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

A. M. Womack, P. E. Artaxo, F. Y. Ishida, R. C. Mueller, S. R. Saleska, K. T. Wiedemann, B. J. M. Bohannan[1] and J. L. Green

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.

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

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Abstract

Fungi are ubiquitous in the atmosphere and may play an important role in atmospheric processes. We investigated the composition and diversity of fungal communities over the Amazon rainforest canopy and compared these communities to fungal communities found in terrestrial environments. We characterized the total fungal community and the metabolically active portion of the community using high-throughout DNA and RNA sequencing and compared these data to predictions generated by a mass-balance model. We found that the total community was primarily comprised of fungi from the phylum Basidiomycota. In contrast, the active community was primarily composed of members of the phylum Ascomycota and included a high relative

abundance of lichen fungi, which were not detected in the total community. The relative abundance of Basidiomycota and Ascomycota in the total and active communities was consistent with our model predictions, suggesting that this result was driven by the relative size and number of spores produced by these groups. When compared to other environments, fungal communities in the atmosphere were most similar to communities found in tropical soils and leaf surfaces. Our results demonstrate that there are significant differences in the composition of the total and active fungal communities in the atmosphere, and that lichen fungi, which have been shown to be efficient ice nucleators, may be abundant members of active atmospheric fungal communities over the forest canopy.

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1 Introduction

Fungi are critical to the functioning of terrestrial ecosystems and may also play an important role in the functioning of the atmosphere. Fungi are abundant and ubiquitous in the atmosphere, with an estimated global land surface emission rate of 50 Tg/year for fungal spores alone (Elbert et al, 2007). Fungal bioaerosols are not only abundant but also affect physical and chemical processes in the atmosphere. Fungal spores, cellular fragments, and cell-free biological particles have the potential to affect precipitation by acting as ice and cloud condensation nuclei (Després et al., 2012; Morris et al., 2013; Pouleur et al., 1992; Richard et al., 1996), and metabolically active fungi sampled from the atmosphere are capable of transforming compounds known to play a major role in atmospheric chemistry, including carboxylic acids (Ariya, 2002; Côté et al., 2008; Vaïtilingom et al., 2013), formaldehyde, and hydrogen peroxide (Vaïtilingom et al., 2013).

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The *in situ* function of airborne fungi will depend on the physiological state of fungal cells. Metabolically active vegetative cells have the potential to transform atmospheric compounds and ultimately alter atmospheric chemistry, whereas for dormant spores this metabolic capability is greatly reduced (Sussman and Douthit, 1973). The ice nucleation efficiency of fungal cells also likely depends on their physiological state; vegetative cells derived from potentially active fungi are more efficient ice nucleators than spores. Vegetative forms of *Fusarium* (a filamentous fungi) as well as several lichen fungi have been shown to nucleate ice at temperatures as warm as -1°C (Després et al., 2012) (Supplement figure 1), and ice nucleation by hyphae has been observed at -

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2.5°C. In contrast, dormant spores – particularly those with surface hydrophobins – are generally poor ice nucleators. For example, ice nucleation of rust (*Puccinia*) spores requires temperatures lower than -10°C (Morris et al., 2013), and *Cladsporium* spores nucleate ice at temperatures of approximately -28.4°C (Iannone et al., 2011).

Despite its importance, we know relatively little about the physiological state of fungal cells in the atmosphere. Specifically, we know little about the taxonomic composition of metabolically active airborne fungi and how this compares to the composition of the total fungal community. One way to survey the total and active communities is to measure community composition from rDNA (i.e. rRNA genes) and rRNA in ribosomes. Sequencing rDNA provides information about the total community, which includes both active and dormant individuals, whereas rRNA sequences provide information about the potentially active community, because ribosomes are more abundant in active cells than dormant cells (Prosser, 2002). This approach has been applied to study active fungal communities in soils and on decaying plant material (Baldrian et al., 2012; Barnard et al., 2013, 2014; Rajala et al., 2011) but has not been applied to fungal communities in the atmosphere.

Information about the taxonomic composition of airborne fungi that are present in different physiological states can be used to advance atmospheric science. For example, such data can be used to improve estimates of the ice nucleating capacity of fungal bioaerosols. Historically, the composition of fungal communities in the atmosphere has been measured using culture-based approaches such as the abundance of colony forming units of specific taxa. This has led some scientists to conclude that fungal communities in the atmosphere have a low capacity for ice nucleation because taxa that appear abundant using plate counts have a low ice nucleation efficiency (Iannone et al., 2011, but see Pummer et al., 2013). This data may be misleading, as the vast majority of fungi require identification using culture-independent approaches (Borneman and Hartin, 2000). Today, culture-independent identification of active fungal taxa sampled from the atmosphere can be used to direct selective culturing of potentially important fungi in the laboratory, where their ice nucleation efficiencies and their metabolic capabilities can be further tested.

In this study, we used culture-independent approaches to measure the composition of total and active atmospheric fungal communities *in situ* and a mass-balance model to aid in the

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interpretation of our results. Our study system is the atmosphere above the Amazon rainforest canopy. We chose this system because fungal bioaerosols make up a substantial proportion of aerosol particulate matter over the Amazon (Elbert et al., 2007; Heald and Spracklen, 2009) and are estimated to be a dominant force responsible for cloud formation over the Amazon (Pöschl et al., 2010). We used a combined approach of DNA and RNA sequencing to address the following questions: 1) What is the composition of total airborne fungal communities? 2) What is the composition of active airborne fungal communities? 3) What likely drives differences in the composition of the total and active airborne fungal communities? 4) Is the diversity and structure of fungal communities in the atmosphere similar to that found in terrestrial environments?

2 Methods

2.1 Sample collection

Sampling was conducted on the ZF2 K34 flux tower (S -2.60907, W -60.20917, 67 m a.s.l.) in the Reserva Biologica do Cueiras in central Amazonia, about 60 km NNW of Manaus, Brazil. The site is operated by the Instituto Nacional de Pesquisas da Amazonia (INPA) under the Large Scale Biosphere-Atmosphere Experiment in Amazonia (LBA) program (Martin et al., 2010). Tower height is approximately 54 m. Surrounding vegetation is undisturbed, mature, terra firme rainforest, with a leaf area index of 5–6 and an average canopy height of 30 m. Samples were collected at the end of dry season over four days, December 8-11, 2010, from a height of 48m above the forest floor. Environmental conditions during the four-day sampling period were typical for the location in early December with partial clouds and temperatures ranging from approximately 28.5°C to 32.1°C. Heavy rain and thunderstorms occurred on 12/8 and 12/11. Aerosol samples were collected using SKC Biosamplers (BioSampler SKC Inc.). Samplers were filled with 20 mL of a water-based preservation solution (LifeGuard Soil Preservation Solution, MO BIO Laboratories, Inc) to prevent DNase and RNase activity and maintain cells in stasis to allow accurate community profiling of the total and active fungal community. Twelve impingers were operated at 12.5 L/min from approximately 9:00 am – 4:00 pm each day. At the end of each day, the sampling liquid from all impingers was pooled and stored at -20°C. Impingers were

cleaned each day by rinsing in 70% ethanol followed by sterilization using a portable pressure cooker.

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2.2 Nucleic acid isolation and cDNA synthesis

Samples were transported on ice to the University of Oregon where the liquid sample from each day was separated into two aliquots, one to be used for DNA extraction and the other for RNA extraction. The divided samples were filtered through sterile, individually wrapped, 0.22 µm cellulose nitrate filters (Nalgene Analytical Test Filter Funnels, Thermo Fisher Scientific). DNA was extracted from filters using the MO BIO PowerWater DNA Isolation Kit according to the manufacturer's instructions with a 100 µl elution volume. RNA was extracted from filters using the MO BIO PowerWater RNA Isolation Kit with the following modifications. The DNase steps included in the kit were omitted. RNA was eluted in 50 µl. The extracted RNA was treated with DNase I (RNase-free) (Fermentas International, Inc) according to the manufacturer's instructions. DNase reactions were cleaned (Zymo Research Clean and Concentrate-5) and eluted into 50 µl. cDNA was synthesized from the total RNA extract using the SuperScript II First-Strand Synthesis System (Invitrogen, Life Technologies Corporation) with random hexamers. All RNA was converted into cDNA in six synthesis reactions and one reverse transcriptase negative control reaction. Three field blanks were generated by filtering unused LifeGuard Solution through new, sterile filters. Blanks were processed in parallel to the RNA and DNA samples including extraction, PCR amplification, and library preparation. Following library preparation, blank samples were visualized on an agarose gel and no visible bands were observed.

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2.3 Library preparation and sequencing

To increase the concentration of cDNA to levels required for sequencing, we used multiple displacement amplification (GenomiPhi V2, GE Healthcare) according to the protocol described in Gilbert *et al.* (2010) including second-stand synthesis, amplification, and de-branching of amplification products. The fully de-branched products were sheared by sonication (24 cycles, 30 seconds each) using the Bioruptor sonication system (Diagenode). cDNA fragments were end-repaired (End-It DNA End-Repair Kit, Epicentre Biotechnologies), cleaned and concentrated (Zymo Research Clean and Concentrate-5) and eluted in 40 µl. A-overhangs were added to the

end-repaired fragments using Klenow exo(-) (Epicentre Biotechnologies) in a 50 µl reaction. Reaction products were cleaned and concentrated (Zymo Research Clean and Concentrate-5). Standard paired-end, barcoded Illumina adaptors (Supplement table 1) were ligated to the fragments using T4 ligase (Fermantas). Reaction products were cleaned and concentrated (Zymo Research Clean and Concentrate-5) and eluted in 12 µl. To enrich fragments with ligated adaptors, PCR amplification was performed using primers containing the flowcell adaptor and complementary to the Illumina sequencing primer (Supplement Table 1). PCR reactions were performed using Phusion DNA polymerase (New England Biolabs) with 12 µl template, 10 µl 5x HF buffer, 1 µl 10 mM dNTPs, 2 µl 10 mM primer mix, 0,5 µl enzyme and 25.5 µl water for a final reaction volume of 50 µl. PCR cycling conditions were as follows: 30 seconds denaturation at 98°C followed by 18 cycles of 98°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds following by a final extension at 72°C for 5 minutes. PCR products were size fractionated by gel electrophoresis (2.5%, low-melt agarose). Products in the range of 150-500 bp were excised, and DNA from the excised gel pieces was extracted (QiagenMinElute Gel Extraction) and eluted into 20 µl. DNA was quantitated using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies Corporation) and combined in equal molar concentrations. Shotgun metatranscriptome libraries were sequenced (150 base pairs, paired-end) on the Illumina HiSeq 2000 (Illumina, Inc.) platform at the University of Oregon Genomics Core Facility. LSU rDNA amplicons were sequenced (250 base pairs, paired-end) on the Illumina MiSeq platform at the Dana-Farber Cancer Institute Molecular Biology Core.

