

The manuscript "Fertilizer regimes reshape microbial interaction networks without altering sugarcane rhizosphere diversity" by Zamora-Leiva et al. addresses an interesting and relevant question of how long-term NPK fertilization affects the microbial community in sugarcane rhizosphere soils. The core question is interesting and the data from a long-term study site in tropical agroecosystems is valuable. The writing is generally clear. However, the manuscript has substantial methodological concerns that need to be addressed before it is suitable for publication. The per-treatment network analysis which represents the central claim of the paper, is unclear and statistically weak with $n=3$ samples. Key methodological details (like replication plots, samples, stats) need clarification.

Section-by-Section Comments:

1. Introduction

Ln 79-81: Sentence is not clear. Please rephrase.

2. Materials and methods

Section 2.1.1:

- The paper states the trial was established in 1998 but planting in the current study cycle was in spring 2020 with sampling in March 2022. Was there a gap in management between 1998 and 2020? How many cycles have occurred and what was the fertilization history in prior cycles? Please include this information; important for interpreting "legacy effects."

Section 2.1.2:

- N5 treatment labels do not match in text and Table 1. Please check this throughout the manuscript.
- Table 1 and 2 : Include the unit kg/ha in the table headers.
- Ln 142: Instead of "control 1" and "control 2", suggest mentioning "control" and "blank" here in brackets as those are the terms used in figures and tables.
- For "Blank" (rhizosphere without fertilization): Was this plot never fertilized since 1998? Or not fertilized during the three fertilizer applications for other plots? Was it part of the same experimental block? This control is important but underspecified.
- Ln 143: Are the three replicates per treatment true biological replicates (independent plots) or subsamples from the same plot?

Section 2.1.3:

- This section begins mid-sentence ("These were shaken to remove excess soil..."). This needs to be corrected.
- Not clear at all how the rhizosphere was collected. Please include details on how the rhizosphere was operationally defined for sampling and how it was collected. It's not clear if only the rhizosphere is being compared for fertilization treatments.
- How many root systems were pooled per replicate?
- What was the sampling depth?
- Ln 152: What do the authors mean by "enriched soil samples"?

Section 2.1.4:

- Ln, 163: Was DNA extracted from the rhizosphere or bulk soil?

- How many replicates per treatment?

Section 2.1.5:

- Ln 184: How many samples with <1,000 reads were excluded? This is critical as there appears to be only 3 reps per treatment.
- Ln 185: "Samples were analyzed with both unassigned ASVs removed and retained" — Which version is reported in the results? Removing unclassified ASVs before alpha and beta diversity calculations would bias the results. Please clarify which analyses were conducted with "unassigned ASVs removed" and how that will affect the conclusions.
- Ln 192: Suggest replacing "prokaryotic" with "microbial" for avoiding confusion and staying consistent.
- Ln 194-196: These analyses are mentioned in the methods but completely absent from the results. If differential abundance analyses were performed, the results must be reported. If they yielded no significant results, that itself is a result worth reporting. This needs to be addressed.
- Ln 197: Were the fungal and bacterial datasets merged at the ASV level? Please specify.
- Does the combined network include control samples?
- Ln 205-207: "between taxa". Clarify that the taxa here represent ASVs.
- Ln 205-207: Please justify why only positive correlations were retained.
- Ln 207-209: The construction of per-treatment network is not clear. Please clarify how and why the same rarefaction, correlation, and filtering criteria were applied as the combined network. For each treatment, there are only 3 samples. But the combined network has 33 (?). Was the same significance filtering applied here for $\rho > 0.6$? Were each treatment's 3 samples rarefied to 11,248 reads (same depth, different subset of samples)? Or the full rarefied table is subset to only the 3 samples of that treatment after rarefaction? Were only positive correlations retained here as well? It's not clear to me why they were constructed the same way as the combined network if the comparisons are only based on treatments.

3. Results

Section 3.1

- Table 3 : Include units of measurement for all variables; missing for Ca and Mg
- Ln 232: Suggest using one term: "soil respiration" or "microbial respiration" in text and Table 3.
- "No significant differences among treatments": Which statistical tests were applied to the physicochemical data? What were the p-values? Please include the test and p-values.
- The stats used for both soil properties and microbiome analyses should be mentioned in the methods.

Section 3.2

- Ln 240: This information should go under methods. Clearly specify reps per treatment.

Section 3.5

- Fig 5: The methods say only positive correlations were retained, but the results and figure explicitly describe and display five negative correlations as red edges. Please explain this contradiction.

- Ln 335 - 339: If negative correlations were filtered out by design, how are the authors interpreting this?
- Ln 355-360: "2,116 to 1,450 nodes". Please check the language. The number of edges is being discussed as nodes.
- Figure 6: Why are the edges so high for the per-treatment networks? It looks like a subset of samples (n=3 for any single treatment) produces a network with similar or greater node counts but dramatically more edges than the full combined network. Was the significance filter not applied to per-treatment networks? If so, this would contradict the methods and needs to be clarified. If the networks are built without significance filtering, how are they comparable across treatments?

4. Discussion

Section 4.2

- Ln 437- 439: The unclassified taxa discussion is very interesting. Considering that the percentage is so high for bacteria, how do these findings affect the conclusions about community stability? Does the proportion of unclassified ASVs differ across treatments? It's not clear to me if these taxa were not included in diversity analyses. If not, this would be problematic and bias the results.

Section 4.3

- Ln 462-467: The methods need clarity on how the per-treatment networks are being compared. Also considering that there are only 3 reps per treatment, the interpretation needs to be substantially softened for this section as well as the community composition statements.
- Ln 466: The comparisons of these network properties are not present. Suggest including a table with these measurements.

5. Conclusions

Recommend moderating the language. "long-term fertilization in brown sialitic soils can profoundly influence the organization of rhizosphere microbial communities" is overstated given the methodological caveats, particularly the n=3 per treatment network analysis.