22nd May 2026

Dear editor and reviewers,

We would like to resubmit our revised manuscript to Soil. The manuscript ID is: EGUSPHERE-2026-629

We thank you for reviewing our manuscript and for your valuable comments, which helped us improve it. We have revised the manuscript in accordance with your comments. The revised portions of the manuscript have been highlighted in red.

Our responses (in red font) to the comments are provided below.

We look forward to your response.

Sincerely,

Manuel Paneque (mpaneque@uchile.cl);

Reviewer #2 comments:

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:

- **Introduction**

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

R. We will rephrase to the following text: “This response is influenced by several factors, including soil type (Huang et al., 2025), climate (Du et al., 2025), cropping history (Pan et al., 2014), and management intensity (Suman et al., 2022)”.

- **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."

R. We thank the reviewer for pointing out the need for this important historical context. There were no gaps in management between 1998 and 2020. The experimental plots have been continuously cultivated with sugarcane under consistent 5-year cycles (consisting of one plant cane harvest followed by four ratoon harvests, with one cut per year). Crucially, the specific fertilization treatments assigned to each plot have been strictly and continuously maintained across all prior cycles since the trial's establishment in 1998. Therefore, the 'legacy effects' observed in our 2022 sampling represent the cumulative, uninterrupted 24-year impact of these specific nutrient regimes on the soil and microbiome. We have explicitly incorporated this historical management information into the Methods section (Section 2.1.1) to aid in the correct interpretation of the long-term trial

Section 2.1.2:

- N5 treatment labels do not match in text and Table 1. Please check this throughout the manuscript.

R. We changed N5 treatment labels to "(N50P0K0), only nitrogen"

- Table 1 and 2 : Include the unit kg/ha in the table headers.

R. This will be added to the respecting tables.

- 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.

R. We will accept the suggestion and change the text.

- 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.

R. This plot has never fertilized since 1998, and yes, this plot was part of the same experimental block.

- Ln 143: Are the three replicates per treatment true biological replicates (independent plots) or subsamples from the same plot?

R. Yes, every replicate is biological replicates, since each sample was taken from a different plot

Section 2.1.3:

- This section begins mid-sentence ("These were shaken to remove excess soil..."). This needs to be corrected.

The sentence will be rephrased to: "The sugarcane roots were gently shaken to remove the loosely bound bulk soil, retaining only the firmly adhering soil, which is strictly considered the rhizosphere. These root-adhering soil samples were then carefully collected into 50 mL

Falcon™ tubes (Thermo Fisher Scientific, Waltham, MA, USA) and transported to the laboratory (Zamora-Leiva et al., 2025) until completing the volume with roots".

- 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.

R. We appreciate the reviewer's request for clarity. As mentioned in a previous response, we have now explicitly defined our operational collection method in the text: the rhizosphere was defined as the soil firmly adhering to the roots after gentle shaking. Furthermore, we clarify that the experimental design compares the fertilized rhizosphere treatments against both non-fertilized rhizosphere (control) and non-fertilized bulk soil (blank). This has been clarified in Section 2.1.3.

- How many root systems were pooled per replicate?

R. For each replicate, root systems of individual sugarcane plants were collected until the collecting tube was full and pooled to ensure a representative sample of that specific plot. We have added this detail to the Methods section.

- What was the sampling depth?

R. The sampling depth was approximately 10 cm, which corresponds to the main root exploration zone for sugarcane. This information has been added to Section 2.1.3.

- Ln 152: What do the authors mean by "enriched soil samples"?

R. By enriched soil, we refer to soil sampled at several sites (on a limited sampling space), and then mixed, to better represent the natural heterogeneity of the sampled site. This means that soil sample for which soil chemistry was evaluated is a mixture of different points near (bulk soil) or around the plant (rhizosphere).

The following paragraphs have been revised under the sample collection, for a better understanding of the soil and rhizosphere sampling process:

Individual root system of sugarcane plants was extracted. These were shaken to remove excess soil not adhering to the roots. The plants were stored in 50 ml Falcon™ tubes (Fisher Scientific, Waltham, MA, USA) and transported to the laboratory. Plants were vortexed to dislodge soil particles adhering to the roots and the soil was subsequently used for DNA extraction. Simultaneously, the soil removed from around the roots (within 3 cm) was collected as an enriched rhizosphere soil sample for physicochemical analysis.

