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Interactive comment on "Physical constraints for respiration in microbial hotspots in soil and their importance for denitrification" *by* Steffen Schlüter et al.

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Summary

The manuscript entitled "Physical constraints for respiration in microbial hotspots in soil and their importance for denitrification" by Steffen Schlüter, Jan Zawallich, Hans-Jörg Vogel and Peter Dörsch deals with studying the role of soil structure on the rates and dynamics of respiration and denitrification in a closed system. The authors manufactured porous glass beads as their model system for microbial

hotspots "soil aggregates". Porous glass beads were embedded in background of sand in a closed flasks and individual porous glass beads were incubated with either one of two soil bacterial isolates. One of the isolates, Paracoccus denitrificans perfomes the whole denitrification pathway including N2O reductase to N2 while the other isolate (Agrobacterium tumefaciens) produces N2O as the final metabolite. The idea of using controlled model systems to study the important process of denitrification in soil aggregates is highly novel and the author's attempt is clever! However, the results in the current form of the manuscript remain rather speculative and lack a meaningful transition to relevant scenarios for soil systems. At this stage, I focused on concerns regarding the design of the experiment, results and their interpretations, with minor emphasize on linguistic problems.

Detailed review

Major concerns:

I) Some of experimental decisions require better justifications in the context of denitrification process in natural soil systems. For instance, why two isolates with full or partial denitrification steps are chosen? I could think of this choice as an interesting option when studying the residence times of N2O in soil aggregates but I was confused with lack of justifications for these choices. A similar problem could be stated to the design of layered vs. random microbial hotspots "aggregates". It was nearly impossible to conceptualize how these types of distributions might actually have any relevance for soil systems or representing any specific scenarios with generalizable outcome. In the present form it is quite confusing and hard to understand what is the relevant, unknown question that the study aims to address and how the experimental design helps to navigate in that direction. The conclusions are mostly based on describing the observations and lacks discussion and interpretations to link these specific results to advance general knowledge on soil denitrification /respiration. My understanding is that a simple mathematical model that would explain the basic mechanisms underlying the observations could go a long way to translate the results into general conclusions.

II) While authors attempted to justify the choice of closed system for their experiments, but there are fundamental issues to measure gas fluxes in the closed system. One of the main issues is that the accumulation of gases in the headspace for a long time will result into altering dissolved gas concentration profile within the soil aggregates and the soil profile itself that will ultimately affect the gas fluxes from the soil surface. From the theoretical calculations (unpublished, but shareable upon request), having even quite large headspace (1litter) would affect gas concentration profile and gas storage in soil within less than 10 hours. In other studies to resolve this issue, partial closing of the headspace is suggested (A. Ebrahimi and Or 2018). This is especially important to make sure that the oxygen concentration at the headspace remains unchanged, similar to natural soil profile throughout the experiments. In the current study, it is hard to disentangle the main mechanisms of forming anoxic microsites in the soil profile, if the main reason is because of the physical restrictions and presence of soil aggregates or just simply because of oxygen consumption and lack of oxygen in the headspace.

III) While I would like to be supportive of the idea of microbial hotspots, given the experimental design, it is hard to understand why these porous beads are hotspots for the microbial activity, compared to the full sample (background sand). First, there is no explicit observations of microbial activity at the bead scale. Second, the choice of Sistrom's medium (rich, dissolved medium) insures that substrates are uniformly distributed all over the flask (sand+beads) and knowing the fact that bacterial cells could easy spread all over the flask in the given water contents (A. N. Ebrahimi and Or 2014; Tecon and Or 2016), even if pre-inoculated only within the beads, I would then argue that the substrate and bacteria could actually be quite uniformly

distributed in the flask and turns the whole system and not only beads into "microbial hotspots". This might still be fine, if the argument of "microbial hotspot" is not based on the substrate or bacteria distribution but rather physical regulations that beads impose on the oxygen gradients, leading to anoxic hotspots in the beads. However, this argument is also unlikely given the data presented in Figure 6 that shows similar air connectivity and tortuosity for the hotspots and the full system. Surprisingly, air distance seems to be higher in the full system (Figure 6C) that could mean some regions in the full system (likely the bottom of the flask) could be even more anoxic than the porous beads. I think this aspect of the research will require better explanations of the assumptions and the reasoning behind the experimental design.

Minor concerns:

- In general, the Figures require more comprehensive captions. In the current form, it was painful to get full grasp of the figures without going back and forth into the text to learn the conditions that the experiments were performed.

- I think it would help a lot to use equal range for N2, NO and N2O or plotting the ratios of these gases to the total amount of available nitrogen. It was really hard to compare the rates of these gases to each other given the way the results were presented. Similar comment could be made for O2 and CO2.

- Throughout the manuscript, a few times the arguments were based on assuming that P. dentrificans is slow grower because it produces less CO2. However, this argument would only hold if both strains would have similar yield of converting substrate to biomass and CO2. Otherwise, one may argue P. dentrificans is more efficient on converting substrate to biomass and that is why produces less CO2.

- In Figure 4, scenario with 30 percent WFPS starts with about 200umol more O2 compared to 90 percent WFPS, however at the end of the experiment both scenario produces approximately 450umol CO2 with no O2 left in the flask. I was wondering where does the extra O2 is gone in 30 percent WFPS scenario? It might help to check again the mass conservations for different elements.

- I am also concerned that some of the dynamics that we see for NO and N2O gases are solely driven from the closed-nature of the experimental system. For instance, any drop in the amount of NO and N2O in the headspace observed in Figure 2 to 4 and wouldn't really happen in the open system. This type of artificial storage of reactive gases in the headspace interferes with the important storage mechanism of gases within soil aggregates (Rabot et al. 2015; Rabot, Hénault, and Cousin 2014) that significantly affect the total rate of N2O emission from soil profile (A. Ebrahimi and Or 2018).

Recommendations:

- While my comments may sound rather major, I still believe the study opens up a promising path toward more quantitative understanding of the denitrification process and key players in soil. I think the feature of this study is the quantification of the impact of individual factors (e.g., soil structure, water content, oxygen availability) and offering a well-controlled system with the option of disentangling multiple interacting factors. At this stage, it is fine that the experimental condition does not capture the most common scenarios in natural soil system and it would be insightful if the results would offer generalizable conclusions on the underlying mechanisms. To do this, I recommend that authors put extra work on conceptualizing the role of individual factors on the rates and patterns observed for each of gas

fluxes. I think the ideal way would be implementing a mathematical model or at minimum summarizing the results into a conceptual representation of the whole processes.

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