

Review response on “Comparing modified substrate induced respiration with selective inhibition (SIRIN) and N₂O isotope approaches to estimate fungal contribution to denitrification in three arable soils under anoxic conditions” by Lena Rohe et al.

Referee #1

We thank the reviewer for the overall positive critique and for the good comments that will largely improve the manuscript.

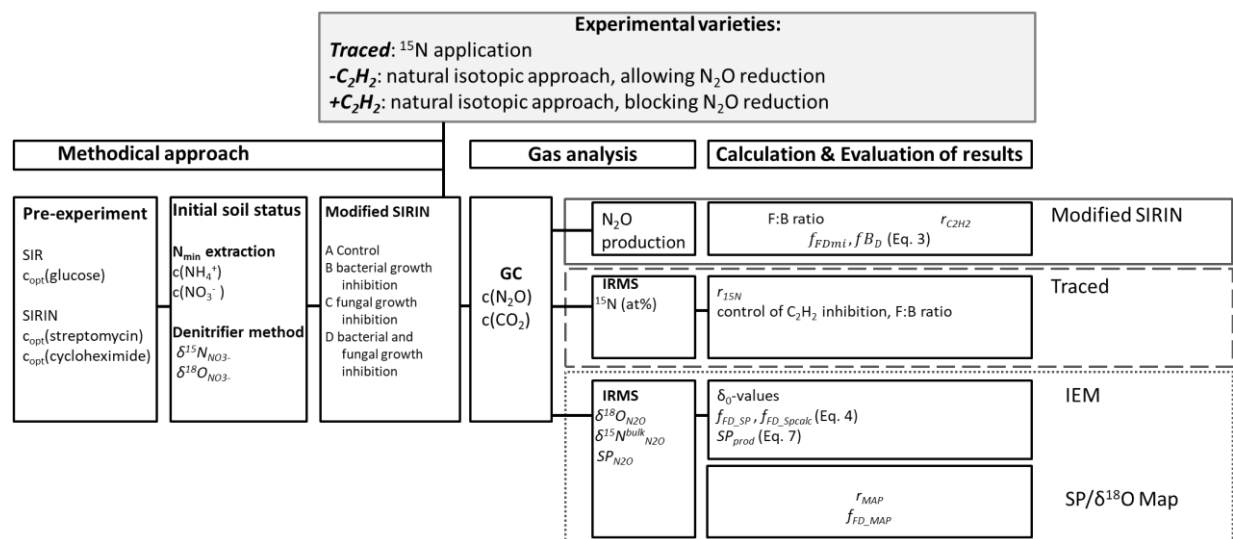
The authors’ answer is in italic font.

Comparing modified substrate induced respiration with selective inhibition 2 (SIRIN) and N₂O isotope approaches to estimate fungal contribution to 3 denitrification in three arable soils under anoxic conditions

Fungal denitrification can make a significant contribution to N₂O production in soils, however emissions are poorly constrained. This study uses a variety of approaches to attempt to quantify the proportion of N₂O produced by fungal denitrification under anaerobic conditions.

The methods are carefully applied however the complex treatment design is challenging to follow and a better overview is needed.

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 variables and abbreviations.



The interpretation and statistical analysis is careful but somewhat basic and empirical – each of the methods is considered separately, and results from one are often used in another (eg. product ratios)

which makes reasoning circular, and assumptions and uncertainties hard to follow. This type of multipronged approach would hugely benefit from a more complex statistical analysis, such as a Bayesian methodology whereby the results from all experiments as well as the uncertainties in many critical parameters from previous studies could all be brought together to gain a much clearer and more robust picture of the results and implications. It would be a great benefit to the paper if the authors would take the opportunity to use such methods to improve the results at this stage, although I suspect they may consider this beyond the scope of the paper and review.

