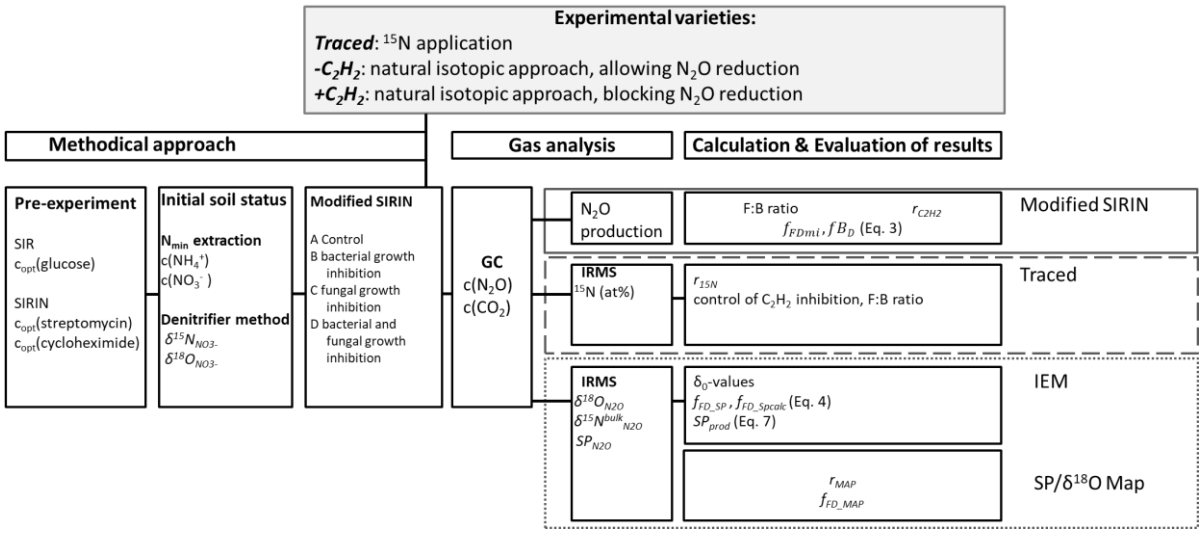
Review response on **"Comparing modified substrate induced respiration with selective inhibition (SIRIN) and N_2O isotope approaches to estimate fungal contribution to denitrification in three arable soils under anoxic conditions"** ***by*** **Lena Rohe et al.**

Anonymous Referee #2

We thank the reviewer for the critical comments on the structure of the manuscript and for the good comments that will largely improve the manuscript.
The authors' answer is in italic font.

The manuscript submitted here presents an interesting combination of approaches for assessing the contribution of fungal denitrification to the N2O. By using some SIRIN and two isotopic techniques (endmember mixing) and SP/delta18O mapping, they conclude that the fungal contribution to N2O fluxes under anaerobic conditions in the three investigated soils is modest. In general, the manuscript is well written, and the methods are well elaborated. I however miss a clear rationale for the study. As a consequence, the reader is not guided through the work, so that it is hard to get the main conclusions of the work, and how the different pieces of the work fit together (i.e. how the methods compare to each other). Below, you can find some critical parts which should be revisited, with regard to objectives, experimental design and methods and discussion of the results. At the end, some more specific comments.
I think the manuscript would benefit from a more straightforward formulation of the objectives. As it is now, the three objectives (L155-162) are hard to differentiate from each other (ie, using three approaches to determine the fungal contribution to N2O efflux; compare the fungal contribution obtained by the three, and evaluate the use of SPN2O values); even more importantly, in the abstract I don0t see a connection with such objectives. You can think on hypotheses -e.g. methods (do not) perform equal-, and ways to test them.

To represent the different methods applied with various measures derived from these methods more comprehensible, we will prepare a scheme to illustrate the methodological procedure. We will change the variables for product ratios of the different methods to r_{15N}, r_{C2H2}, and r_{MAP} and also for fraction (F) to the more common "f" in the revised version. In the revised version we will also provide a table in supplementary material showing the variable and abbreviations. Furthermore, the term "Experiment" will be changed throughout the text, tables and figures to "Soil".



