February 19rd, 2020

Dear editor,

We would like to submit a revised version of our manuscript #EMI-2019-1581 entitled “Cellular life from the three domains and viruses are transcriptionally active in a hypersaline desert community” by Uritskiy, Tisza, Gelsinger, Munn, Taylor, and DiRuggiero for publication by Environmental Microbiology as a Research Article in the special issue on "Halophiles: diversity of habitats and ecophysiology".

We have taken into account the constructive comments of the reviewers to revise extensively this new version of the manuscript. In particular, we have addressed key questions regarding the timing of our sampling, the nature of this hypersaline endolithic habitat, we added metadata for additional environmental context, and reframed the interpretation of our data.

We argue here that this study’s primary significance is characterizing the community-level transcriptional activities this community. While there have been efforts to understand the taxonomic diversity and metabolic potential of endolithic communities at the dry limit for life, their transcriptional activities have not been characterized at a global level. This is the first report of a robust shotgun meta-omic investigation of a hypersaline or endolithic microbiome, linking the genomic and transcriptional elements of the community.

Our study provides a snapshot of the transcriptional activities of halite endolithic microbial communities during the day and the night, revealing that Eukaryotes may be key primary producers in this extreme environment despite being low in genomic abundance. We expand on the existing known diversity of halophilic viruses and demonstrate that they are transcriptionally active in their natural environments. The divergence in the transcriptional landscapes of these segregated communities, compared to the relatively stable metagenomic functional potential, suggests that microbiomes in each salt nodule undergo unique transcriptional adjustments to adapt to local conditions. This study also provides an important experimental approach and foundation to understand the mechanisms by which the microbiome of these extreme systems function and the impacts of an ever-changing environment.

Below we address point-by-point each comment from the reviewers and the corresponding changes we made to the manuscript (in blue). We also provided a copy of the original manuscript on which the changes we have made are clearly shown.

**REVIEWER 1**

**Major concern:**

1. A major concern with regard to the transcriptomics of phototrophic mat communities is that the collection of samples at 9 AM and 9 PM is going to miss the major transcriptional activity of the cyanobacteria. Previous work on the diel cycling of microbial mats (e.g. Liu et al., 2011; Louyakis et al., 2018), have shown that even in the early morning the mats exhibit primarily heterotrophic activity and the peaks of photosynthetic activity occur at midday to late afternoon. Therefore, many of the assumptions made throughout the paper (e.g. 399-401; line 413) could be a product of sampling time points and not reflect the real activity of some parts of the community. As mentioned in lines 385-387, it's very likely that these transcriptional changes were not observed because of the time of day the samples were collected.

Liu, Z., Klatt, C. G., Wood, J. M., Rusch, D. B., Ludwig, M., Wittekindt, N., ... & Bryant, D. A. (2011). Metatranscriptomic analyses of chlorophototrophs of a hot-spring microbial mat. The ISME journal, 5(8), 1279.

Louyakis, A. S., Gourlé, H., Casaburi, G., Bonjawo, R. M., Duscher, A. A., & Foster, J. S. (2018). A year in the life of a thrombolite: comparative metatranscriptomics reveals dynamic metabolic changes over diel and seasonal cycles. Environmental Microbiology, 20(2), 842-861.

Also, in these same studies of diel cycling listed above it was shown the archaea were very active at midnight and at 6 AM so, it’s possible that the archaeal activity discussed on lines 410-412 might also be missed based on the sampling times and should be addressed.

We agree with this reviewer that the choice of time points should have been thoroughly discussed in our manuscript; it is now an integral part of the results and discussion and includes additional environmental metadata. However, the review’s comment that previous work on the diel cycling of microbial mats, with peaks of photosynthetic activity at midday to late afternoon, should apply to communities from halite nodules from the Atacama Desert, is not warranted. On the contrary, there are several arguments to think otherwise:

1. Halite nodules from the Atacama Desert are not microbial mats from aquatic environments and, as such, undergo severe desiccation throughout the day (see newly added weather metadata in Figure S2).
2. Because of the extremely high solar irradiance in the Atacama Desert, halite communities are exposed to more than 2400 µmol photons m-1s-2. As such, a significant decrease in photosynthetic activity has been reported at midday, most likely due to photo-oxidation (Davila et al., 2015).
3. Previous studies indicating timing for maximum expression of photosynthetic genes were not all in agreement: Louyakis et al. (2018) reported a maximum photosynthetic activity at noon for Thrombolite communities of Highborne Cay in the Bahamas, while Liu et al. (2011) showed that transcripts for the bacteriochlorophyll synthesis genes of six phototrophs from YNP microbial mats were most abundant at ~ 9 am (8:40 am) and 9 pm.
4. Distinct temporal regulation of photosynthesis and central carbon metabolism pathways, in response to light, has also been shown in aquatic *Cyanobacteria*, with light-harvesting photosynthetic genes being upregulated early in the light period, and the core oxidative pentose pathway being activated during the dark period (Welkie et al., 2019)

