Dr. Alexandra Linz

University of Wisconsin - Madison

(414) 630-7850

[amlinz@wisc.edu](mailto:amlinz@wisc.edu)

Dr. Ludmila Chistoserdova

Academic Editor

*PeerJ*

September 28, 2018

Dear Dr. Chistoserdova,

Thank you for the opportunity to revise our paper “Connections between freshwater carbon and nutrient cycles revealed through reconstructed population genomes” for submission to PeerJ. The reviewer comments were insightful, and we incorporated many in our revisions. We have included detailed responses to the reviewers’ points below, with our responses marked in red.

A recurring theme in the comments received from the reviewers was that the relevance and importance of our research needed to be more clearly stated. To address these comments, we substantially revised and expanded the introduction, while simultaneously reducing the length of the text by over 1,000 words. The resulting manuscript is more concise and focused.

One reviewer expressed the concern that a combined Results/Discussion section does not conform to PeerJ standards. We advocate keeping these sections combined to reduce redundancy and better communicate the relevance of each result, which were also critiques made by both reviewers. We have identified several recent PeerJ articles with combined Results/Discussion sections (detailed in the response to reviewers below). However, we will concede to your judgement in this matter.

We thank the reviewers for their work towards improving our manuscript. All co-authors have approved these revisions. Please let us know if any further revisions are required.

Sincerely,

Alexandra Linz

Reviewer 1 (Adrienne Narrowe)

Basic reporting No comment.

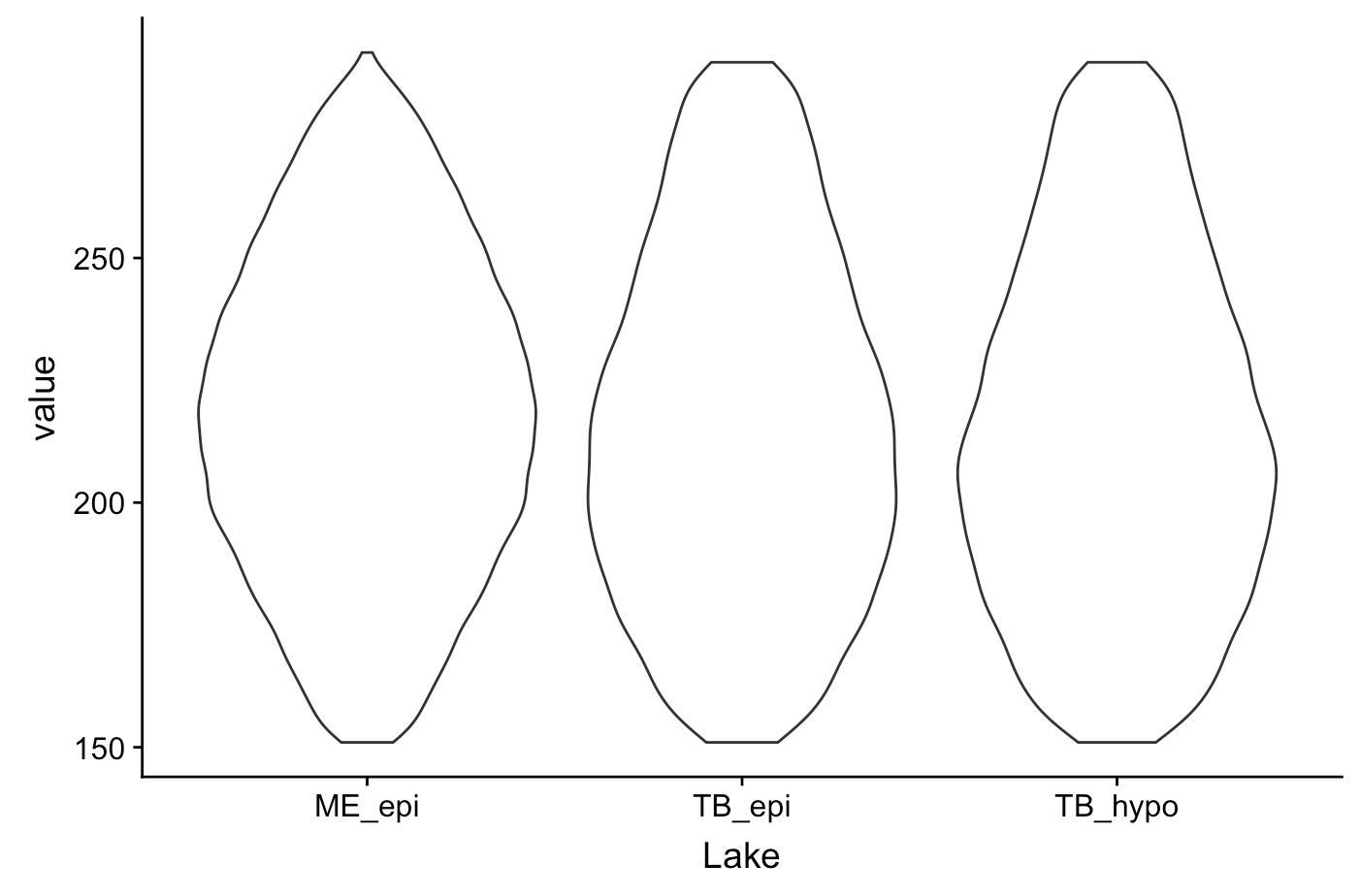
Experimental design No comment.