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The D1-D2 region of the large subunit (LSU) rRNA gene was targeted using PCR with the primers LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR3 (5'-CCGTGTTTCAAGACGGG-3') (http://sites.biology.duke.edu/fungi/mycolab/primers.htm). LSU amplicon libraries were prepared using a two-stage PCR procedure as described in (Kembel and Mueller, 2014) using unique combinatorial barcodes (Gloor et al., 2010) to identify samples (Supplement table 2).

2.4 Sequence pre-processing

2.4.1 Metatranscriptome

Overlapping paired end reads were aligned and joined using fastq-join (https://code.google.com/p/ea-utils/wiki/FastqJoin). Joined reads and non-overlapping single-end reads were trimmed and filtered using PrinSeq (Schmieder and Edwards, 2011). Sequences <75 bp, > 2% Ns, and/or mean quality score <20 were removed. Sequence artifacts defined as exact duplicates with >5,000 sequences were removed. Sequences in the Dec. 10 sample were primarily artifacts, so this metatranscriptome sample was excluded from further analysis. Putative rRNAs in the remaining sequences were identified using SortMeRNA (Kopylova et al., 2012) with the non-redundant version of the following databases: rfam 5.8S (version 11.0) (Burge et al., 2013); Unite (November 2011 version) (Kõljalg et al., 2013), and Silva 18S and Silva 28S (Release 115) (Quast et al., 2013). Of 5,165,185 quality-filtered reads, 1,915,994 with an average length of 137.5 bp were identified as putative rRNAs (Supplement table 3).

2.4.2 LSU amplicons

Forward and reverse barcodes were combined to make a 12 bp barcode on the forward read. Only forward reads derived from the LR3 region were used for analysis. This region has been shown to have high species-level resolution even with short read lengths (Liu et al., 2012).

2.4.3 Multi-environment sequences

LSU sequences from four soil studies (Barnard et al., 2013; Kerekes et al., 2013; Penton et al., 2013, 2014) and one phyllosphere study (Kembel and Mueller, 2014) were compared to air samples collected for this study (Supplement table 4). Raw sequence data and associated metadata were downloaded from publically available databases. 12 bp barcodes were added to all sequences to identify each sample in downstream analysis.

2.5 LSU amplicon and metatranscriptome sequence processing

All sequences were processed in QIIME version 1.7 (Caporaso et al., 2010). Briefly, libraries were individually demultiplexed and filtered for quality. Sequences with an average quality score less than 20, shorter than 150 bp and with greater than 2 primer mismatches were discarded. The same parameters were used across all samples except the metatranscriptome rRNAs were a size cut off of greater than 75 bp was used. In order to decrease computation time, sequences from

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Kembel and Mueller (2014) and Penton *et al*. (2014) were randomly subsampled to 25% and 60% of the total number of sequences, respectively. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using closed reference BLAST (Altschul et al., 1990) against the Ribosomal Database Project Fungal LSU training set 1 (Cole et al., 2014). Taxonomy was assigned to each OTU was that of the most similar representative in the RDP database.

Following sequence processing and quality filtering, a total of 55,414 amplicon and 1,915,994 metatranscriptome LSU sequences generated for this study and 1,577,458 LSU sequences from soil and phyllosphere studies were retained (Supplement table 3). For analyses using only samples from this study, the data were rarefied to 5,300 sequences per sample. For analyses that compare samples in this study to samples from other studies, the data were rarefied to 500 sequences per sample.

2.6 Statistical analyses and data availability

All statistical analyses were conducted in R (R Core Team, 2014) primarily using the `vegan` (Oksanen et al., 2013) package for ecological statistics and the `ggplot2` (Wickham, 2009) package for visualizations.

Sequence files and metadata have been deposited in Figshare (http://dx.doi.org/10.6084/m9.figshare.1335851). Data from other studies used for cross environment analyses are available using the databases and identifiers referenced in the respective manuscripts.

2.7 Mass-balance model

We use a global, well-mixed, one-box material-balance model to predict the relative abundances of fungal cells measured as gene copies sampled in the active and total portions of atmospheric bioaerosols. Model description and details are available in Appendix A.

3 Results & Discussion

3.1 Basidiomycota dominate total airborne fungal communities

Measurements of airborne fungi using culture-based methods such as quantifying spore and colony-forming unit counts have been conducted for centuries (Després et al., 2012). In comparison, there have been few culture-independent studies of the fungal composition of atmospheric samples (e.g. Boreson et al., 2004; Bowers et al., 2013; Fierer et al., 2008; Fröhlich-Nowoisky et al., 2009, 2012; Pashley et al., 2012; Yamamoto et al., 2012). Using a culture-independent approach, we found the composition of total airborne fungal communities primarily included taxa belonging to the phyla Ascomycota and Basidiomycota (Figure 1). This result is similar to what is observed in environments on the Earth's surface (James et al., 2006) and what has been reported in other studies of fungi in the atmosphere (Bowers et al., 2013; Fröhlich-Nowoisky et al., 2009, 2012; Yamamoto et al., 2012).

Basidiomycota dominated the total airborne community in our air samples (mean relative abundance = 90.2±6.9%) (Figure 1). Within the phylum Basidiomycota, Agaricomycetes were the most abundant class in our samples. Agaricomycetes have been previously detected in air samples (Fröhlich-Nowoisky et al., 2012; Woo et al., 2013; Yamamoto et al., 2012) and are common in tropical soils (Tedersoo et al., 2014) and leaf surfaces (Kembel and Mueller, 2014). Within the Agaricomycetes, the most abundant order was the Polyporales (mean = 55.7±2.3%). Polyporales have been detected in culture-independent studies of urban aerosols (Yamamoto et al., 2012) and culturable representatives have been isolated from cloud water (Amato et al., 2007). At the genus level, there were several taxa detect in the total community with ice nucleation activity including Acremonium, Cladosporium, Fusarium, and Rhizopus (Table S4).

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The presence of Agaricomycetes may have implications for atmospheric processes. Ice nucleation efficiency within the Agaricomycetes is variable, with some taxa capable of nucleating ice at temperatures as warm as -17°C (Haga et al., 2014) (Supplement figure 1). These temperatures are warmer than what has been measured for *Penicillium* spores (Iannone et al., 2011) although not as warm as what has been measured for other biological particles including other spore types (Morris et al., 2013), suspensions of *Fusarium* cultures (containing spores and hyphae) (Pouleur et al., 1992), and lichen fungi (Després et al., 2012). Despite the low ice nucleation efficiency of some taxa in this group, given the high abundance of Agaricomycetes over the forest canopy, this group could still have a significant impact on cloud formation and precipitation in the tropics.

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The patterns we report reflect a snapshot in space and time. As in other environmental systems, the composition of total fungal communities in the atmosphere will vary across different spatial and temporal scales. Research has shown, for example, that concentrations of fungal spores in the atmosphere vary diurnally and seasonally. This variation is driven by complex interactions between fungal dispersal mechanisms and environmental conditions, particularly moisture and wind speed. (Lacey, 1996). Our samples were collected during the day, and spores released by mechanical disturbances often peak in abundance in the air during midday when wind speeds are highest (Lacey, 1996). Taxa that require dry conditions for dispersal also tend to release spores during the day, and taxa that require high relative humidity, including many Basidiomycota, tend to release spores at night when humidity is highest (Elbert et al., 2007; Lacey, 1996). In addition to humidity, precipitation events can also affect the dispersal of fungi. Overall concentrations of spores have been shown to increase in the atmosphere due to convective instability preceding thunderstorms (Burch and Levetin, 2002), and Ascomycota concentrations increase during and immediately after rainstorms (Elbert et al., 2007). Our samples were collected at the end of the dry season. If we had sampled during the wet season, it is possible we would have observed a higher relative abundance of Ascomycota in the total community since the dispersal of Ascospores has been shown to increase before and after rain storms.

3.2 Ascomycota dominate active airborne fungal communities

The composition of total and active fungal communities over the Amazon rainforest canopy significantly differed (ADONIS, $R^2 = 0.342$, $p = 0.029$). The active community in the atmosphere over the forest canopy was dominated by Ascomycota (mean relative abundance = 80.4±20%) (Figure 1). Basidiomycota comprised a smaller fraction of the sampled genes (mean = 7.3±6.8%) with the remainder of identified sequences belonging to the phyla Chytridiomycota and Glomeromycota. This result makes sense in light of the natural histories of many of the Ascomycota, which have single-celled or filamentous vegetative growth forms that are small enough to become aerosolized, whereas many of the Basidiomycota are too large to be easily aerosolized, other than in the form of metabolically inactive spores. It is possible that if we had sampled at night rather than during the day, we would have observed a higher relative abundance of Basidiomycota in the active community. The abundance of vegetative Ascomycota fragments

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may peak during the day when wind speeds are high, assuming they are passively dispersed by wind and convection (as opposed to active mechanisms many fungi use to disperse spores). And, Basidiospores have been shown to be particularly abundant in the Amazon atmosphere at night (Elbert et al., 2007). As with the total community, we expect that the composition of active fungal communities in the atmosphere will vary across different spatial and temporal scales.

The most abundant classes of Ascomycota detected were Sordariomycetes (mean = 27.1±6.6%), and Lecanoromycetes (mean = 17.5±7.6%). Sordariomycetes have been previously detected in culture-independent air samples (Fröhlich-Nowoisky et al., 2009, 2012; Yamamoto et al., 2012) and have been shown to be abundant on tropical tree leaves (Kembel and Mueller, 2014) and tropical soils (Peay et al., 2013). In most ecosystems, Sordariomycetes are endophytes, pathogens, and saprotophs (Zhang et al., 2007). Xylariales, which includes both endophytes and plant pathogens (Zhang et al., 2007), was the most abundant order within the Sordariomycetes in our samples. Several genera with known ice nucleation capability were detected in the active community including Agaricus, Amanita, Aspergillus, Boletus, Lepsita, Mortierella Puccinia, Rhizopus, and the lichen fungus Cladonia (Table S4). Below we focus our discussion on the class Lecanoromycetes, an understudied but potentially important group of fungi in the atmosphere.

Lecanoromycetes were the second most abundant class of Ascomycota detected over the rainforest canopy. This group has been detected in other culture-independent studies of fungi in the atmosphere (Fröhlich-Nowoisky et al., 2012; Yamamoto et al., 2012). The Lecanoromycetes contain 90% of the lichen-associated fungi (Miadlikowska et al., 2007). Lichens are a symbiosis between a fungus and a photosynthetic partner such as eukaryotic algae or cyanobacteria. Lichens are known to be hardy and may be particularly well-adapted for long distance transport and metabolic activity in the atmosphere. Lichens are often the dominant life forms in environments that have conditions similar to those found in the atmosphere, including low water (Kranner et al., 2008) and nutrient availability, wide temperature variations, and high UV irradiance (e.g. Solhaug, Gauslaa, Nybakken, & Bilger, 2003) (Onofri et al., 2004).