We will revise the abstract and introduction. The abstract will be revised by including "Three approaches were established (modified SIRIN approach, endmember mixing approach (IEM) and the SP/$\delta^{18}O$ mapping approach (SP/$\delta^{18}O$ Map) to estimate the N_2O production by a fungal community in soil: i) A modification of the SIRIN approach was used to calculate N2O evolved from selected organism groups, ii) SP_{N2O} values from the acetylated treatment were used in the isotope endmember mixing approach (IEM), and iii) the SP/$\delta^{18}O$ mapping approach (SP/$\delta^{18}O$ Map) was used to estimate the fungal contribution to N_2O production and N_2O reduction under anaerobic conditions from the non-acetylated treatment to investigate the fungal fraction contributing to N_2O from denitrification in different soils independently. Furthermore, experiments with clear results in determined fungal fraction contributing to N_2O from denitrification using SIRIN will be used to compare SP_{N2O} values of the fungal fraction with fungal SP_{N2O} endmember values previously reported in the literature." in l. 37 ff.

We will include the following hypothesises to the introduction (l. 156 ff.):" We hypothesized that the fungal fraction contributing to N_2O from denitrification in different soils using a modified SIRIN approach and isotopic methods will be correlated but not match exactly due to limited inhibitability of microbial communities and variability in SP_{N2O} endmember values. Furthermore, experiments with clear results in determined fungal fraction contributing to N_2O from denitrification using SIRIN will yield fungal SP_{N2O} endmember values within the range of values previously reported in the literature.". Thus, we will change the objectives of the study to (l. 159 ff.): "Therefore, this study aims at (i) determining the fungal contribution to N_2O production by denitrification under anoxic conditions and glucose addition using three arable soils and approaches (modified SIRIN, IEM and the SP/$\delta^{18}O$ Map), and to assess the reliability in soil studies and thus assess factors of potential bias of the methods and (ii) to estimate the SP_{N2O} values from a fungal soil community and thus to evaluate the transferability of the pure culture range of the fungal SP_{N2O} endmember values."

In the abstract section, we will also clarify the conclusion (l. 43 ff.): "All three approaches tested revealed a small fungal contribution to N_2O fluxes (F_{FD}) under anaerobic conditions in the soils tested. Quantifying the fungal fraction with modified SIRIN was in most cases not successful. In only one soil, F_{FD} of modified SIRIN was 0.28±0.09, which was possibly overestimated as it was higher than the results obtained by IEM and SP/$\delta^{18}O$ Map (FD of 0 and 0.20, respectively)."

According to this, the Conclusion section will be changed to (l. 836 ff.): "However, it has to be pointed out, that quantifying the fungal fraction with modified SIRIN was only possible with one soil and was possibly overestimated. According to this, the SP_{N2O} values of fungal N_2O calculated from the SIRIN treatment did not appear to be a valid estimate of this value and need further evaluation.".

It is also not clear to me the reasoning for the selection of the three soils and how this relates to the objectives, so it is hard to evaluate the suitability of the approach. Is it just to get an idea of variability? In L165 you stated that the soils differ in texture to provide different conditions for denitrification, so that might be the reason behind. The experiments take place under anoxic conditions, so texture might not be that relevant, and one may argue that, for example, different C sources for denitrification maybe more important, or different proportion of fungal vs. bacterial microbial biomass. However, the variability across soils, or the potential role of texture on the results is not discussed at all. Furthermore, one of the soils was sampled twice. Why? What is the difference between experiment 1 and 4? Is it about seasonality? Which kind of information did you want to obtain, and which kind of lessons you learned in hand of the results? I am missing this information in the discussion.

Three different soils were assumed to provide various conditions for denitrification and thus also different environments for microbial communities. Thus, the three soils were not selected to analyse effects of land-use type or soil types, but aimed to compare the different methods (modified SIRIN and isotope approaches) to analyse denitrification. Therefore three soils

harbouring different microbial communities were used to estimate differences in results of the used methods among soils (as described in section 2.1).
The three soils differed in texture, but also in C content, C/N ratio of C_{org} and pH (Table 1) and we identified differing microbial biomass values. Thus we assumed variable community structures and as a consequence, differences in fungal to bacterial abundances were assumed. We thus did not focus on textural effects on denitrification, but aimed to find differences in fungal abundance in soil. We will include this as follows: "Three soils differing in texture, C_{org} content, C/N ratio of C_{org} and pH were chosen assuming that the soils harbour different denitrifying communities, i.e., different fractions of bacteria and fungi contributing to denitrification."