Validity of the findings Linz et al. present additional analyses of 3 time-series metagenomic datasets, which were collected over several years from 2 fractions of a humic lake, Trout Bog (3 years) and 1 fraction of eutrophic Lake Mendota (5 years). They provide 1) gene-centric analyses of the metagenomes (pooled by fraction) examining the differential distribution of a set of functional marker genes, and 2) population-genome-centric analyses of metagenome-assembled-genome bins (MAGs). With the marker-gene analyses they infer differences in multiple metabolic pathways among the lakes and water column fractions. They then use the MAGs to associate the identified processes with specific taxa, finding that certain metabolic processes appear to co-occur within taxa, and that common processes across sites are associated with (and attributed to) differing phylogenetic groups. This manuscript is very well written and organized and materials and scripts have been provided to make it reproducible. The following two main concerns regarding their analyses and interpretations should be addressed:

1) 'Community Functional Marker Gene Analysis' Lines 179-194

• A technical concern with the marker-gene analysis is that the authors are using merged reads (Lines 143-147) for which they predict ORFs and use blast to assign function. Previously the authors stated that the merged reads range from 150-290bp (Lines 110-111). The authors do not indicate the read lengths used for sequencing, this should be added to the relevant supplementary information, or if all are using the same read lengths (2x150?), this can be added to the text. Even if these are all using the same read-lengths, please test that the distribution of merged read lengths does not differ significantly across sample groups. I am concerned that a skewed distribution toward shorter merged read lengths would result in the underprediction/failure to detect marker genes, particularly longer ones. If your method already controls for this, please add a statement explaining how.

Read lengths in both lakes are 2x150bp - this has been added to the text in Line 108 and Supplemental Table S2, and we thank Dr. Narrowe for noting this omission. We analyzed the distribution of merged read lengths in each site and found that the median length of merged reads in Lake Mendota was 218bp, 214bp in Trout Bog’s epilimnion, and 212bp in Trout Bog’s hypolimnion. While the distributions of read lengths in each site were significantly different using the Wilcoxon signed rank test, skew in the distributions is slight (see figure below). As the site with the shortest read lengths (Trout Bog’s hypolimnion) also tended to have the most marker gene hits, we do not feel that underprediction due to differences in read lengths is a concern in this case.



• Even once this is resolved, I am confused by the reporting of the marker gene analysis. It is unclear why the authors used LEfSE to identify significant differences in gene 'abundance' among sites (Line 149), yet Figure 1 employs and presents results from pairwise Wilcoxon rank sum tests. I understand that in the second case the authors are combining multiple genes together to simplify the presentation; however in the case of citrate lyase, Figure 1 indicates that the two comparisons are significant by this combined test, but the LEfSE results for aclA and aclB report "not\_significant" for both (TB layers, ML/TB epi) comparisons. Please explain this discrepancy. Do your conclusions vary based on which results you are reporting? Additionally, please add to Supplemental Data S2 a notation so that it is clear which markers are combined to generate the categories on the y-axis in Figure 1. Should you choose to display only the LEfSE results, a form similar to that used in Figure 2 might be better showing the actual genes and their groupings.

We agree that presenting both methods is confusing and have chosen to present only the Wilcoxon signed-rank test results, as this is the simpler test of the two. LEfSe also employs the Wilcoxon test after an initial screen for genes with potentially significant differences in distributions between sites. However, since our list of genes to test is relatively small, we can run the Wilcoxon test without a preceding screening step.