Therefore, the time points selected for this study, 9 am (~3 hours after first light; corresponding to high photosynthetic activity in Liu et al., 2011) and 9 pm (~3 hours after complete darkness; corresponding to low photosynthetic activity in Louyakis et al., 2018, but activation of the oxidative pentose phosphate pathway in Welkie et al., 2019), were likely to show significant difference in photosynthetic activity.

The same argument can also be applied to the archaea present in the halite communities, which mostly haloarchaea with light-driven proton pumps, making them likely to react to light. In contrast, the archaea described in Louyakis et al. (2018), referenced by this reviewer (methanogens and sulfate reducers), are not known to be involved in light-related processes.

While sampling at time points with the lowest and highest RH in the halite nodules might have provided relevant information on microbial activity with regard to water availability, this study intended to characterize photosynthetic adaptations to light exposure. It is also important to keep in mind that those experiments are very challenging because of the very small amount of material that can be collected at each time point; replicate rocks have to be collected, fragments scraped, and enough material stored under appropriate conditions. Once a rock has been “harvested” is cannot be used again.

To conclude, we agree with this reviewer that a possible explanation for not detecting differential expression between time points might stem from our selection of sampling time points. However, inter-nodule variation in transcriptional profiles cannot be discounted, especially in view of the arguments discussed above, arguments based on previous work and on our knowledge of this hypersaline system. All these points are now clearly laid out in the discussion of the manuscript.

2. Additionally, it appears no environmental measurements were taken (e.g. light PAR readings or oxygen measurements for the time points) in addition to the measurements listed on line 365). Based on diel measurements taken from other systems (e.g. Yellowstone mats, hypersaline and marine microbialites), it is very likely the study has not captured the major photosynthetic activity of the cyanobacterial community; therefore, as stated earlier many of the assumptions made in this study are not supported and that likely explains the lack of significant differences in the transcriptional activity between the 9 AM and 9 PM samples.

We would like to emphasize here that halite nodules represent a very different system than freshwater microbial mats and marine microbialites, for which metatranscriptomic studies have been performed. Most importantly, halite communities can be subjected to long periods of desiccation, severely restricting photosynthetic activity, regardless of the presence of light (Wierzchos et al., 2006). To answer this reviewer’s comment, we included a supplementary figure with temperature and air relative humidity (RH) measurements taken over the course of a month (at the time of sampling), and PAR measurements for a similar period but a different year. These measurements show that the 9 am time point had high sunlight (PAR: 1571 µmol photons m-1s-2), and the evening time point was in complete darkness. We apologize that this information was not available in the original submission, and made changes accordingly.

3. As there appears to be extensive heterogeneity with the samples collected and the fact they were collected over 50 m^2, perhaps a supplemental map or figure to show the collection locations of these samples would be valuable to the reader. Were there any geographical features (e.g. water availability, changes in surface characteristics, shading throughout the day) that would account for these differences in heterogeneity? There have several papers on mats that have discussed this aspect and perhaps a more detailed description of where the mats were collected and their environmental context could be elaborated.

Also, as the legend in FigS1 discusses “pairs of neighboring samples”, again a map of sample location might be useful to know how close the samples were from each other and better inform the reader.

We would like to emphasize that halite nodules are significantly different from microbial mats. Halite nodules are salt rocks, roughly 20-30 cm in size, scattered over large flat areas of the Atacama Desert, called Salars. They are subjected to the same lighting and atmospheric relative humidity, in contrast to the layer of a microbial mat where light intensity and composition varies with depth. However, the shape of the nodules and their internal structure can greatly vary with significant consequences on conditions within. We included a supplemental figure with a photo of the sampling site to give context to the reader. We also address, in great depth, the possible sources of heterogeneity in the discussion.

Our wording of the Fig. S1 caption led to a misunderstanding, we meant “pairs of replicate libraries” and clarified the caption to avoid confusion.

