

## Response to referee comments and suggestions on bg-2020-469', by Prass et al.

### Manuscript format description:

Black text shows the original referee comment, blue text shows the authors response, and red text shows quoted manuscript text. Changes to the manuscript text are shown as *italicized and underlined*. We used bracketed comment numbers for referee comments (e.g., [R1.1]) and author's responses (e.g., [A1.1]). Line numbers refer to the discussion/review manuscript.

### Anonymous Referee #1,

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Prass et al. provide an analysis of the domain level diversity of bioaerosols from the Amazon rainforest. This dataset provides a window into an area of the atmosphere considered to be less affected by human pollution, which could help deconvolve what microbes are “naturally” in the atmosphere vs there by human-activities. It also provides a dataset that focuses on intact cells compared to sequencing methods that could also include eDNA.

We appreciate the constructive comments by Referee #1, which have been considered carefully and helped to improve the quality of our manuscript. The referee's comments and our responses are outlined in detail below:

### Major comments:

[R1.1] The manuscript would be improved by more discussion of other bioaerosol-microbe focused papers and how they relate to the current findings, such as Souza et al. 2019 (referenced but not discussed), Stern et al. 2021; Env. Sci.&Tech. Zhen, Sci. Total Environ. 2017; Yamaguchi, N. Sci. Rep. 2012. What is known about the different domains and their potential ecosystem roles or residence times?

[A1.1] Thanks for the suggestion. More papers on the role of fungal and bacterial bioaerosols were implemented in the discussion as specified in detail below:

(P5, L16-P6, L3): These numbers are in good agreement with estimated and measured concentrations in previous bioaerosol studies (e.g., Burrows et al., 2009b; Fröhlich-Nowoisky et al., 2016). For instance, our measured  $N_{BAC}$  values fall within the estimated range of bacterial cell concentrations for forest ecosystems (i.e.,  $3.3\text{--}8.8 \cdot 10^4 \text{ m}^{-3}$ ) according to Burrows et al. (2009a). Furthermore, a predominance of Eukaryotes in the Amazon was shown before e.g. by Souza et al. (2019) and Elbert et al. (2007), which is consistent with our results.

(P6, L28-P7, L13): While the results presented here emphasize such potential links between the variability in bioaerosol concentrations and meteorological environmental parameters (which are speculative so far), the statistical basis of these initial FISH results is too small to constrain these relationships. Accordingly, an investigation of bioaerosol emission mechanisms in relation to the local and regional meteorology requires more extended follow-up FISH studies. *In contrast to the bioaerosol burden mainly originating from forest emission during clean wet season conditions, an investigation of long-range transport-related changes in the air microbiome might be of interest, for instance with respect to dust-associated bacteria as found by Yamaguchi et al. (2018) and Prospero et al. (2005).*

[R1.2] The study seems to pause right when something unique to FISH (the future work proposed at the end of page 10) could be presented. It is unclear why only FISH was utilized. The current standard for airborne microbiology appears to be sequencing based (see papers in point 1), which provides higher taxonomic resolution than the domain level FISH analysis conducted in this manuscript. 16S rRNA qPCR is more robust than microscopy for counting as well. The manuscript lacks an explanation why sequencing techniques were not applicable for this system (why wasn't FISH and sequencing done?) and should justify the decision to only look at domain level diversity (why weren't more FISH probes used?).