Regarding the discrepancy in citrate lyase, the abundances of these marker genes are relatively low, and we suspect that combining both marker genes pushed this category over the edge for significance. We have removed this category from Figure 1. We have also added a category column to Data S2 (renumbered as Data S4).

Specific changes in the text reflecting the removal of LEfSe significance testing:

L148: removed sentence “Significant differences in gene frequency between sites were identified using LEfSE (Segata et al., 012).

L186-188: removed “Many genes differed significantly by site, indicating contrasting gene content between lakes and layers (Data S3). To further infer differences in microbial metabolism…”

L316: removed “In contrast, citrate lyase, the marker gene for the reverse TCA cycle, was observed most frequently in Trout Bog’s hypolimnion.”

L322-324: removed “As Chlorobium is a strictly anaerobic lineage, the presence of citrate lyase in these populations may explain why this gene was observed more frequently in metagenomes from Trout Bog’s hypolimnion.”

2) Overall, the remaining descriptions of the metabolic pathways identified within the MAGs were good but could benefit from additional interpretation. There were several paragraphs where it felt that the authors were describing at length the results, which are nicely displayed in Figure 2, but I was left wondering 'why do I care?' Please place these discussions in the environmental context. A particular example is the paragraph on the cytochrome oxidases. To summarize, my recommendation is to revisit these paragraphs and confirm the conclusions were strongly stated and the linkages to differing chemistries across sites made effectively.

Per this and Reviewer 2’s comments, we have made efforts throughout the text to better discuss the relevance of our results. We have substantially revised the introduction with more information on why our results are important to the field (discussed in Reviewer 2’s section). We also added or improved topic sentences, deleted results that did not add to the main message, and stated conclusions more directly, particularly in the section on central metabolism.

L180: added “Because Lake Mendota and Trout Bog have contrasting water chemistry, we expected that microbial metabolisms would differ between lakes, and that these differences would be reflected in metagenomic gene content.”

L226: added “It [nitrogen availability] is often a determining factor of trophic status in a lake and a risk factor for the development of toxic cyanobacterial blooms (Smith, 2003; Beversdorf, Miller & McMahon, 2013).”

L259: 1st sentence changed to “We noted a high frequency of genes related to polyamine biosynthesis and degradation in our MAGs.”

L409: 1st sentence changed to “We investigated the types of cytochrome oxidases encoded in our MAGs to compare oxidative phosphorylation between lakes and layers.”

L415-421: deleted (information about cytochromes not relating to oxygen concentrations)

L425-431: deleted (information about hydrogenase types not discussed in text)

L453: added “The pathways predicted in our MAGs suggest which low molecular weight compounds may be important carbon substrates in freshwater.

L461: removed “The Methylophilales MAGs also likely degrade methylamines, based on the presence of genes encoding the N-methylglutamate pathway or the tetrahydrofolate pathway (Latypova et al., 2010).”

L470: 1st sentence changed to “Because our metagenomes comprise a time series, we can investigate potential changes in function over time using our MAGs and functional marker genes.”

Comments for the Author Additional points to address:

In 'How Representative are the MAGs?' (Lines 195-223) and 'Using MAGs to track population abundances over time' (Lines 469-491):

• The authors make a qualitative comparison at the phylum level across the datasets and methods indicating that the assignments "largely" agree. What is the point to this analysis? If anything this analysis underscores the bias associated with all methods of describing microbial community composition and undermines the implicit assumption across the manuscript that the MAGs are representative of the community, and therefore are accountable for the observed differences in marker genes. Please summarize why this comparison matters and how it supports the conclusions reached in this manuscript. Please explain explicitly at the end of this paragraph what the main takeaway from this analysis is and how it is related to the rest of the observations. Or remove.

We agree with Dr. Narrowe that the assumption that our MAGs are representative of the larger community is incorrect and misleading, and have worked to remove that assumption throughout the main text. Still, we feel that reporting the results of 16S rRNA gene amplicon sequencing is important to allow comparison of our dataset to previous studies of freshwater communities that used only 16S rRNA gene amplicon sequencing and to confirm that the years included in our dataset are not “abnormal” in terms of microbial community compared to existing longer-term 16S rRNA gene amplicon analyses.

We have worked to clarify that distinction in the text and have split Figure S2 into two separate plots (new Figure S2 and S3) to avoid inviting comparisons between community composition via MAGs and 16S rRNA gene amplicon data.

