 (Table 3) and ε_n values were calculated individually for each sample with Eq. (11) from the $\delta_0{}^{18}O(N_2O/H_2O)$ values. In each scenario ε_n was equally distributed between NIR and NOR according to Eq. (10), so that $\varepsilon_{NIR} = \varepsilon_{NOR}$. For our samples we know the value of total isotope exchange (x determined with $\Delta^{17}O$ method), but we do not know at which enzymatic step(s) this exchange occurred. Since the isotope exchange has very different impact on the final $\delta^{18}O(N_2O)$ when associated with NIR or NOR, we can obtain this information by comparing different scenarios (Table 4). In Scenario 1 the total isotope exchange is associated with the first reduction step NIR and in Scenario 2, with the final reduction step NOR. In Scenario 3 the total isotope exchange is equally distributed between both steps NIR and NOR according to Eq. (9) so that $x_{NIR} = x_{NOR}$. ~~Actually, in this study we cannot precisely determine the enzymatic step where the isotope exchange occurs, but rather the relative relation between the both isotope effects. Namely, in Scenario 1 the exchange effect associated with x_{NIR} precedes the branching effect at NOR (ε_{NOR}) and, conversely, in Scenario 2 the exchange isotope effect associated with x_{NOR} occurs later than the both branching effects (ε_{NIR}, ε_{NOR}). Hence, in Scenario 1 the ε_{NOR} has more direct impact on the final $\delta^{18}O(N_2O)$ whereas in Scenario 2 the last fractionation step is due to ε_w (Eq. (11)). Therefore, applying different scenarios results in different values of calculated ε_n (Table 4).~~

In this study, we could not determine at which enzymatic step isotope exchange occurs, but only its impact on the implied isotope effects. Namely, in Scenario 1 the exchange effect associated with x_{NIR} precedes the branching effect at NOR (ε_{NOR}) and, conversely, in Scenario 2 the exchange isotope effect associated with x_{NOR} occurs after both branching effects (ε_{NIR}, ε_{NOR}). Hence, in Scenario 1 ε_{NOR} has a more direct impact on the final $\delta^{18}O(N_2O)$ whereas in Scenario 2 the last fractionation step is related to ε_w (Eq. (11)). Therefore, applying different scenarios results in different values for the calculated ε_n (Table 4).

The narrowest range of variations of the calculated ε_n values was obtained in Scenario 1. For Exp 1 they vary around 0, similarly to the results presented in Table 3, which indicates that this model and the equations applied for $\delta^{18}O$ method (Eq. (12)) are actually the same. For

Exp 2 the calculated ε_n values are negative for sandy soils (Exp 2.1 and 2.2) from -9.1 to -6.2 ‰ and positive for other soils with lower values for silt loam from 1.6 to 3.8 ‰ and higher for organic soil from 3.8 to 18.1 ‰ (Table 4). Variations of calculated ε_n values are much larger in Scenario 2 with ~~especially very~~a particularly wide range for Exp 1 from -72.8 to +38.5 ‰. For Exp. 2, a similar trend as in Scenario 1 is observed, with negative values for sandy soils (down to -20.0 ‰) and highest values for organic soil (up to 37.1 ‰). The absolute values are generally larger and the variations among them are thereby increased when compared to Scenario 1. The strongly negative ε_n values obtained ~~in Scenario 2 are rather out of the plausible range of values.~~for Scenario 2 are outside the range of plausible range based on previous determinations (Casciotti et al., 2007; Rohe et al., 2014a). Moreover, for the last sample of Exp 1 where x=1 this scenario fails in finding the ε_n value for D=0, because for ~~the~~ complete isotope exchange ~~at x_{NOR}~~by NOR, the associated branching isotope effect has no impact on the final $\delta^{18}O(N_2O)$. ~~However, the residual D = 0.2 ‰ is very low, which do not exclude this scenario.~~ But still Scenario 1 is more plausible because (i) the overall ε_n variations are smaller and (ii) we do not find extremely negative values. Results from Scenario 3 are situated in the middle of Scenario 1 and 2, and show larger variations than Scenario 1, but without the extreme outliers, hence can be also a plausible model. From comparison of these scenarios we can say that ~~the~~ isotope exchange is ~~definitely~~likely associated with NIR and may also partially take place at ~~both steps~~ NOR (but not ~~solely at~~ NOR~~.~~ alone). This reinforces the previous findings from pure culture studies which suggested the majority of isotope exchange associated mainly with nitrite reduction (Garber and Hollocher, 1982; Rohe et al., 2014a). Moreover, each scenario indicates clearly a much lower branching effect for the two sandy soils in Exp. 2 when compared to silt loam and organic soil. This is the reason behind the different slope of correlation $\delta_0^{18}O(N_2O/H_2O)$ *vs.* x in Fig. 4 for sandy soils. Lower ε_n values mean that N_2O is less enriched in ^{18}O in relation to soil nitrate and lower x results in smaller increase in $\delta^{18}O(N_2O)$ values, which was observed for sandy soils (Fig.4).

For each scenario our model indicated rather lower ε_n values than previously assumed (Casciotti et al., 2007; Rohe et al., 2014a). But actually, the isotope effect determined by Casciotti et al. (2007), +25 to +30 ‰, takes only the intra-molecular branching effect into account, because in the bacterial denitrification method the whole nitrate pool is quantitatively consumed, hence the inter-molecular isotope effect cannot manifest. Therefore,

the values found by Casciotti et al. (2007) represent the maximal possible branching effect. In the experiment presented by Rohe et al. (2014a) only very little ~~of~~ added substrate was reduced, hence we should also observe the inter-molecular isotope effects. Indeed, the ~~values for ε_{NIR} were~~model applied by Rohe et al. (2014a) indicated lower magnitudes for net branching, down to +10 ‰ for ε_{NIR} and 0 ‰ for ε_{NAR} ~~was assumed 0 ‰.~~. This may suggest that the net branching effect decreases with smaller reaction rates because of inter-molecular isotope ~~fractionation.~~effects. But are ~~the~~ negative net branching effects actually possible? ~~It could be the case only if~~ The answer is yes, provided that the inter-molecular effect exceeds the intra-molecular effect, *i.e.* the former must be more negative than -30 ‰. An idea about the magnitude of the ~~intra-molecular~~intermolecular effect can be obtained from the change in isotopic signature of the remaining nitrate, since this reflects the enrichment in residual nitrate-^{18}O due to ~~intra-molecular~~intermolecular effects. In pure culture studies this effect ranges from -23 to -5 ‰ (Granger et al., 2008), but in soil incubations values as low as -37 ‰ ~~have~~has been observed (Exp. 1F in Lewicka-Szczebak et al. (2014)). Hence, slightly negative net ε_n ~~is~~values are theoretically possible, but up to a few ‰ for each enzymatic step, which gives the minimal overall ε_n of about -10 ‰. Therefore, the results of Scenario 2 must be rejected, whereas the values found in Scenario 1 are most plausible.