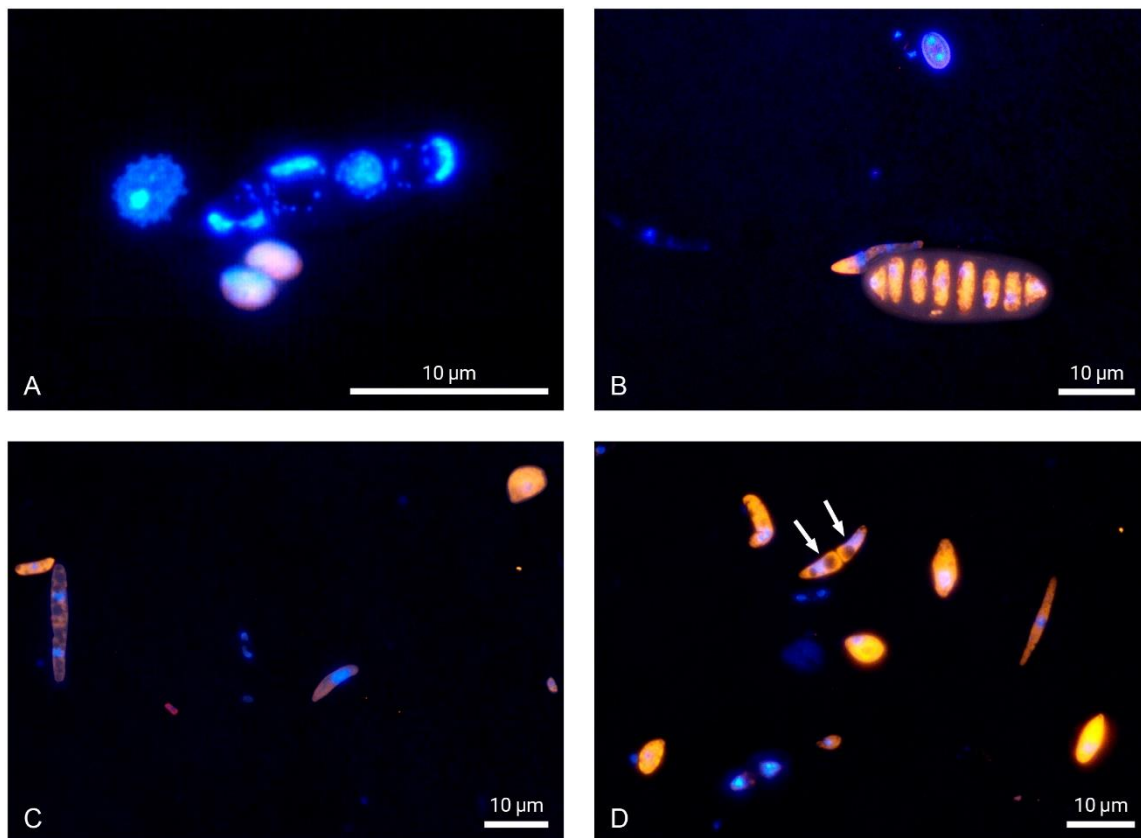
[A1.2] We thank Reviewer #1 for this comment. The reviewer questions here several aspects that are obviously not clear enough in the current version of the manuscript. Accordingly, we took this criticism into account to further clarify and sharpen our argumentation. Specifically, we added a new section into the supplement (S1.4), which address and justify in detail the various aspects brought up by the reviewers. We further adjusted and optimized several statements in the main text of the manuscript. In [R.1.2], the reviewer combines several questions, which are answered separately below.

“It is unclear why only FISH was utilized. The current standard for airborne microbiology appears to be sequencing based (see papers in point 1), which provides higher taxonomic resolution than the domain level FISH analysis conducted in this manuscript.

We are aware that sequencing-based techniques provide a higher taxonomic resolution. The aim of this study, however, was not to seek for the highest taxonomic resolution. In our opinion, the information that FISH provides is more comprehensive compared to sequencing-based methods: besides bioaerosol identification (as a function of FISH probe choice) and enumeration, it also enables the direct visualization of individual particles. Especially in the

field of bioaerosols, with limited knowledge about transport modes/mixing state and the potential influence of particles' size, shape, and surface structure on atmospheric processes, we believe that the visualization can be an advantage. Just as reviewer # 3 stated; „the ability to observe and enumerate assemblages of different composition (for example, assemblages that consist of only bacteria or assemblages that consist of eukaryotes and bacteria) is a clear strength of this technique. Figure 4H is a beautiful example.”

To highlight this strength of the FISH approach we added a new figure (Figure 4) showing further microscopic images of Amazonian bioaerosols and included a description:



**Figure 4.** Microscopic images of fluorescence signals after DNA staining with DAPI (blue) and FISH (eukaryotes in orange and bacteria in red). Bioaerosol samples were collected at 5 m height. Except for one bacterial bioaerosol in panel C, all other fluorescent bioaerosol signals in these panels were attributed to the eukaryotic domain.

(P 10 L23-P11 L14): *Finally, the microscopic visualization of cells after staining also provides qualitative insights into the Amazonian bioaerosol population, which is a strength of the FISH-approach. Figure 4 shows selected examples of bioaerosols typically found at 5 m height at the ATTO site. Most of the bioaerosol visualized in Figure 4 belong to the eukaryotic domain. Some of them could also be identified as spores based on morphological criteria (Gregory et al., 1973; Lacey and West, 2007). Figure 4A further illustrates the importance of a careful*

fixation and permeabilization prior to hybridization to enable the entrance of the FISH-probe into the cells. Here, the fern spores – identified by their typical spike-like surface structure according to Lacey and West (2007) – show nearly no orange fluorescence indicating a lack of hybridized eukaryotic probe due to insufficient cell lysis. In comparison, the ascospore in Figure 4B shows intense orange fluorescence as a sign of successful hybridization. However, signal intensities may vary also due to different rRNA contents as a matter of metabolic activity (positive signal but overall low fluorescent intensity of the spore on the left site in Figure 4C). Here, the manual microscopic inspection is of advantage as parameters such as particle size, morphology, surface structure, and fluorescent color can be considered beyond fluorescence intensity, to discriminate biological from non-biological and potentially autofluorescent particles. In terms of counting statistics, the manual enumeration can be beneficial as particles yielding two or more DAPI stained cores can be identified as single bioaerosol as shown in form of an ascospore (white arrows) in Figure 4D.

