January 23rd, 2020

Dear editor,

We would like to re-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 extensively revise this new version of the manuscript. In particular, we have addressed key questions regarding the timing of the sampling, added metadata for additional environmental context, and reframed the interpretation of our data.

One of the primary concerns of Reviewer 1 was the choice of sampling time points, which could have led to our inability to detect differential transcript expression. While we acknowledge that this was not adequately addressed in the original manuscript, we argue that our choice of 9 am and 9 pm sampling times was based on existing literature (including papers sited by the reviewer) as well as *in-situ* climate measurements. We added appropriate metadata and discussion to the manuscript, and provide more details for the reviewers below. The morning time point was characterized by bright light (1500 µmol photons m-2 s-1) and allowed the microbial communities two hours to respond to the sunrise. This time point was also deliberately taken before the photo-oxidation, which has been documented to occur around midday and early afternoon as a result of high solar irradiance (Davila 2015). On the other hand, the evening time point was taken in complete darkness, and likewise allowed the microbiota two hours to adjust their transcriptional profiles to these conditions.

We would also like to emphasize to the reviewers that halite nodules are not microbial mats but endolithic habitats in an extreme desert and, as such, undergo significant desiccation throughout the day. Our choice of sampling times was optimized to capture cellular activity during the optimal light and dark time periods. As for our inability to detect transcriptional differences between these time points, in the manuscript we clearly argue that this was likely not from the lack of real adaptation differences, but rather due to high inter-replicate variability stemming from the divergence of transcriptional profiles of separate halite nodules.

We argue here that this study’s primary significance is in regard to how microbial communities from extremely arid environments interact and coordinate their activities. While there have been efforts to understand the taxonomic diversity and metabolic potential of endolithic communities, 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 from an extreme desert ecosystem, 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 the main 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 MS. We also provided a copy of the original manuscript on which the changes you 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 MS and as it is now an integral part of the MS, including environmental metadata. However, the review’s comment that previous work on the diel cycling of microbial mats showing 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 figure.
2. Because of the extremely high solar irradiance halite communities are exposed to (>2400 µmol photons m-1s-2), 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 exactly) and 9 pm.

Therefore, the time points selected for this study, 9 am (~2 hours after first light and with high RH; corresponding to high photosynthetic activity at the high light morning in Liu et al., 2011) and 9 pm (~2 hours after complete darkness; corresponding to low photosynthetic activity in Louyakis et al., 2018), 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 are all 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 involved in any process related to light.

While sampling at time points with the lowest and highest RH in the halite nodules would could have provided relevant information on microbial activity with regard to water availability, the study intended to characterize photosynthetic adaptations to light and not to water availability. 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. As such, the previous attempts at extracting RNA from desiccated halite has not been successful.

We would also like to point out that in the discussion we clearly argue that our inability to find significant differential expression is not likely to stem from the lack of any real transcriptional adaptations between these time points. Instead, it is likely that the high inter-nodule variance in transcriptional profiles overshadowed any signal between these time points.

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.

Here again, we would like to emphasize that halite nodules represent a very different system than freshwater microbial mats and marine microbialites, for which metatranscriptomic studies have been performed. For one, halite communities can be subjected to long periods of desiccation (Wierzchos et al., 2006), severely restricting photosynthetic activity, regardless of the presence of light. However, for the reasons stated above, this was not the objective of this work. To answer this reviewer’s comment, we included a supplementary figure with temperature and 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 at the 9 am and 9 pm time points, RT and temperature conditions were relatively similar, however, 9 am time point had sunlight light (PAR: 1500 µmol photons m-1s-2; sunrise was at 7:30 am), and the evening time point was in complete darkness (sunset was at 8:20 pm). 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 are not microbial mats. Halite nodules are salt rocks, roughly 10-20 cm in size, scattered over large flat areas of the Atacama Desert, called Salars. They are subjected to the same lighting and air RH, in contrast to the layer of a microbial mat where light intensity and composition varies with depth. 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 of 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 like fixes significant amounts of carbon for the rest of the community. 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 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 is only colonized on the bottom of rocks, hypoliths. We agree that the endolithic environment is dramatically different than in other substrate, 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.

• Line 337 – I think you mean DESeq2

Yes, fixed

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

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 MS, we hope that you will reconsider our MS for publication in EMI.

Sincerely,

Jocelyne DiRuggiero, Ph.D.

Johns Hopkins University