L195: “How Representative are the MAGs?” changed to “Overview of the MAGs dataset”

L212-220: removed (compared taxonomy of MAGs to 16S rRNA gene amplicon results)

L220: added “We also compared 16S rRNA gene amplicon sequencing data from the same timeframe as the metagenomes to confirm that the microbial community composition for these lakes and years was not “abnormal” compared to previously published studies.”

L222: added “... and about [the taxonomic compositions of] freshwater communities in general (Newton et al., 2011).”

L222: removed “The detection of similar phyla using both methods suggests that our MAGs are representative of the resident microbial communities.”.

• If the authors would like to rely on this similarity to ascribe the observed marker-gene abundances to the MAGs with similar pathways, this could be better achieved by making a direct comparison of the merged reads (marker genes) identified with those encoded in the MAGs in order to quantify which are actually associated with the assembled MAGs and what proportion remained unassembled. I believe this is what the authors are trying to do with the final analysis of the paper, linking nitrogenase marker genes to the dominant cyanobacterial MAG, however in that analysis it is also unclear what exactly is being measured and compared. It seems that the authors are correlating the abundances of nitrogenase genes with the abundances of the Cyanobacteria MAG. Why then not make a direct comparison of sequences? Lines 485-6, state "it is possible that other diazotrophic populations were more abundant, or that the nitrogenase subunits were derived from populations that did not assemble into MAGs." The authors should have the data available to actually test this statement. Please explain if there is some reason why this analysis could not be done, otherwise I think it would strengthen these observations if it is possible to directly attribute the nitrogenase counts to the MAGs rather than making the indirect link presented in Figure 4.

If we use nitrogenase genes from our MAGs as our subject, we would expect to see the same trends over time as in our MAGs because abundance of the genes would be driven by abundance of the populations represented by the MAGs. Using more general marker genes for nitrogen fixation gives us a better chance at capturing greater diversity than just Cyanobacteria. Our main point with this figure is that we found highly correlated trends using two different reference sequences.

We did try using genes encoding nitrogenase subunits from our Cyanobacteria MAGs as Dr. Narrowe suggests, and found an increase of approximately 2 orders of magnitude in the abundances of these genes compared to the “general” marker genes. However, using the MAG nitrogenases in our marker gene reference set and then correlating the results to the relative number of reads mapped to each MAG is too circular. In any case, the observed trend over time was the same as the broader nitrogenase marker gene set. Because of these similar results and the logic explained above, we prefer to present the genes from the general marker gene BLAST.

We have worked to clarify what is being measured and compared in Fig. 4 in both the main text and figure legend.

L474-479: deleted and replaced with “We compared read coverage-based abundance of the dominant *Cyanobacteria* MAG to the normalized number of BLAST hits in the metagenomes from abundant functional marker genes encoding nitrogenase subunits…”

L482-491: deleted and replaced with “As expected, we detected significant correlations (p< 0.05) between MAG abundance and nitrogen fixation marker genes in 2008, 2011, and 2012. In these years, the dominant *Cyanobacteria* MAGs were predicted to fix nitrogen based on gene content, while the dominant MAGs in 2009 and 2010 were not predicted to fix nitrogen. In agreement with this, the numbers of hits for the nitrogenase marker genes in 2008 and 2010 were an order of magnitude lower than the numbers of hits in 2008 and 2012. While genome incompleteness precludes us from concluding that the potential for nitrogen fixation in Lake Mendota based on metagenomic gene content was lower in 2009 and 2010 because the dominant *Cyanobacteria* populations were not diazotrophic, it does corroborate a strong link between *Cyanobacteria* population dynamics and nitrogen fixation in this ecosystem (Beversdorf et al., 2013). This could also have implications for cyanotoxin production, since nitrogen stress has been linked to toxin production (Beversdorf et al., 2015).”

L576-589: deleted and now reads “To investigate potential functional changes over time in Lake Mendota, we compared the abundances of *Cyanobacteria* MAGs (approximated using read coverage normalized by genome length) to the abundance of nitrogen fixation marker genes (approximated using the number of BLAST hits in metagenamenes normalized by metagenome size). Only the most abundant *Cyanobacteria* MAG is shown for each year (panels A-E) because a single MAG was more abundant than the rest in each observed year. The marker genes used were TIGR1282, TIGR1286, and TIGR1287, encoding subunits of Mo-Fe nitrogenase, as these were the most frequently observed nitrogenase markers in the Lake Mendota metagenomes (panels F-J). Significantly correlated trends over time were observed between the MAGs and the nitrogenase marker genes in 2008, 2011, and 2012. In years where there was no significant correlation, the dominant MAG did not contain genes indicative of the nitrogen fixation pathway. This suggests that *Cyanobacteria* dynamics may be linked to the potential for nitrogen fixation in Lake Mendota.”