The enriched soil (control and blank) samples comprised five subsamples of approximately 200 g, which were combined and mixed to better represent the sample area. Subsequently, the enriched soil samples were used for chemical analysis.

Section 2.1.4:

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

R. For both. We will add "rhizosphere and control".

- How many replicates per treatment?

r. DNA was extracted from both kinds of samples, with three replicates per treatment. Hence, the text will be re write as follows: “For each rhizosphere and control sample, genomic DNA was extracted (three replicates for treatment) from a total of 0.5 g of fresh soil using the DNeasy Powersoil DNA isolation kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions”.

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.

R. We appreciate the reviewer’s careful attention to our sample size. We confirm that no samples were excluded based on this criterion. The <1,000 reads threshold is a standard quality-control step built into our bioinformatic pipeline; however, all 39 sequenced samples comfortably exceeded this minimum read count and were fully retained for downstream analyses. We have clarified this in the manuscript to assure readers that all three replicates per treatment remained intact.

- 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.

R. We analyze the samples with and without unassigned ASVs to evaluate if there were differences on differential abundance when unassigned ASVs were removed. However, we understand that the exclusion of ASVs could affect the results, especially of alpha and beta diversity. What we finally did on the manuscript was to analyze the complete set of data, but if unassigned data occupy much space on the figures, we eliminate them graphically.

- Ln 192: Suggest replacing “prokaryotic” with “microbial” for avoiding confusion and staying consistent.

R. This will be replaced in the text.

- 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.

R. Indeed, the analysis was made with no significant results, so we decided to not make any figure and to not give importance in the text. However, we thank you for the valuable comment, and we will add some discussion on this matter. So, the text following text will be added at the end of section 3.3: “To further investigate potential treatment-driven shifts at specific taxonomic levels, differential abundance analyses were conducted using LEfSe and ANCOM-BC2. Notably, neither approach identified any taxa with statistically significant differential abundance among the evaluated fertilization regimes (LDA score < 2.0; adjusted $p > 0.05$). This lack of significant biomarkers further corroborates the overall compositional stability of the sugarcane rhizosphere microbiome across treatments."

- Ln 197: Were the fungal and bacterial datasets merged at the ASV level? Please specify.

R. Yes, fungal and bacterial were merged as ASV levels. This will be explicated on the method section with the following addition to the text: "For the co-occurrence network and overlapping community analyses, the bacterial and fungal datasets were merged at ASV level"

- Does the combined network include control samples?

Yes, the combined network includes all samples, including controls. Based on comments of another revisor, we modified the network and included only one network with all samples. Then, the nodes of the network were depicted with colors related to a specific nutrient, as shown on figure 5 (new).

- Ln 205-207: "between taxa". Clarify that the taxa here represent ASVs.

Taxa will be replaced with ASVs.

- Ln 205-207: Please justify why only positive correlations were retained.

Initially, we only retain positive correlations because were only few negatives. Now, on the new network, we retain all the correlations (positive and negative).

- 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.

We sincerely thank the reviewer for this critical observation, which prompted us to deeply re-evaluate our network methodology.

First, regarding the rarefaction step: to ensure standardized sequencing effort across the entire study, the full combined ASV table (all samples) was rarefied to an even depth of 11,248 reads prior to any subsetting or downstream analysis.

Second, and more importantly, we fully agree with the reviewer's implicit concern regarding the statistical validity of computing per-treatment networks with only 3 samples using strict correlation thresholds (rho > 0.6). Small sample sizes inherently inflate the risk of spurious correlations. To completely eliminate this bias and address the reviewer's concern, we have removed the per-treatment networks from the revised manuscript.

Instead, we adopted a more robust topological approach: we constructed a single, unified Global Network incorporating all samples across all treatments. This large sample size provides the statistical power necessary to validate strict filtering criteria (Spearman rho > 0.6, $p < 0.01$). To evaluate the treatment-specific effects (such as the impact of N or P fertilization) without compromising network statistics, we now map the significantly enriched taxa (biomarkers identified via LEfSe) directly onto this robust Global Network framework (see revised Figure X). This allows us to observe how different fertilizers recruit specific modules within the stabilized rhizosphere core community, resolving the methodological concerns while strengthening our ecological discussion.