Regarding the quantification of bioaerosols, we are convinced that FISH is a technique that provides robust concentrations of airborne cells for clearly defined taxonomic (or functional) groups on the basis of direct enumeration (instead of calculation like done for qPCR). Therefore, we purposefully decided to choose FISH probes to target the domain levels and to obtain number concentrations of Bacteria, Archaea, and Eukaryotes. To clarify this aspect, the following text has been added / section has been modified:

(P10, L23-24): Finally, the microscopic visualization of cells after staining also provides qualitative insights into the Amazonian bioaerosol population, which is a strength of the FISH-approach.

(P14, L2-12): Our study showed that FISH has great analytical potential in bioaerosol analysis. It combines bioaer-osol identification, quantification, as well as visualization and, thus, provides insights into the concentration levels and spatiotemporal variability of specific and clearly defined organism groups with-in the bioaerosol population. Besides airborne abundances, only little is known about single particle properties such as identity, mixture or size. Here, we propose FISH to be a promising tool, to complement the methods currently established for environmental bioaerosol analysis (Sect. S1.4). As this is the first study using FISH for Amazonian bioaerosol analysis, we decided for three broad taxonomic probes, to create a first overview on domain level before exploring the bioaerosol population at a higher taxonomic resolution. The Amazonian bioaerosols were investigated on domain level by quantifying eukaryotic, bacterial, and archaeal cells as well as the overall concentrations of airborne cells as a function of time and height within and above the forest canopy.

Further the reviewer stated “16S rRNA qPCR is more robust than microscopy for counting as well.” We oppose the reviewer’s position here. (i) Generally, nucleic acid staining is a widely used, well established and a rather simple approach for total cell quantification (e.g., Kepner and Pratt, 1994; Matthias-Maser and Jaenicke, 1995; Eduard and Heederik, 1998; Amato et al., 2007; Yamaguchi et al., 2012; Liu et al., 2019). Since a DAPI counter-staining (as well as a comparison to the aerosol online instruments) has been conducted here and yielded very consistent concentrations overall, we are confident that our FISH counts are robust. (ii) Further, we sought for a quantification of bioaerosols (on domain level) that also provides information on the *airborne state* of the cells. Thus, we chose a combination of sampling (membrane filters) and analysis through visualization (FISH with microscopy) that largely conserves airborne cluster of cells or agglomerates of cells and non-biological particles. For a sequencing-based approach (e.g. qPCR), information on cells clusters or agglomerates would be entirely lost after sampling (high-volume filter samplers or impingers), DNA extraction, and sequencing. (iii) Further, sequencing requires DNA extraction, either after resuspension from HiVol filters or directly from impinger samples (cell suspensions). In both cases, uncertainties remain on whether the DNA extraction occur quantitatively (due to thick cell walls and unequal cell lysis), which might bias the finally calculated cell number concentration. The choice of the extraction protocol might introduce an additional source of cell number underestimation and/or overestimation of certain clades. Gene copy number variations are still a challenge in the accurate estimation of microbial cell concentrations. In addition, the presence of eDNA can further complicate the quantification using qPCR, which might have a significant influence, especially when it comes to low-biomass aerosol samples (Luk et al., 2018).

Supplement, P4, L11- P6, L11:

#### *S1.4 Bioaerosol analysis methods*

*Environmental bioaerosol populations comprise highly complex and diverse particle mixtures. As a consequence, the choice of analysis method is not trivial and has to be made carefully to avoid biases caused by e.g., differences in particle size, metabolic state, or physical and chemical properties. A long time, cultivation was the method of choice for bioaerosol analysis. Since less than 1% of all bioaerosols are assumed to be culturable, this technique was more and more superseded by new analytical methods based on DNA analysis or real-time autofluorescence detection. To find the most suitable analysis technique, the main investigation target has to be defined first. We suggest the categorization into three objectives: 1. quantification, 2. identification, and 3. qualitative analysis of bioaerosols.*