Another notable trait of lichens is their efficient ice nucleation capacity. Although there have been no investigations specifically on the most abundant lichen species detected in this study, *Physcia stellaris* (mean = 8.3±3.8%) and *Rinodina milvina* (mean = 4.8±3.4%), there have been multiple studies of the ice nucleation efficiency of many other lichen fungi species. Ice nucleation

activity of lichens has been measured at temperatures warmer than -8°C, including 13 of 15 taxa tested by Henderson-Begg and colleagues (Henderson-Begg et al., 2009) and 9 of 15 taxa tested by Kieft (Kieft, 1988). These studies have demonstrated that lichens are among the most efficient biological ice nucleators. Therefore, their presence in the atmosphere may have a significant impact on cloud formation and precipitation. This ice nucleation capacity may also enable lichens to control the extent of their dispersal through the atmosphere. It is possible that lichens achieve this by nucleating ice formation, which leads to precipitation and ultimately deposition. This phenomenon has been shown to occur in some phytopathogenic bacteria (Morris et al., 2008, 2010) and may occur in fungi as well (Morris et al., 2013).

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3.3 Dominance of Basidiomycota in total communities and Ascomycota in active communities is consistent with mass-balance predictions

Our mass balance model (Appendix A) predicted Basidiomycota would dominate the total community because they produce orders of magnitude more spores and have smaller aerodynamic diameters compared to Ascomycota. Consistent with this prediction, the total airborne community was dominated by Basidiomycota in our air samples (mean relative abundance = 90.2±6.9%) (Figure 1). There have been some empirical studies reporting the opposite pattern, with a higher relative abundance of Ascomycota compared to Basidiomycota (Bowers et al., 2013; Fierer et al., 2008; Pashley et al., 2012). There has been one study focused on airborne fungal communities in the Amazon Basin (Fröhlich-Nowoisky et al., 2012). Although the site of this study was the atmosphere above a rural pasture (versus a tropical rainforest, as in our study) these investigators also found that Basidiomycota dominate airborne fungal communities

Our mass-balance model explains the differences in composition between the total and active air communities. However, some of the differences we observed may be partially attributable to the use of different approaches in characterizing the total and active communities. In this study, the total community was characterized by PCR-based amplification and sequencing of LSU genes, whereas the active community was characterized through random sequencing of all the RNA present in the samples. Shotgun metatranscriptome sequencing and PCR-based community characterization approaches each have their own biases (Hong et al., 2009; Morgan et al., 2010).

Our data suggest that the selection of LSU primers led to biased results. For example, the high relative abundance of lichen fungi (class Lecanoromycetes) in the active community was unexpected because this group was not detected in the total community and has only been detected in low abundance in other PCR-based studies of fungi in the atmosphere (Fröhlich-Nowoisky et al., 2012). We tested the primer pair used in this study (LR0R-LR3) using the SILVA TestPrime tool (Klindworth et al., 2013) and found coverage of the Lecanoromycetes with this primer pair was 71.4%. Within the class Lecanoromycetes, the order Teloschistales, which contains the most abundant species detected in the active community, would not have been detected with this primer pair. However, the general pattern that Ascomycota were much less abundant than Basidiomycota in the total community is not likely due to primer bias as overage of the phylum Ascomycota by the LR0R-LR3 primer pair is 85.5% according to TestPrime. Our findings underscore the value of using a combination of PCR-based and shotgun-based sequencing approaches, particularly in environments that are understudied and where little is known about microbiome structure and function.

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3.4 Fungal air communities above the forest canopy are most similar in composition to tropical phyllosphere and soil communities

We compared total and active fungal air communities to communities from tropical, temperate, and tundra soils and from the surfaces of tropical tree leaves. Community composition significantly differed across environment types (ADONIS, R^2 = 0.167, p = 0.001), and fungal communities in the atmosphere were compositionally distinct from communities in other environments (Figure 2). Ascomycota was the most abundant phylum across all soil and phyllosphere samples (soil mean relative abundance = 78.4±14.9%, phyllosphere = 90.9±4.9%) followed by Basidiomycota (soil mean relative abundance = 19.0±14.9%, phyllosphere = 7.4±4.5%) (Figure 3). We expected communities to be distinct across habitat types because environmental conditions may differ across the habitat types and select for different communities. However, in the atmosphere, dispersal and mixing of fungi from multiple habitat types may be driving the observed community composition differences instead of environmental selection.

The diversity of fungal communities in the atmosphere is within the range of diversities reported for terrestrial environments, including those of tropical leaf surfaces, tropical soils, temperate

grassland soils, and tundra soils. Overall taxonomic richness, defined as the number of OTUs, significantly varied among environment types (ANOVA, $F(5,237) = 66.89$, $p < 0.001$) (Supplement figure 2). Tukey's HSD post-hoc comparisons indicated that the richness of air communities, both total and active, was greater than tundra soil communities and did not significantly differ from temperate grassland soil communities. In general, air communities were less diverse than tropical forest phyllosphere and soil communities with the exception of tropical forest soils and active air communities, which did not significantly differ. Similar patterns have been observed in soil communities where taxonomic richness in arctic soils was significantly lower than soils from temperate and tropical ecosystems (Fierer et al., 2012).

Total air communities were most similar to tropical phyllosphere communities (mean Sørensen similarity = 0.015±0.009; Tukey's HSD, $p < 0.001$) and active air communities were most similar to tropical soil communities (mean Sørensen similarity = 0.010±0.007, Tukey's HSD, $p < 0.001$) (Supplement figure 3). This suggestion makes sense since fungal spores and hyphae are relatively large aerosol particles with short residence times in the atmosphere, limiting opportunities for long-distance dispersal. While these results are suggestive, detailed information is lacking regarding the potential influence of terrestrial source environments and their role in structuring airborne fungal communities.

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4 Conclusion

Fungi in the atmosphere play an important role in atmospheric processes including precipitation development through ice nucleation. This is of particular significance in the atmosphere over the Amazon rainforest canopy where fungi constitute a large fraction of the total aerosol content (Elbert et al., 2007; Heald and Spracklen, 2009) and precipitation is aerosol-limited (Pöschl et al., 2010). Our study represents the first culture-independent survey of fungal communities over the Amazon rainforest canopy. It is also the first to measure metabolically active microbial communities in the atmosphere using an RNA-based approach. Using this RNA-based approach, we found evidence for the presence of potentially active fungi in the atmosphere, including lichen fungi (class Lecanonomycetes) and the following genera: Agaricus; Amanita; Aspergillus; Boletus; Cladonia; Lepsita; Mortierella; Puccinia; and Rhizopus. While an understanding of the

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structure of fungal communities in the atmosphere is beginning to emerge, studies on the function of these communities have lagged behind. We suggest that future research focus on understanding the functional capacity of airborne microbes with traits particularly well-suited for survival and metabolic activity in extreme environments. As with any environment, understanding both the structure and function of microbial communities in the atmosphere is needed to assess their potential impact on ecosystem processes such as water and carbon cycling. This study opens the door for future investigations of the diversity and function of fungal communities in the atmosphere.

Author contributions

A. M. Womack conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, and reviewed drafts of the paper. P. E. Artaxo conceived and designed the experiments. F. Y. Ishida collected the samples and reviewed drafts of the paper. R. C. Mueller conceived and designed the experiments, reviewed drafts of the paper and contributed reagents/materials/analysis tools. S. R. Saleska conceived and designed the experiments. K. T. Wiedemann collected the samples. B. J. M. Bohannan conceived and designed the experiments, collected the samples, and reviewed drafts of the paper. J. L. Green conceived and designed the experiments, wrote the paper, reviewed drafts of the paper, and contributed reagents/materials/analysis tools.

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Figures

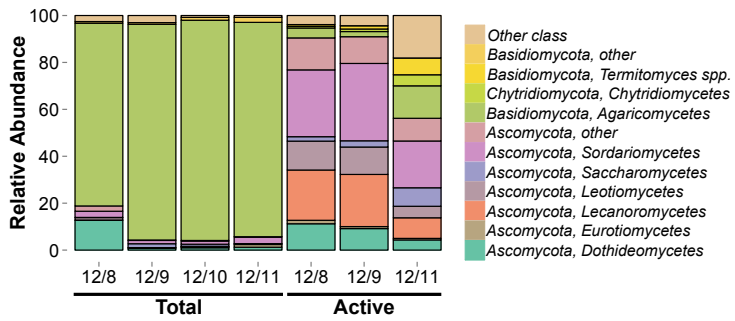

Figure 1. Basidiomycota dominate the total fungal community (mean relative abundance = 90.2±6.9%). Bars are colored according to class-level taxonomic assignments. Taxonomy was assigned to representative sequences from each OTU.

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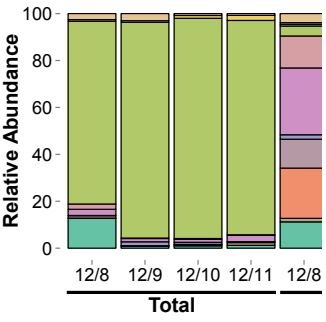

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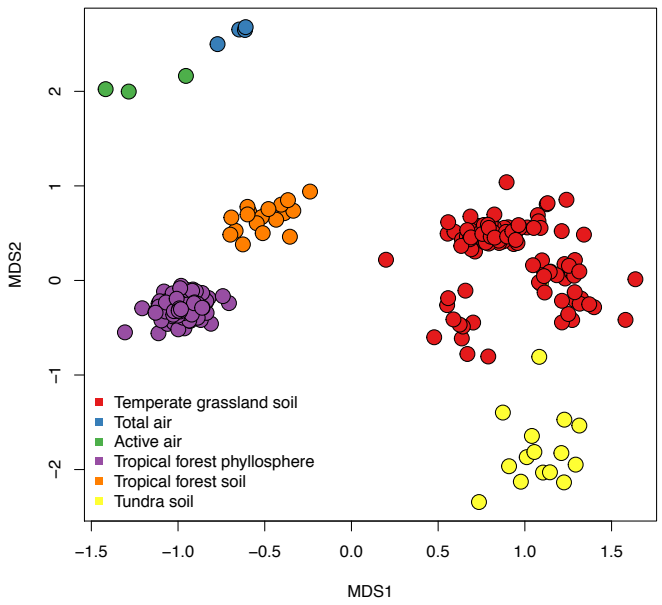

Figure 2. OTU-based community composition significantly differed across environment types (ADONIS, R^2 = 0.167, p = 0.001). Total and active communities in air samples (upper left) clustered together and separate from other environments indicating these communities are distinct from communities found in soils and on leaf surfaces. Sørensen similarities are depicted, ordinated via NMDS.