As you said, the microbial inhibitors did not have the expected effects. This is evident not only for N2O, but also for CO2. I understand this kind of results are disappointing when investing large efforts in conducting the analysis. But this is a key issue which deserves more attention, since it has important implications for the relevance of the whole study. For example, L609-610 read: "the SIRIN results [: : :] were rather unsatisfactory and led to unsolved questions" (a similar statement at the end of the discussion, L827-830). This is quite a statement which, to be honest, it is not reflected in the abstract, which describes that, for the one soil where it was possible to quantify the fungal contribution, this was 28%, higher than what obtained by the other methods. Thus, as a reader I would infer that SIRIN might overestimate the fungal contribution to the N2O fluxes, which is quite a different conclusion compared to "SIRIN results were rather unsatisfactory". On the contrary, the conclusions have a totally different approach, focusing almost only in the caveats of the SIRIN approach.

We agree to clearly state in the manuscript that SIRIN was not successful, because we have the large amount of non-inhibitable production and the only result for Experiment 2 is actually very unsure. Thus, we decided to delete the attempt to calculate SP_{FD} using the SIRIN results by resolving Eq. 4 for SP_{FD} (section 2.5.2) since this yielded biased results. Consequently, section 3.6 (SP_{N2O} values of N_2O produced by the fungal soil community) will be deleted. We will focus on SP_{N2O} values from the fungal fraction in the discussion as follows (l. 829): "The critical question whether the isotopic signatures of fungal N_2O determined in pure culture studies are transferable to natural soil conditions cannot be fully answered with this study due to large uncertainties associated with the results of the SIRIN method making it inappropriate to calculate the SP_{N2O} values of the fungal soil fraction. Further experiments would be needed with improved selective inhibition to assure that SP_{N2O} values known from pure cultures or soil isolates (Sutka et al., 2008; Rohe et al., 2014a; Maeda et al., 2015) are true for fungi in selective groups in soil as well.

As mentioned above we will change the abstract as follow (l. 43 ff.): "All three approaches tested revealed a small fungal contribution to N_2O fluxes (F_{FD}) under anaerobic conditions in the soils tested. Quantifying the fungal fraction with modified SIRIN was in most cases not successful. In only one soil, F_{FD} of modified SIRIN was 0.28±0.09, which was possibly overestimated as it was higher than the results obtained by IEM and SP/$\delta^{18}O$ Map (FD of 0 and 0.20, respectively)."

By the way, you said you tested the concentrations of inhibitors applied; thus, were the preliminary tests performing better than the "real runs"? How did you test the optimal concentration? In general, when presenting the results of the different methods and discussing them, I missed a profound analysis on which method should be applied, what the cons and pros are and whether methods provide complementary information, which would support the simultaneous use. As it is now, they are presented almost separately, thus failing in in the objective which can be derived from the tile: "comparing : : :approaches to estimate fungal contribution to denitrification : : :"). And I

honestly consider this is a serial issue, especially because you are making use of some of the outputs of selected methods as input for the rest of the methods, making all of them dependent to each other. It is only a suggestion, but consider including some table or graph with the main features of each methods and the key info, so that a reader can get an overview at first glance

As described in section 2.2.1 the pre-experiments were conducted as described in the original method to analyse F:B ratio by substrate induced respiration with selective inhibition. Unfortunately, only CO_2 production was analysed under oxic conditions and we did not test the optimum conditions under anoxic conditions. Additionally, due to the oxic conditions, N_2O production was not measured in this pre-experiment. We will describe the differences between this pre-experiment and the incubation experiment presented in more detail in the method section. Regarding the different methods used (modified SIRIN and isotope approaches), we want to emphasize that the both isotope approaches, IEM and SP/$\delta^{18}O$ Map, were independent on results of the modified SIRIN approach. While SP_{N2O} values from the acetylated treatment were used in IEM, the SP/$\delta^{18}O$ Map was used to estimate the fungal contribution to N_2O production and N_2O reduction from the non-acetylated treatments. As described above we will point on this in the revised objectives of the study.