The reviewer is correct, that the manuscript and evaluation of the different measures derived from analysis would benefit from a more detailed data analysis including estimations of uncertainties of the different methods tested. However, as stated in the text the methods tested had high uncertainties that could not clearly be quantified with the presented approaches due to too few data. Only one out of four modified SIRIN experiments revealed a result for the fungal fraction contributing to denitrification. For Bayesian probability the very small number of values and the large uncertainties would result in a very wide probability distribution. We are aware that the different approaches have high uncertainties, especially deriving from partly ineffective inhibition of microbial groups, but we think that a further analysis of uncertainties would not contribute to improve understanding of the present data as we have only a small data set to test and compare different methods in parallel.

The use of English in the paper is not too bad, but would really improve following careful copyediting by a native speaker – it is often awkward and difficult to follow.

We apologize for linguistic errors. We will revise the manuscript carefully.

Overall the paper is of a good scientific quality and worthy of publication, which I recommend once the comments in this review have been addressed.

- Specific comments:

- o L266: How did you calibrate N₂O isotopic values? Where values and/or precision dependent on N₂O concentration? Was interference from or dependency of isotope ratios on CO₂, H₂O or any other gas observed?

The isotopic analysis will be described in more detail in the revised version as follows: “A laboratory standard N₂O gas was used for calibration, having $\delta^{15}\text{N}_{\text{N}_2\text{O}}^{\text{bulk}}$, $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ and $SP_{\text{N}_2\text{O}}$ values of -1.06 ‰, 40.22 ‰, and -2.13 ‰, respectively, in three concentrations (5, 10 and 20 ppm).” Additional information on traps used will be added: “H₂O and CO₂ were trapped with magnesium perchlorate and Ascarite, respectively, to prevent any interference with N₂O analysis.”

- o L290: I guess D is abiotic production, eg. chemodenitrification and similar. But if D is abiotic production and not any kind of artefact, why does it matter if D is lower than A, B and C for this calculation? And why is the denominator A-D? The equation then surely gives fungal production as a proportion of biotic denitrification production rather than as a proportion of total production, which would be more relevant?

The equation for calculating fractions of sources is adapted from the original SIRIN method by Anderson & Domsch 1973. The calculation is based on the assumption that the fraction of N₂O of treatment D is present in all other treatments as well (A, B, C), representing non-inhibitable sources. Thus, calculating (A-D) as denominator enables to calculate the contribution of N₂O production by bacteria or fungi to the proportion of N₂O from bacteria plus fungi. The

method is also based on the assumption that only/mainly bacteria and fungi contribute to N₂O production.

The reviewer is correct, that abiotic N₂O production may be one source in modified SIRIN treatment D. Additionally to that source, we also cannot exclude N₂O production from organisms that were either not inhibited by the antibiotics (e.g. archaea or incompleteness of selective inhibition) or ineffectiveness when organisms are active but not growing. Both inhibitors block the protein biosynthesis and thus are ineffective for ongoing processes. This was discussed in detail in section 4.2. As stated in the Material and Method section (l. 281 ff.), the dominator has to be A-D underlying the assumption that the proportion of undefined sources in D contribute to N₂O in the other three treatments as well. In the Method and Result section, we will include a reference to the discussion section: "A detailed discussion of inhibitor effects and difficulties with organisms that were not inhibited or abiotic sources is presented in section 4.1 and 4.2".

o L379: Why would production rates change with time throughout the incubation? Why did you only use the 10 h time to compare?

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 avoid 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 CO₂ 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. As stated before, antibiotics inhibit the protein biosynthesis, and therefore an increase in microbial growth should be reached by changing the environment. We will expand this by "Thus, short-time incubation should cause changes in environment for microorganisms and initiate growth on the one hand, while it should avoid 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.

o Table 2 / Results S 3.1: Rates for D are clearly not negligible, in fact usually on the order of around half of the total N₂O production. I don't see this as a big problem for Eq. 3, as I stated earlier, but it is a significant problem for the use of Eq. 4, which assumes mixing of only FD and BD endmembers.