4. In Figure 2A it doesn’t appear that any of the eukaryotes other than Chlorophyta listed in Figure 1 are included in Figure 2A and it wasn’t clear why this information was not included.

We apologize for the confusion – the original Figure 1 showed the taxonomy before additional taxonomic annotation based on binning results. The default IMG annotation was not able to assign accurate taxonomy to many eukaryotic contigs, so all contigs belonging to the *Dolichomastix* MAG were annotated as Chlorophyta. Figure 1 has been updated and the information about the taxonomy assignment of eukaryotic contigs was added to the methods. Since Chlorophyta (*Dolichomastix* MAG) constituted >98% of all Eukaryotic contigs (the rest were unannotated), we only included this phylum in Figure 2.

5. Line 416-417: there have been other studies on hypersaline mat ecosystems where the transcriptomics of the eukaryotes have been reported and so this statement is not accurate. For example, please see Edgcomb et al., 2014.

Edgcomb, V. P., Bernhard, J. M., Summons, R. E., Orsi, W., Beaudoin, D., & Visscher, P. T. (2014). Active eukaryotes in microbialites from Highborne Cay, Bahamas, and Hamelin Pool (Shark Bay), Australia. The ISME Journal, 8(2), 418.

We clarified the statement to refer to *shotgun* metatranscriptomics, for which our study is the first in a natural halophilic or endolithic environment, and included the suggested citation when discussing past amplicon-based metatranscriptomic research into halophilic Eukaryotes.

**Minor Comments**

We apologized for submitting a MS that did not conform to the format and standards of EMI. We made the change and corrected the mistakes pointed out by this reviewer and made great efforts in proof-reading the MS.

1. There were extensive formatting issues associated with the references. It would seem that the Environmental Microbiology formatting was not used. For example, when more than two authors are present then “et al.,” should be used (in italics). There were several instances where all the last names of more than two were listed (examples include but not limited to lines 63, 71, 80, 93….). Also, in several cases, the first initial of the author was included (e.g. line 87, 98, 545), none of the ‘et al.’ used were in italics. When multiple references were cited, often the years did not go in chronological order. It is suggested that the Environmental Microbiology output style be downloaded and used for your citation manager (e.g. Endnote) so that the citations are corrected.

Acknowledged and fixed

2. The order of the figures in the supplemental material was not presented in the order discussed. Figure S1 was actually the last supplemental figures mentioned in the text. Another example was that Figure S3C, D was discussed before S3A, B (lines 183-185). I might also recommend organizing the panels of the figures as you discuss them. For example, in the figure legend, you describe panel C before B.

Fixed

3. In general, it is recommended to run the paper through the program grammarly.com (or something equivalent to catch some of these smaller writing and grammar issues). Also, spaces between numbers and units are needed throughout the manuscript.

We did and are now routinely using Grammarly.

4. Typically, numbers less than 10 are spelled out (unless they are associated with a unit). For example, in lines 518, 519, 526, the numbers should be spelled out as they are not associated with units and are below 10.

Fixed

5. In general, several abbreviations in the figure legends are not spelled out in the legends. For example in Figure S2, there is no descriptor on the color legend. What does 1.5 mean? Is that fold change as if it is transcripts per million as the figure legend suggests? In general, abbreviations such as MAG or TPM need to be spelled out to help the readers. Also in Figure S2, what does the T17 mean for all the labels and the other numbers?

Fixed. The T17 in Fig. S2 corresponds to the unique identifier for the project – these are the exact names of the MAGs, as submitted for annotation, and we would prefer not to change them. We clarified this in the legend.

6. The font size of Figures 1, 2 and 3 was rather small and should be increased. Even at 200% magnification, it was difficult to read and if published it would be very difficult to read when in print. Typically, the instructions to authors indicate a minimum font size of 6 pt should be used.

Fixed.

7. Suggestion: Lines 401-403: The discussion about differences in cyanobacterial metagenome and metatranscriptome has been previously published and discussed in microbial mats as well if you want to provide a mat example.

Mobberley, J. M., et al., (2015). Inner workings of thrombolites: spatial gradients of metabolic activity as revealed by metatranscriptome profiling. Scientific reports, 5, 12601.

This was added in the discussion as well.

**Additional suggestions**: Done

Line 24 – suggest removing “already”

Line 26 – should be “performed”

Line 34 – in my copy the sentence was in blue, perhaps change to black

Line 56 – should be “gives” as metatranscriptome is the subject.

Line 106 – reference is needed at the end of this statement, but this result could be a product of the time of day when this sample was collected.