We will revise the conclusion section and include: "Based on the presented results we conclude that the modified SIRIN approach presented here is not appropriate to estimate the contribution of selected communities (bacteria or fungi) on denitrification from soil. Both isotope approaches (IEM and SP/$\delta^{18}O$ Map) revealed similar and reasonable results of the fungal fraction contributing to denitrification and thus could be recommended in future studies. However, further studies would be needed to cross-validate methods, e. g. with such as improved inhibitor approaches or molecular-based methods."

Specific comments:

L48: What do you mean by "under conditions ensuring larger fungal N2O fluxes"?

When supplying C sources other than glucose, the fungal growth might be greater compared to that with glucose. Apart from that, in a future study one could analyse the microbial community first and by this identify soils with approved high fungal abundance or maybe even a high fungal denitrifier abundance before applying such experiments. We will clarify this in the revised text by including "…by adding C sources preferred by fungi…" to the sentence in the abstract.

L165-169: A more detailed description of the soil sampled will help. In general, the use of experiment/soil/treatment and variety is confusing, e.g. the same soil is used in two "experiments" (see above) and variety might refer to the use of c2h2 or 15N tracer.

As described in a comment above the different soils were not chosen to analyse soil factors controlling denitrification, but were chosen to provide variable microbial communities with assumingly variations in fungal and bacterial ratios contributing to denitrifying community. In the revised version the term "Experiment" will be changed throughout the text, tables and figures to "Soil".

Table 1: inorganic N is expressed in mg/L, which is fine for solutions, but not for de-scribing soils. The usual unit for me is mg N /kg soil. Please check consistence of these numbers.

Thanks for pointing this out. In the revised version, this will be corrected.

See my comments below, these values can have high temporal variability, with consequences on your analysis. Further to the table: what is "natural soil NO3-"? In general, how stable are these numbers for the Braunschweig soils, and for the rest of the sites? They are arable soils, probably subject to fertilization.

This is correct. The measurements for Braunschweig soil were performed in samples from 2012. Especially these values will vary within one year in arable soils. However, we amended the soil with C and N, thus changing the current state of the soil before incubation. Although

soil properties and microbial community or biomass may have changed over time, we thus assumed pre-incubating the soil for seven days, applying C and N, and changing the environmental conditions during denitrification induced a rapid growth of specific organisms. Consequently, we were aware, that the denitrifying community and the abundance of these organisms in incubation experiments may differ from the community in the field. We will include a discussion on this point to section 4.1 in the discussion section (l. 621. ff.).

L243: What is the rationale of having target soil densities? Do they correspond to the field bulk density?

We did not analyse the bulk density of the tested soils. We repacked the soils according to the expected bulk densities based on texture, i.e. 1.6 g cm^{-3} for a sandy soil and 1.3 g cm^{-3} for a silt loam. We will change the respective sentence in the Material and Methods section as follows (l. 244 ff.): "During packing, the soil density was adjusted to an expected target soil density of 1.6 g cm^{-3} in Experiment 1, 2 and 4 and of 1.3 g cm^{-3} in Experiment 3."

L248/L378/Figure 1: How did you calculate the N2O (and CO2) fluxes? Since you flushed with N2, I presume that, for t=0, you used a background concentration of 0 for both N2O and CO2 and then calculated the rate of change after 6, 8 or 10 hours (or 2, 4 and 8 in experiment 4). However, you mentioned average production rates (L378, L390); thus, where does the average come from? As you show in Figure 1, rates vary in some cases by more than 100% depending on the incubation length. So, what is your view on this and, more importantly, what is your suggestion for future experiments?

We calculated the N_2O production rates by averaging the measured N_2O production over 6, 8 or 10 hours (or 2, 4 and 8 in experiment 4). As you described, we calculated rates between the time point of flushing with N2 (t=0) and 6, 8 or 10 hours (or 2, 4 and 8 in experiment 4). Thus, we did not calculate the difference in rates between two time points, but averaged over the time point of incubation (0-6, 0-8 or 0-10 hours or 0-2, 0-4 and 0-8 in experiment 4). However, although we calculated these average values, the production rates differed largely between the time points.