3.6 Significance for quantification and differentiation of soil denitrification

From the presented results it is most surprising and incomprehensible, why the same soils show various extents of isotope exchange with soil water, and especially, why this exchange was high and stable ~~in~~under static ~~experiment~~anoxic conditions and ~~decreases by dynamic~~significantly lower in flow-through incubations. Most probably, in the static inhibited experiments denitrification is the only N_2O producing process and in the ~~dynamic~~flow-through uninhibited incubations other N_2O producing processes may significantly contribute to N_2O production. These incubations were performed initially under oxic conditions, which were switched to anoxic conditions after three days. However, all the results presented here originate from this anoxic phase, since the N_2O production during oxic phase was too low for $\Delta^{17}O$ analyses. Hence, the potentially contributing processes might be fungal denitrification, co-denitrification, nitrifier denitrification or dissimilatory nitrate reduction to ammonium (DNRA). ^{15}N site preference ($\delta^{15}N^{sp}$) may be used as a tracer to distinguish some of these processes. It is known that fungal denitrification and nitrification are characterized by significantly higher $\delta^{15}N^{sp}$ values (33 to 37 ‰ (Rohe et al., 2014a; Sutka et al., 2008; Sutka et

al., 2006)) when compared to bacterial denitrification and nitrifier denitrification (-11 to 0 ‰ (Sutka et al., 2006; Toyoda et al., 2005)). To check the hypothesis of mixing of N_2O from various sources we plotted $\delta_0{}^{18}O$ (N_2O/H_2O) values against $\delta_0{}^{15}N^{sp}$ values of produced N_2O (Fig. 5).

[Fig. 5]

It can be clearly noticed that the results from the inhibited experiment (Exp 1, red symbols) fit perfectly into the field of bacterial denitrification. Similarly, the results of sandy soils from the Exp 2 show a slightly wider range, but still are typical for bacterial denitrification. In contrast, silt loam soil (Exp 2.3, 2.4) and ~~the~~ organic soil (Exp 2.5, 2.6) both show increased $\delta_0{}^{18}O(N_2O/H_2O)$ and $\delta_0{}^{15}N^{sp}$ values which are very well correlated. This could indicate that in Exp 2 another process characterized by high $\delta^{15}N^{sp}$ and $\delta^{18}O$ values has significant contribution to total N_2O production by these two soils. This could be nitrification, which is rather not plausible due to the anoxic conditions, or fungal denitrification. But it remains unclear why this was not observed in the inhibited static ~~experiment~~incubation for the same soil (silt loam). C_2H_2 inhibition do not affect fungal denitrification (Maeda et al., 2015) as far as NO_3^- and NO_2^- availability is not restricted by inhibited nitrification. However, in the ~~dynamic experiments~~flow-through incubations, the first oxic phase might have activated other microorganisms, possibly preferentially fungi. This could explain that their contribution is observed only in Exp 2 but not in Exp 1. Such an activation of denitrification by oxygen supply has been documented for one fungus species (Zhou et al., 2001).

We verified if the correlation presented in Fig. 5 could have resulted from calculation ~~artefacts~~artifacts, since all of the higher $\delta_0{}^{18}O(N_2O/H_2O)$ and $\delta_0{}^{15}N^{sp}$ values were corrected for N_2O reduction (according to the method described in Sect. 2.5). This correction method does not provide very precise results, since the isotope effects associated with N_2O reduction are not entirely stable and predictable (Lewicka-Szczebak et al., 2015; Lewicka-Szczebak et al., 2014). Therefore, we have checked if this correlation may be only a calculation artifact and recalculated the values assuming larger range of isotopic fractionations (±5 ‰, resulting in $\varepsilon^{15}N^{sp}(N_2/N_2O)$ from -10 to 0 ‰ and $\varepsilon^{18}O(N_2/N_2O)$ from -20 to -6 ‰). Results show that

the correlation may slightly change in slope (from 0.41 to 0.85), intercept (from -10.4 to -18.0) and significance (R^2 from 0.64 to 0.91). But it always keeps the same trend, *i.e.* for the Exps 2.3 - 2.6 we obtain in any case correlated increase of $\delta_0^{15}N^{sp}$ and $\delta_0^{18}O$ (N_2O/H_2O) (see grey dashed lines in Fig. 5), proving that the indication for further contributing processes cannot be an ~~artefact~~artifact of the correction approach. For these experiments (2.3-2.6) in our model calculations (Table 4) always higher ε_n values were found when compared to Exp 1 and 2.1-2.2. Also for pure culture studies of fungal denitrification the ε_n values determined by a similar ~~modelling~~modeling approach were higher, up to 30 ‰ (Rohe et al., 2014a). This would support the hypothesis on fungal denitrification contribution.

3.7 Source of $\Delta^{17}O$ in atmospheric N_2O

In Exp 1 the $\Delta^{17}O(N_2O)$ values obtained from all measured N_2O samples were very low. Moreover, we also included the treatment with chemical nitrate as fertilizer, characterised by slightly negative $\Delta^{17}O$ excess~~,~~ (of ca.-1.5‰), and the produced N_2O did not show any positive $\Delta^{17}O$ excess (~~Table 1~~results not shown). The produced N_2O is always characterised by smaller ^{17}O-excess ($\Delta^{17}O$ values closer to 0) than in the source nitrate (Table 1). These results indicate that denitrification produces N_2O of randomly distributed oxygen, due to mostly very high extent of isotope exchange with soil water and the consequent loss of ^{17}O excess of nitrate. However, in Exp 2 numerous samples showed lower extent of isotope exchange, down to 50 %, and the ^{17}O excess of nitrate is partially transferred to N_2O, resulting in $\Delta^{17}O(N_2O)$ up to 5 ‰. This indicates that denitrification may be potentially the source of atmospheric N_2O with ^{17}O excess, as previously supposed (Kaiser et al., 2004; Michalski et al., 2003), but the magnitude of this excess is largely reduced by the exchange of oxygen isotopes with randomly distributed soil water.

~~5.~~4. Conclusions

It can be supposed that bacterial denitrification in soils is characterised by quite stable $\delta_0^{18}O(N_2O/H_2O)$ of 17.5 ± 1.2 ‰ due to the nearly complete O isotope exchange and constant isotope effect associated with this exchange. Hence, when N_2O producing processes other than heterotrophic processes are negligible, $\delta_0^{18}O(N_2O)$ can be well predicted. Conversely, $\delta_0^{18}O(N_2O/H_2O)$ values larger than 19 ‰ are probably indicative for the contribution of other processes. ~~But~~However, more work on oxygen isotope effects during N_2O production ~~of those~~

~~other processes~~by various microorganisms is needed to obtain robust estimate of their contribution. It is necessary to conduct experiments to determine the possible range of $\delta_0^{18}O(N_2O/H_2O)$ for ~~other~~different N_2O ~~producing~~forming processes. From the studies available until now, we can make a first estimate for $\delta_0^{18}O(N_2O/H_2O)$ characteristic of fungal denitrification of (48.2 ~~±~~ ±3.7) ‰ (when disregarding two most extreme values; for all results (47.4 ~~±~~ ±10.3) ‰) (Rohe et al., 2014a). This value is very different from the $\delta_0^{18}O(N_2O/H_2O)$ of bacterial denitrification determined here, i.e. (17.5 ~~±~~ ±1.2 ~~‰) which~~) ‰. This opens up a new perspective of applying $\delta_0^{18}O(N_2O/H_2O)$ for differentiation between fungal and bacterial denitrification.