• Line 213 - "...consistent with a higher likelihood of recovering MAGs from the most abundant populations in the community." Please provide either a reference or analysis to support this statement or remove it. It has been demonstrated that high-abundance populations can in fact be underrepresented in assembly (PMID 26033198 and others) due to technical issues with assembling strain variants. Rather, it is often the opposite, where low abundance, low-diversity populations are the most amenable to assembly from metagenomic data. Finally as the authors also noticed, some taxa are not identified via 16S rRNA amplicon sequencing despite their representation in the metagenome. (for example PMID 26083755 and others)

We thank Dr. Narrowe for these papers, as we had heard the statement about a higher likelihood of recovering MAGs from abundant populations anecdotally. We have removed this statement from the manuscript (L212-214).

• Line 221 - 16S rRNA amplicon sequencing not just "16S" please.

This has been fixed throughout the text.

• Lines 244-246. Why does the observed diversity suggest horizontal gene transfer? Are these taxa not typically associated with nitrogen fixation? Is this diversity particularly surprising for some reason? Does analysis of the MAGs suggest these particular genes have been horizontally transferred?

Horizontal gene transfer is a hypothesis for the explained diversity, but the reviewer is correct that more analyses are needed to lend support for this hypothesis. As such, we have removed discussion of horizontal gene transfer from the manuscript (L244-246). We are excited to follow up on these suggestions in a future study.

L244-246: now reads “The increased diversity of diazotrophs in Trout Bog compared to Lake Mendota suggests that nitrogen fixation may be a more advantageous trait in humic lakes than in eutrophic lakes.”

• Line 326: "The co-occurrence of fixation pathways in these pathways in these populations are especially interesting given their relatively high abundance in their respective lakes." Why is this especially interesting? Please elaborate.

While we are not trying to say that our MAGs are representative of their communities, we know from previous literature that Cyanobacteria and Chlorobi are often abundant members of freshwater communities. The high abundances of these microbes makes their processes more likely to be relevant on an ecosystem scale. We have clarified this in the text.

L326-327: changed to “As both *Chlorobi* and *Cyanobacteria* are often abundant members of freshwater communities (Eiler & Bertilsson, 2004; Peura et al., 2012), their fixation capabilities may be relevant on an ecosystem scale.”

• Line 328-336: The discussion of the presence of rbcL in Chlorobium, is lacking some connections. What is the genomic context of rbcL in non-Chlorobiales and in the Chlorobium isolates. Is the context described for these new MAGs totally different from these other observations?

We specifically highlight *rbcL* in *Chlorobiales* because published research on cultured isolates of *Chlorobiales* has found no evidence of the Calvin Cycle in this group and has determined that this particular gene likely has a function unrelated to carbon fixation. In bacteria that fix carbon using the Calvin Cycle, genes encoding the RuBisCO enzyme are usually found together in an operon, typically containing genes encoding the large RuBisCO subunit (*rbcL*), the small RuBisCO subunit, and a putative expression protein. Many (but not all) of our MAGs predicted to possess the Calvin Cycle contain operons with this structure. Our MAG does not contain this operon structure. Therefore, we do not conclude that our MAG represents a population of *Chlorobiales* with the Calvin Cycle even though our pathway analysis predicted this function based on gene content.

As the genomic context of *rbcL* is not critical to this discussion, we have removed it to avoid confusing readers and combined this paragraph with the one above (L332-334).

• Line 344- "Unexpectedly, an Acidobacteria MAG from the Trout Bog epilimnion also contained genes suggesting aerobic anoyxgenic phototrophy." And? Why is this specifically called out - please explain why this is novel or interesting and worth the mention.

Aerobic anoxygenic phototrophy has not previously been demonstrated in members of *Acidobacteria*. While our data does not conclusively prove that they have this capability, it does suggest that further research should be done to investigate this potential function in *Acidobacteria*. We have clarified this point in the text.

L344-345: now reads “However, an *Acidobacteria* MAG from the Trout Bog epilimnion also contained genes suggesting AAP, which to our knowledge has not previously been found in this phylum.”

• Lines 352-398: Complex carbon degradation:

GH are necessary for complex-carbon degradation, but are also employed for structural purposes within an organism, this should be acknowledged, as the abundance and diversity of GH identified here are being used as a proxy for complex carbon degradation, and this may not be accurate.

