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Interactive comment on "Soil properties impacting denitrifier community size, structure, and activity in New Zealand dairy-grazed pasture" *by* Neha Jha et al.

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Anonymous Referee #2

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Scientific significance: Does the manuscript represent a substantial contribution to scientific progress within the scope of Biogeosciences (substantial new concepts, ideas, methods, or data)?

The manuscript is aiming at unravelling the relationships between denitrifier community structure and environmental parameters in pasture soils. It is well within the focus of the journal. The methods used are solid but not cutting edge and suited to

answer some of the questions. However, the experimental design is not perfect for the big aim of understanding the connections between nitrous oxide emissions, denitrifier community structure composition and soil type and land management.

Author's Response: As in our responses to R1 above, we concede that our aim of understanding the link between the structure, abundance, and activity of denitrifiers based on soil physicochemical characteristics may not directly 'enhance our ability to promote complete denitrification in order to reduce N2O emissions from pastoral agriculture' and we have now revised the introduction to reflect this.

Scientific quality: Are the scientific approach and applied methods valid? Are the results discussed in an appropriate and balanced way (consideration of related work, including appropriate references)?

In principal I think the study has great potential but in present form suffers a little from too many variables between the different soils and not enough samples/replicates of similar soils to resolve their influences.

Author's Response: We present n=6 for all soil physicochemical datasets and n=3 for molecular microbial datasets. However molecular work was based on 6 separate DNA extractions followed by pooling 2 extractions/PCR amplification in attempt to better represent potential spatial variability among replicates.

I further have a slight problem with the determination of copy numbers for functional genes and using these numbers as 'abundances' of the organisms. The denitrifiers could be the same percentage of the total population in all soils and it would make sense to at least also determine the copy numbers of the bacterial 16S rRNA gene with a general primer set. Then there are still issues with gene copy number per genome, functional gene/16S rRNA gene ratio in a genome and such left, which would be harder to account for.

Author's Response: Yes, agreed. This problem is inherent in many qPCR studies

of functional genes. We have revised the methods and results sections to reflect this limitation of our approach. In particular, we have moved figure 4a to the supplementary data so that our results and discussion focus on the nos: nir ratio only. Because these genes do not always (but can) co-occur within an organism their ratio may better reflect cell numbers of complete: incomplete denitrifiers. Of course, this assumes similar PCR bias among the different primer sets, but that assumption applies equally to amplification of a "housekeeper gene" like 16S rRNA or rpoB.

From an organismic point of view it has to be considered that the nirS/K and nosZ genes are not distributed completely independent. They are linked in organisms that can perform the full denitrification pathway. Therefore it is quite surprising that the NMS analysis of nosZ (Fig. 3c) doesn't show any clustering while nirS/K did. Would it be possible to identify the T-RFs of nirS/K that have similar distribution patterns over the samples than those from nosZ? That way only subsets of T-RFs could be analyzed in order to determine how the soil parameters influence their presence/abundance.

Author's Response: This is an interesting suggestion. It would certainly shed light on the how complete denitrifiers respond to varied soil conditions. However, this is really a separate question from the one we pose here because complete denitrifiers are typically only a small subset ($\sim$ 0.5%; Deslippe et al. 2014) of all denitrifiers in New Zealand pasture soils. Should we follow this suggestion, we would miss incomplete denitrifiers, which are equally likely to be affected by the soil physicochemical characteristics we study here, and they are especially of concern for GHG emissions.

The discussion is a bit lackluster and is missing a part in which the results are discussed in the frame of the bigger question, nitrous oxide emissions. Especially as the results of the study seem to suggest that all the soil parameters collected do not explain the distribution and abundance of the nosZ gene over the different soils. How does this fit with the question? I would have expected a more thorough discussion of this, also the potential pitfalls of the methods used that could have influenced this result (primer bias, etc.).

Author's Response: Yes agreed. We have thoroughly revised the discussion section and we now more fully address reasons that the distribution and abundance of nosZ genes respond primarily to SWC and Olsen P in our study.

