<u>Response to reviewer</u>

Reviewers' comments:

Anonymous Referee #1: The manuscript discusses the effects of mineral N fertilization rates and manure amendment on soil N_2O emissions across barley, wheat, and sorghum. The study leverages soil physicochemical analysis, nitrification- and denitrification-related genes, and gas emissions to assess the impact of fertilization strategies on N use efficiency and N_2O emissions. The long-term aspects of the study site, the monthly variations of N_2O emissions across crops, and the genetic components of N transforming pathways, provide a rich, publishable study. However, although the data is interesting, I do think this manuscript is not in good shape yet.

A: Thanks for the reviewer's thorough and constructive feedback. We have thoroughly reviewed comments and addressed all the critical points raised by the reviewer. Detailed responses are provided below.

1. The manuscript's writing style, particularly in the Introduction and Discussion sections, is disjointed and verbose. The introduction is wordy with some redundancies. For example, the first and second paragraphs could be consolidated.

A: Thank you for highlighting this. We have strengthened the Introduction and Discussion sections. We have shortened the text by modifying and removing less relevant sentences or sentence fragments. We have additionally included sentences into both sections (for example added factors that influence N-cycle processes). Also, we have consolidated some paragraphs in both Introduction and Discussion sections.

For example, we have consolidated first and second paragraph in Introduction section as reviewer suggested and removed less relevant sentences: "*The rising demand for agricultural commodities and the management of agroecosystems are important factors contributing to global environmental problems. Increasing crop yield while reducing pollution from agricultural production is crucial (Abdalla et al., 2019; Tilman et al., 2011). Global food demand projections suggest a 50% increase in agricultural production by 2050 (compared to 2012) to feed the fast-growing human population (FAO, 2017). Enhancing agricultural production involves actions such as expanding agricultural land, applying more fertilisers, and using water resources and fertilisers more effectively (Tian et al., 2021). In today's agricultural practises, the applied N with fertilisation is often excessive for plant needs (Robertson and Vitousek et al., 2009; Zhou et al., 2016). About half of the applied N to the fields is not taken up by crops (Coskun et al., 2017); which may lead to N loss in the surrounding environment. Key processes for soil N loss are denitrification, ammonia oxidation, N leaching, erosion of soil and ammonia (NH_3) volatilisation (Thomson et al., 2012). This results in adverse ecological impacts, such as eutrophication of aquatic ecosystems and increased gaseous emissions of N into the atmosphere (Cameron et al., 2013; Liu et al., 2017; Whetton et al., 2022).*"

In Discussion section, we have removed the repetitive parts and improved flowing of the text. We have also consolidated paragraphs, where discussion of the same topic was previously divided into multiple, short paragraphs.

2. The factors that influence nitrification, denitrification, comammox, and DNRA should be provided.

A: We have made adjustments, and the introduction now incorporates the factors that influence the above-mentioned processes. We have included in the Introduction section the following paragraphs:

"*Synthetic fertilizers containing NH_3 offer an immediate substrate for ammonia oxidizers, thus accelerating the nitrification process (Ayiti & Babalola, 2022). Also, fertilizers that raise soil pH can significantly enhance the nitrification rate, as increasing soil pH from 4.8 to 6.7 can boost nitrification rates by 30 times (DeForest & Otuya, 2020).*"

"*Carbon to nitrogen ratio (C/N) and C/NO_3^- are recognised as the main environmental factors controlling, which nitrate-reducing process is favoured as DNRA and denitrifying microbes compete for NO_3^- and carbon sources (Bai et al., 2020). DNRA is dominant in the presence of a high C/N ratio and low NO_3^- availability, while the denitrification process favours a low ratio of C/N and C/ NO_3^- (Bai et al., 2020; Pandey et al., 2020).*"

3. Background information about effects of different crops on N_2O emissions should be provided.

A: Thank you for drawing attention to this. To our understanding, there is a limited number of studies where comparisons between crop species and N_2O emission have been made. We have included additional information, and now the Introduction section contains details about the effects of crops on N_2O emissions. We have included the following paragraph in the Introduction section:

"Only a limited number of studies have compared N_2O emissions between different crop species. Abdalla *et al*. (2022) found that crop type has significant effect ($p<0.05$) on the BNE values from soil. However, study including 372 sites showed that cover crops did not have significant ($p>0.05$) effect on direct N_2O emissions (Abdalla *et al*., 2019)."

4. Additionally, the manuscript should clarify why nitrification is considered a primary source N_2O fluxes. Though nitrification is the dominant step over denitrification in the soil, N_2O is not the major product of nitrification.