- **Results**

Section 3.1

- Table 3: Include units of measurement for all variables; missing for Ca and Mg

R. This will be added to the manuscript

- Ln 232: Suggest using one term: “soil respiration” or “microbial respiration” in text and Table 3.

R. This recommendation will be accepted and “Soil respiration” will be used on both line 232 and

- "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.

R. We have updated the Methods and Results sections to explicitly state that soil physicochemical properties were analyzed using a one-way ANOVA followed by a post-hoc Tukey's test). The overall p-values for all comparisons were >0.05, confirming no significant differences. We also add columns to the supplementary table 1 with data about statistical test.

- The stats used for both soil properties and microbiome analyses should be mentioned in the methods.

R. We completely agree. We have added a dedicated "Statistical Analysis" subsection at the end of the Materials and Methods. This section comprehensively details the tests used for soil properties (ANOVA), alpha diversity (Kruskal-Wallis), beta diversity (PERMANOVA), and network correlations (Spearman with Benjamini-Hochberg FDR correction).

Section 3.2

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

Agree. This information should be moved to methods section.

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.

We sincerely thank the reviewer for pointing out this issue. Originally, we make the figure with positive and negative correlations but decided to just show the positive for the negative are only a few. Now, we recreate the network with all correlations.

- Ln 335 - 339: If negative correlations were filtered out by design, how are the authors interpreting this?

R. We remade the complete global network (Figure 5), now including both positive and negative correlations. As a result, we have updated our interpretation in the discussion. Negative correlations (which represent a minority of edges) are now properly interpreted as potential competitive exclusion for resources or distinct niche preferences among specific microbial taxa, while positive correlations reflect the dominant cooperative or shared environmental responses.

- Ln 355-360: "2,116 to 1,450 nodes". Please check the language. The number of edges is being discussed as nodes.

R. We thank the reviewer for catching this typographical error. The word "nodes" was mistakenly used instead of "edges". Regardless, as we have completely replaced the per-treatment networks with a unified Global Network (as addressed in major comments), this specific section and its corresponding numbers have been entirely rewritten to describe the topological features of the new robust global network.

- 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?

R. Figure 6 was completely remade.

- **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.

R. This is a critical point, and we apologize for not making it clearer. To prevent any bias, all unclassified ASVs were strictly retained for all alpha and beta diversity analyses, as well as for network construction. They were only filtered out later, strictly for taxonomic-assignment visualizations, to ensure that we discussed identifiable biological entities. Because they were included in the diversity metrics, we think that our conclusions regarding overall community stability remain mathematically sound and unbiased. We have added a clarifying sentence in the Methods and Discussion to ensure this distinction is clear.

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.

R. We completely agree with the reviewer's methodological concern regarding the n=3 per-treatment networks. As detailed in our previous responses, we have completely removed the small per-treatment networks to avoid spurious correlations. Consequently, we have deleted the speculative comparisons between them. The interpretation in this section has been entirely rewritten and focuses strictly on the robust Global Spearman Network (n=39), mapping treatment-specific responsive taxa (biomarkers) onto this statistically validated topology.

- Ln 466: The comparisons of these network properties are not present. Suggest including a table with these measurements.

R. Since we have removed the problematic per-treatment networks from the study, a comparative table of their properties is no longer applicable. Instead, we have provided the topological properties (e.g., modularity, average degree, clustering coefficient) for the new, unified Global Network in the main text to validate its structural robustness.

- **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.

R. We agree that the previous phrasing was too strong, especially in light of our initial methodology. With our updated global network approach (n=39), the statistical caveats have been resolved; however, our new data actually supports a more nuanced conclusion. We have moderated the language in the Conclusions to state: *"Long-term fertilization in brown sialitic soils does not completely restructure the overall rhizosphere community; rather, specific nutrient applications (such as Phosphorus) act as targeted drivers that recruit and organize specialized trans-domain accessory microbiomes within a highly stable core network."*