The reviewer is correct; the large amount of N₂O produced in treatment D is clearly problematic to interpret data of the other treatments. As presented in Eq. 3, the fungal or bacterial proportion is estimated by taking the production of N₂O from treatment D into account. However, this can only be estimated for N₂O production but not for isotopic values of different treatments. As stated in the results and discussion (section 4.4), the SP_{N₂O} values did not largely differ between the SIRIN treatments A-D. Thus we are aware of an uncertainty that is difficult to be estimated and we will describe this in more detail in the revised version.

o L451: Yes, it sounds like they are a valid estimate of emitted N₂O ie. without reduction, however the IEM still suffers from the problem of unrepresented processes as evidenced by significant fluxes from D.

In section 4.2 the inhibitor effects on N₂O production and interpretation of data were discussed with focussing on treatment D. It is correct that the large N₂O production of non-inhibitable sources (D) was too large in Experiment 1, 3, and 4 to estimate f_{FD} . Calculation of f_{FD} resulted in a large range and was of course uncertain, as N₂O production in treatment D was large, although it was significantly smaller than that of treatment A. However, we decided 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 also 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 result.

As stated in section 4.2, we assumed a similar presence and activity of non-inhibitable N₂O sources on all four treatments and only small variations of SP_{N_2O} values among the four modified SIRIN treatments indicated, that bacteria mainly contributed to N₂O production. This was discussed in detail in section 4.4. The IEM, however, relies on isotopic values (SP_{N_2O}) known from pure culture studies.

o L458: This maybe suggests a problem with either the product ratio or the fractionation factor?

The fractionation factor for N₂O reduction (-6‰) was adapted from published data (Yu et al. 2020) and not directly estimated in the present study. Thus your suggestion might be true and the fractionation factor for N₂O reduction may slightly deviate from the literature value. Apart from that we calculated with average values of SP_{N_2O} and product ratio_{15N}, which of course contributes to deviations between measured and calculated values. A comment on this will be added to section 4.4. The fractionation factor of about -6‰ is an estimate representing a range of measured fractionation factors in soil and pure culture experiments (e.g. Ostrom et al. 2007). Decreasing this average fractionation factor (-6‰) leads to increasing SP_{prod} values, what in turn would result in values more similar to SP_{N_2O} values of variety -C₂H₂. We will describe this possible uncertainty of the fractionation factor used in the present study in more detail in the discussion section of the revised version.

o L474: If inhibition was not successful, there would be less N₂O following inhibition than was really produced (eg. lower denominator of Eq. 6), and the calculated product ratio would be larger than it should be. This seems to be the case in most of Expt 2 and in Expt 4 but in Expt 1 and 3 the opposite is observed. Why would you observe this effect, which is really strong for Expt 1? An unaccounted for process in tracing? Or an additional impact of +C₂H₂ on N cycling that is not just due to reduction? Also, it seems like you don't have complete inhibition for 2 and 4 – maybe 10% not inhibited – how much may this affect results?

It is true, from Eq. 6 (product ratio = $N_2O_{-C_2H_2} / N_2O_{+C_2H_2}$) unsuccessful blockage of N₂O reduction would result in smaller $N_2O_{+C_2H_2}$ values, resulting in larger product ratios. It is well known that N₂O blockage with C₂H₂ is very challenging, but due to the comprehensive experimental setup we did not conduct a control of effective blockage of N₂O reduction using C₂H₂ with ¹⁵N labelling. However, in the present study we used the comparison of product ratios derived from approaches with and without acetylene and the ¹⁵N tracing approach. Comparison of both calculations of product ratio as well as possible artefacts of C₂H₂ blockage was discussed in section 4.3 (NO oxidation and incomplete diffusion of C₂H₂). To clarify this,

we will add "It was possible to assess the completeness of blockage of N₂O reduction by C₂H₂ 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 (¹⁵N and C₂H₂) were in similar ranges for Experiment 2, 3, and 4, while only Experiment 1 revealed differences in the two calculated product ratios. Additionally, the microbial activity may slightly differ among replicates. Regarding the standard deviations, both product ratios were indistinguishable for treatments (Exp. 2, experiment 4 treatment A, B, D).

o S3.4: This suggests that inhibition may have downstream effects on N cycling, eg. through inhibited processing of N species that are important as substrates for other processes. This could be a really significant problem for all your experiments, which all rely to some extent on SIRIN, and warrants a great deal more discussion.