Line 133 – end of the sentence “within then” seems to be missing part of the sentence.

Line 143 – eukaryotic should be in lowercase “Eukarya” is the domain name.

Line 220 – recommended adding the location of Lake Tyrell in Australia as readers might not be familiar with this location.

Line 499 – comma needed after “Here” (again running this through grammarly.com will help catch these issues).

Line 537 – recommended not starting a sentence with a number. If one has to start a sentence with a number, then the number needs to be spelled out.

**REVIEWER 2**

Although the study is novel and the results interesting, the manuscript needs significant reorganization to improve readability, and considerable minor improvements to language and grammar. From the quality of the writing I find it extremely doubtful that all authors read the final version of the manuscript. I’ve documented a few examples of the language issues below, but only a few examples.

We apologize for submitting a MS that did not conform to the format and standards of EMI. We made great efforts in proof-reading this new version of the MS and used Grammarly as a final editing tool.

**Suggested major revisions:**

• Reorganize the manuscript to improve flow and readability. Right now the reader bounces back and forth between the different components of the community, and the different analyses. It’s hard to see the big picture or appreciate the key points.

We re-organized the manuscript, in particularly the results section, to improve the flow and to underline better or key findings.

• The authors need to emphasize that transcripts are not an indicator of activity. For example, the transcriptome can be frozen in time in (e.g.) hypersaline environments.

This is a very good point and we made sure to address it in the opening paragraphs of the introduction to avoid any confusion.

• One of the major conclusions of the paper – that the observed eukaryote might play an outsized role in carbon fixation in the environment despite low abundance – contradicts earlier statements that the high transcript numbers associated with this community member can be explained by high basal metabolism and large cell volume (line 422).

The cell size and other factors discussed in the paper do likely explain the high transcript count, however, this does not lessen the finding that the alga produces the vast majority of the community’s photosynthetic transcripts and fixes significant amounts of carbon. We added a clarifying statement explaining this in the discussion.

• Organelle genomes are certainly available for at least O. tauri (line 238).

Thank you for pointing this out. We added appropriate comparisons and references to the manuscript.

**Suggested minor revisions:**

• The whole first paragraph seems unnecessary. The novelty of this study is it’s interrogation of halite nodule communities, so start with a description of those and why it’s important to understand them.

We whole heartily agree with this statement and have rearranged the introduction to reflect the primary novelty of this study.

• In the introduction the halite crystal environment seems to be confused with the classic endolithic environment. Yes, halite nodules are rocks, but they differ in really important ways from quartz rocks or other endolithic environments. They are far more porous, have large and well defined crystals (and thus boundaries), and, most importantly, are formed through evaporative processes. This has a huge impact on the interior bio-physio-chemico environment.

We respectively disagree with this reviewer; halite nodules are evaporitic endoliths. While their substrate composition is unique, mostly NaCl, there are considered an endolithic habitat to the same extent as other evaporitic substrates, such as gypsum, are considered endolithic habitats. In contrast, quartz rocks are only colonized on the bottom and, as such, are defined as hypolithic environments. We do agree with this reviewer that the halite endolithic environment is different than other endolithic substrates, which is, of course, reflected by a microbiome dominated by archaea. We added a description of the halite endolithic habitat to the introduction and discuss major differences with other systems in the discussion.

• Line 203 – viral transcripts are certainly suggestive of an active lytic cycle but are not definitive. To make this case you would need to know something about the growth rate.

This is a very good point that we have added to the discussion.

• Line 235 – the way the chromosome length is stated makes it appear that the chromosome is complete. If it were truly this length, that would be an astonishing and provocative finding.

The length is correct, genome completeness estimates based on marker genes were at ~50%. We moved up this sentence to clarify this point.

• Line 337 – I think you mean DESeq2

Yes, fixed

• Line 349 – variance has a specific mathematical meaning, do you mean variation?

Yes, fixed

• Fig. 6 – caption is insufficient to understand the plot.

Caption was extended.

• Examples of grammatical errors, please thoroughly check manuscript:

Done. Extensive editing was done of the new version.

* Line 37 – eliminate “of” after “framework”
* Line 56 – “gives” not “give”
* Line 57 – “in a” before “previous”

The authors have made their data available, and the data seems to be uploaded correctly (thanks!).

Thank you!

In view of the replies to comments and changes to our manuscript, we hope that you will consider this new version for publication in EMI.

Sincerely,

Jocelyne DiRuggiero, Ph.D.

Johns Hopkins University