Online autofluorescence detectors such as the Rapid-E (Plair), BioScout (EnviroNics, Ltd.) and spectral intensity bioaerosol sensor (Droplet Measurement Technologies) are especially useful for long-term quantification of bioaerosols, as no time-consuming laboratory sample analysis is needed. Furthermore, data is generated in comparably high time resolution. Nevertheless, data provided by these detectors has to be evaluated carefully, as bioaerosol number concentrations based on autofluorescence detection are prone to biases caused by bioaerosols' diverse autofluorescence intensities and wavelength range as well as interferences with autofluorescence from inorganic aerosols (e.g., Pöschl et al., 2010; Huffman et al., 2010; Savage and Huffman, 2018). In contrast, methods based on DNA analysis are focused on the specific identification of bioaerosols. By now, a broad range of tools are used, such as metagenome sequencing, metabarcoding, rRNA sequencing, or qPCR. The different techniques have in common that the taxonomic resolution is determined by the choice of the target sequence. Next to the taxonomic identification, microbial abundances can be quantified indirectly, e.g., by calculating microbial cell numbers out of detected gene copy numbers. These calculations are statistically robust, as DNA analysis requires high load of biological sample material on one hand, and automated high-throughput instruments enable a quick analysis of large sample numbers on the other hand. However, they can be biased by multiple copy numbers of marker genes. With the microscopic analysis of FISH, we combine the quantification and identification with a qualitative analysis. Even though the manual enumeration of fluorescent single particles is time consuming (automated counting can speed up the analysis), we suppose the advantages to outweigh this drawback: i) The quantification is based on direct enumeration of fluorescent cell signals and therefore assumed to be very accurate. ii) A countercheck with DAPI staining provides additional safety. iii) Particle loss during laboratory analysis is assumed to be minimized, as bioaerosols are identified directly on the filter as collection medium. For qPCR or flow cytometric analysis a re-suspension into liquid and a cell concentration is required after bioaerosol filtration, which enhances the chance of particle loss, especially in terms of charged and/or hydrophobic bioaerosols such as certain molds. Bioaerosol collection directly into liquid (e.g., impingement) could solve this issue. However, varying collection efficiency due to liquid evaporation over time, changes in chemical composition (e.g., pH or fixative concentration) as well as microbial growth within the liquid have to be taken into account.

Generally, it is important to note that the large bioaerosol diversity imposes significant analytical challenges in terms of sound bioaerosol analysis. There is no general "method of choice" for bioaerosol analysis, but various different approaches, that have advantages and

drawbacks. Accordingly, number and mass concentrations derived from different measurement techniques and sampling locations are comparable only within certain limits and similarities as well as deviations have to be evaluated carefully (see Table S3). We suppose that FISH, which was considered before but never established for environmental bioaerosol investigations, can advance the range of tools and techniques by combining the three major goals that are identification, enumerations and qualitative analysis.

The manuscript lacks an explanation why sequencing techniques were not applicable for this system (why wasn't FISH and sequencing done?)

We absolutely agree that sequencing in parallel to the FISH sampling would have opened perspectives for interesting comparisons. The main reason why sampling for sequencing was not conducted in this study were the generally challenging field logistics and work at the ATTO site, which requires careful planning of all activities. This refers especially to activities on the tall tower. In this 'FISH-focused field campaign' in 2018 analyzed here, we put a clear focus on the implementation and testing of the FISH sampling protocol, using 3 sampling heights. Based on the interesting and encouraging results obtained here, we conducted a follow-up campaign in 2019 including filter sampling for sequencing, which is currently subject of in-depth analysis.

We added a reference that gives an overview about potential bioaerosol analysis techniques and linked the new supplement paragraphs in the main article:

(P3, L14-18): Particularly scarce are techniques that provide atmospheric number concentrations for specific and clearly defined organism groups within the bioaerosol population (e.g., Kabir et al., 2020; Mbareche et al., 2017; Sect. S1.4).

[R1.3] What is the standard microbial density of a given aggregate? Are all domains found in physical association? Separate? How are they dispersed on the aggregate? This would be something a sequencing-only study could not provide and better justify the methods used. There are qualitative statements to this effect that could be expanded upon (pg 10 lns 13-16).

[A1.3] It is not quite clear what the reviewers means with "microbial density of a given aggregate". We assume that this refers to degree of aggregation of the cells. If Reviewer #1 is asking for a ratio (such as cells per agglomerate) this is nothing we measured here. We complemented the paragraph in the article as follows:

(P12, L 811): In the course of the microscopic analysis Archaea were found as single particles only. Fungal spores were occasionally found in physical association with bacteria (as shown

*in Figure 4G and H) or with other fungal spores.* The vast majority of cells, however, was observed as separated cells, which suggests that under the given wet season conditions the bioaerosol components are largely externally mixed.

