Characterization of active and total fungal communities in the atmosphere over the Amazon rainforest

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Reviewer responses

We thank two anonymous referees for their thoughtful and constructive comments. Detailed responses and revisions are given below. Referee text is in black and our responses are in blue.

Responses to Anonymous Referee #1

One important limitation of the study is the sampling extent, which was restricted to four days at the end of the dry season. Tropical fieldwork is difficult, but how representative is this four-day period? How does seasonality influence the ratio of viable to dormant fungal tissue? How are the natural histories of the dominant taxa driving the total versus active patterns influenced by seasonality? Could one make the argument that decomposition rates are higher during the rainy season and therefore one might expect higher numbers of active Polyporales cells? The authors should address this sampling limitation - or at least address the potential role of seasonality in the introduction/discussion.
We appreciate the reviewer's questions about the role of seasonality in shaping the biodiversity patterns of fungal communities in the atmosphere. Our goal in this study was to first characterize the diversity of the active and total community within a single season, as this has never been done to our knowledge. We agree that a discussion of temporal variation is pertinent to our study and for the development of future studies. We have expanded section 3.1 and 3.2 to include a discussion of the temporal variation and how it may have affected our findings. We have also added information about local environmental conditions during the time of sampling to the methods section. Overall, conditions during the sampling period were typical for the location at that time of year.

Another question that cannot be addressed with this sampling scheme is the diurnal versus nocturnal shift in fungal composition - both in terms of OTUs but also active/dormant state. What cues do fungi use to release spores and how would this influence the active/dormant ratio in the atmosphere? This provides another opportunity to flesh out the discussion, which is currently limited in scope.
We agree that future studies should investigate temporal variation in fungal diversity at multiple scales - both diurnally and seasonally, as discussed above. We have added text about diurnal variation in the active community to section 3.2 and text about seasonal variability in the total community to section 3.1

Presumably control filters were used? If so please include results.
Yes, controls were used. We added information about blank filters to the methods section of the manuscript.

The fact that the samples are dominated by Ascomycota and Basidiomycota is not informative. There are three fungal Phyla if one does not include the Imperfect Fungi (which are typically thought to be ascomycetes that that lost sexual state). Some information at taxonomic

resolution that is informative is provided (e.g., Polyporales) - the authors should provide more of this context by listing the other families - beyond what is provided in Fig 1.
We have added a table with the relative abundances of families to the Supplemental information (Table S4).

P9: I do not follow the logic of the sentence starting on line 7. Just because these are wood decay fungi, it does not necessarily follow that they are from a local source given that there is much evidence (some of which should be cited in this manuscript) for extremely long distance dispersal through the lower atmosphere.
We agree that without more detailed data and additional analyses, we cannot conclude the wood decay fungi found in our sample were derived from local sources. We have removed this text from the manuscript.

In order to provide more support for this idea that inputs of fungi to the atmosphere are from local, rather than distant, sources could the authors compare sequence similarity of some of the more common OTUs in both their study and the reference tropical soil database they are using? Greater sequence similarity - and not simply community composition, would provide evidence for the supposition that resident fungal communities were in fact the source of atmospheric spores. Without this evidence, the supposition should be removed.
This is an excellent suggestion. However, this type of analysis is not possible with the publicly available data included in this study. The reason is that the targeted LSU gene region was not consistent across these datasets. To clarify this point, we have included the gene regions used in each study in the new table S4. Because we are unable to provide a more detailed analysis to support the suggestion that fungal communities in the atmosphere over the Amazon are structured by local (as opposed to long distance) sources, we have removed this portion of the discussion from the text.

Fig 3 (and supplemental 3) - given the error bars associated with the active atmospheric fungal community, it does not appear that it differs from grassland or tundra soil and therefore is not more similar to tropical soil. Please address.
In figure 3 we show that the phylum-level composition of the active community more closely resembles soil and phyllosphere communities than does the total community. We do not use Figure 3 to show active communities are more similar to phyllosphere communities than they are to soil communities because there is not likely to be a differences at this coarse taxonomic level. However, at the OTU level we did find that the total community was most similar to tropical phyllosphere and the active community was most similar to tropical soil and phyllosphere. We have included relevant statistics to support these findings in the results and in the legend for figure S3.

