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 #2,

Received: 19 Feb 2021

M. Prass et al. present a paper entitled « Bioaerosols in the Amazon rain forest: Temporal variations and vertical profiles of Eukarya, Bacteria and Archaea". They measured the concentrations of these types of bioaerosols during a campaign of six days (sampling every day) at three levels of altitude (5, 30 and 325 m) on the Amazon Tall Tower Observatory (ATTO) during typical wet season conditions.

The obtained results are of interest as the scientific community needs data on bioaerosol concentrations in various sites and under defined atmospheric conditions in order to understand the transport and potential roles of bioaerosols in atmospheric processes (cloud chemistry and microphysics, precipitation formation…). However, I have some major concerns about the introduction, discussion and conclusion of the paper, these parts of the paper should be partly rewritten before the paper is published.

We thank Referee #2 for the evaluation of our work. We appreciate the critical and constructive analysis and answered the comments in detail below:

Major comments:

[R2.1] Abstract line 5-6, Introduction P3 lines 30-37, P4 lines 1-3, Conclusion P12 1-5, 32 (etc..): The authors focus the interest of their paper on the use of the FISH method as an "analytical novelty "and a great improvement to analyze and quantify bioaerosols.

First, this method is not new and was used, even on bioaerosols, quite a long time ago (see for instance Lange et al. Application of Flow Cytometry and Fluorescent In Situ Hybridization for

Assessment of Exposures to Airborne Bacteria, Appl. Environ. Microbiol. 1997, 63, 1557–1563).

[A2.1] We appreciate that the reviewer questions whether the scientific novelty might be overstated. We checked the manuscript – and especially the sections that the reviewer pointed out – critically again. We came to the conclusions that these statements are generally appropriate and not overstated. Nevertheless, we found room for further clarification and revised the text accordingly as outlined below. Specifically, the reviewer refers to the following statement here:

(i) Abstract, L5-6: We used fluorescence *in situ* hybridization (FISH), a molecular biological staining technique largely unexplored in aerosol research, to investigate the sources and spatiotemporal distribution of Amazonian bioaerosols on domain level.

(ii) P3, L31-37 & P4, L1-3: The analytical and scientific novelty of this study is threefold: First, it widens the spectrum of techniques for bioaerosol investigations by exploring the analytical potential of fluorescence in situ hybridization (FISH) in this field. FISH is a molecular genetic technique for the specific staining of cells by targeting characteristic RNA or DNA sequences with complementary and fluorescently labeled nucleotide probes (e.g. Amann and Fuchs, 2008). In terrestrial and marine microbiology, FISH has become an important technique in identification and enumeration of microbial organisms with numerous applications (e.g., Pernthaler et al., 2004; Christensen et al., 1999). However, applications in bioaerosol research have remained remarkably sparse (e.g., Lange J. L., 1997; Yoo et al., 2017; Harrison et al., 2005). Our results demonstrate that FISH has great potential in bioaerosol analysis as it provides number concentrations of specific organism classes (i.e., from domain down to species level) and, therefore, combines *bioaerosol identification, enumeration, and visualization.*

(iii) P14, L1-5: Our study showed that FISH has great analytical potential in bioaerosol analysis. It combines bioaerosol identification, quantification, *as well as visualization* and, thus, provides insights into the concentration levels and spatiotemporal variability of specific and clearly defined organism groups within the bioaerosol population.

We do not see that these sections would claim that FISH is new. In fact, several references – also two studies on previous FISH applications on bioaerosols (Yoo et al., 2017 and Harrison et al., 2005) are cited. The number of FISH studies on bioaerosols is very small, however, which is addressed by saying that the combination of FISH and bioaerosols is largely unexplored. In terms of novelty of this study we state that the study widens the spectrum of techniques for bioaerosol investigations by exploring the analytical potential of fluorescence in situ hybridization (FISH) in this field, which seems not to claim that we "introduced" or "developed" FISH for bioaerosol analysis here.