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Table 1. Exp 1 results: soil moisture (expressed as water filled pore space: WFPS), N_2O+N_2 production rate (expressed as mass of N as sum of N_2O and N_2 per mass of dry soil per time), ^{17}O excess in soil nitrate ($\Delta^{17}O(NO_3)$) and in N_2O ($\Delta^{17}O(N_2O)$) with calculated exchange with soil water (x), and oxygen isotopic signature ($\delta^{18}O$) of soil nitrate (NO_3^-), soil water (H_2O) and N_2O with calculated isotope ratio difference between soil water and N_2O (~~$\delta^{18}O$~~$\delta_0{}^{18}O$ (N_2O/H_2O)). For samples with non-inhibited N_2O reduction the N_2O mole fraction ($f(N_2O)$) was taken into account to calculate the $\delta^{18}O$ unaffected by N_2O reduction ($\delta_0{}^{18}O(N_2O)$) and the respective $\delta_0{}^{18}O(N_2O/H_2O)$. Only Chile Saltpeter treatments are presented, for which the individual determination of x was possible. Part of the data from Exp 1.1 ($\delta^{18}O(NO_3^-)$, $\delta^{18}O(H_2O)$, $\delta^{18}O(N_2O)$) was already published in (Lewicka-Szczebak et al., 2014).

~~WFPS [%]~~treatment WFPS [%]	inhibition	N_2O+N_2 production rate [µg kg^{-1} h^{-1}]	$\Delta^{17}O(NO_3^-)$ [‰]	$\Delta^{17}O(N_2O)$ [‰]	x [%]	$\delta^{18}O(NO_3)$ [‰]	$\delta^{18}O(H_2O)$ [‰]	$\delta^{18}O(N_2O)$ [‰]	$f(N_2O)$[a]	$\delta_0{}^{18}O(N_2O)$[b] [‰]	$\delta_0{}^{18}O(N_2O/H_2O)$ [‰]
Exp 1.1 a, loamy sand, 8 ºC											
~~79~~80		114	11.9 ± 0.6	0.4 ± 0.5	96.2 ± 4.7	38.8±0.5	-9.2±0.5	13.4 ± 0.2	0.84±0.04	10.4	19.7 ± 0.5
~~79~~80	C_2H_2	107	11.9 ± 0.6	0.8 ± 0.4	93.1 ± 3.1	38.8±0.5	-9.2±0.5	10.4 ± 0.1	1	10.4	19.8 ± 0.5
80		125	11.9 ± 0.6	0.8 ± 0.2	92.7 ± 1.1	37.5±0.5	-13.5±0.5	8.4 ± 0.3	0.84±0.04	5.4	19.1 ± 0.6
80	C_2H_2	126	11.9 ± 0.6	0.3 ± 0.7	96.2 ± 3.4	37.5±0.5	-13.5±0.5	5.7 ± 0.0	1	5.7	19.4 ± 0.5
Exp 1.1b, loamy sand, 22 ºC											
~~78~~80		427	10.4 ± 0.8	0.4 ± 0.2	95.7 ± 1.8	42.6±0.5	-9.2±0.5	12.5 ± 0.2	0.85±0.06	9.6	19.0 ± 0.5
~~79~~80	C_2H_2	362	10.4 ± 0.8	0.4 ± 0.0	96.4 ± 0.2	42.6±0.5	-9.2±0.5	9.5 ± 0.0	1	9.5	18.9 ± 0.5
~~79~~80		429	10.4 ± 0.8	0.2 ± 0.1	98.2 ± 1.5	42.1±0.5	-13.5±0.5	7.5 ± 0.1	0.85±0.06	4.7	18.4 ± 0.5

80	C_2H_2	370	10.4 ± 0.8	0.5 ± 0.1	94.8 ± 0.5	42.1±0.5	-13.5±0.5	4.5 ± 0.1	1	4.5	18.3 ± 0.5
Exp 1.1 c, silt loam, 22 ºC											
80		266	9.2 ± 1.3	0.0 ± 0.2	99.5 ± 0.9	31.8±0.5	-2.6±0.5	26.4 ± 0.1	0.57±0.03	16.4	19.1 ± 0.5
~~81~~80	C_2H_2	257	9.2 ± 1.3	0.4 ± 0.1	95.3 ± 1.4	31.8±0.5	-2.6±0.5	15.9 ± 0.1	1	15.9	18.5 ± 0.5
~~82~~80		271	9.2 ± 1.3	0.1 ± 0.2	98.6 ± 1.3	31.8±0.5	-8.7±0.5	20.7 ± 0.2	0.57±0.03	10.8	19.7 ± 0.5
~~82~~80	C_2H_2	251	9.2 ± 1.3	0.4 ± 0.1	95.0 ± 1.5	31.8±0.5	-8.7±0.5	9.8 ± 0.1	1	9.8	18.7 ± 0.5
Exp 1.2 a, loamy sand, 22 ºC											
~~78~~80	C_2H_2	126	3.4 ± 0.5	n.d.	n.d.	6.5±0.5	-10.4±0.5	6.3 ± 0.1	1	6.3	16.9 ± 0.5
~~66~~65	C_2H_2	112	3.4 ± 0.5	0.2 ± 0.3	92.6 ± 8.5	6.5±0.5	-10.1±0.5	6.9 ± 0.2	1	6.9	17.2 ± 0.5
~~52~~50	C_2H_2	50	3.4 ± 0.5	0.0 ± 0.3	95.8 ± 3.9	6.5±0.5	-8.9±0.5	7.6 ± 0.3	1	7.6	16.6 ± 0.6
~~79~~80	C_2H_2	161	3.4 ± 0.5	n.d.	n.d.	6.5±0.5	-5.0±0.5	10.5 ± 0.0	1	10.5	15.6 ± 0.5
~~64~~65	C_2H_2	102	3.4 ± 0.5	0.2 ± 0.2	92.7 ± 5.2	6.5±0.5	-5.7±0.5	11.6 ± 0.1	1	11.6	17.5 ± 0.5
~~52~~50	C_2H_2	74	3.4 ± 0.5	0.2 ± 0.2	94.5 ± 5.1	6.5±0.5	-6.6±0.5	10.7 ± 0.1	1	10.7	17.4 ± 0.5
~~81~~	~~C_2H_2~~	~~158~~	~~1.5 ± 0.9~~	~~n.d.~~	~~n.d.~~	~~3.3±0.5~~	~~5.0±0.5~~	~~10.8 ± 0.2~~	~~1~~	~~10.8~~	~~15.9 ± 0.5~~
~~64~~	~~C_2H_2~~	~~77~~	~~1.5 ± 0.9~~	~~0.2 ± 0.3~~	~~84.4 ± 23.3[-e]~~	~~3.3±0.5~~	~~5.7±0.5~~	~~11.0 ± 0.0~~	~~1~~	~~11.0~~	~~16.8 ± 0.5~~
~~50~~	~~C_2H_2~~	~~46~~	~~1.5 ± 0.9~~	~~0.4 ± 0.3~~	~~68.9 ± 19.3[-e]~~	~~3.3±0.5~~	~~6.6±0.5~~	~~9.4 ± 0.5~~	~~1~~	~~9.4~~	~~16.1 ± 0.7~~
Exp 1.2 b, silt loam, 22 ºC											
~~77~~80	C_2H_2	137	2.6 ± 0.4	0.2 ± 0.2	90.6 ± 7.3	3.2±0.5	-8.1±0.5	8.3 ± 0.1	1	8.3	16.5 ± 0.5