A: In arable soil, nitrification tends to be a more important process than denitrification as arable soils are usually well-aerated and have sufficient oxygen to support nitrifying microbes. Yes, we agree that this part needs further clarification. In the manuscript, we mean that nitrification potential was higher than the denitrification one. We have rephrased sentences containing the word "dominance" throughout the manuscript in the Abstract, Discussion and Conclusions section. For example, we included the following sentence in the Conclusions section:

"*N_2O emissions were mostly caused by nitrification, with potential contribution from denitrification, comammox and DNRA processes.*"

5. Hypothesis 5 (do you really need that many hypotheses?) is not a testable/measurable hypothesis, and how would the authors define "prospective"? Adaptability? Yield? N use efficiency? Water use efficiency?

A: We have reduced the number of hypotheses. We agree with reviewer`s comment that hypothesis "*sorghum is a prospective crop to cultivate in temperate climate*" could not be fully proven with presented results. We excluded this hypothesis from the paper.

6. My major concern in M&M is the experimental designs. It's more like a pseudo-replicated (the three study plots within each crop are not independent) instead of a completed randomized block design by looking at Fig 1.

A: We will use linear mixed-effects models in the revised version of the manuscript to overcome the possible problem of the pseudo-replicates. We use it to test statistical differences between N emissions of different fertilisation rates in plots with different crop types. We use spatial (different fertilisation rate) and temporal (sampling dates) effects as random effects. This model will help account for both fixed and random effects inside the experimental design, which provides better analysis of data.

7. Besides, the authors should consider providing more information about manure amendments, like the major source, the CN ratio of the manure, and whether the manure application is just one-time for this experiment or it's a part of long-term experiments (if so, the manure application started since which year?

A: Study is made on long-term three-field crop rotation experiment established in 1989 and all fertilization treatments are applied continuously from start. Manure treatment is amended with solid farmyard manure (ca 40 t ha^{-1}) in every third year before sorghum/potato. The farmyard manure is cattle dung with straw bedding, freely fermented before use 6-8 months in heap. We have added this information to the Material and Methods section. Additionally, we have added chemical properties (C, N, P, K, dry matter) of the last manure that was added in year 2022 and also last ten year average chemical properties of the manure in the Supplementary materials.

8. A climate diagram or bi-weekly/monthly precipitation amount should also be provided to align with soil moisture (Fig. S6) and N_2O emissions plots (Fig. 5).

A: Thank you for the comment! We have included a climate diagram for the year 2022 in the Supplementary materials section.

9. Other soil properties like pH, texture, and bulk density, which influence nutrient dynamics and gas emissions, are also crucial and should be included in the study.

A: The soil type is Glossic Retisols associated with is a Stagnic Luvisol. Texture by FAO classification is sandy loam: 57.86% sand (>0.063 mm), 33.58% silt (0.063–0.002 mm) and 8.55% clay (<0.002 mm). Soil bulk density was in range of 1.5 to 1.6 g cm^{-3} with slightly lower values for manure treatment plots. The average pH levels were 5.4 for barley plots, 5.3 for wheat plots, 5.6 for sorghum plots without manure amendment, and 6.2 for sorghum plots with manure amendment. We have added the above-mentioned information to the Material and Methods section.

10. The author should report soil organic C instead of total C.

A: Soil organic carbon is already included in the analyses; it is just named a little differently because of the method used: HWEOC – hot-water extractable organic carbon. We can rename it to SOC if the reviewer requests

11. I don't understand why the authors use PCA instead of simpler methods like bar charts to present soil C, N, and inorganic N in different sites.

A: PCA gives a good overview of the data and helps to observe trends and patterns. For example, lines 200-203: "*Fertilised plots had higher soil N_{tot}, C_{tot}, NO_3^{-}-N and NH_4^{+}-N content compared to non-fertilised plots according to the principal component analysis (PCA) (Figure 2). For sorghum without manure amendment plots (Figure 2C), $NO3^{-}$-N and $NH4^{+}$-N contents were more different from each other compared to sorghum with manure amendment plots, where NO^{-}_{3}-N and NH_4^{+}-N contents were relatively similar (Figure 2D).*"

However, as the reviewer requested, we have added additional figures in the Supplementary materials section to represent TN and TC data in different sites.

12. I also think the authors should consider using other approaches (like structural equation models or approaches that can consider contributions from multiple factors) in addition to ANOVA and Pearson correlation to analyze their data. N cycling is complicated and has been influenced by many factors including vegetation, texture, soil moisture (precipitation), temperature, soil fertility and C concentration, and management practices like tillage, fertilization, etc. Simple correlation analysis may not always be the best way to capture those complicated interactions. And since the authors measured N_2O emissions with time, I think they should analyze the data by different time periods instead of just cumulative fluxes.