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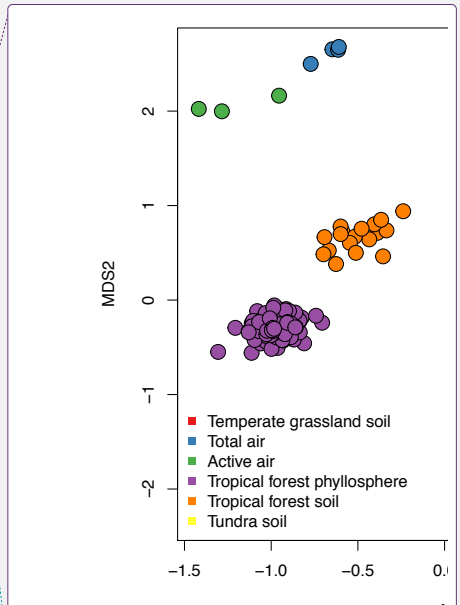

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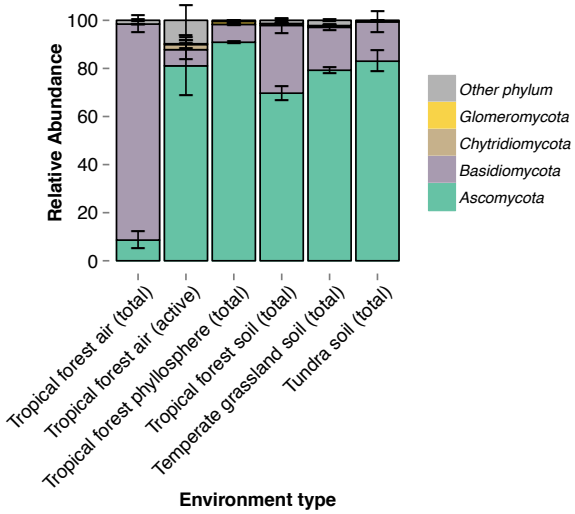

Figure 3. Relative abundances of fungal phyla across environment types. The active atmospheric fungal community over the Amazon rainforest was more similar in phylum-level composition to fungal communities found in tropical soils and on plant leaves than was the total community. Error bar represent standard deviations.

Appendix A

Mass-balance model

We use a global, well-mixed, one-box material-balance model to explain the relative abundances of fungal cells measured as gene copies sampled in the active and total portions of atmospheric bioaerosols. By material-balance, for any taxon i within a biological community, the change in time in the abundance of fungal gene copies, N_i, must be equal to the difference in source and sinks:

$$\frac{dN_i}{dt} = \sum sources - \sum sinks \quad \text{(A1)}$$

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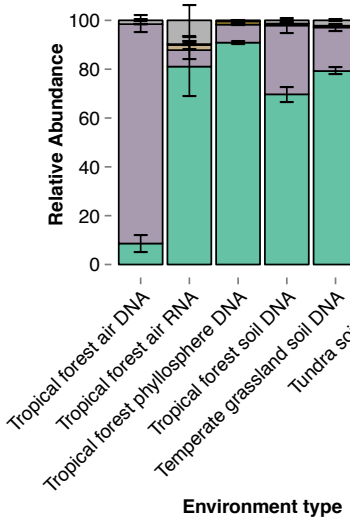

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Here we assume sources are equal to the emission of fungal gene copies from the Earth's surface into the atmosphere, E_i (gene copies/hour). We assume sinks are equal to deposition of fungal gene copies out of the atmosphere back to the Earth's surface, $D_i = N_i k_i$, (gene copies/hour), where k_i (1/hour) represents a first order deposition coefficient. We can rewrite Equation (A1) as:

$$\frac{dN_i}{dt} = E_i - N_i k_i$$

We expect the terms E_i and k_i to vary as a function of life history traits including the method of cell release into the atmosphere, the physiological state of sampled cells, and the aerodynamic diameter of fungal taxa. In this case, Equation (A2) does not directly represent the entire airborne fungal gene copy abundance. We assume that a first order approximation of fungal bioaerosol behavior may be obtained by subdividing the particle distribution into two modes: vegetative cells, $N_{i,veg}$, and spores, $N_{i,spores}$. We thus model fungal gene copy abundance as:

$$N_i = N_{i,veg} + N_{i,spores}$$

We can then write and solve parallel versions of Equation (A2) for each mode. At steady state, the expected gene copy abundance taxa *i* in each mode is:

$$N_{i,veg} = \frac{E_{i,veg}}{k_{i,veg}}$$

$$N_{i,spore} = \frac{E_{i,spore}}{k_{i,spore}}$$

Our interest lies in the two most common fungal phyla sampled in the atmosphere: Ascomycota, N_A, and Basidiomycota, N_B. To make predictions about the expected relative abundance of gene copies in these two groups, we make informed assumptions about the relative magnitude of their respective emission and deposition rates. We begin by considering fungal spores. Although a few empirical studies have suggested that Ascomycota are more abundant than Basidiomycota in likely source environments including tropical soils (Kerekes et al., 2013) and leaf surfaces (Kembel and Mueller, 2014), Basidiomycota (e.g. Agaricomycetes, the most abundant class of Basidiomycota in our samples) produce orders of magnitude more spores per individual than Ascomycota (Elbert et al., 2007; Pringle, 2013). For this reason, we assume the emission rate of Basidiomycota spores is much greater than that of Ascomycota spores:

$$E_{A,spores} \ll E_{B,spores}$$

Culture-based microscopy data suggests that spores of Ascomycota are typically larger than spores of Basidiomycota (Elbert et al., 2007; Ingold, 2001; Yamamoto et al., 2014). Owing to the difference in spore size, we expect deposition rate of Ascomycota spores to be greater than that of Basidiomycota spores:

$$k_{d,A,spores} > k_{d,B,spores}$$

Based on these assumptions, it follows that the expected number of Ascomycota spores in the atmosphere will be less than the number of Basidiomycota spores:

$$\frac{E_{A,spore}}{k_{A,spore}} \ll \frac{E_{B,spore}}{k_{B,spore}}$$

or

$$N_{A,spores} \ll N_{B,spores}$$

We next consider fungal vegetative cells. Vegetative forms of Ascomycota are generally smaller than vegetative forms of Basidiomycota (Moore et al., 2011). Many Ascomycota grow as filaments or single cells which are small enough to be aerosolized (Després et al., 2012). In contrast, many Basidiomycota grow as mushrooms, which are too large to be aerosolized (although debris from mushrooms and their mycelia can be aerosolized). Due to this difference in the vegetative forms of each group, we expect emission rate of vegetative Ascomycota to be greater than Basidiomycota:

$$E_{A,veg} > E_{B,veg}$$

No comparative data currently exists on the relative deposition rate of vegetative cells across fungal taxa. Research has shown that at the phylum level, the aerodynamic diameter of Ascomycota is greater than that of Basidiomycota, resulting in a greater deposition rate overall for Ascomycota (Yamamoto et al. 2014). However, this work did not differentiate between vegetative cells and spores, and there is no *a priori* reason to assume that the deposition rate of Ascomycota vegetative cells are less than or greater to that of Basidiomycota cells. For this reason, we make the null assumption that the deposition rate of each group is equal:

$$k_{d,A,veg} = k_{d,B,veg}$$

Based on these assumptions, we expect the number of vegetative Ascomycota genes to be greater than the number of vegetative Basidiomycota genes:

$$\frac{E_{A,veg}}{k_{A,veg}} > \frac{E_{B,veg}}{k_{B,veg}}$$

or

$$N_{A,veg} > N_{B,veg} \quad \text{(A3)}$$

Equation (A3) predicts that Ascomycota will dominate the active fungal community in the atmosphere.

Finally, we relate the abundance of Ascomycota and Basiodiomycota gene copies in their totality to ask if $N_A < N_B$ or $N_A \geq N_B$. $N_A < N_B$ if and only if:

$$N_{A,veg} + N_{A,spores} < N_{B,veg} + N_{B,spores}$$

Rearranging terms and dividing both sides of the equation by $N_{B,spores}$ yields the inequality:

$$\frac{N_{A,veg} - N_{B,veg}}{N_{B,spores}} + \frac{N_{A,spores}}{N_{B,spores}} < 1$$

or

$$\frac{N_{A,veg} - N_{B,veg}}{N_{B,spores}} < 1 - \varepsilon$$

where $\varepsilon = \frac{N_{A,spores}}{N_{B,spores}}$. Empirical data on the discharge of Ascomycota and Basidiomycota spores from fruiting bodies suggests that $\varepsilon \leq 0.01$ (Elbert et al. 2007). In this case $N_A < N_B$ if and only if:

$$\frac{N_{A,veg} - N_{B,veg}}{N_{B,spores}} < 0.99 \quad \text{(A4)}$$

We expect Equation A4 to hold due to the likelihood that spores greatly out number vegetative cells in the atmosphere in both phyla. Spores can be actively discharged into the air, whereas vegetative cells are not actively propelled into the atmosphere and require aerosolization by

mechanical forces like wind. Furthermore, empirical data suggests that vegetative cell fragments constitute a small fraction (0.2-16% (Green et al., 2011)) of the total fungal biomass in the atmosphere. For these reasons, we predict that

$$N_A < N_B$$

Based on the conclusions of this model, we expect Basidiomycota will dominate the total community, and Ascomycota will dominate the active community in the atmosphere. We note there are many limitations to our model. First, we model fungal gene copy abundances assuming a well-mixed atmosphere at steady state. Yet the atmosphere is a highly heterogeneous and dynamic environment; the sampled air volume was likely neither well mixed nor at steady state over the time intervals we measured. Second, we use a global model with emission and deposition as the sole input and output, whereas a local model that incorporated site-specific environmental fate and transport terms would likely provide more accurate expectations. Third, due to a paucity of data, our estimates of fungal gene abundance levels rely on assumptions about the emission and deposition rates of vegetative cells and spores across fungal taxonomic groups. Empirically derived estimates of these model parameters would significantly improve our approach. Fourth, we do not know to what extent vegetative cells and spores are associated with other particulate matter and how this affects their deposition and emission rates. Adopting an approach to empirically estimate the aerodynamic diameter of these fungal cell types across taxonomic groups would allow for improved estimates of deposition rates (Yamamoto et al., 2014).

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

A. M. Womack, P. E. Artaxo, F. Y. Ishida, R. C. Mueller, S. R. Saleska, K. T. Wiedemann, B. J. M. Bohannan[1] and J. L. Green

Responses to editor comments

Detailed responses and revisions are given below. Editor text is in black and our responses are in blue.