~~60~~65	C_2H_2	130	2.6 ± 0.4	0.2 ± 0.1	92.2 ± 3.7	3.2±0.5	-7.1±0.5	9.8 ± 0.1	1	9.8	17.1 ± 0.5
~~46~~50	C_2H_2	121	2.6 ± 0.4	0.1 ± 0.1	96.5 ± 4.3	3.2±0.5	-5.9±0.5	12.5 ± 0.2	1	12.5	18.6 ± 0.5
~~77~~80	C_2H_2	111	2.6 ± 0.4	-0.1 ± 0.1	99.1 ± 1.6	3.2±0.5	-1.6±0.5	15.1 ± 0.2	1	15.1	16.7 ± 0.6
~~62~~65	C_2H_2	132	2.6 ± 0.4	0.0 ± 0.1	98.4 ± 1.6	3.2±0.5	-1.8±0.5	15.2 ± 0.2	1	15.2	17.0 ± 0.5
~~49~~50	C_2H_2	106	2.6 ± 0.4	-0.2 ± 0.0	100.0 ± 1.8	3.2±0.5	-2.0±0.5	15.7 ± 0.3	1	15.7	17.7 ± 0.6
	~~77~~	~~124~~	~~1.3 ± 0.8~~	~~0.3 ± 0.3~~	~~72.4 ± 25.7~~[e]	~~2.0±0.5~~	~~1.6±0.5~~	~~15.1 ± 0.1~~	~~1~~	~~15.1~~	~~16.8 ± 0.5~~
	~~63~~	~~133~~	~~1.3 ± 0.8~~	~~0.0 ± 0.4~~	~~98.7 ± 31.3~~[e]	~~2.0±0.5~~	~~1.8±0.5~~	~~14.9 ± 0.1~~	~~1~~	~~14.9~~	~~16.8 ± 0.5~~
	~~47~~	~~125~~	~~1.3 ± 0.8~~	~~0.3 ± 0.3~~	~~72.5 ± 22.7~~[e]	~~2.0±0.5~~	~~2.0±0.5~~	~~15.9 ± 0.1~~	~~1~~	~~15.9~~	~~18.0 ± 0.5~~

[a] $c(N_2O)/[c(N_2)+c(N_2O)]$: based on parallel ^{15}N treatment (last sampling results)

[b] N_2O reduction not inhibited, the values are corrected taking into account product ratio and isotope fractionation, according to Rayleigh fractionation $^{18}\varepsilon(N_2/N_2O)$ values taken from Lewicka-Szczebak et al. (2014): -17.4 ‰ (see Sect. 2.5 for details)

~~[e] results disregarded because of large errors which are due to too small ^{17}O excess in the substrate~~

Table 2. Exp 2 results: soil moisture (expressed as water filled pore space: WFPS), N_2O+N_2 production rate (expressed as mass of N as sum of N_2O and N_2 per mass of dry soil per time), ^{17}O excess in soil nitrate ($\Delta^{17}O(NO_3)$) and in N_2O ($\Delta^{17}O(N_2O)$) with calculated exchange with soil water (x) and oxygen isotopic signature ($\delta^{18}O$) of soil nitrate (NO_3), soil water (H_2O) and N_2O. All $\delta^{18}O(N_2O)$ values were corrected taking into account ~~product ratio~~N_2O mole fraction ($f(N_2O)$) to calculate the ~~$\delta^{18}O(N_2O)$~~ values unaffected by N_2O reduction ($\delta_0{}^{18}O$-(N_2O)) and the respective $\delta_0{}^{18}O(N_2O/H_2O)$.

WFPS [%]	N_2O+N_2 production rate ~~[mg~~ [µg kg^{-1} h^{-1}]	$\Delta^{17}O(NO_3^-)$ [‰]	$\Delta^{17}O(N_2O)$ [‰]	x [%]	$\delta^{18}O(NO_3^-)$ [‰]	$\delta^{18}O(H_2O)$ [‰]	$\delta^{18}O(N_2O)$ [‰]	$f(N_2O)$ [a]	$\delta_0{}^{18}O$ (N_2O) [b] [‰]	$\delta_0{}^{18}O$ (N_2O/H_2O) [‰]
Exp 2.1, sand										
73.6 ± 0.7	91	10.8 ± 0.3	2.7 ± 0.4	73.9 ± 4.2	34.3 ± 1.7	-8.6 ± 0.5	12.1 ± 0.2	0.95 ± 0.01	11.5 ± 0.2	20.2 ± 0.5
			2.6 ± 1.1	74.4 ± 11.0			11.0 ± 0.4	0.92 ± 0.01	10.0 ± 0.5	18.8 ± 0.7
Exp 2.2 loamy sand										
70.4 ± 0.9	49	11.9 ± 0.3	3.7 ± 0.4	66.9 ± 3.1	43.0 ± 2.4	-7.4 ± 0.5	18.4 ± 2.7	0.80 ± 0.05	15.7 ± 2.1	23.3 ± 2.2
			3.3 ± 0.2	71.2 ± 1.6			15.7 ± 0.9	0.83 ± 0.02	13.5 ± 0.7	21.0 ± 0.8
Exp 2.3 silt loam										
78.4 ± 1.9	80	11.3 ± 0.2	5.2 ± 0.2	52.0 ± 2.2	43.1 ± 2.3	-5.3 ± 0.5	43.8 ± 2.2	0.32 ± 0.03	29.4 ± 2.6	34.9 ± 2.6
			5.3 ± 0.1	50.4 ± 1.4			46.1 ± 3.9	0.29 ± 0.10	30.4 ± 0.2	35.9 ± 0.5
Exp 2.4 silt loam										
73.6 ± 1.8	52	12.1 ± 0.3	3.5 ± 0.5	69.9 ± 4.0	52.0 ± 3.3	-5.0 ± 0.5	30.1 ± 0.4	0.68 ± 0.02	25.4 ± 0.7	30.5 ± 0.9

			5.0 ± 0.5	56.3 ± 4.1			37.7 ± 4.1	0.63 ± 0.07	31.9 ± 4.3	37.1 ± 4.3
Exp 2.5 organic										
86.5 ± 1.8	743	7.8 ± 0.2	2.3 ± 1.1	68.1 ± 13.8	30.4 ± 0.6	-6.4 ± 0.5	26.4 ± 5.3	0.60 ± 0.02	20.0 ± 5.1	26.6 ± 5.1
			2.3 ± 0.8	68.2 ± 9.5			37.7 ± 2.9	0.51 ± 0.02	29.3 ± 3.3	36.0 ± 3.3
Exp 2.6 organic										
78.7 ± 0.4	1198	12.5 ± 0.7	1.1 ± 0.2	90.2 ± 1.8	43.6 ± 5.6	-6.7 ± 0.5	18.5 ± 0.0	0.82 ± 0.02	16.1 ± 0.2	22.9 ± 0.6
			2.3 ± 0.3	78.8 ± 3.0			25.6 ± 0.8	0.74 ± 0.05	21.9 ± 1.6	28.7 ± 1.7

[a] $c(N_2O)/[c(N_2)+c(N_2O)]$: based on direct GC measurements in N_2-free atmosphere

[b] initial $\delta^{18}O$ values of unreduced N_2O calculated according to Rayleigh fractionation, $^{18}\varepsilon(N_2/N_2O)$ values taken from Lewicka-Szczebak et al. (2015): -12 ‰ (see Sect. 2.5)

Table 3. Isotopic fractionation factors calculated based on Exp 1 results with Eq. (12) (see text for details). Results presented separately for Exp 1.1 and 1.2 and mean values for both.