The reference (Lange et al., 1997) that Reviewer #2 mentioned has been added on P43, L1. We would like to point out here that all previous studies that we are aware of and that used FISH for bioaerosol analysis did not focus on bioaerosol samples from natural habitats. Instead they addressed laboratory-generated bioaerosols as well as 'artificial' environments such as swine bars. This is part of the reason for our statement that this field of applications is largely unexplored. Clearly, the bioaerosol populations in e.g. swine bars and the Amazonian atmosphere are quite different (e.g., diversity, concentration, emission patterns, etc.). Accordingly, an achievement and novelty of our work was the adaptation of existing FISH protocols (e.g., fixation, permeabilization and hybridization) to the highly diverse mixture of atmospheric bioaerosols, which has been outlined transparently in the manuscript as detailed experimental protocols for further use by other researchers.

Along the lines of [R2.1] and [A2.1], we have implemented the following changes in the manuscript for further clarification:

We added the reference Referee #2 mentioned in:
(P3 L32- P4, L2): The analytical and scientific novelty of this study is threefold: First, it widens the spectrum of techniques for bioaerosol investigations *in environmental samples* by exploring the analytical potential of fluorescence *in situ* hybridization (FISH) in this field. FISH is a molecular genetic technique for the specific staining of cells by targeting characteristic RNA or DNA sequences with complementary and fluorescently labeled nucleotide probes (e.g., Amann and Fuchs, 2008). In terrestrial and marine microbiology, FISH has become an important technique in identification and enumeration of microbial organisms with numerous applications (e.g., Pernthaler et al., 2004; Christensen et al., 1999). However, applications in

bioaerosol research have remained remarkably sparse (e.g., Lange et al., 1997; Yoo et al., 2017; Harrison et al., 2005)

[R2.2] Second, although this method is valuable to distinguish different types of bioaerosols using specific probes (bacteria, eukaryotes and archaea, or different genera, species, etc….), it is a very laborious and time consuming approach when combined with microscopy, in addition it may presents some artifacts and so the quantification of bioaerosols must be first validated using another well-established method.
This is actually recognized by the authors (P 4 lines 19-23). In this paper they used DAPI staining combined with microscopic observation to quantify the total number of cells and validate their data. Also this method is very laborious and time consuming. DAPI and FISH combined with microscopic observations require a great number of counts to avoid errors and are also dependent on the observer faculties, as explained p16 lines 11-15, 31-35.

[A2.2] DAPI staining combined with microscopic detection of fluorescent signal is a well-established method which was used in studies for the quantification of bioaerosols before. Therefore, we indeed regard these numbers as a valuable and robust reference for the FISH counts. Furthermore negative tests were performed by analyzing blanks as well as by the application of the non-specific probe. Positive test by applying the bacterial probe to filters covered with *E.coli* were performed to ensure the hybridization with the fluorescent probes was successfull. In addition, the total aerosol counts in the same size range by the OPS give a statistic robust estimation of (bio-)aerosol number concentrations.
The statistical evidence of the FISH and DAPI counts as well were achieved by counting not less than 500 particles, a number that was established by microbiologists using the method for marine science for many years now (Kirchman et al., 1982; Pernthaler et al., 2003).

[R2.3] I do recognize the efforts of the authors and the serious of their job, that finally gave valuable data. However, we cannot consider this method as the "future of the bioaerosol characterization and quantification" as there are alternative methods which are now currently used.
[A2.3] As already explained as answer to comment from Referee # 1 [A1.2],
we wouldn't dedicate FISH as "the only future of bioaerosol analysis", but [P3, L31] it widens the spectrum of techniques for bioaerosol investigations in environmental samples by a visualization and localization tool.

(P15, L3-6): Finally, the taxonomic resolution of this study operates exclusively on domain level. Future studies should use the analytical potential of FISH by targeting organism classes on lower taxonomic levels (e.g., theoretically down to species level) *in combination with sequencing-based techniques.*

(P14, L32-34): For this purpose, a broader statistical basis of FISH results and comparisons with bioaerosol analysis techniques *(such as sequencing or qPCR)* along with meteorological observations is needed.