The mass balance approach is a useful contribution to this manuscript and while some very broad assumptions are made given that comparisons are being made between two Phyla (!), it is a useful exercise.
Thank you.

The figure legends are all lacking. Please include relevant statistics, error bar details, etc.
Figure legends have been edited to include relevant information and statistics.

Responses to Anonymous Referee #2

Introduction: The authors should carry out a thorough literature search on fungal ice nuclei and rewrite this part of the introduction.
We wish to thank Reviewer #2 for their suggestions and guidance regarding the literature on ice nucleation by fungi in the atmosphere. We have incorporated all of the references suggested in the reviewer responses below and also revised the introductory text as recommended.

page 7178, line 26: reference needed for the 50Tg yr^{-1} statement
We added the citation for Elbert et al., 2007.

page 7179, line 2-3: non of the cited references actually showed that fungal spores and fragments affect precipitation
We changed the wording to reflect uncertainty about whether spores and fragments affect precipitation *in situ*.

page 7179, line 11-13: reference needed for the statement that vegetative cells are more active than spores. In contrast to bacterial ice nuclei, most of the fungal ice nuclei seem not to be anchored in the fungal cell wall and can be easily washed of the mycelium/spores. These cell-free ice nuclei should be mentioned.
We have added a reference (Sussman and Douthit,1973) and changed wording in the text to indicate that metabolic processes do occur in spores, but that these processes operate in spores at a much lower rate than in vegetative cells. We have included text about cell-free ice nuclei in the introduction.

page 7179, 13-16: Please rewrite. The mycelium forming state is the vegetative form of the fungus. Pouleur et al 1992 studied suspensions of Fusarium cultures (containing mycelium and spores) as well as filtrates (containing cell-free ice nuclei).
We thank the reviewers for clarifying the results of the Pouleur et al 1992 study. We have removed the text and reference on lines 15-16 referring to nucleation by hyphae. We also edited the text on line 27 of page 7186 to include spores as well as hyphal fragments.

Page 7179, line 19-20: Please cite the right reference. Iannone et al., 2011 worked with Cladosporium and did not study Penicillium.
We changed “Penicillium” to “Cladosporium” in the manuscript.

Page 7180, line 7-15: It is not clear what the authors try to say here. Recent estimates of the ice nucleation capacity of fungal bioaerosols based on culture-based approaches – the abundance of CFU- . . .. . .have a low ice nucleation efficiency . Iannone at al, did not estimate the ice nucleation capacity based on the abundance of CFU. However, there are studies published where atmospheric fungi were cultured and screened for their ice nucleation activity (e.g. Huffman et al., 2013, ACP, Pummer et al., 2013, BG). These studies should be cited and discussed.
We have reworded the text for clarify our point. We did not intend to state that Iannone et al., 2011 measured ice nucleation capacity based on numbers of CFUs, rather that Iannone et al., 2011 measured the ice nucleation capacity of organisms (*Cladosporium* species) that culture-based studies (counting CFUs) have shown to be abundant in the atmosphere. We also added the Pummer et al., 2013 reference as an exception to our point that many studies measuring ice nucleation activity have been done with taxa that are not necessarily abundant in the atmosphere.

Methods: Please add information about blank samples for the entire study. How many and what kind of blanks were taken during sampling? How were the samplers cleaned (sterilized) between different samples? How was the cellulose nitrate filter pretreated to ensure that it's DNA free before filtering? How many of such filter blanks were included in the extraction? What are the results of the analysis of blank samples?.....
We added information about filter blanks, cellulose nitrate filters, and sampler cleaning to the methods section of the manuscript.

Results and discussion: Page 7186, line 22-24: The authors refer to figure S1, but there are no data from Haga et al, 2014 in this figure. Please correct.
The figure has been edited and now includes data from Haga et al. 2014.

Page 7186, line 25: Please correct as Iannone et al., 2011 did not work with Penicillium or add the right reference for Penicillium.
Corrected to reference Iannone et al. 2011 study of *Cladosporium* spores.