	ε_w [‰]	ε_n [‰]
Exp 1.1	17.44 ± 0.71	0.74 ± 0.70
Exp 1.2	17.50 ± 0.67	-0.39 ± 0.66
mean all	17.48 ± 0.66	0.03 ± 0.86

Table 4. Oxygen fractionation model based on the results obtained ($\delta_0^{18}O(N_2O)$) and isotope exchange (x) determined by $\Delta^{17}O$ method~~)~~ and ε_w = 17.5 ‰ determined from Exp 1 data (Table 3). Scenarios with varied ε_n values and x_{NIR} or x_{NOR} (fraction of isotope exchange associated with NIR or NOR) are compared. *D* is the difference between measured $\delta^{18}O$ of N_2O and the calculated $\delta^{18}O$ of N_2O in a particular scenario.

		Scenario 0: $x = x_{NIR}$ or x_{NOR} **$\varepsilon_n = 0$** ε_w = 17.5 [‰]		**Scenario 1:** $x_{NIR} = x$; $x_{NOR} = 0$ ε_n fitted ε_w = 17.5 [‰]		**Scenario 2:** $x_{NIR} = 0$; $x_{NOR} = x$ ε_n fitted ε_w = 17.5 [‰]		**Scenario 3:** $x_{NIR} = x_{NOR}$ ε_n fitted ε_w = 17.5 [‰]	
	~~calculated $\delta^{18}O(N_2O)$ [‰]~~	*D*	ε_n	*D*	ε_n	*D*	ε_n	*D*	ε_n	*D*
Exp 1.1a	~~10.5~~	0.2	**0.3**	0.00	**2.3**	0.00	**1.0**	0.00
	~~5.4~~	0.6	**1.2**	0.00	**16.0**	0.00	**5.3**	0.00
Exp 1.1b	~~9.6~~	0.1	**0.2**	0.00	**2.7**	0.00	**0.9**	0.00
	~~6.1~~	-1.2	**-2.3**	0.00	**-22.6**	0.00	**-8.6**	0.00
Exp 1.1c	~~15.7~~	0.2	**0.4**	0.00	**4.7**	0.00	**1.7**	0.00
	~~10.1~~	0.0	**0.1**	0.00	**0.6**	0.00	**0.2**	0.00
Exp 1.2a	~~7.4~~	-0.3	**-0.5**	0.00	**-3.7**	0.00	**-1.6**	0.00
	~~8.6~~	-0.8	**-1.5**	0.00	**-18.4**	0.00	**-6.2**	0.00
	~~11.5~~	0.3	**0.6**	0.00	**4.5**	0.00	**1.9**	0.00
	~~10.7~~	0.2	**0.3**	0.00	**2.7**	0.00	**1.0**	0.00
Exp 1.2b	~~8.9~~	-0.4	**-0.7**	0.00	**-4.0**	0.00	**-1.9**	0.00
	~~9.9~~	0.1	**0.2**	0.00	**1.7**	0.00	**0.7**	0.00
	~~11.3~~	1.4	**2.6**	0.00	**38.5**	0.00	**12.1**	0.00
	~~15.8~~	-0.7	**-1.3**	0.00	**-72.8**	0.00	**-12.5**	0.00
	~~15.5~~	-0.3	**-0.6**	0.00	**-19.3**	0.00	**-4.2**	0.00
	~~15.5~~	0.2	**0.4**	0.00	**0.0**	0.22	**0.0**	0.22
Exp 2.1	~~15.8~~	-4.0	**-6.2**	0.00	**-14.7**	0.00	**-10.0**	0.00

	~~15.6~~	-5.3	**-8.2**	0.00	**-19.9**	0.00	**-13.4**	0.00
Exp 2.2	~~21.3~~	-5.2	**-7.6**	0.00	**-15.0**	0.00	**-11.0**	0.00
	~~19.8~~	-6.1	**-9.1**	0.00	**-20.0**	0.00	**-14.1**	0.00
Exp 2.3	~~27.3~~	2.5	**3.2**	0.00	**4.9**	0.00	**4.0**	0.00
	~~27.8~~	3.0	**3.8**	0.00	**5.7**	0.00	**4.7**	0.00
Exp 2.4	~~24.6~~	1.1	**1.6**	0.00	**3.4**	0.00	**2.4**	0.00
	~~30.0~~	2.2	**2.9**	0.00	**4.8**	0.00	**3.8**	0.00
Exp 2.5	~~17.4~~	2.8	**4.2**	0.00	**8.5**	0.00	**6.2**	0.00
	~~17.4~~	12.2	**18.1**	0.00	**37.1**	0.00	**27.0**	0.00
Exp 2.6	~~14.2~~	2.2	**3.8**	0.00	**20.9**	0.00	**10.2**	0.00
	~~17.9~~	4.2	**6.8**	0.00	**19.1**	0.00	**12.2**	0.00

Figures captions:

Figure 1. Oxygen isotope fractionation during denitrification as a result of branching effects (~~ε_n~~ε_{NAR}, ε_{NIR}, ε_{NOR}) und exchange effects (ε_w) associated with the following enzymatic reaction steps: NAR, NIR and NOR.

Figure 2. Correlation between oxygen isotopic signatures of N_2O and soil water expressed in relation to soil nitrate, the equation of linear fit allows for estimation of isotope exchange with soil water (slope of the linear fit) and the associated isotope effect (intercept of the linear fit). In red the influence of N_2O reduction on the method performance is presented - red X points represent the samples with not inhibited N_2O reduction (note that the slope and intercept are very different), whereas the red + points stand for the same samples after mathematical correction of N_2O reduction effect (as described in Sect. 2.5) which fit very well to the samples where N_2O reduction was inhibited. Data from Exp 1.

Figure 3. Relation between relative isotope ratio differences between produced N_2O and soil water ($\delta_0{}^{18}O(N_2O/H_2O)$ and between produced N_2O and soil nitrate ($\delta_0{}^{18}O(N_2O/NO_3^-)$, on the right $\delta^{18}O$ values of the initial soil nitrate for different treatments. $\delta^{18}O$ values of the initial soil water ranged between -13.5 and -1.6 ‰ (see Table 1) and its variation had no impact on $\delta_0{}^{18}O(N_2O/H_2O)$. Open symbols: ~~addition of~~treatments with synthetic nitrate as fertilizer, filled symbols: ~~addition of~~treatments with natural Chile saltpeter as fertilizer. Data from Exp 1.

Figure 4. $\delta_0{}^{18}O(N_2O/H_2O)$ as a function of isotope exchange extent, x (determined with $\Delta^{17}O$ method). Red symbols: Exp 1, black symbols: Exp 2; open symbols: incubations with lower WFPS (70 %), filled symbols: incubations with higher WFPS (80 %). Note that same symbols shapes always represent the same soil.

Figure 5. Relation between $\delta_0{}^{15}N^{sp}$ of produced N_2O and relative ratio difference between produced N_2O and soil water ($\delta_0{}^{18}O(N_2O/H_2O)$). Red symbols: Exp 1, black symbols: Exp 2; open symbols: incubations with lower WFPS (70 %), filled symbols: incubations with higher WFPS (80 %). Note that same symbols shapes always represent the same soil. Grey dashed lines represent the possible range of linear fit when extreme values of isotope effects for N_2O reduction are assumed in correction calculations (Eq. (5~~)) - see discussion.~~)). Range of values for fungal denitrification from Rohe et al. (2014a).