Responses to Editor Comments

I find the manuscript as written to be methodologically sound and a nice contribution to an understudied area of Earth science. I would prefer that the authors provide references for the following recently-added statement: Research has shown, for example, that concentrations of fungal spores in the atmosphere vary diurnally and seasonally.
We added a citation for Ingold 1971.

I would also remove the distinction between wet and dry season in this case; rainfall is abundant during both seasons but dry season storms tend to be more intermittent and energetic. This counters the previous statement about convective instability before thunderstorms as they influence atmospheric spore concentration and composition.
We agree and have removed the text discussing potential changes in Ascomycota abundance during the wet season.

Likewise, the following statement needs references and less speculation: 'This result makes sense in light of the natural histories of many of the Ascomycota, which have single-celled or filamentous vegetative growth forms that are small enough to become aerosolized, whereas many of the Basidiomycota are too large to be easily aerosolized, other than in the form of metabolically inactive spores. It is possible that if we had sampled at night rather than during the day, we would have observed a higher relative abundance of Basidiomycota in the active community. The abundance of vegetative Ascomycota fragments may peak during the day when wind speeds are high, assuming they are passively dispersed by wind and convection (as opposed to active mechanisms many fungi use to disperse spores).' Such dynamics depend on the ability of the canopy to be flushed by turbulence and is the topic of an in review paper for the ZF2 tower: Gerken, T., Chamecki, M., Fuentes J.D.: Air parcel residence times and chemical processing of biogenic hydrocarbons within forest canopies. In review for: Agricultural and Forest Meteorology
We have edited the second paragraph of results section 3.2 to reduce speculative language. As suggested, we added text to acknowledge that temporal dynamics of fungi in the atmosphere above the canopy likely depend on the residence times of air parcels within and below the canopy as well as the ability of fungi to disperse through the canopy.

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

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Abstract

Fungi are ubiquitous in the atmosphere and may play an important role in atmospheric processes. We investigated the composition and diversity of fungal communities over the Amazon rainforest canopy and compared these communities to fungal communities found in terrestrial environments. We characterized the total fungal community and the metabolically active portion of the community using high-throughout DNA and RNA sequencing and compared these data to predictions generated by a mass-balance model. We found that the total community was primarily comprised of fungi from the phylum Basidiomycota. In contrast, the active community was primarily composed of members of the phylum Ascomycota and included a high relative abundance

of lichen fungi, which were not detected in the total community. The relative abundance of Basidiomycota and Ascomycota in the total and active communities was consistent with our model predictions, suggesting that this result was driven by the relative size and number of spores produced by these groups. When compared to other environments, fungal communities in the atmosphere were most similar to communities found in tropical soils and leaf surfaces. Our results demonstrate that there are significant differences in the composition of the total and active fungal communities in the atmosphere, and that lichen fungi, which have been shown to be efficient ice nucleators, may be abundant members of active atmospheric fungal communities over the forest canopy.

1 Introduction

Fungi are critical to the functioning of terrestrial ecosystems and may also play an important role in the functioning of the atmosphere. Fungi are abundant and ubiquitous in the atmosphere, with an estimated global land surface emission rate of 50 Tg/year for fungal spores alone (Elbert et al, 2007). Fungal bioaerosols are not only abundant but also affect physical and chemical processes in the atmosphere. Fungal spores, cellular fragments, and cell-free biological particles have the potential to affect precipitation by acting as ice and cloud condensation nuclei (Després et al., 2012; Morris et al., 2013; Pouleur et al., 1992; Richard et al., 1996), and metabolically active fungi sampled from the atmosphere are capable of transforming compounds known to play a major role in atmospheric chemistry, including carboxylic acids (Ariya, 2002; Côté et al., 2008; Vaïtilingom et al., 2013), formaldehyde, and hydrogen peroxide (Vaïtilingom et al., 2013).

The *in situ* function of airborne fungi will depend on the physiological state of fungal cells. Metabolically active vegetative cells have the potential to transform atmospheric compounds and ultimately alter atmospheric chemistry, whereas for dormant spores this metabolic capability is greatly reduced (Sussman and Douthit, 1973). The ice nucleation efficiency of fungal cells also likely depends on their physiological state; vegetative cells derived from potentially active fungi are more efficient ice nucleators than spores. Vegetative forms of *Fusarium* (a filamentous fungi) as well as several lichen fungi have been shown to nucleate ice at temperatures as warm as -1°C (Després et al., 2012) (Supplement figure 1), and ice nucleation by hyphae has been observed at -

2.5°C. In contrast, dormant spores – particularly those with surface hydrophobins – are generally poor ice nucleators. For example, ice nucleation of rust (*Puccinia*) spores requires temperatures lower than -10°C (Morris et al., 2013), and *Cladsporium* spores nucleate ice at temperatures of approximately -28.4°C (Iannone et al., 2011).

Despite its importance, we know relatively little about the physiological state of fungal cells in the atmosphere. Specifically, we know little about the taxonomic composition of metabolically active airborne fungi and how this compares to the composition of the total fungal community. One way to survey the total and active communities is to measure community composition from rDNA (i.e. rRNA genes) and rRNA in ribosomes. Sequencing rDNA provides information about the total community, which includes both active and dormant individuals, whereas rRNA sequences provide information about the potentially active community, because ribosomes are more abundant in active cells than dormant cells (Prosser, 2002). This approach has been applied to study active fungal communities in soils and on decaying plant material (Baldrian et al., 2012; Barnard et al., 2013, 2014; Rajala et al., 2011) but has not been applied to fungal communities in the atmosphere.

Information about the taxonomic composition of airborne fungi that are present in different physiological states can be used to advance atmospheric science. For example, such data can be used to improve estimates of the ice nucleating capacity of fungal bioaerosols. Historically, the composition of fungal communities in the atmosphere has been measured using culture-based approaches such as the abundance of colony forming units of specific taxa. This has led some scientists to conclude that fungal communities in the atmosphere have a low capacity for ice nucleation because taxa that appear abundant using plate counts have a low ice nucleation efficiency (Iannone et al., 2011, but see Pummer et al., 2013). This data may be misleading, as the vast majority of fungi require identification using culture-independent approaches (Borneman and Hartin, 2000). Today, culture-independent identification of active fungal taxa sampled from the atmosphere can be used to direct selective culturing of potentially important fungi in the laboratory, where their ice nucleation efficiencies and their metabolic capabilities can be further tested.

In this study, we used culture-independent approaches to measure the composition of total and active atmospheric fungal communities *in situ* and a mass-balance model to aid in the interpretation of our results. Our study system is the atmosphere above the Amazon rainforest canopy. We chose this system because fungal bioaerosols make up a substantial proportion of aerosol particulate

matter over the Amazon (Elbert et al., 2007; Heald and Spracklen, 2009) and are estimated to be a dominant force responsible for cloud formation over the Amazon (Pöschl et al., 2010). We used a combined approach of DNA and RNA sequencing to address the following questions: 1) What is the composition of total airborne fungal communities? 2) What is the composition of active airborne fungal communities? 3) What likely drives differences in the composition of the total and active airborne fungal communities? 4) Is the diversity and structure of fungal communities in the atmosphere similar to that found in terrestrial environments?

2 Methods

2.1 Sample collection

Sampling was conducted on the ZF2 K34 flux tower (S -2.60907, W -60.20917, 67 m a.s.l.) in the Reserva Biologica do Cueiras in central Amazonia, about 60 km NNW of Manaus, Brazil. The site is operated by the Instituto Nacional de Pesquisas da Amazonia (INPA) under the Large Scale Biosphere-Atmosphere Experiment in Amazonia (LBA) program (Martin et al., 2010). Tower height is approximately 54 m. Surrounding vegetation is undisturbed, mature, terra firme rainforest, with a leaf area index of 5–6 and an average canopy height of 30 m. Samples were collected at the end of dry season over four days, December 8-11, 2010, from a height of 48m above the forest floor. Environmental conditions during the four-day sampling period were typical for the location in early December with partial clouds and temperatures ranging from approximately 28.5°C to 32.1°C. Heavy rain and thunderstorms occurred on 12/8 and 12/11. Aerosol samples were collected using SKC Biosamplers (BioSampler SKC Inc.). Samplers were filled with 20 mL of a water-based preservation solution (LifeGuard Soil Preservation Solution, MO BIO Laboratories, Inc) to prevent DNase and RNase activity and maintain cells in stasis to allow accurate community profiling of the total and active fungal community. Twelve impingers were operated at 12.5 L/min from approximately 9:00 am – 4:00 pm each day. At the end of each day, the sampling liquid from all impingers was pooled and stored at -20°C. Impingers were cleaned each day by rinsing in 70% ethanol followed by sterilization using a portable pressure cooker.

2.2 Nucleic acid isolation and cDNA synthesis

Samples were transported on ice to the University of Oregon where the liquid sample from each day was separated into two aliquots, one to be used for DNA extraction and the other for RNA extraction. The divided samples were filtered through sterile, individually wrapped, 0.22 µm cellulose nitrate filters (Nalgene Analytical Test Filter Funnels, Thermo Fisher Scientific). DNA was extracted from filters using the MO BIO PowerWater DNA Isolation Kit according to the manufacturer's instructions with a 100 µl elution volume. RNA was extracted from filters using the MO BIO PowerWater RNA Isolation Kit with the following modifications. The DNase steps included in the kit were omitted. RNA was eluted in 50 µl. The extracted RNA was treated with DNase I (RNase-free) (Fermentas International, Inc) according to the manufacturer's instructions. DNase reactions were cleaned (Zymo Research Clean and Concentrate-5) and eluted into 50 µl. cDNA was synthesized from the total RNA extract using the SuperScript II First-Strand Synthesis System (Invitrogen, Life Technologies Corporation) with random hexamers. All RNA was converted into cDNA in six synthesis reactions and one reverse transcriptase negative control reaction. Three field blanks were generated by filtering unused LifeGuard Solution through new, sterile filters. Blanks were processed in parallel to the RNA and DNA samples including extraction, PCR amplification, and library preparation. Following library preparation, blank samples were visualized on an agarose gel and no visible bands were observed.