A: Yes, we agree that SEM would have been a good option, and that is why we tested it as well. We tried SEM with a small set of soil chemical parameters (the ones that are the main substrates/controllers of nitrification and denitrification, adding nitrification and denitrification as latent variables), but the results were not clear. We have not tried it yet with microbiological parameters, but we will try it as well, and if that seems to work, we will add those results to the manuscript. Additionally, we will include more complicated modeling through linear mixed-effects modeling.

13. It's also odd for me to compare sorghum + manure with barley/wheat without manure application in Fig 6. It should be barley vs sorghum vs wheat as one part, and sorghum vs sorghum w/ manure as another part.

A: We have chosen to present all treatments and crop types together in Figure 6, because it provides an overview of the differences across all crop types and treatments. Additionally, we wanted to limit the number of figures, as we have already seven figures in the main text and six figures in the Supplementary materials section.

14. The Discussion sections probably need some major work. There are many repetitive parts (N_2O emissions increased with high mineral N application) and many statements are contradicted with each other in the current version. For example, the authors said there is no correlation between soil moisture levels and N_2O emissions or functional marker gene abundances. Then the authors note that the lowest levels of N_2O emissions and functional marker gene abundances occurred during periods of low soil moisture.

A: In the Discussion section, we removed repetitive content and improved the flow of the text. Additionally, we consolidated paragraphs where the discussion of the same topic was previously divided into multiple short paragraphs.

We were not able to see any correlations between soil moisture levels and N_2O emissions. Although we were able to see visually in the middle of July low N_2O emissions in Figure 5 in the main text and low soil moisture content in Figure S6 in the Supplementary materials section. Also, we were able to see low gene abundances in some genes in Figures S1, S2, S3 and S4 in the Supplementary materials section.

15. Another example is the authors said no significant influence of crop type on N_2O emissions, then the authors suggested sorghum as a potential crop in Northern Europe as sorghum maintained low N_2O emissions.

A: Yes, there was no significant influence of crop type on N_2O emissions. Probably the effect of fertiliser was much greater than the effect of crop type, therefore it was not visible. The suggestion is based on the smaller N losses compared to other two crop types in table S3 in the Supplementary materials section.

16. The authors also said N_2O emissions increased with fertilization rates for wheat and barley plots, but the statistical results in Fig 6 showed no significant differences between N0 and N80.

A: Indeed, there is no significant differences between fertilisation rates N0 and N80 for wheat and barley plots in Figure 6A. However, there is a significant difference between fertilisation rates N0 and N160 in barley and wheat plots. Also, we can see significant differences between rates N80 and N160 for both barley and wheat plots.

17. I also don't know how to use the ratio of gene copy numbers to infer the resources of N_2O. Both nitrification and denitrification contribute to N_2O emissions. "dominance" might overstate the results given the weak strength of the correlation. Nitrification and denitrification are complex processes influenced by a variety of environmental and microbial factors. This correlation alone does not conclusively establish dominance or any cause-effect relationship.

A: The *amoA*/*nir* ratio indicates the potential activity of nitrifying and denitrifying microorganisms as it shows abundance of these microbes. We agree that it might be too strong to use word "dominance." We have corrected this throughout the entire manuscript. For example, we included the following sentence in the Conclusions section:

"*N_2O emissions were mostly caused by nitrification, with potential contribution from denitrification, comammox and DNRA processes.*"

We included following sentence in the Discussion section: "*The significant positive correlation between the ratio of amoA/nir and N_2O emissions ($\rho= 0.20$, $p<0.001$) indicates that nitrification potential was higher than denitrification potential and thereby N_2O emissions were mainly related to nitrification in the soil.*"

18. It always needs extra caution on suggestions replacing current crops with sorghum. Assuming that sorghum with enhanced biological nitrification inhibition properties could reduce N_2O given the same levels of N as other crops (corn, wheat, barley, etc) is applied, how much grain demand could be met by sorghum when considering large-scale implementation of the practice? Instead, including sorghum in the existing crop rotation and understanding its subsequent effects on N dynamics seems a more practical approach.

A: Yes, we agree that it must be checked further to determine if it would be suitable for large-scale implementation. However, we were more suggesting that it could be a great alternative in the near future from the perspectives of climate and yield. We have smoothed our phrases and added that including sorghum in the existing crop rotation and its subsequent effects on N dynamics seems like a good option.

19. Please use upper case L to represent liter.

A: We corrected it.

20. L52: 70% of N fertilizers were lost due to nitrification and denitrification? You just said about half of applied N to the field is not taken by plants (L44). In addition, how about N leaching and volatilization?