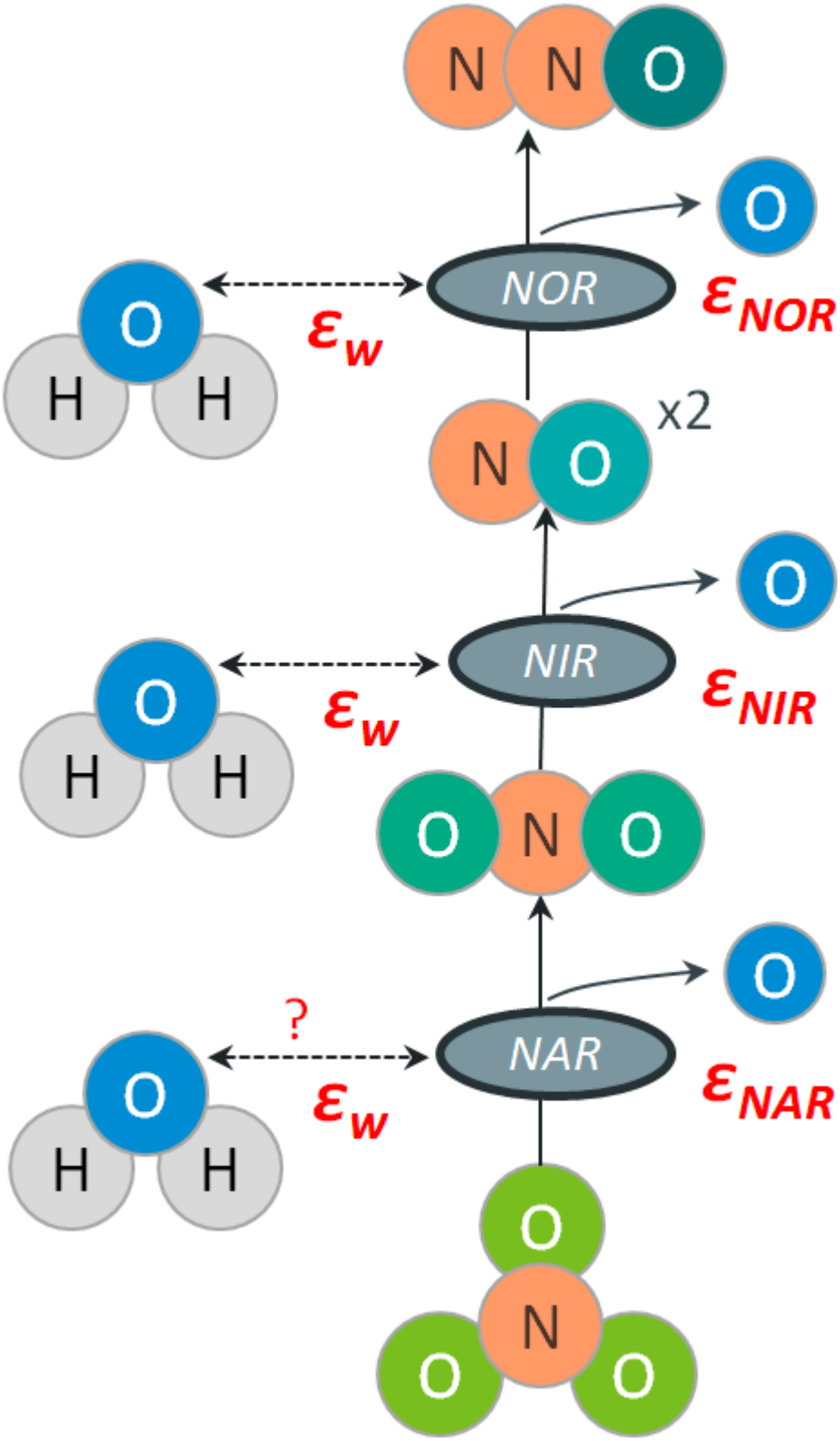
N N O
O
NOR
ε_{NOR}
O H H
ε_w
x2
N O
O
NIR
ε_{NIR}
O H H
ε_w
O N O
O
?
NAR
ε_{NAR}
O H H
ε_w
O
N
O O

Fig.1

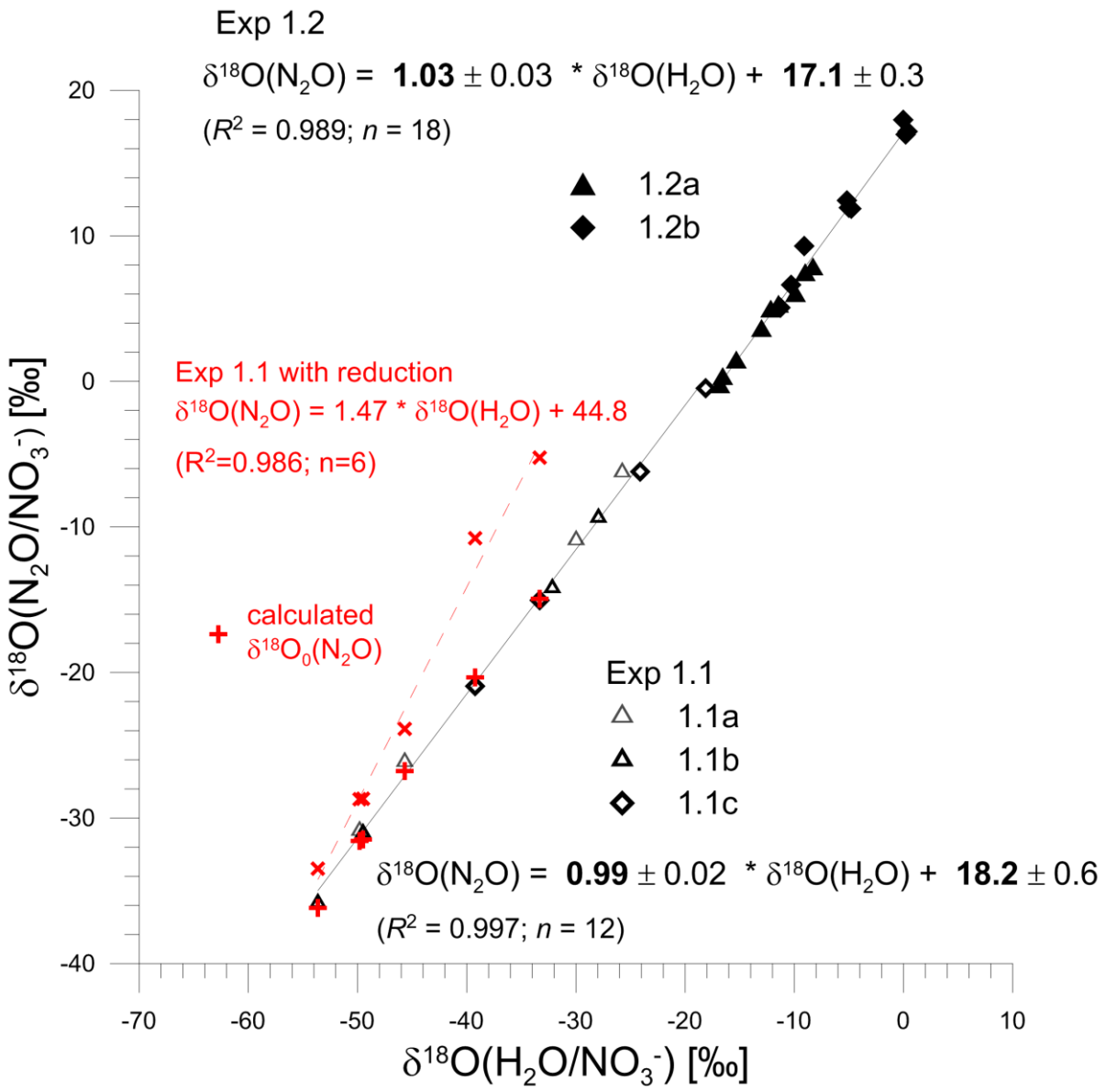
Exp 1.2
δ18O(N2O) = 1.03 ± 0.03 * δ18O(H2O) + 17.1 ± 0.3
(R2 = 0.989; n = 18)
1.2a
1.2b
Exp 1.1 with reduction
δ18O(N2O) = 1.47 * δ18O(H2O) + 44.8
(R2=0.986; n=6)
calculated
δ18O0(N2O)
Exp 1.1
1.1a
1.1b
1.1c
δ18O(N2O) = 0.99 ± 0.02 * δ18O(H2O) + 18.2 ± 0.6
(R2 = 0.997; n = 12)
δ18O(N2O/NO3-) [‰]
δ18O(H2O/NO3-) [‰]
20
10
0
-10
-20
-30
-40
-70
-60
-50
-40
-30
-20
-10
0
10