2.3 Library preparation and sequencing

To increase the concentration of cDNA to levels required for sequencing, we used multiple displacement amplification (GenomiPhi V2, GE Healthcare) according to the protocol described in Gilbert *et al.* (2010) including second-stand synthesis, amplification, and de-branching of amplification products. The fully de-branched products were sheared by sonication (24 cycles, 30 seconds each) using the Bioruptor sonication system (Diagenode). cDNA fragments were end-repaired (End-It DNA End-Repair Kit, Epicentre Biotechnologies), cleaned and concentrated (Zymo Research Clean and Concentrate-5) and eluted in 40 µl. A-overhangs were added to the end-repaired fragments using Klenow exo(-) (Epicentre Biotechnologies) in a 50 µl reaction. Reaction products were cleaned and concentrated (Zymo Research Clean and Concentrate-5). Standard paired-end, barcoded Illumina adaptors (Supplement table 1) were ligated to the fragments using T4 ligase (Fermantas). Reaction products were cleaned and concentrated (Zymo

Research Clean and Concentrate-5) and eluted in 12 µl. To enrich fragments with ligated adaptors, PCR amplification was performed using primers containing the flowcell adaptor and complementary to the Illumina sequencing primer (Supplement Table 1). PCR reactions were performed using Phusion DNA polymerase (New England Biolabs) with 12 µl template, 10 µl 5x HF buffer, 1 µl 10 mM dNTPs, 2 µl 10 mM primer mix, 0,5 µl enzyme and 25.5 µl water for a final reaction volume of 50 µl. PCR cycling conditions were as follows: 30 seconds denaturation at 98°C followed by 18 cycles of 98°C for 30 seconds, 65°C for 30 seconds and 72°C for 30 seconds following by a final extension at 72°C for 5 minutes. PCR products were size fractionated by gel electrophoresis (2.5%, low-melt agarose). Products in the range of 150-500 bp were excised, and DNA from the excised gel pieces was extracted (QiagenMinElute Gel Extraction) and eluted into 20 µl. DNA was quantitated using a Qubit 2.0 Fluorometer (Invitrogen, Life Technologies Corporation) and combined in equal molar concentrations. Shotgun metatranscriptome libraries were sequenced (150 base pairs, paired-end) on the Illumina HiSeq 2000 (Illumina, Inc.) platform at the University of Oregon Genomics Core Facility. LSU rDNA amplicons were sequenced (250 base pairs, paired-end) on the Illumina MiSeq platform at the Dana-Farber Cancer Institute Molecular Biology Core.

The D1-D2 region of the large subunit (LSU) rRNA gene was targeted using PCR with the primers LR0R (5'-ACCCGCTGAACTTAAGC-3') and LR3 (5'-CCGTGTTTCAAGACGGG-3') (http://sites.biology.duke.edu/fungi/mycolab/primers.htm). LSU amplicon libraries were prepared using a two-stage PCR procedure as described in (Kembel and Mueller, 2014) using unique combinatorial barcodes (Gloor et al., 2010) to identify samples (Supplement table 2).

2.4 Sequence pre-processing

2.4.1 Metatranscriptome

Overlapping paired end reads were aligned and joined using fastq-join (https://code.google.com/p/ea-utils/wiki/FastqJoin). Joined reads and non-overlapping single-end reads were trimmed and filtered using PrinSeq (Schmieder and Edwards, 2011). Sequences <75 bp, > 2% Ns, and/or mean quality score <20 were removed. Sequence artifacts defined as exact duplicates with >5,000 sequences were removed. Sequences in the Dec. 10 sample were primarily artifacts, so this

metatranscriptome sample was excluded from further analysis. Putative rRNAs in the remaining sequences were identified using SortMeRNA (Kopylova et al., 2012) with the non-redundant version of the following databases: rfam 5.8S (version 11.0) (Burge et al., 2013); Unite (November 2011 version) (Kõljalg et al., 2013), and Silva 18S and Silva 28S (Release 115) (Quast et al., 2013). Of 5,165,185 quality-filtered reads, 1,915,994 with an average length of 137.5 bp were identified as putative rRNAs (Supplement table 3).

2.4.2 LSU amplicons

Forward and reverse barcodes were combined to make a 12 bp barcode on the forward read. Only forward reads derived from the LR3 region were used for analysis. This region has been shown to have high species-level resolution even with short read lengths (Liu et al., 2012).

2.4.3 Multi-environment sequences

LSU sequences from four soil studies (Barnard et al., 2013; Kerekes et al., 2013; Penton et al., 2013, 2014) and one phyllosphere study (Kembel and Mueller, 2014) were compared to air samples collected for this study (Supplement table 4). Raw sequence data and associated metadata were downloaded from publically available databases. 12 bp barcodes were added to all sequences to identify each sample in downstream analysis.

2.5 LSU amplicon and metatranscriptome sequence processing

All sequences were processed in QIIME version 1.7 (Caporaso et al., 2010). Briefly, libraries were individually demultiplexed and filtered for quality. Sequences with an average quality score less than 20, shorter than 150 bp and with greater than 2 primer mismatches were discarded. The same parameters were used across all samples except the metatranscriptome rRNAs were a size cut off of greater than 75 bp was used. In order to decrease computation time, sequences from Kembel and Mueller (2014) and Penton *et al*. (2014) were randomly subsampled to 25% and 60% of the total number of sequences, respectively. Sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using closed reference BLAST (Altschul et al., 1990) against the Ribosomal Database Project Fungal LSU training set 1 (Cole et al., 2014). Taxonomy was assigned to each OTU was that of the most similar representative in the RDP database.

Following sequence processing and quality filtering, a total of 55,414 amplicon and 1,915,994 metatranscriptome LSU sequences generated for this study and 1,577,458 LSU sequences from soil and phyllosphere studies were retained (Supplement table 3). For analyses using only samples from this study, the data were rarefied to 5,300 sequences per sample. For analyses that compare samples in this study to samples from other studies, the data were rarefied to 500 sequences per sample.

2.6 Statistical analyses and data availability

All statistical analyses were conducted in R (R Core Team, 2014) primarily using the `vegan` (Oksanen et al., 2013) package for ecological statistics and the `ggplot2` (Wickham, 2009) package for visualizations.

Sequence files and metadata have been deposited in Figshare (http://dx.doi.org/10.6084/m9.figshare.1335851). Data from other studies used for cross environment analyses are available using the databases and identifiers referenced in the respective manuscripts.

2.7 Mass-balance model

We use a global, well-mixed, one-box material-balance model to predict the relative abundances of fungal cells measured as gene copies sampled in the active and total portions of atmospheric bioaerosols. Model description and details are available in Appendix A.

3 Results & Discussion

3.1 Basidiomycota dominate total airborne fungal communities

Measurements of airborne fungi using culture-based methods such as quantifying spore and colony-forming unit counts have been conducted for centuries (Després et al., 2012). In comparison, there have been few culture-independent studies of the fungal composition of atmospheric samples (e.g. Boreson et al., 2004; Bowers et al., 2013; Fierer et al., 2008; Fröhlich-Nowoisky et al., 2009, 2012; Pashley et al., 2012; Yamamoto et al., 2012). Using a culture-independent approach, we found the composition of total airborne fungal communities primarily included taxa belonging to the phyla

Ascomycota and Basidiomycota (Figure 1). This result is similar to what is observed in environments on the Earth's surface (James et al., 2006) and what has been reported in other studies of fungi in the atmosphere (Bowers et al., 2013; Fröhlich-Nowoisky et al., 2009, 2012; Yamamoto et al., 2012).

Basidiomycota dominated the total airborne community in our air samples (mean relative abundance = 90.2±6.9%) (Figure 1). Within the phylum Basidiomycota, Agaricomycetes were the most abundant class in our samples. Agaricomycetes have been previously detected in air samples (Fröhlich-Nowoisky et al., 2012; Woo et al., 2013; Yamamoto et al., 2012) and are common in tropical soils (Tedersoo et al., 2014) and leaf surfaces (Kembel and Mueller, 2014). Within the Agaricomycetes, the most abundant order was the Polyporales (mean = 55.7±2.3%). Polyporales have been detected in culture-independent studies of urban aerosols (Yamamoto et al., 2012) and culturable representatives have been isolated from cloud water (Amato et al., 2007). At the genus level, there were several taxa detect in the total community with ice nucleation activity including Acremonium, Cladosporium, Fusarium, and Rhizopus (Table S4).

The presence of Agaricomycetes may have implications for atmospheric processes. Ice nucleation efficiency within the Agaricomycetes is variable, with some taxa capable of nucleating ice at temperatures as warm as -17°C (Haga et al., 2014) (Supplement figure 1). These temperatures are warmer than what has been measured for *Penicillium* spores (Iannone et al., 2011) although not as warm as what has been measured for other biological particles including other spore types (Morris et al., 2013), suspensions of *Fusarium* cultures (containing spores and hyphae) (Pouleur et al., 1992), and lichen fungi (Després et al., 2012). Despite the low ice nucleation efficiency of some taxa in this group, given the high abundance of Agaricomycetes over the forest canopy, this group could still have a significant impact on cloud formation and precipitation in the tropics.

The patterns we report reflect a snapshot in space and time. As in other environmental systems, the composition of total fungal communities in the atmosphere will vary across different spatial and temporal scales. Research has shown, for example, that concentrations of fungal spores in the atmosphere vary diurnally and seasonally (Ingold, 1971). This variation is driven by complex interactions between fungal dispersal mechanisms and environmental conditions, particularly moisture and wind speed. (Lacey, 1996). Our samples were collected during the day, and spores released by mechanical disturbances often peak in abundance in the air during midday when wind

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speeds are highest (Lacey, 1996). Taxa that require dry conditions for dispersal also tend to release spores during the day, and taxa that require high relative humidity, including many Basidiomycota, tend to release spores at night when humidity is highest (Elbert et al., 2007; Lacey, 1996). In addition to humidity, precipitation events can also affect the dispersal of fungi. Overall concentrations of spores have been shown to increase in the atmosphere due to convective instability preceding thunderstorms (Burch and Levetin, 2002), and Ascomycota concentrations increase during and immediately after rainstorms (Elbert et al., 2007).

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3.2 Ascomycota dominate active airborne fungal communities

The composition of total and active fungal communities over the Amazon rainforest canopy significantly differed (ADONIS, $R^2 = 0.342$, $p = 0.029$). The active community in the atmosphere over the forest canopy was dominated by Ascomycota (mean relative abundance = 80.4±20%) (Figure 1). Basidiomycota comprised a smaller fraction of the sampled genes (mean = 7.3±6.8%) with the remainder of identified sequences belonging to the phyla Chytridiomycota and Glomeromycota. This result makes sense in light of the natural histories of many of the Ascomycota, which have single-celled or filamentous vegetative growth forms that are small enough to become aerosolized, whereas many of the Basidiomycota are too large to be easily aerosolized (Moore et al., 2011), other than in the form of metabolically inactive spores.