We thank Dr. Narrowe for pointing this out, and have added this caveat to the text.

L366: added “However, it is important to keep in mind that GHs can also play structural roles in microbial cells in addition to the degradation of complex carbon substrates (Henrissat & Davies, 1997).”

In lines 338-391, the authors indicate that the relative numbers of GH identified may not be relevant, but then proceed to talk primarily about the most frequently identified GH families (lines 394-6; legend to Figure 3b).

While we do not have much faith in the relative abundances of GH families identified, enough informal readers asked for this information that we included it anyway. However, we agree with Dr. Narrowe that this is likely misleading for readers and have removed discussion of the most abundant GHs from the text (L385-396) and removed panels B-D in Fig. 3.

Finally, the authors identify GH % values approaching 5%. This seems high (PMID 22536372, Figure 4). Both this study and that of He et al. (presumably using similar methods) identified these high percentages. If these are really accurate percentages, this seems like it would be a big deal and should be called out. I wonder if there is some technical artifact related to assembly that might be inflating this value. If so, I don't think that it changes the comparisons of these MAGs to each other, but to other genomes using different methods it might not be an appropriate comparison. Please either a) acknowledge this large discrepancy and comment on its possible source (biological or technical) or b) show from the literature how this is not an unusually high value.

Dr. Narrowe makes an excellent point that these values are rather high. We did use the same method as in He et al., which was annotation via dbCAN. This software was recently updated (<https://www.ncbi.nlm.nih.gov/pubmed/29771380>, July 2018) and now has better support for prokaryotic draft genomes. We re-annotated carbohydrate active enzymes using dbCAN2 and found slightly lower (up to 4%) coding densities. Still, this is a rather high value, and we have commented on its possible source in the text.

Incidentally, using dbCAN2 instead of dbCAN increased the correlation between GH density and diversity from 0.39 to 0.92.

L382: added “Members of *Verrucomicrobia* have been previously identified as potential polysaccharide degraders in freshwater, although our coding densities for this phylum are higher than others reported (Martinez-Garcia et al., 2012a). This may be due to differences in trophic status between our lakes and those previously studied, or it may be that MAGs capture more pan-genomic content than isolate or single amplified genomes.”

Regarding this analysis in Figure 3 B, C, D please add additional categories to the bar chart. Surely there are others that might be biologically interesting to include here. The large 'other' bar does not provide much information.

Due to the earlier comment concerning over-interpreting GH abundances, and our concerns about the accuracy of inferring function from GH families, we have removed panels B-D from Figure 3.

• Lines 493-494. These results have shown significant differences in marker gene abundances indicating POTENTIAL differences in microbial nutrient cycling. Please adjust.

This has been changed.

Other comments on Supplementary files:

S1- Please check that the data for the following samples is correctly entered, as these lines differ in format from the other samples. HZIF, HZHX

This is correct. These samples were done in an earlier batch as “practice samples” to make sure that the sequencing protocol worked.

S2 - Please add titles indicating which data is shown in panels A and B so that this is clear even without the legend. What is y-axis showing in panel B? This is unclear.

We split each panel of Figure S2 into two supplemental figures, Figures S2 and S3. Each now has its own legend with more information about how the data were generated and presented.

S6 - This table is incredibly sparse and not especially useful in this format. Please consider melting in R and retaining only those comparisons with non-zero ANI values. As a reduced 3-column file, this would be sortable and more readily usable for people who will make use of this excellent MAG dataset in the future.

This is a good point, and we have made this change.

Reviewer 2 (Anonymous)

Basic reporting

While the English was clear and professional, I found the writing style at times repetitive. For example, three out of four paragraphs in the ‘Nitrogen cycling’ section begin with ‘To identify…’. I also think that sections can be shortened (e.g. ‘Community Functional Marker Gene Analysis’, l. 178-194 which seems largely redundant with the methods section).

We have worked to reduce redundancy both in the sections called to attention here and throughout the text, as well as removing reporting of results that are less central to our main message to make more room for discussion, as Reviewer 2 suggests below.

L183-191: moved to Methods. We did not delete this section outright because we feel the information here is necessary for reproducibility.