Fig.2

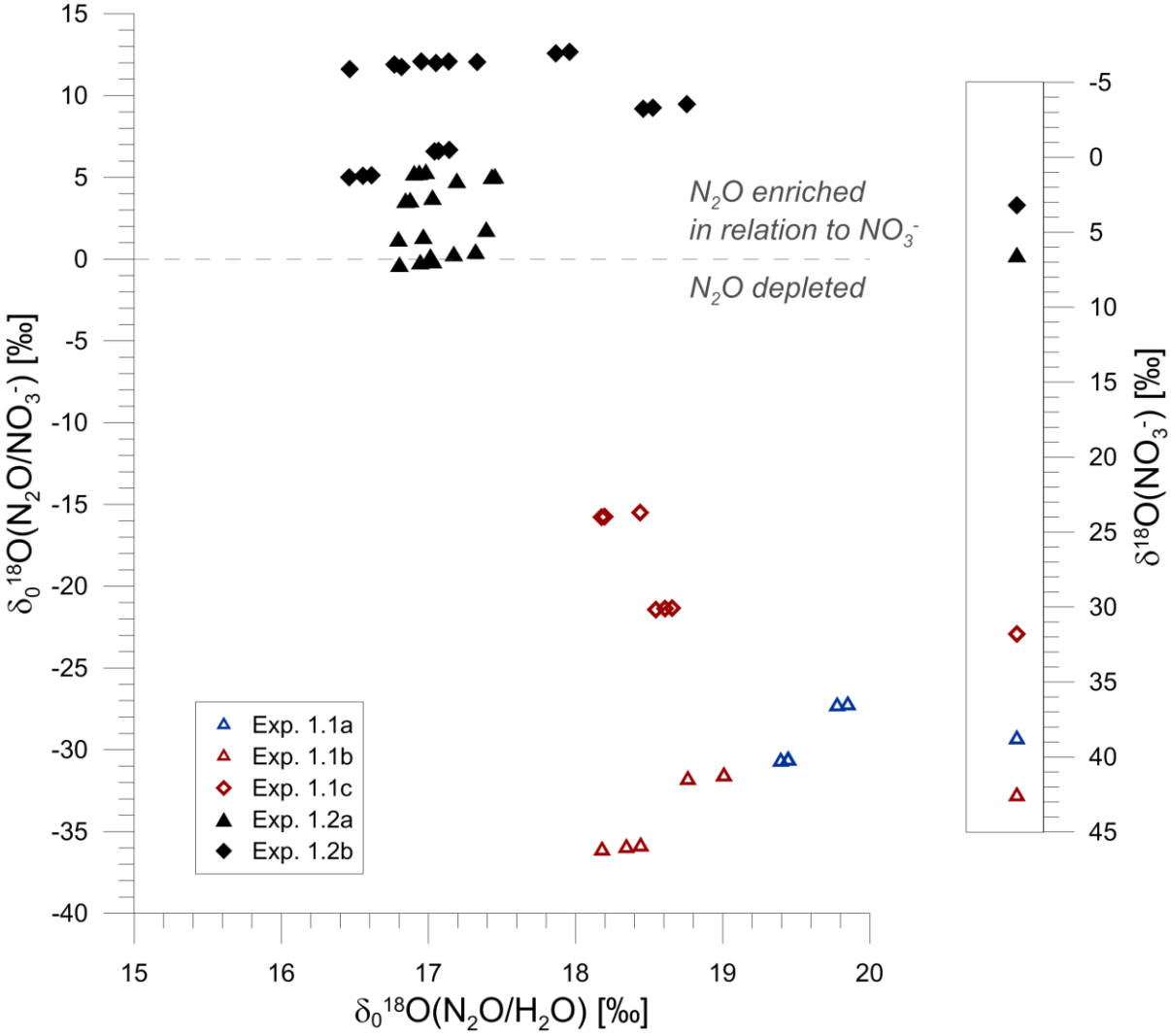
15
10
5
0
-5
-10
-15
-20
-25
-30
-35
-40
15
16
17
18
19
20
δ0 18O(N2O/H2O) [‰]
δ0 18O(N2O/NO3-) [‰]
N2O enriched
in relation to NO3-
N2O depleted
Exp. 1.1a
Exp. 1.1b
Exp. 1.1c
Exp. 1.2a
Exp. 1.2b
-5
0
5
10
15
20
25
30
35
40
45
δ18O(NO3-) [‰]