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Similar to the total community, we expect the compositions of active fungal communities in the atmosphere likely vary over different time scales. For example, had we sampled at night rather than during the day, we may have observed a higher relative abundance of Basidiomycota in the active community. This could be due to an increase in the concentration of basidiospores combined with a decrease in vegetative Ascomycota at night because basidiospores are abundant in the Amazon atmosphere at night (Elbert et al., 2007) and the detection of ribosomes within spores could lead to an increase in the observed relative abundance of Basidiomycota in the active community. We would also expect the relative abundance of Ascomycota to decrease at night when wind speeds typically decrease, particularly considering that many vegetative Ascomycota fragments are passively dispersed by wind and convection (as opposed to active mechanisms many fungi use to disperse spores). However, these patterns will depend on the relative abilities of spores (Gilbert & Reynolds, 2005) and fragments to disperse beyond the understory as well as the residence times of

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air parcels below and within the canopy. It will be fruitful for future studies to dynamically sample both above and below the canopy to elucidate the mechanisms driving temporal variation in fungal communities in the atmosphere.

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The most abundant classes of Ascomycota detected were Sordariomycetes (mean = 27.1±6.6%), and Lecanoromycetes (mean = 17.5±7.6%). Sordariomycetes have been previously detected in culture-independent air samples (Fröhlich-Nowoisky et al., 2009, 2012; Yamamoto et al., 2012) and have been shown to be abundant on tropical tree leaves (Kembel and Mueller, 2014) and tropical soils (Peay et al., 2013). In most ecosystems, Sordariomycetes are endophytes, pathogens, and saprotophs (Zhang et al., 2007). Xylariales, which includes both endophytes and plant pathogens (Zhang et al., 2007), was the most abundant order within the Sordariomycetes in our samples. Several genera with known ice nucleation capability were detected in the active community including Agaricus, Amanita, Aspergillus, Boletus, Lepsita, Mortierella Puccinia, Rhizopus, and the lichen fungus Cladonia (Table S4). Below we focus our discussion on the class Lecanoromycetes, an understudied but potentially important group of fungi in the atmosphere.

Lecanoromycetes were the second most abundant class of Ascomycota detected over the rainforest canopy. This group has been detected in other culture-independent studies of fungi in the atmosphere (Fröhlich-Nowoisky et al., 2012; Yamamoto et al., 2012). The Lecanoromycetes contain 90% of the lichen-associated fungi (Miadlikowska et al., 2007). Lichens are a symbiosis between a fungus and a photosynthetic partner such as eukaryotic algae or cyanobacteria. Lichens are known to be hardy and may be particularly well-adapted for long distance transport and metabolic activity in the atmosphere. Lichens are often the dominant life forms in environments that have conditions similar to those found in the atmosphere, including low water (Kranner et al., 2008) and nutrient availability, wide temperature variations, and high UV irradiance (e.g. Solhaug, Gauslaa, Nybakken, & Bilger, 2003) (Onofri et al., 2004).

Another notable trait of lichens is their efficient ice nucleation capacity. Although there have been no investigations specifically on the most abundant lichen species detected in this study, *Physcia stellaris* (mean = 8.3±3.8%) and *Rinodina milvina* (mean = 4.8±3.4%), there have been multiple studies of the ice nucleation efficiency of many other lichen fungi species. Ice nucleation activity of lichens has been measured at temperatures warmer than -8°C, including 13 of 15 taxa tested by Henderson-Begg and colleagues (Henderson-Begg et al., 2009) and 9 of 15 taxa tested by Kieft

(Kieft, 1988). These studies have demonstrated that lichens are among the most efficient biological ice nucleators. Therefore, their presence in the atmosphere may have a significant impact on cloud formation and precipitation. This ice nucleation capacity may also enable lichens to control the extent of their dispersal through the atmosphere. It is possible that lichens achieve this by nucleating ice formation, which leads to precipitation and ultimately deposition. This phenomenon has been shown to occur in some phytopathogenic bacteria (Morris et al., 2008, 2010) and may occur in fungi as well (Morris et al., 2013).

3.3 Dominance of Basidiomycota in total communities and Ascomycota in active communities is consistent with mass-balance predictions

Our mass balance model (Appendix A) predicted Basidiomycota would dominate the total community because they produce orders of magnitude more spores and have smaller aerodynamic diameters compared to Ascomycota. Consistent with this prediction, the total airborne community was dominated by Basidiomycota in our air samples (mean relative abundance = 90.2±6.9%) (Figure 1). There have been some empirical studies reporting the opposite pattern, with a higher relative abundance of Ascomycota compared to Basidiomycota (Bowers et al., 2013; Fierer et al., 2008; Pashley et al., 2012). There has been one study focused on airborne fungal communities in the Amazon Basin (Fröhlich-Nowoisky et al., 2012). Although the site of this study was the atmosphere above a rural pasture (versus a tropical rainforest, as in our study) these investigators also found that Basidiomycota dominate airborne fungal communities

Our mass-balance model explains the differences in composition between the total and active air communities. However, some of the differences we observed may be partially attributable to the use of different approaches in characterizing the total and active communities. In this study, the total community was characterized by PCR-based amplification and sequencing of LSU genes, whereas the active community was characterized through random sequencing of all the RNA present in the samples. Shotgun metatranscriptome sequencing and PCR-based community characterization approaches each have their own biases (Hong et al., 2009; Morgan et al., 2010). Our data suggest that the selection of LSU primers led to biased results. For example, the high relative abundance of lichen fungi (class Lecanoromycetes) in the active community was unexpected because this group was not detected in the total community and has only been detected

in low abundance in other PCR-based studies of fungi in the atmosphere (Fröhlich-Nowoisky et al., 2012). We tested the primer pair used in this study (LR0R-LR3) using the SILVA TestPrime tool (Klindworth et al., 2013) and found coverage of the Lecanoromycetes with this primer pair was 71.4%. Within the class Lecanoromycetes, the order Teloschistales, which contains the most abundant species detected in the active community, would not have been detected with this primer pair. However, the general pattern that Ascomycota were much less abundant than Basidiomycota in the total community is not likely due to primer bias as overage of the phylum Ascomycota by the LR0R-LR3 primer pair is 85.5% according to TestPrime. Our findings underscore the value of using a combination of PCR-based and shotgun-based sequencing approaches, particularly in environments that are understudied and where little is known about microbiome structure and function.

3.4 Fungal air communities above the forest canopy are most similar in composition to tropical phyllosphere and soil communities

We compared total and active fungal air communities to communities from tropical, temperate, and tundra soils and from the surfaces of tropical tree leaves. Community composition significantly differed across environment types (ADONIS, $R^2 = 0.167$, $p = 0.001$), and fungal communities in the atmosphere were compositionally distinct from communities in other environments (Figure 2). Ascomycota was the most abundant phylum across all soil and phyllosphere samples (soil mean relative abundance = 78.4±14.9%, phyllosphere = 90.9±4.9%) followed by Basidiomycota (soil mean relative abundance = 19.0±14.9%, phyllosphere = 7.4±4.5%) (Figure 3). We expected communities to be distinct across habitat types because environmental conditions may differ across the habitat types and select for different communities. However, in the atmosphere, dispersal and mixing of fungi from multiple habitat types may be driving the observed community composition differences instead of environmental selection.

The diversity of fungal communities in the atmosphere is within the range of diversities reported for terrestrial environments, including those of tropical leaf surfaces, tropical soils, temperate grassland soils, and tundra soils. Overall taxonomic richness, defined as the number of OTUs, significantly varied among environment types (ANOVA, $F(5,237) = 66.89$, $p < 0.001$) (Supplement figure 2). Tukey's HSD post-hoc comparisons indicated that the richness of air communities, both

total and active, was greater than tundra soil communities and did not significantly differ from temperate grassland soil communities. In general, air communities were less diverse than tropical forest phyllosphere and soil communities with the exception of tropical forest soils and active air communities, which did not significantly differ. Similar patterns have been observed in soil communities where taxonomic richness in arctic soils was significantly lower than soils from temperate and tropical ecosystems (Fierer et al., 2012).

Total air communities were most similar to tropical phyllosphere communities (mean Sørensen similarity = 0.015±0.009; Tukey's HSD, $p < 0.001$) and active air communities were most similar to tropical soil communities (mean Sørensen similarity = 0.010±0.007, Tukey's HSD, $p < 0.001$) (Supplement figure 3). This suggestion makes sense since fungal spores and hyphae are relatively large aerosol particles with short residence times in the atmosphere, limiting opportunities for long-distance dispersal. While these results are suggestive, detailed information is lacking regarding the potential influence of terrestrial source environments and their role in structuring airborne fungal communities.

4 Conclusion

Fungi in the atmosphere play an important role in atmospheric processes including precipitation development through ice nucleation. This is of particular significance in the atmosphere over the Amazon rainforest canopy where fungi constitute a large fraction of the total aerosol content (Elbert et al., 2007; Heald and Spracklen, 2009) and precipitation is aerosol-limited (Pöschl et al., 2010). Our study represents the first culture-independent survey of fungal communities over the Amazon rainforest canopy. It is also the first to measure metabolically active microbial communities in the atmosphere using an RNA-based approach. Using this RNA-based approach, we found evidence for the presence of potentially active fungi in the atmosphere, including lichen fungi (class Lecanonomycetes) and the following genera: Agaricus; Amanita; Aspergillus; Boletus; Cladonia; Lepsita; Mortierella; Puccinia; and Rhizopus. While an understanding of the structure of fungal communities in the atmosphere is beginning to emerge, studies on the function of these communities have lagged behind. We suggest that future research focus on understanding the functional capacity of airborne microbes with traits particularly well-suited for survival and

metabolic activity in extreme environments. As with any environment, understanding both the structure and function of microbial communities in the atmosphere is needed to assess their potential impact on ecosystem processes such as water and carbon cycling. This study opens the door for future investigations of the diversity and function of fungal communities in the atmosphere.

Author contributions

A. M. Womack conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, and reviewed drafts of the paper. P. E. Artaxo conceived and designed the experiments. F. Y. Ishida collected the samples and reviewed drafts of the paper. R. C. Mueller conceived and designed the experiments, reviewed drafts of the paper and contributed reagents/materials/analysis tools. S. R. Saleska conceived and designed the experiments. K. T. Wiedemann collected the samples. B. J. M. Bohannan conceived and designed the experiments, collected the samples, and reviewed drafts of the paper. J. L. Green conceived and designed the experiments, wrote the paper, reviewed drafts of the paper, and contributed reagents/materials/analysis tools.

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Figures

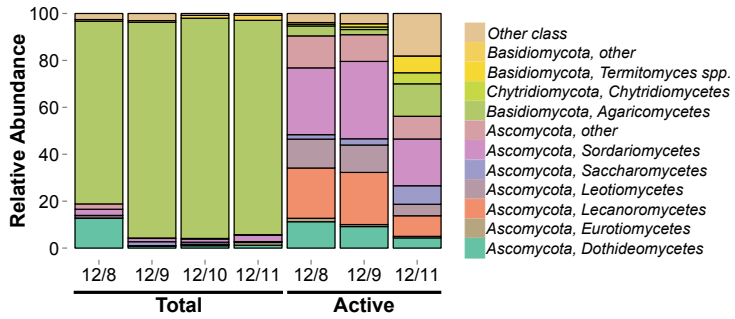

Figure 1. Basidiomycota dominate the total fungal community (mean relative abundance = 90.2±6.9%). Bars are colored according to class-level taxonomic assignments. Taxonomy was assigned to representative sequences from each OTU.