[R2.4] The authors could refer to a recent review by Kabir et al. (Recent Advances in Monitoring, Sampling, and Sensing Techniques for Bioaerosols in the Atmosphere, ACS Sens. 2020, 5, 1254−1267). In my opinion two main approaches can be interesting:

1- Flow Cytometry: this method is not new (see Lange et al 1997 cited above) but is very efficient, cheap and quick, in addition improvements of flow cytometers open new possibilities. Many samples can be analyzed; it allows to quantify the total number of cells but also to distinguish different types of cells using specific probes (bacteria, fungal spores, genera ….), or dead and alive cells using viability markers, or photosynthetic cells using specific wavelengths of emission of these organisms. This analyze can be performed directly on atmospheric samples with intact cells in water medium (rain, snow, aerosols in impingers, resuspension of aerosols from filters…). See for instance some papers:

Chena and Li. Real-time monitoring for bioaerosols—flow cytometry, Analyst, 2007, 132, 14–16, DOI: 10.1039/b603611m.
Liang et al., Rapid detection and quantification of fungal spores in the urban atmosphere by flow cytometry, J. Aerosol Sci. 2013,66, 179–186.
Jang et al., Application of Cytosense flow cytometer for the analysis of airborne bacteria collected by a high volume impingement sampler, J. Microbiol. Methods, 2018,
154, 63-72.
Negron et al. Using flow cytometry and light-induced fluorescence to characterize the variability and characteristics of bioaerosols in springtime in Metro Atlanta, Georgia. Atmos. Chem. Phys., 2020, 20, 1817–1838, https://doi.org/10.5194/acp-20-1817-2020.
Dillon et al., Cyanobacteria and Algae in Clouds and Rain in the Area of puy de Dôme, Central France, Appl. Environ. Microbiol. ,2020 , 87 , e01850-20. doi: 10.1128/AEM.01850-20

2- qPCR: This molecular approach is more recent but has been used intensively in recent papers, it allows all types of quantifications (total number of cells, specific counts…). This approach is also quick, cheap and easy to run. See for instance (among many others):

Tignat-Perrier et al., Global airborne microbial communities controlled by surrounding landscapes and wind conditions, Sci. Rep., 2019, 9, 14441 , https://doi.org/10.1038/s41598-019-51073-4.

Some papers combine flow cytometry and qPCR, and even new-generation sequencing approaches (metagenomics), see for instance (among others):

Dillon et al., Cyanobacteria and Algae in Clouds and Rain in the Area of puy de Dôme, Central France, Appl. Environ. Microbiol., 2020 , 87 , e01850-20. doi: 10.1128/AEM.01850-20.

[A2.4] We thank Referee #2 for the given reference, it was added in the introduction:

(P3, L14-16): Particularly scarce are techniques that provide atmospheric number concentrations for specific and clearly defined organism groups within the bioaerosol population (e.g., *Mbareche et al., 2017; Kabir et al., 2020; Sect. S1.4).*

We agree, flow cytometry and qPCR hold several advantages that make them suitable especially for quantification of bioaerosols in large sample sets. However, one of this study's scopes was the exploration of FISH for environmental bioaerosol analysis. We particularly supposed the visualization to be a promising tool, to enable the investigation of bioaerosol travel modes (e.g., agglomeration), a characteristic that cannot be approached by flow cytometry or qPCR.

Comparisons of different analysis technique can be found in the literature, e.g., Ghosh et al. (2015) summarized a handy table providing basic advantages and drawbacks of different techniques. Nevertheless, we took R2.4 as a motivation to carefully compare FISH, qPCR, and flow cytometry ourselves during the review process. Please, find attached a table summarizing the method characteristics.

Another reason FISH seemed promising for us was the convincing sampling technique: With this study, we aimed to determine the characteristic cross section of a diel bioaerosol population during the wet season. Accordingly, sampling periods of approximately 24 hours were striven for. The dry collection by direct filtration onto polycarbonate membranes was our collection

method of choice as it minimizes the chance of unwanted microbial growth on or within the collection medium during that relatively long sampling time, as it would be expectable when sampling into liquids. Additionally, particles can be analyzed on the filters directly. A re-suspension and concentration by centrifuging, as required for sequencing or flow cytometric analysis, enhances the chance of particle loss, especially in terms of charged and/or hydrophobic bioaerosols such as certain molds (Mbareche et al., 2019).