This is true and is a common problem of all inhibitor approaches. Treatments B, C and D revealed that a large number of organisms or processes were not fully inhibited in the presented experiment. This was discussed in detail in the discussion section (section 4.2) and this discussion will be expanded. However, the estimations based on stable isotope approaches do not rely on SIRIN results. As the results of both approaches are only compared, this is not a problem for this approach. This will be further clarified in the discussion.

It is correct, that inhibitors can also be used as C sources for microbial growth and therefore it is absolutely necessary to keep incubation time as short as possible to prevent changes in microbial communities due to species development, also 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 CO₂ 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."

o S3.5: As a rule of thumb, I would have thought that the further the points are from the BD origin, the more FD would be calculated. This appears to be the case for 4 -C₂H₂ but for 3 -C₂H₂ the calculated FFD values are very low. Why is this? Also, most of your points are close to the origin of BD. Can you use uncertainties in isotope measurements and in endmember values to put uncertainty ranges on the FFD estimates? And can you give a minimum FFD that you would detect by this approach? I think given the uncertainties in every term you would need a relatively strong contribution, eg. 20%, for it to be visible.

The very precise uncertainty analysis of the isotope mapping approach (SP/¹⁸O Map) is a complex issue and was published recently (Wu et al., 2019). The uncertainties are indeed large when we take into account all the possible sources of errors. We will include this information with the relevant citation in the discussion.

The points for 4-C₂H₂ and 3-C₂H₂ are the values for the treatments without inhibition of N₂O reduction. So, these points are shifted from the bD field mostly due to N₂O reduction. SP/¹⁸O Map allows for differentiation of N₂O reduction and N₂O fungal admixture. And for these treatments, we calculate that the possible f_{FD} fraction is up to 9 and 20%, respectively. Table 4 presents a summary of the calculation results of different cases (for the range of literature values for SP_{BD} values and possible different ¹⁸O(H₂O) values. This table can be used as estimation of the possible uncertainty of this approach. The range for calculated f_{FD}

values varies up to 20% (for Exp4), so this is the absolute uncertainty of this method in this case study. This discussion with assessment of uncertainty will be added in the manuscript.

o S3.6: These are much lower than the endmember you used for FD. How does this impact your other results? If the fungal endmember was lower than you assumed, the FFD from both IEM and mapping approaches would have been underestimated. Indeed following half your calculated endmembers (4 of 8 are negative) FD and BD could be indistinguishable isotopically. Why do you think your endmembers are so low? Could this relate to underexpression when substrates are limiting, or some other effect?

Fungal endmember values obtained by modified SIRIN were biased by the high uncertainty of the SIRIN approach. As stated above we therefore decided to delete the attempt to calculate SP_{FD} values. Therefore, we had to rely on the values known from literature and further experiment would be needed to compare these values with SP_{N_2O} values known from selective organisms or from a soil culture. These could be investigated by mixing various fungal species known to occur in soil or by isolating fungal communities from soil and conduct experiments under anoxic conditions with supply of electron acceptors and C sources to investigate denitrification. With these incubations, parallel ^{15}N tracing experiments should be conducted to confirm denitrification as the dominating process. However, we think we should not vary SP_{N_2O} values for isotope mapping or IEM, but will discuss this point in section 4.4. As stated in section 4.4, we assume bacterial dominance in the denitrifying community and thus a large uncertainty in the estimated fungal fraction. Using a fungal fraction with high uncertainty would thus results in imprecise SP_{N_2O} values.

o L723: Well, except that the FD endmembers you found were much lower than expected...?