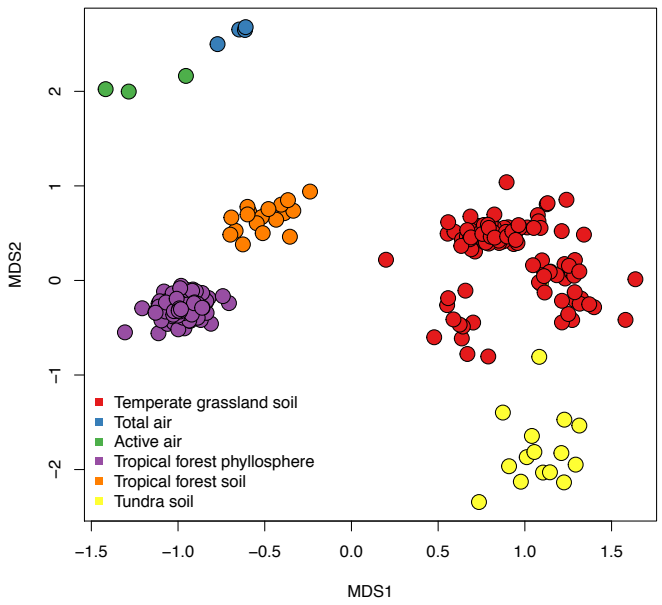

Figure 2. OTU-based community composition significantly differed across environment types (ADONIS, R^2 = 0.167, p = 0.001). Total and active communities in air samples (upper left) clustered together and separate from other environments indicating these communities are distinct from communities found in soils and on leaf surfaces. Sørensen similarities are depicted, ordinated via NMDS.

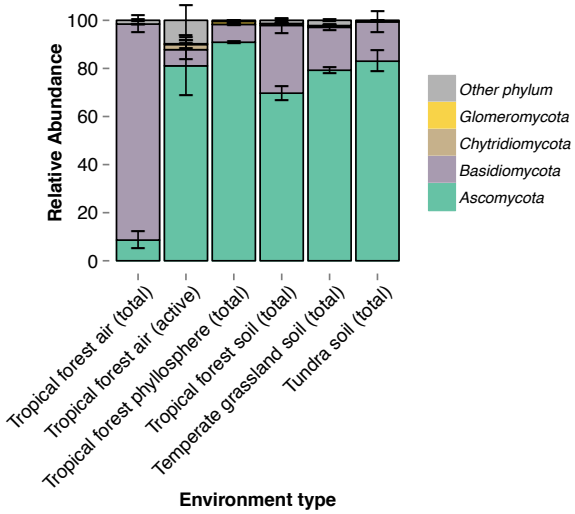

Figure 3. Relative abundances of fungal phyla across environment types. The active atmospheric fungal community over the Amazon rainforest was more similar in phylum-level composition to fungal communities found in tropical soils and on plant leaves than was the total community. Error bar represent standard deviations.

Appendix A

Mass-balance model

We use a global, well-mixed, one-box material-balance model to explain the relative abundances of fungal cells measured as gene copies sampled in the active and total portions of atmospheric bioaerosols. By material-balance, for any taxon *i* within a biological community, the change in time in the abundance of fungal gene copies, N_i, must be equal to the difference in source and sinks:

$$\frac{dN_i}{dt} = \sum sources - \sum sinks \quad \text{(A1)}$$

Here we assume sources are equal to the emission of fungal gene copies from the Earth's surface into the atmosphere, E_i (gene copies/hour). We assume sinks are equal to deposition of fungal gene

copies out of the atmosphere back to the Earth's surface, $D_i = N_i k_i$, (gene copies/hour), where k_i (1/hour) represents a first order deposition coefficient. We can rewrite Equation (A1) as:

$$\frac{dN_i}{dt} = E_i - N_i k_i$$

We expect the terms E_i and k_i to vary as a function of life history traits including the method of cell release into the atmosphere, the physiological state of sampled cells, and the aerodynamic diameter of fungal taxa. In this case, Equation (A2) does not directly represent the entire airborne fungal gene copy abundance. We assume that a first order approximation of fungal bioaerosol behavior may be obtained by subdividing the particle distribution into two modes: vegetative cells, $N_{i,veg}$, and spores, $N_{i,spores}$. We thus model fungal gene copy abundance as:

$$N_i = N_{i,veg} + N_{i,spores}$$

We can then write and solve parallel versions of Equation (A2) for each mode. At steady state, the expected gene copy abundance taxa *i* in each mode is:

$$N_{i,veg} = \frac{E_{i,veg}}{k_{i,veg}}$$

$$N_{i,spore} = \frac{E_{i,spore}}{k_{i,spore}}$$

Our interest lies in the two most common fungal phyla sampled in the atmosphere: Ascomycota, N_A, and Basidiomycota, N_B. To make predictions about the expected relative abundance of gene copies in these two groups, we make informed assumptions about the relative magnitude of their respective emission and deposition rates. We begin by considering fungal spores. Although a few empirical studies have suggested that Ascomycota are more abundant than Basidiomycota in likely source environments including tropical soils (Kerekes et al., 2013) and leaf surfaces (Kembel and Mueller, 2014), Basidiomycota (e.g. Agaricomycetes, the most abundant class of Basidiomycota in our samples) produce orders of magnitude more spores per individual than Ascomycota (Elbert et al., 2007; Pringle, 2013). For this reason, we assume the emission rate of Basidiomycota spores is much greater than that of Ascomycota spores:

$$E_{A,spores} \ll E_{B,spores}$$

Culture-based microscopy data suggests that spores of Ascomycota are typically larger than spores of Basidiomycota (Elbert et al., 2007; Ingold, 2001; Yamamoto et al., 2014). Owing to the difference in spore size, we expect deposition rate of Ascomycota spores to be greater than that of Basidiomycota spores:

$$k_{d,A,spores} > k_{d,B,spores}$$

Based on these assumptions, it follows that the expected number of Ascomycota spores in the atmosphere will be less than the number of Basidiomycota spores:

$$\frac{E_{A,spore}}{k_{A,spore}} \ll \frac{E_{B,spore}}{k_{B,spore}}$$

or

$$N_{A,spores} \ll N_{B,spores}$$

We next consider fungal vegetative cells. Vegetative forms of Ascomycota are generally smaller than vegetative forms of Basidiomycota (Moore et al., 2011). Many Ascomycota grow as filaments or single cells which are small enough to be aerosolized (Després et al., 2012). In contrast, many Basidiomycota grow as mushrooms, which are too large to be aerosolized (although debris from mushrooms and their mycelia can be aerosolized). Due to this difference in the vegetative forms of each group, we expect emission rate of vegetative Ascomycota to be greater than Basidiomycota:

$$E_{A,veg} > E_{B,veg}$$

No comparative data currently exists on the relative deposition rate of vegetative cells across fungal taxa. Research has shown that at the phylum level, the aerodynamic diameter of Ascomycota is greater than that of Basidiomycota, resulting in a greater deposition rate overall for Ascomycota (Yamamoto et al. 2014). However, this work did not differentiate between vegetative cells and spores, and there is no *a priori* reason to assume that the deposition rate of Ascomycota vegetative cells are less than or greater to that of Basidiomycota cells. For this reason, we make the null assumption that the deposition rate of each group is equal:

$$k_{d,A,veg} = k_{d,B,veg}$$

Based on these assumptions, we expect the number of vegetative Ascomycota genes to be greater than the number of vegetative Basidiomycota genes:

$$\frac{E_{A,veg}}{k_{A,veg}} > \frac{E_{B,veg}}{k_{B,veg}}$$

or

$$N_{A,veg} > N_{B,veg} \quad \text{(A3)}$$

Equation (A3) predicts that Ascomycota will dominate the active fungal community in the atmosphere.

Finally, we relate the abundance of Ascomycota and Basiodiomycota gene copies in their totality to ask if $N_A < N_B$ or $N_A \geq N_B$. $N_A < N_B$ if and only if:

$$N_{A,veg} + N_{A,spores} < N_{B,veg} + N_{B,spores}$$

Rearranging terms and dividing both sides of the equation by $N_{B,spores}$ yields the inequality:

$$\frac{N_{A,veg} - N_{B,veg}}{N_{B,spores}} + \frac{N_{A,spores}}{N_{B,spores}} < 1$$

or

$$\frac{N_{A,veg} - N_{B,veg}}{N_{B,spores}} < 1 - \varepsilon$$

where $\varepsilon = \frac{N_{A,spores}}{N_{B,spores}}$. Empirical data on the discharge of Ascomycota and Basidiomycota spores from fruiting bodies suggests that $\varepsilon \leq 0.01$ (Elbert et al. 2007). In this case $N_A < N_B$ if and only if:

$$\frac{N_{A,veg} - N_{B,veg}}{N_{B,spores}} < 0.99 \quad \text{(A4)}$$

We expect Equation A4 to hold due to the likelihood that spores greatly out number vegetative cells in the atmosphere in both phyla. Spores can be actively discharged into the air, whereas vegetative cells are not actively propelled into the atmosphere and require aerosolization by mechanical forces like wind. Furthermore, empirical data suggests that vegetative cell fragments

constitute a small fraction (0.2-16% (Green et al., 2011)) of the total fungal biomass in the atmosphere. For these reasons, we predict that

$$N_A < N_B$$

Based on the conclusions of this model, we expect Basidiomycota will dominate the total community, and Ascomycota will dominate the active community in the atmosphere. We note there are many limitations to our model. First, we model fungal gene copy abundances assuming a well-mixed atmosphere at steady state. Yet the atmosphere is a highly heterogeneous and dynamic environment; the sampled air volume was likely neither well mixed nor at steady state over the time intervals we measured. Second, we use a global model with emission and deposition as the sole input and output, whereas a local model that incorporated site-specific environmental fate and transport terms would likely provide more accurate expectations. Third, due to a paucity of data, our estimates of fungal gene abundance levels rely on assumptions about the emission and deposition rates of vegetative cells and spores across fungal taxonomic groups. Empirically derived estimates of these model parameters would significantly improve our approach. Fourth, we do not know to what extent vegetative cells and spores are associated with other particulate matter and how this affects their deposition and emission rates. Adopting an approach to empirically estimate the aerodynamic diameter of these fungal cell types across taxonomic groups would allow for improved estimates of deposition rates (Yamamoto et al., 2014).