A: Sorry for the confusion. We have rephrased it more clearly to avoid any misunderstanding. We have added information about other possible N losses pathways such as N leaching and volatilization in the Introduction section. The text has been revised as follows:

"*The key microbial processes leading to soil N loss are nitrification and denitrification (Thomson et al., 2012). In agriculture, N fertilisers added to the soil can be lost due to these processes (Saud et al., 2022).*"

"*About half of the applied N to the fields is not taken up by crops (Coskun et al., 2017); which may lead to N loss in the surrounding environment. Main soil nitrogen loss mechanisms include denitrification, ammonia oxidation, N leaching, erosion of soil and ammonia (NH_3) volatilisation (Thomson et al., 2012).*"

21. L94: IOSDV: Should put full name first and abbreviation in the parentheses.

A: We corrected it.

22. L96: on crop type? Did you mean on crop responses of various crops?

A: Yes, exactly. The sentence has been revised to make it clearer as follows:

"*The experiment was set up as a three-field crop rotation experiment in 1989 to investigate the long-term effects of mineral and organic fertilisation on crop responses of various crops and soil properties.*"

23. Table S1:. Please express the unit of herbicide application as L ha-1. Should be the same order as other figures: barley - Sorghum – wheat

A: Thank you for pointing this out. We have made the changes accordingly in the Supplementary materials section.

24. L120: Ø: diameter? is this 65 L the entire volume of PVC collars+lid? Looks like the volume is not consistent across treatments due to chamber extension, which create another sources of variable

A: It was not possible to measure different crops with the same 65 L chamber because the height of the crop exceeded chamber size.

Yes, 65 L is the entire volume of polyvinyl chloride chamber with collar. Chamber extensions were used for some treatments of sorghum on four sampling days. As chamber extensions increase the total volume of the chamber, it is essential to adjust the calculations accordingly. On all four occasions, the use of chamber extensions is considered in the calculations.

25. L188: What's biomass yield produced? Biomass production?

A: We agree that the term is confusing. We have corrected the manuscript according to the reviewer`s suggestion.

26. Figures: The figures should be labeled in order in the Result section. Fig S5 comes first in this draft so it should be S1. Similarly, Fig S6 -> S2. And Figure S1-4 should be S3-6. And please use the correct format for unit, like using mg kg-1 instead of mg/kg

A: Thanks for the comment. We have now labeled all the figures accordingly and corrected the format for unit.

27. L221: The unit of Y axis in Fig 3B is not concentration.

A: Accepted. The word "concentration" has been replaced with "content."

28. Fig 3a: Using ton ha-1 in the y axis may better align with the context

A: Using ton ha^{-1} can also be a good choice. However, we chose to leave the unit unchanged for now but can make the adjustment if needed.

29. Fig 5: precipitation data should be provided.

A: As mentioned above in comment number 8, we have included a climate diagram for the year 2022 in the Supplementary materials section.

30. L267: The statement that cumulative barley

A: Unfortunately, we do not understand the reviewer`s comment. It seems that part of the sentence is missing.

31. Fig 6: I don't understand the reason for estimating N_2 emissions in this study.

A: The reason for estimating N_2 emissions in this study was done to understand more widely the N cycling, for example denitrification. By including the functional genes responsible for denitrification in the study, additional estimation of N_2 emissions can provide insights into the denitrification process.

32. Table 1: I am not sure if the reader needs to know sum of squares, means square, w2. And it's odd that manure amendment is significant for N_2O emissions in Table 1 but not in Fig 6.

A: We have removed sum of squares, means square, ω^2 as reviewer suggested. In Figure 6, there is simple testing of statistical significance between different fertilisation rates and crop types. However, in the table 1, we have considered interactions between N_2O emissions and different factors and measured the effect size of fertilization rate, manure amendment, and crop type on N_2O emissions.

33. L334: It seems long-term manure application showed no significant difference in NO_3, NH_4, N_2O, and N_2? That is odd. In Fig 6A, soils under 231 kg N ha^{-1} (N0 at sorghum w/ manure) treatment produce lower cumulative N_2O compared to those under 80 kg N ha^{-1} (N80 at sorghum). That required some explanations.

A: We will add additional explanations.

34. L339: This paragraph needs further expended. We generally expect organic fertilization would increase SOC, total N, yield, and N2O due to direct C & N (both labile and recalcitrant) inputs. Same as L379, what's the potential reason for different results in this study and previous studies?

A: We will add additional explanations.

35. L381: there are no significant difference between sorghum and sorghum + manure in N0 and N160 (Fig 6A).

A: Manure addition was significant according to the Table 1 in the main text, although it was not significant within all the fertiliser addition rates (Figure 6A).

36. L434: if there is a liner response, the authors should provide p-value and r2

A: Thanks for pointing that out. We have removed sections from the manuscript that refer to a linear response between fertilisation rate and N_2O emissions due to the comments of anonymous referee nr 3.