3) [R1.4] It is unclear why there is an improved understanding of bioaerosols from the number of bacteria present as opposed to total number of microbes present. For example, the paper states that these data provide constraints on mixing information, but it is not known if the bacteria are the same or different throughout. Is it the same population of bacteria that travel through the different heights? Or entirely different bacteria?

[A1.4] This study gives first insights into the general mixing of bioaerosols on domain level. However, these results don't allow assumptions about different types or populations of bacteria at different heights. The probe used here is applied for the general identification of bacteria to create an overview image.

In general, we expect that other bacterial probes, on higher taxonomic resolution, could answer the question of Reviewer #1. However, bacteria were found in relatively low abundance throughout this study. Accordingly, we expect too low bacterial numbers on the filters used here to be hybridized with a more specific probe but still ensuring robust statistics. As a result, the sampling volume would need to be increased in order to proceed with this approach.

#### **Minor and specific comments:**

[R1.5] Methods - It is not clear how the determination for the genome size of all bacteria and archaea was made. Was this an average of all the genomes? How might this vary between cells?

[A1.5] We have clarified this aspect and improved the paragraph "Quantification of atmospheric DNA concentration" in the appendix. Specifically, we included a link to the NCBI database that was used for the genome size determination.

To (P19, L13-19): *The genome sizes were determined as follows: Souza et al. (2019) found Proteobacteria and Actinobacteria to be the dominant phyla within the airborne Amazonian bacterial population. The median genome sizes found in the NCBI database were ~ 4.8 Mb and ~4.3 Mb for Proteobacteria and Actinobacteria, respectively. (<https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/proteobacteria>). By discussing these numbers and the results by Landenmark et al., 2015 with ecologists from the Max Planck*



Institute for marine Ecology in Bremen, the approximate bacterial and archaeal genome size were defined as 4 Mb for bioaerosols.

[R1.6] The SD is very high compared to the average (Table 1). How many filters were counted? A supplemental table of each count conducted per time/height would be useful. It would be useful to know the variability in counting a given filter (variability in counts per field of view) separately from the deviation between filters counted for a given experimental filter (Table 1).

[A1.6] One filter was collected and counted per sampling height and sampling period. That results in 16 filters that were analyzed in total, five of those were collected at 5 m height and at 325 m height, respectively, and 6 were collected at 60 m height. These numbers as well as the FISH and DAPI raw counts per filter are presented in Table S1 in the supplement.

Naturally, the SD is higher for samples with low numbers of raw counts, for example the SD for archaeal counts is higher than for eukaryotic counts. In general, we assume the SD to be an acceptable range.

The parameter to ensure robust statistics was not the number of fields of view, but the number of DAPI stained particles that were enumerated (also given in table S1). To avoid confusion, we therefore refrain from presenting the number of fields of view (FOV) in an additional table. However, we understand the interest of Reviewer #1. The following numbers might give Reviewer #1 an impression regarding the number of FOV: at 5 m height on average 17 FOV were inspected, at 60 m height on average 28 FOV were inspected and at 325 m height on average 26 FOV were inspected, each per FISH probe. In total 1140 FOV were analyzed for the data presented in the study.

[R1.7] Man-made could be replaced with anthropogenic or human.

[A1.7] We replaced “man-made” by “human” and “anthropogenic” as suggested.

(P3, L22): unperturbed by human emissions

(P3, L27): the nowadays pervasive anthropogenic emissions and activities

[R1.8] Figure 2 – why are there no “unknowns” for Mar 1 and Mar 2?

FISH numbers are never a 100 % reflection of the reality but it can suffer from false negative but also false positive counts, as mentioned in the article. This can be due to different artefacts such as e.g. low signal intensity due to little number of bound fluorescent molecules and on the other hand also unspecific binding of probes or autofluorescent particles. For March 1 and March 2, we expect this to be the case. Especially archaeal numbers were comparably high at 60 m. The probe ARCH915 was reported to be prone to unspecific binding (Pernthaler et al.,

2002). We assume that this in combination with the low counting statistics for Archaea might be the reason for the “missing” unknowns during these two sampling days. We completed the respective footnote in the article, to point that our more clearly:

(P6, footnote): In fact, we refrain from interpreting NARC in great detail in this work due to the low statistics. Furthermore, the probe ARCH915 used here was found to hybridize with some Bacteria, which could lead to false-positive signals. We assume that this could have been the case on March 1 and March 2 at 60 m sampling height, leading to no “unknowns” in respect to DAPI numbers.

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