Page 7187- 7188: Can the authors clarify why they focus only on the lichen forming fungi as ice nucleation active? Some known ice nucleation active other fungi like Fusarium spp. and Isaria farinosa belong to the class Sordariomycetes, the most abundant (sequence or OTU level?) class found in this study. Did the authors find Fusarium or Isaria in their data set? What about ice nucleation active fungi of other classes or phyla like Penicillium sp., Acremonium implicatum (new name Sarocladium implicatum) or Mortierella alpina? Can the authors give some more details? I suggest also including these fungi in figure S1.
We chose to focus on the lichen fungi because this group is understudied in the atmosphere and because many LSU PCR primers, including those used in this study, are not able to detect many members of this group. Many of the lichen fungi can nucleate ice at relatively warm temperatures (e.g. >-10°C) meaning that ice nucleation can occur over a broad range of temperatures. This suggests that this group of fungi has a greater potential to effect precipitation than many other fungi with colder ice nucleation temperatures. We have added text to the manuscript clarifying why we chose to focus on the lichen forming fungi at the end of the second paragraph of section 3.2.

All analyses were performed at the OTU level. We did not detect *Isaria, Penicillium,* or *Acremonium* in our samples. We did detect a *Fusarium* in the total community and *Mortierella* in the active community.

We edited figure S1 to include the suggested taxa. In addition, we constructed a table (Table S4) with the abundances in air samples of the IN taxa included in figure S1 and added text referring to the table to the results section.

Page 7189: It is indeed surprising that the lichen fungi found in the active community were not found in the total community. For me it is not clear why the authors used LSU-amplicon sequencing for DNA but shotgun sequencing for RNA? Can the authors clarify?
The blue sky aim of our study was to characterize gene expression of microorganisms in the atmosphere using shotgun metatranscriptomics. We worked with Dr. Jason Stajich (Associate Professor, UC Riverside), an expert in fungal genomics, to obtain annotations of the protein coding genes detected in our metatranscriptomic dataset. However, due to the short sequence read length (150 bp) and the lack comprehensive fungal reference databases and bioinformatics tools for dealing with shotgun sequence data from Eukaryotes, we were unable to extract

detailed gene expression profiles from the sample data. Given that there is a significant knowledge gap about the composition of active and total fungal communities in the atmosphere, we annotated rRNA sequences obtained from the metatranscriptome data to characterize taxonomic composition of the active community. We then sequenced the LSU rDNA genes from DNA extracted from the same samples to characterize the total community. Ideally, we would have also amplified and sequenced the same LSU region from the RNA, but we did not have enough RNA remaining after the metatranscriptome libraries were prepared. While these differences in data types limit the analyses that can be conducted and the conclusions that can be drawn, we have taken steps to minimize discrepancies such the use of reference-based OTU clustering.

Figure captions: All captions must be optimized. It is not possible to understand the figures with the current captions.
We have added relevant information and statistics to the figure legends.

Figure 1: Is that on sequence or OTU level?
Taxonomy was assigned at the OTU level. This has been added to the figure legend.

Figure 3: In the figure is written that it is DNA and RNA. Do the authors actually mean sequences or OTU? What kind of error is shown here?
Error bars on stacked bars are standard deviations. This information was added to the figure legend. “DNA” and “RNA” in the figure labels were changed to “Total” and “Active”, respectively.

Figure S1: This figure seems incomplete and partly wrong. Why are there several Penicillium alli or Puccina sp.? What are the differences? Both cited references do not say anything about ice nucleation active Penicillium alli. Please add the missing references. Photobionts are not fungi and should be deleted from this figure. I suggest to include data from other studies like e.g. Huffman et al., 2013, Haga et al., 2014, Fröhlich-Nowoisky et al., 2015, . . .
Several species including *P. allii* and *Puccinia sp.* occur multiple times because they were measured in different studies. Text has been added to the figure legend to explain this. The label “Penicillium allii” should read “Puccinia allii”. The figure has been corrected to reflect this. In addition, we added data to the figure from the studies suggested by the reviewers including Haga et al. 2014, Fröhlich-Nowoisky et al. 2015, and Huffman et al. 2013.

Tables: Table S4: I suggest to add the number of OTU for each study. Furthermore the information of the sequencing method would help (amplicon (which region)? Metagenome? Metatranscriptome?,..)
Table S4 has been revised as recommended. We added columns for total number of OTUs and gene region.

Other comments/typos: Methods, page 7182, line 27: typo: it should be extension
Corrected.