Presentation quality: Are the scientific results and conclusions presented in a clear, concise, and well-structured way (number and quality of figures/tables, appropriate use of English language)?

The quality of the presentation is lacking a little with sentences that sometimes need re-reading before they make sense. Minor grammar mistakes here and there can be found too as well as layout issues.

Author's Response: We have given the manuscript a general overhaul and respond to specific issues in detail below.

The figures are not always as informative as they could be.

Figure 1 doesn't resolve the differences between the sites closely located next to each other well. It gives a general impression where the sites are located but why not move it to SOM and then add three zoomed in insert maps that resolve the three local areas where the samples were taken better?

Author's Response: Thanks we have revised this figure.

Figure 2 is really busy, especially with the legend for each dot. As the color code already defines which sampling site they are from, why not just put the numbers for the replicates on? And I don't think it adds anything to know which exact replicates are closer together as it is not mentioned elsewhere in the manuscript. So it might be an idea to leave the annotations in the figure off altogether and just rely on the color code explained in the legend. Further, the circles defining the clusters should not cross the borders of the ordination.

Author's Response: Done. Good suggestion, thanks.

Figure 3 is again pretty busy and would need some cleaning up. It would also make sense to stick to the same symbols/colors as in Fig. 2. Fig. 3 b is pretty meaningless as the majority of samples can't be resolved in the presented ordination. Here the question is if an outlier analysis could be used to remove the data points at the edges of the ordination. If not, I would suggest to at least show an ordination with only the data points that cluster tightly together in the SOM to resolve potential trends in this subset of samples that is not affected by the 'outliers'.

Author's Response: In this version of the manuscript we have recreated figure 3 (now figure 4). It now retains the same symbol colours as in figure 2, but has different symbol shapes to communicate the soil groups (based on the PCA result). We disagree that nirS ordination (formerly fig3b) is meaningless because it illustrates that nirK community structure responded to the same physicochemical characteristics (SWC and Olsen P) as nirS communities did, which is a major point of the manuscript. However we acknowledge that the importance of this result was not sufficiently described in the previous results section nor was it adequately discussed. Consequently, in this version of the manuscript we have corrected those issues as well. While we disagree that outlier analysis is appropriate in this case (removal of HR and PL soils constitutes a 20% data reduction), as requested we have, added an ordination of the nirK data without PL and HR soils to the supplementary materials, which shows that Olsen P and soil water variables remain the primary driver of nirK community structure, even for this reduced dataset. Likewise, we have added this information to the results and discussion.

The data presented in table 2 would also make a nice figure, maybe even in combination with Fig. 4.

Author's Response: Agreed. Since the patterns of significance were similar for gene richness, evenness and diversity we chose, (for the sake of simplicity) to make a figure illustrating only gene richness by soil group. We have moved table 2 to the supplementary section.

Specific comments

Multiple pages: gene names are normally all italicized, also e.g. the 'K' from 'nirK'

Author's Response: Thanks, we have now thoroughly checked the manuscript for italicized gene name.

p 3, l 16: Sampling was conducted between August and December. Where there any kind of controls to test for seasonality effects?

Author's Response: Our aim was to sample from the range of soil conditions that occur on NZ pasture farms. It was therefore important to sample in both wet and dry seasons. However it was not our intention to characterise the amplitude of seasonal variation within any given soil, and so we did not design controls that would allow us to assess seasonal variation. However, to ensure that our sampling spanned the range of soil moistures that are typical for pasture soils in NZ we sampled the soils that were expected to be wettest (OH and TeK) in winter and the soils that were expected to be driest in summer (PS, LM MF) the other soils were sampled in between these. We have clarified this in the methods section

p 3, l 18: Were the 25 soil cores per replicate homogenized and mixed during the process of sieving?

Author's Response: Yes. Thanks for pointing out that this was unclear. This information has been added to the methods section.

p 3, l 18: Were all samples besides the ones for molecular data stored at 4 âŮęC? If some of the analyses were done 6 months later I would be worried about changes in the soils as microbial activity will continue, although much slower.