This paragraph focusses on treatment A (without inhibition), and we interpreted the low SP_{N_2O} values as indicative for bacterial dominance of N_2O production. We will clarify this in the revised version. The ^{15}N tracing approach revealed that other processes than denitrification played no or only a minor role and. However, to clarify we will change the beginning of this section (4.4) as follows: "As discussed above, all modified SIRIN treatments of Experiment 1, 3 and 4 were largely affected by N_2O from non-inhibitable organisms or processes which of course has an impact on SP_{N_2O} values of all SIRIN. This made it inappropriate to calculate SP_{N_2O} values for active bacteria or fungi (modified SIRIN B-C).

o L768: I don't think you do show this, because you had really large variability in your FD map values, and no clear quantitative answer for fFD because you had no clear endmember for soil water.

This is correct, the variability among experiments (soils) was large, although it was much smaller among samples from one experiment, with exception of Experiment 4 ($-C_2H_2$). In the revised version we will point out that in the presented application of $SP/\delta^{18}O$ MAP we fitted $\delta^{18}O$ values of water, but calculation would be more precise when measuring $\delta^{18}O$ values of water during comparable experiments.

• Minor comments:

o L60: This description of denitrification should be the first sentence in the paragraph.

We will change this in the revised version.

o L149-156: This discussion of whether fungal soil and pure culture values agree seems logically to fit before the more detailed introduction to IEM and mixing line approaches. Overall the introduction is

a little hard to follow – it would be good to really think about the logical flow of the concepts from least to most complex and structure the intro accordingly.

We will revise the introduction. Thank you for the constructive comment.

o L216-236: A table summarising the treatments and abbreviations used throughout would be very useful here. It is very confusing at the moment and needs to be laid out much more clearly.

Thanks for this constructive and helpful suggestion. We will insert such a table in the supplementary material of the revised version and provide a scheme showing approaches and different measured values in the material and method section (as shown above).

o L239: The word “Experiment” here is confusing since it is really four different soils, right? It would be better to call the different soils “Soil 1” and so on. Also, why does Soil 4 get more fertiliser added?

In the revised version the term “Experiment” will be changed throughout the text, tables and figures to “Soil”.

o L300: f rather than F would be a more common abbreviation for fraction. Also, this assumes no abiotic denitrification.

Thank you for this advice. We will change it in the revised version. We will also include the information that non-inhibitable organisms contributing to N₂O production and abiotic processes are not included in the assumptions of Eq. 4.

o S2.5.1 is very hard to follow because of the treatment designations. Again, a table earlier in the methods is needed, much more clearly linking each specific treatment combination to a clear abbreviation code.

Thank you, as described above we will insert such a table.

o L322: Product ratio is much too long to be used repeatedly as a variable, maybe just r_{P} or r_{P} similar?

Thank you for this advice. We will change it to $r_{15\text{N}}$, $r_{\text{C}_2\text{H}_2}$, and r_{MAP} in the revised version.

o S3.2.2: -C₂H₂ is basically a control compared to +C₂H₂ and it seems like logically it should be discussed first.

You are right. We will change the order of 3.2.1 and 3.2.2 in the revised version.

o L645: Partial pressure effects would potentially also be expected to affect N₂O production, but you saw an increase in N₂O production with time?

This is true and unfortunately we did not analyse the partial pressure of CO₂ during incubation. However, due to the inhibitor application, the incubation time was rather short and we can only assume that the N₂O production was not largely affected since we found increasing production rates over time.

o L4.2: Also potentially abiotic production.

In the revised version we will include information on this as follows: “Additionally, abiotic N₂O production cannot be quantified with the experimental setup, but might be contributing to each inhibitor treatment.”