As we modified the conditions for microorganisms directly before the incubation and accumulation of N_2O started (i.e. mixing the soil, adding water, nitrate, CO_2, flushing the headspace with N_2) we expected high activity of a large fraction of microorganisms. The antibiotics inhibit protein biosynthesis, and therefore we aimed to increase microbial growth by changing the environment. We will explain this in more detail in section 4.1: "Thus, short-time incubation should cause changes in environment for microorganisms and initiate growth on the one hand, while it should prevent the use of inhibitors as C sources by organisms on the other." However, it is well known from a previous study (Ladan & Jancinthe 2016) that incubations with selective growth inhibitors over a too long period result in non-plausible artefacts. We will include this point in the discussion section.

The incubation time of the presented study was relatively short (10 hours) compared to other studies focussing on denitrification. However, when using inhibitors it is absolutely necessary to keep incubation time as short as possible to prevent changes in microbial communities due to species development of non-inhibited organisms. This was already described by Anderson & Domsch 1973 and mentioned in l. 631 ff.:"Anderson and Domsch (1975) stated already that CO_2 production of initially active organisms can only be ensured up to six or eight hours of experimental duration and biomass activity is changed by both inhibitors." The reason for this is that inhibitors can also be used as C sources for microbial growth.

In consequence, we would aim for improved inhibitor effectiveness in future studies, but would recommend relatively short incubation times to avoid that microorganisms could use the inhibitors or dead cells as energy sources.

L254: How can you have a fixed measurement precision across different incubation lengths? How was this calculated? The precision is some orders of magnitude lower than the calculated fluxes, but

there are some large variations within the same treatment. Is this solely due to spatial variability? Or are you presenting only the analytical precision and leaving out some other sources of uncertainty?

The precision for GC and IRMS analysis is the analytical precision of measurements derived from analysing laboratory standards of different concentrations. We will describe this in more detail in the revised version. Variations within treatments derive from spatial variations and replicate incubation that may differ in microbial activity and thus denitrifying activity.

L289: I am not sure I understood this section. I suspect you used two approaches, but they are presented in a mixed way. With the IEM, one calculates the fungal contribution to N2O by solving the equation 4 using the SP of the N2O produced in the acetylene treatment (variety A) and the assumed SP for fungal and bacteria (33.6 for fungi, and -7.5 to +3.7 per mil for bacteria). The N2O from the acetylene is used to eliminate the distortion coming from N2O reduction in the non-acetylene treatment. But according toL304-307, you solve the equation for SP of fungi using FFD. Please clarify this, since itis highly misleading as it is now.

This is correct. We will put more emphasis on the precise description of calculations.

L352: Where did you get the amount of N in unfertilized soils from? Table 1?

You are correct; these data are provided in Table 1. We will include this information.

L445: How did you assess the success of the acetylene blockage? A rough look to table 2 suggests that is experiment 2 which did not work. In general, this is a fundamental problem, since you don't know the n2o reduction rate a priori (it is precisely the info you want to obtain), unless e.g. application of 15N labelled substrate is combined with N2 isotopic analyses (what you did in section 3.2.3, but I don0t see results for the acetylene treatment, or for N2).

Unfortunately, Table 2 does not give information on completeness of blockage since in natural soils the product ratio can vary between the full range of 0 and 1. With ^{15}N tracing we did not conduct a treatment with acetylene, but only without acetylene to estimate the N_2O reduction to N_2 by analysing ^{15}N in N_2O and N_2. Nevertheless, we were able to assess the completeness of blockage of N_2O reduction by C_2H_2 with the experimental setup. This was done by comparing product ratios calculated from $+C_2H_2$ and $-C_2H_2$ treatments with product ratio calculated from ^{15}N treatments (section 3.3 Table 3). To clarify this, we added "It was possible to assess the completeness of blockage of N_2O reduction by C_2H_2 with the experimental setup by comparing product ratios among methods." to l. 337. Although estimated from parallel incubations, i. e. different incubation vessels, both product ratios (^{15}N and C_2H_2) were in similar ranges for Experiment 2, 3, and 4, while only Experiment 1 revealed differences in the two calculated product ratios.

L450: Significance level was established at 0.1 (L366) so p = 0.037 is significant

Thank you for the remark. We will change it in the revised version.

L461: What are the implications for exp. 4? Was there a significant amount of NO3- available in the soil which may compromise your results? Interestingly, many replicates were not analysed; why? For those values coming from two replicates, why didn't you include the standard deviation (as you did in Table 2)?