L230: sentence removed

L247-258: paragraph on denitrification and urea removed, one sentence moved to previous paragraph

L273-285: paragraph on genomic signatures of nitrogen limitation removed

L352-361: paragraph on allochthonous vs. autochthonous carbon removed (topic now in revised introduction)

L404-408: sentence removed

L436: topic sentence revised to reference allochthonous vs. autochthonous carbon

L438-440: sentence removed

L440-445: condensed into fewer sentences

The introduction is rather short. It should be extended to include hypotheses about the data and to expand on the motivation of the present study. For example, the manuscript could be set up as a study of methodology (how does marker gene analysis perform compared to MAGs) or of comparing lakes with different environmental conditions (Mendota vs. Trout Bog) or in terms of the time series. I found that many of the results presented were not clearly connected to the introduction, making it difficult for me to evaluate their importance.

Given this feedback and suggestions from Dr. Narrowe, we rewrote our introduction to better set up our results. We intended this study to add to our knowledge of freshwater microbes and their biogeochemical transformations, which would be of benefit to ecosystem-level models. We also worked to introduce more of our results in the introduction.

The original outline of our introduction was:

* Previous knowledge of freshwater microbes
* Importance of microbes in nutrient cycling
* Previous studies using our metagenomic time series
* Summary of this study

The introduction now reads:

* Importance of lakes for global biogeochemical cycling
* Current carbon and nutrient categorizations are too broad for microbes
* Adding microbially-relevant categories would improve models
* Linking taxonomy to function at the community level is difficult, but genomic data can help
* Summary of this study, with more detail on the sections in Results/Discussion

Results and Discussion are merged into one section, which does, as far as I know, not conform to PeerJ structural standards. I think it would help the manuscript if results and discussion would be teased apart because it would allow the authors to highlight main results and discuss their relevance in detail more.

Specifically, the PeerJ author guidelines state: “*PeerJ* covers a wide range of fields and although we can accommodate a variety of ‘standard sections’, we recommend that the following Standard Sections, in this order, are used wherever possible.” We feel that combining our Results and Discussion sections reduces redundancy, which Reviewer 2 also points out as a concern with this manuscript, and improves reader comprehension as we can discuss the importance of a result as soon as we report it. We have endeavored to better highlight our main results in the Introduction and Results/Discussion as Reviewer 2 suggests.

We have also identified several other PeerJ papers that use a combined Results/Discussion section:

<https://peerj.com/articles/3982/>

<https://peerj.com/articles/5431/>

<https://peerj.com/articles/5410/>

The figures were clear, relevant and of high quality.

Thank you!

Experimental design

The research is original and within the scope of the journal. As mentioned above, I found the research question not to be clearly defined. The research was conducted to a high technical and ethical standard and the methods are described well. However, I would encourage the author to explain the experimental design in more detail (e.g. how many samples per year in the time series).

We have added more information based on both reviewers’ comments to the Methods section. The number of samples sequenced per year in each lake has been added to Supplemental Table S2.

Validity of the findings

The data are robust and the analysis statistically sound. However, I think overall it could be presented in less detail, allowing more room for fewer results to be discussed in detail and linked to hypotheses. The conclusions are a bit muddy, because a clear research question is lacking.

We have removed several paragraphs of results, as described in the above comment about shortening results sections. We have also worked to clarify conclusions for each section (see Dr. Narrowe’s comments).

Comments for the Author

The authors investigate 141 metagenomics datasets sampled as a time series from two lakes utilizing both functional marker gene and metagenomic binning approaches. As expected, the results obtained from this impressive dataset are complex and not easily summarized. However, I do feel that the presented manuscript fell a bit short in aiding the reader to understand these complexities and evaluate their importance in light of the existing literature. Specifically, I found that the manuscript in its present form lacked hypotheses about the work presented. As a consequence, the presented results come across a bit like a long list of findings, but their relevance is hard to gage.

We have rewritten the introduction to highlight the knowledge gap in linking microbial taxa to biogeochemical transformations, and stated how this knowledge would improve the predictive power of biogeochemical models at the ecosystem level. We also added a paragraph to the introduction explaining how the results we chose to present from this large dataset contribute to closing this knowledge gap. Many of the revisions made to address comments from Dr. Narrowe also address Reviewer 2’s concerns.

- Title: The title is a bit vague. What is connected exactly and what is the nature of these connections.

We understand Reviewer 2’s comment that the use of the term “connections” in the title is not well explained. We have removed the first two words of the title to address this and to improve conciseness: it now reads “Freshwater carbon and nutrient cycles revealed through reconstructed population genomes.”

- L. 44-50: The flow of this paragraph is a bit strange.

This paragraph was removed while rewriting our introduction.

- L. 115: Do primers for the different V regions have comparative biases? Otherwise, it might be questionable to compare 16S taxonomies between lakes.