Author's Response: The soils used in this study were collected over a nearly 6-month period. After each soil was sampled it was immediately sieved and pH, nitrate (NO3–) & ammonium (NH4+) –N (mineral-N), total nitrogen (TN), total carbon (TC), Olsen phosphorus (P), microbial biomass carbon (MBC), soluble C, and denitrification en-

zyme activity (DEA) were measured within 1-2 weeks. Measurements of DEA and MBC were prioritized so that they typically occurred within the first few days after sieving. However, after the first two sets of soils were sampled, a technical problem with our analytical set-up caused delay in measurements of MBC for nearly 3 months. Given that it was not possible to go back to farms and resample all soils (as their physico-chemical properties were likely to have changed in this time, we remeasured MBC on the initially sampled and stored soils after 3, 4 and 7 months, we determined that no significant changes in MBC occurred between the time period of 3 and 7 months. We therefore report MBC data for all soils that were stored between 4 and 6 months. We understand that this issue can be confusing to readers so we have clarified this in the methods section, as simply as possible.

p 6, l 8 ff/table 2/figure 3: The number of T-RFs used for the NMS analysis seems to be quite low and in the case of nirK also pretty different between the samples. This could result in problems with the ordination that is hard to evaluate. It would be nice to report stress values and also provide the data matrices used for the NMS analysis in the SOM so the reader can evaluate them.

Author's Response: Thank you for this useful comment. Total T-RF richness was nirS=52, nirK=53, nosZ=47, which is quite typical for T-RFLP studies of functional genes. However, you are correct that the minimum and maximum number of T-RF varied among samples, which could possibly have contributed to instability in the NMDS ordinations we present. Final stress for the three ordinations in fig 3 were as follows: nirS=12.5, nirK=5.5, nosZ=9.4. So this was clearly not a major problem in our datasets. Nevertheless this is a good point and we have added this information to the discussion and SOM sections. We would also point out that the new T-RF richness figure (and specifically the size of the error bars on the histograms), which we have produced in response to your earlier comment, will also help our readers to evaluate variability in gene richness among samples in our study.

p 8, l 14: Wouldn't it have been possible to avoid uneven grazing and excretal deposi-

tion by fencing off an area a couple of weeks prior to sampling? Or at least try to avoid these spots by a careful screening of the area to find representative spots?

Author's Response: All of the pastures sampled in this study were fenced from livestock and none had been grazed within 8 weeks of sampling. Thank you for pointing out this omission; this has now been added to the methods section. As explained in the methods section we also avoided any (old) dung patches when sampling, as bovine gut bacteria could have contaminated the soil sample if we had pushed the soil corer through a dung pile, and so we did not do this.

p 9, l 10 ff: I am not sure why the authors are so surprised by this. The sampling procedure (25 cores combined) should diminish the signals from different microniches and create an integrated signal.

Author's Response: True, but as we say we would then expect nirS and nirK to be negatively correlated overall. No significant negative correlation between nirS and nirK suggests independent environmental or stochastic controls on the size of these populations. This section has been expanded in the revised discussion.

p 10, l 21: 'saturated': I assume with water?

Author's Response: Yes, clarified.

p 10, l 24 ff: If the adsorption of copper is the reason that there is less nitrous oxide reduction, then why are there active nirKs, which also have copper as co-factor? There must be another explanation for this observation or could a reduction in the copy numbers of nirK be observed in these soils as well?

Author's Response: Yes, thanks for pointing out that our argument was confusing. We have revised the conclusions to make the point clearer. We did not intend to suggest that adsorption of copper is the reason that there is less nitrous oxide reduction in allophanic soils, but rather less nitrite reduction. We agree that because allophanic soils adsorb copper, they are likely to select against nirK denitrifiers. We expected this to

reduce the overall number of genes encoding nitrite reductase in group 1 soils, but we didn't observe this (Fig 4). We have revised the discussion section of the manuscript to include this point. The point of interest in the conclusion section is that, the nos:nir gene ratio data we show agrees with previous work by our group showing that allophanic soils emit greater N2O: (N2O + N2O) relative to other soil types.

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