The soil of experiment 1 and 4 was investigated two times with the aim to identify differences in fungal and bacterial contribution of denitrification. Unfortunately we did not analyse NO_3^- or microbial biomass in 2011 (exp. 4). However, we supplied NO_3^- in excess and we can assume a homogeneous distribution of NO_3^- added due to the experimental procedure. Thus, NO_3^- supply should not affect fungal or bacterial contribution on denitrification in this setup.

Thank you for the remark. We will include the standard deviation of the two samples in the revised version and indicate that only two samples were analysed.

L479: you probably mean eq. 8

Yes, thanks for spotting this. We will correct this in the revised version.

Table 4 and 5: Why did you set the negative values to 0 in Table 5, but not in Table 4? Further to table 5: How are the ranges calculated? Are they coming from the different replicates, or from different SP and delta18O, or both?

We agree that this was imprecisely described and thus included information on the resulting ranges in the table description. Additionally, we will uniformly set negative fractions to zero in the tables.

Section 4.1 and 4.2 should be better streamlined. Actually, section 4.1 refers almost completely to inhibitors (which is section 4.2)

This will be done in the revised version.

L642: It is not clear to me whether high partial pressure induces physiological changes or rather provokes methodological artifacts (or both). In the former case, respiratory effects might also influence denitrification activity. In the latter case, do you expect an effect on diffusion?

Higher partial pressure could result in lower diffusivity of gases from the soil. Unfortunately, we did not analyse the partial pressure of CO_2 during incubation. However, the incubation time was rather short and thus we can assume from published values and own experience that N_2O production was not largely affected since we found increasing production rates over time.

L653: The role of abiotic processes should be briefly discussed here.

Thank you for this remark. Although it is known that abiotic denitrification may occur under the presented conditions it is not possible to quantify N_2O produced from abiotic processes with the used setup. We will include the possibility of co-occuring abiotic N_2O production in section 4.1 as follows: "Additionally, abiotic N_2O production cannot be quantified with the experimental setup, but might be contributing to each inhibitor treatment."

L674-678: Experiments 1 and 4 were performed on the same soils, but you got completely different results. And this applies for the mismatch between tracers and acetylene method (only in treatment 1, as you say), but also for the tracers results per se (70-80% N2O production ratios in exp. 1, 50-60% in exp. 4). How do you explain this?

Samples were taken at different time points and microbial community may change in seasons during the year. We assume that variations in microbial communities and abundances may be the reasons for the differences in results. We will include this information in the discussion section.

We already discussed possibilities of variations in microbial groups for differences in pathways between Experiment 1 and 4 in section 4.6 as follows: "The question arises, why hybrid N_2O formation was only found when the loamy sand was sampled in summer (June, Experiment 4) but not when it was sampled during winter (December, Experiment 1). Amounts of substrates for co-denitrification, i.e. NO_2^- and NH_4^+ or certain organic N compounds, could have been different due to seasonal effects. Moreover, seasonal impacts on microbial communities could have been relevant. Since these factors were not assessed in our study and their impact on co-denitrification is still poorly understood, it is currently not possible to give an answer here."

L701: Include the papers you refer to.

We will include the requested papers: "(e. g. Laughlin and Stevens, 2002; Ladan and Jacinthe, 2016; Chen et al., 2014)".

L702-706: The explanation is right, but I suspect you have many uncertainties in the application of inhibitors. Take into account that CO2 release was not affected by the inhibitors, and N2O not as high

as expected, so you may have a significant contribution of non-inhibitable organisms, so that the substrate effect on N2O reduction rates may not be that important

Thank you, we will describe this point rather generally and rephrase the sentence as "Consequently, inhibiting bacterial denitrification by SIRIN would lower the flux of fungal N_2O". To clarify we will include the references as requested in the comment above: (e. g. Laughlin and Stevens, 2002; Ladan and Jacinthe, 2016; Chen et al., 2014).

L724-727: The whole sentence is contradictory. Is the SP not an isotopic approach? Please, clarify

We apologize for the imprecise description. The sentence will be changed as follows: "In many soil incubation studies with inhibited N_2O reduction very small SP_{N2O} values have been found that were within the range of bacterial pure cultures (Lewicka-Szczebak et al., 2015; Lewicka-Szczebak et al., 2017; Senbayram et al., 2018).