Fig.3

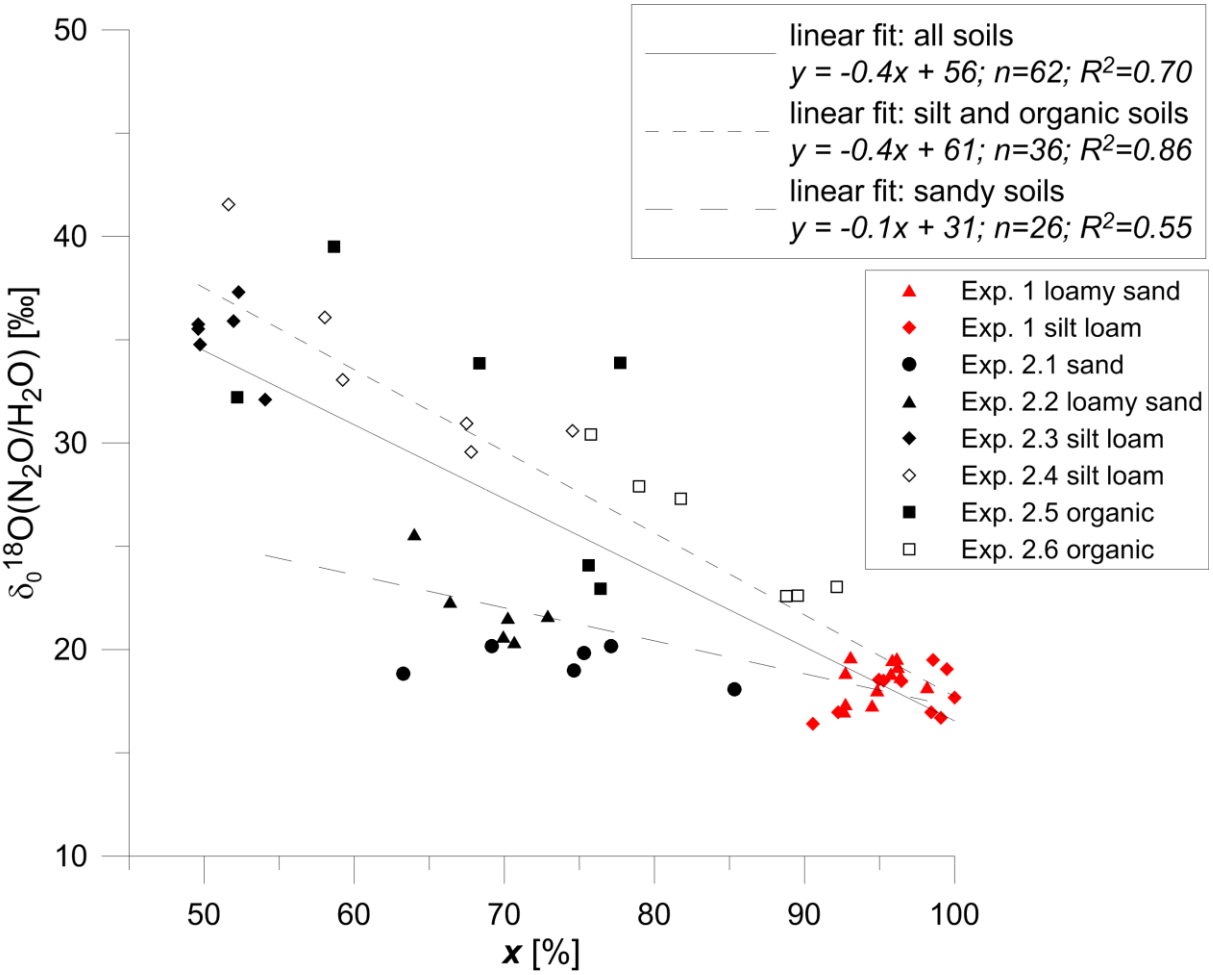
linear fit: all soils
y = -0.4x + 56; n=62; R2=0.70
linear fit: silt and organic soils
y = -0.4x + 61; n=36; R2=0.86
linear fit: sandy soils
y = -0.1x + 31; n=26; R2=0.55
Exp. 1 loamy sand
Exp. 1 silt loam
Exp. 2.1 sand
Exp. 2.2 loamy sand
Exp. 2.3 silt loam
Exp. 2.4 silt loam
Exp. 2.5 organic
Exp. 2.6 organic
δ0 18O(N2O/H2O) [‰]
x [%]
50
40
30
20
10
50
60
70
80
90
100

Fig.4

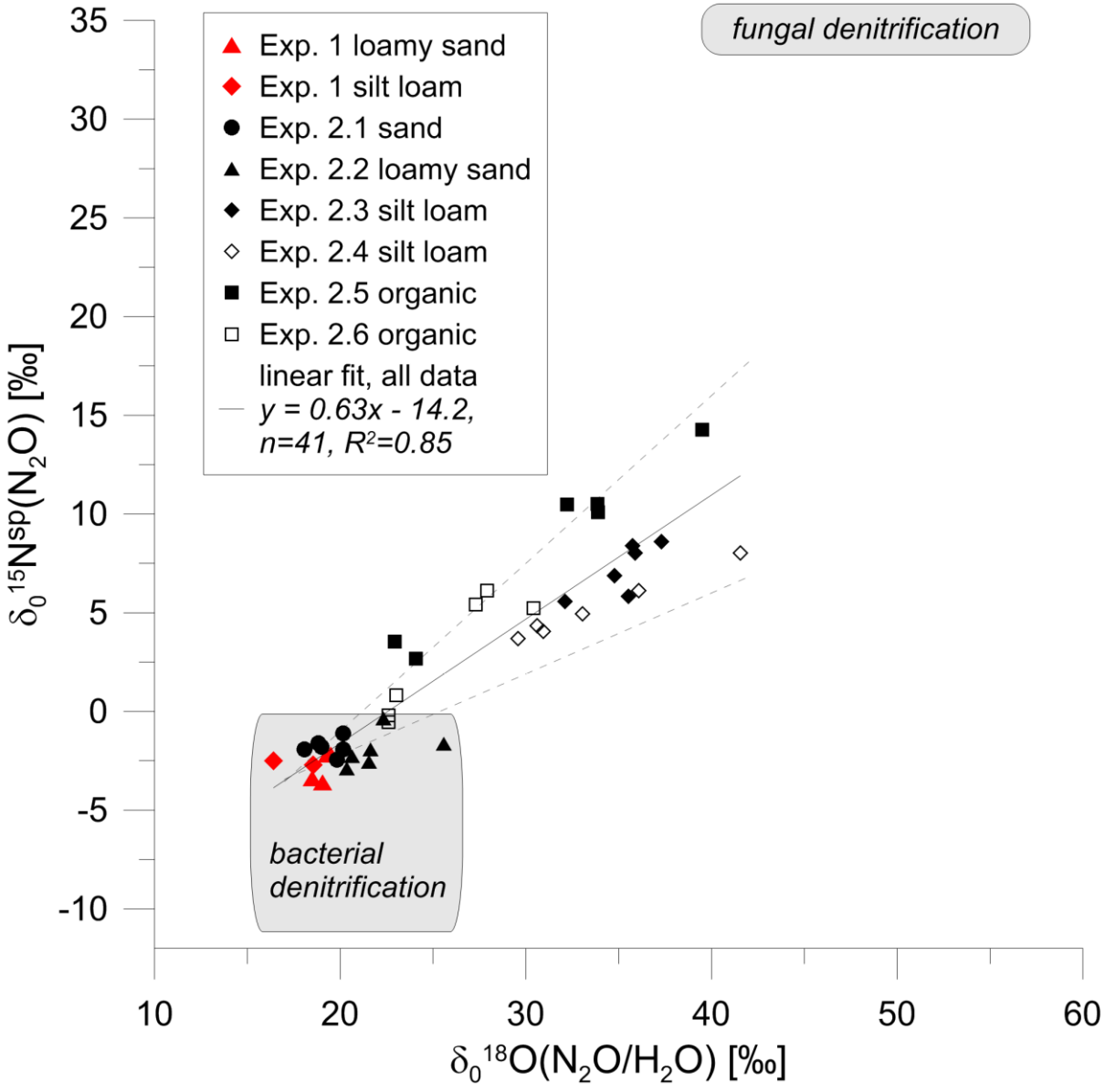
fungal denitrification
Exp. 1 loamy sand
Exp. 1 silt loam
Exp. 2.1 sand
Exp. 2.2 loamy sand
Exp. 2.3 silt loam
Exp. 2.4 silt loam
Exp. 2.5 organic
Exp. 2.6 organic
linear fit, all data
y = 0.63x - 14.2,
n=41, R²=0.85
bacterial denitrification
δ0 15Nsp(N2O) [‰]
δ0 18O(N2O/H2O) [‰]
35
30
25
20
15
10
5
0
-5
-10
10
20
30
40
50
60

Fig.5