The two different primer sets do likely have differing biases. Because of this comment and Dr. Narrowe’s comments on the 16S rRNA gene amplicon analysis, we have removed much of our comparison and discussion of this data and report it only to provide context and to link our dataset to previous 16S rRNA gene amplicon sequencing studies of freshwater.

- L. 121-124: I don’t understand the sentence. Please clarify.

This sentence describes a new method for classifying 16S amplicons using a custom, small database in combination with a larger, more general database. As this method has been published since the time of submission, we have reduced this sentence and instead cite the reference for this method.

L121-124: now reads “Unclustered, unique sequences were classified using a custom database of freshwater 16S rRNA gene sequences (Newton et al., 2011) and the Greengenes database (DeSantis et al., 2006) with the classification pipeline TaxAss (Rohwer et al., 2018).

- L. 132: Please clarify how many datasets were pooled? Based on which criteria?

This information has been added to Supplemental Table S2.

L126-127: now reads “To recover MAGs, metagenomic reads from the same sampling sites (Mendota’s epilimnion, Trout Bog’s epiliminion, and Trout Bog’s hypolimnion) were pooled (Table S2) and then assembled as previously described (Bendall et al., 2016; Roux et al., 2017).

- L. 155: The sentence is redundant with respect to the previous paragraphs.

L153-155: moved to previous section on Assembly and Binning.

L155: removed

- L. 180-194: This paragraph should be significantly shortened or incorporated into the methods section.

L183-191 in this paragraph were moved to the Methods section on the functional marker gene analysis.

- L. 208ff.: This part should likely be moved to the methods section.

We have moved most of this paragraph to the Methods section on Assembly and Binning.

- L. 226: Vague. What specific differences and why?

This sentence was removed per Dr. Narrowe’s comments on highlighting the relevance of each result. It has been replaced with a discussion on why nitrogen availability is important in freshwater.

- L. 233: I don’t think O2 is the explanation here, lots of organisms have the ability to fix N2 in the presence of oxygen, including cyanos.

This is a fair point, and we have removed this sentence.

- L. 246: Interesting. But could it not also have evolved independently? Is there support for HGT (e.g. nitrogen fixation genes are very conserved between different taxa).

As addressed in Dr. Narrowe’s comments, we do not have sufficient evidence to discuss HGT in this context, and have removed this sentence.

- L. 273-285: I don’t understand the point of this paragraph. I recommend removing it.

We have removed this paragraph on genomic signatures of nitrogen limitation to reduce the number of results presented and better discuss those remaining in the text.

- In general in the results/Discussion sections: background and motivation to study a specific pathway is given after a result is introduced. Maybe expand on the background info in the introduction.

As previously outlined, we rewrote our introduction to include each following results section. Still, we prefer to maintain some background and motivation in each results section to improve readability.

- L. 271: The hypothesis is not mentioned before.

We now mention this hypothesis in the rewritten introduction.

- L. 328-336: This paragraph adds little to the results. I recommend removing.

This is the paragraph on the *rbcL* homolog in *Chlorobiales* called to attention by Dr. Narrowe. We removed most of this paragraph and combined the remaining sentences into the previous paragraph. However, we do feel it is important to state clearly that we are not predicting the presence of the Calvin Cycle in *Chlorobiales,* despite the presence of this homolog.

- A table summarizing the results for functional marker genes and MAGs might be useful.

We have added a new supplemental document, Dataset S6, which summarizes predicted pathways in MAGs individual instead of aggregated by phylum. The data for the functional marker gene analysis beyond what is already present in Dataset S4 is a rather large matrix and is already available in our GitHub repo at <https://github.com/McMahonLab/MAGstravaganza/blob/master/Data_files/Functional_marker_gene_analysis/marker_gene_table.txt>. In an effort to provide more of our data in an accessible format wherever possible, we have also included the dbCAN2 output (summarized in Figure 3) as supplemental Dataset S7.

- L. 351-361: This section is about glycoside hydrolases. So I’d remove the opening paragraph, as it’s not helpful. And change the title to reflect what this section is about.

We have removed the opening paragraph of this section and briefly discussed its topic in the new introduction. We changed the title of this section from “Complex Carbon Degradation” to “Glycoside Hydrolases” as Reviewer 2 suggests.

- L. 469: This is the first time this is mentioned. Set the result up in the introduction with background information and a hypothesis.

We now mention this result and the motivation for this analysis in the second to last paragraph of the rewritten introduction.