[R2.5] To conclude, I propose the authors rewrite parts of their manuscript taking into account these alternative approaches, that I think are the future instead of FISH. They will allow to analyze many more samples in a much shorter time.
Actually it is also a weak point of this paper to have only a six-day sampling period, with three altitudes, instead of larger series of data that could bring more information and robust statistical analyses. The obtained data are interesting, valuable but very limited.

[A2.5] Please, find the answer to the comment [R2.5] in [A3.2].

[R2.6] Another general comment is that lots of data were collected in this Amazon campaign thanks to this very well equipped observatory site (ATTO) but they were not really exploited to explain the observed trends concerning bio-aerosols quantification and distribution. Again this is due to the too small number of samples, the discussion part is thus very speculative.

[A2.6] We agree that the comprehensive data collected at the ATTO site on long-term basis is very valuable for the interpretation of targeted bioaerosol studies, such as the one presented in this manuscript. Evidently, some measurements are more closely related to the results here and thus have greater value for the interpretation than others. Here, selected and related data sets from online aerosol instruments were implemented that provide a valuable context for the pilot project collecting DAPI and FISH results. Specifically, data on aerosol concentration and size distributions (from SMPS and OPS instruments) were used (see. Fig. 2 and 3). Meteorological data are part of Fig. 2. The sampling period is embedded in the long-term perspective of climatic anomalies in Fig. S2. Air mass movement and through backward trajectory analysis is presented in Fig. S1. The reviewer is right, that the comparatively short length of the sampling period does not allow long-term correlations with e.g. meteorological parameters (e.g. bioaerosol release as a function of RH). Still, the comprehensive data context from ATTO allowed us to draw some general conclusions that we do not regard as speculative.

Minor and specific comments:

[R2.7] P3 lines 16-20: the cited literature is too limited; it has to be completed.

[A2.7] We cite three review papers at this point, to avoid naming only few field studies in the broad field of valuable work that has been done. To clarify that further references can be found in the review papers, we added the following appendix:

(P3, L17-19): The number of bioaerosol field observations worldwide is constantly increasing (Després et al., 2012; Fröhlich-Nowoisky et al., 2016; Šantl-Temkiv et al., 2019, *and references therein*) with bioaerosol studies in regions that are essential for the climate system being particularly relevant.

[R2.8] P8 line 2-8: It is important to assess the viability of the cells but the method used by the authors is not very accurate because a cell can be "intact" from a microscopic point of view but not alive or active. Alternative "live and dead cell" assay is much more adapted as well as ATP (adenosine triphosphate) or CTC (5-cyano-2,3-ditolyl tetrazolium chloride) assays. Could the authors comment this point?

[A2.8] One of the three scopes of this study was the elaboration of the FISH method's potential in bioaerosol analysis. The analysis provided number concentration of potentially intact cells, which in combination with the total aerosol counts could be used to estimate the fraction of fragments. It is true, viability is important in the broader picture of bioaerosol analysis, but it was beyond the focus of this study.

Furthermore, filter samples collected in the Amazon had to be fixed immediately after sampling to avoid microbial growth that biases the concentrations and to enable the entrance of the fluorescent dye and fluorescently labeled nucleotide probes. The fixation is of high importance, especially because samples were collected in a remote collection, causing long travel times when samples are temporarily stored on ice instead of -20°C. Ergo, in the course of DAPI and FISH analysis, the cells were per se dead.

[R2.9] Figure 4 G and H: the authors show the presence of agglomerates composed of clusters of bacteria and fungal spores in samples taken as the lower altitude. They assume that these aggregates are due to their higher sedimentation tendency and lower atmospheric residence times. This can be one of the hypotheses, but it could also be due to the closer presence of ground and trees, source of dust (from the soil) or biofilm agglomerates (from the phyllosphere). Your opinion?

[A2.9] We agree, this is an important information that should be included here:

(P12, L15-17): An enrichment of larger particles at the lower heights *is probably a result of the large number of emission sources underneath the canopy in combination with* higher sedimentation tendency and lower atmospheric residence times of large particles.

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