Response to Reviewer 1

First of all, we want to thank the reviewer #1 for meticulously reading our manuscript and for providing the critical review to improve the manuscript. Below, we include the response to comments and concern of reviewer #1.

Reviewer comment: "Further, serial (unsupported) assumptions about microbial physiology are embedded in this manuscript, particularly with respect to identifying and quantifying the collected airborne microbiological agents based on presumed genomic characteristics. Important genomic characteristics have either been overly simplified or unfortunately omitted in critical contexts that are needed to support the heart of the work. These (over)simplifications and omissions make it difficult to sustain the author's conclusions given the data they acquired, presented and analyzed (juxtaposition of WIBS, FCM and EPM). The generalization that whole cell bioaerosols can be reliably deconstructed into two pools based on any non-normalized index of DNA/RNA content cannot be not supported by basic microbial (and plant) physiology and the data presented here."

Response: *SYTO-13 stains DNA and RNA nucleic acids (Lebaron et al.,2001; Troussellier et al.,1999; Comas-Riu et al., 2002; https://www.thermofisher.com {Cell-Permeant Cyanine Dyes: The SYTO Nucleic Acid Stains}), and the resulting fluorescence intensity is directly related to the nucleic acid content. Previous literature clearly shows that SYTO-13 can effectively distinguish between HNA and LNA bacterioplankton and phytoplankton populations in fresh and seawater environments, and results are comparable to SYBR green II and SYBR green I, more specific DNA probes (Lebaron et al., 2001; Bouvier et al., 2007). Furthermore, the genome size of sorted HNA and LNA populations in fresh water have shown HNA populations will likely have a larger genome than LNA populations (Schattenhofer et al., 2011). That said, we do not claim that specific types of airborne microorganisms (e.g. bacteria, fungal spore, pollen) were quantified based on the staining intensity, as genome sizes of bacterial and fungal spores may overlap. However, the FCM also detects the physical particle size, which is considerably different between bacteria and fungal spores. Size and fluorescence intensity combined then allow the differentiation, which we denote as the low nucleic acid (LNA) and high nucleic acid (HNA) populations. Therefore, this distinction of stained bioparticles appears to be robust.*

Atmospheric samples are different from aquatic samples in composition and particle sizes, but overall the classification of the HNA and LNA populations is based on the fact that SYTO-13 directly stains nucleic acids and it is well established and accepted in the flow cytometry community. Furthermore, quantifying the DNA and RNA content of specific HNA and LNA populations to determine, for instance, which are bacterial vs. fungal, constitutes the design and optimization of a protocol for in-situ sorting, and quantification, and subsequent molecular analysis of the sorted populations, which is the material for another publication. This should be the next step to have a more specific FCM microbial quantification, but in no way should invalidate our conclusions.

Reviewer Comment: "The premise itself is tentative given the somewhat sensational statement that airborne microbes in a "broad" RH range were in-fact monitored, where 40's% < RH < 80's%. A majority of the observations reported (table 1) were under conditions near 50% RH (± 9%); this

RH is not near saturation conditions, nor is it near desiccating conditions; indeed, many would consider this a "midrange" of relative humidity. In this analytical context, (aut)ecological context or comparative environmental context, by no means is a couple of months of (bio)aerosol sampling conditions in Atlanta "ensuring a wide range of PBAP population(s), state(s) or concentration(s)" (page6)."

Response: *Observations are indeed limited to 15 sampling events conducted in Atlanta, GA during Spring 2015, and we have edited our statements to more precisely reflect what was performed. 24 hour averaged temperature and relative humidity were calculated in order to determine the prevailing temperature and relative humidity (RH) during each sampling event given that meteorological conditions during the sampling time (4hr average) will not necessary represent the meteorological conditions of the whole sampling day. In addition, the residence time of microorganisms (e.g. bacteria and fungal spores) in the atmosphere is larger than SpinCon II sampling time (4hr), which means microorganisms aerosolized the night before or hours before sampling started could still be collected (Delort and Amato, 2018 – Microbiology of Aerosols-Section2.3.4: Residence time, transport history, and emission models). However, the temperature and relative humidity did vary during these 15 sampling events, as shown in the table below. Humid and warm days (April-7: Max RH- 97%, April-14: Max RH-93%, April-15: Max RH-91%) after rain events observed max RH above 90%. In contrast, multiple dry days (e.g. April-8, April-21, April-22, May-13) experienced minimum RH below 30%. It is important to highlight the main reason of the RH and temperature categories is to better understand the substantial change in composition observed between the LNA and HNA populations between sampling events, but we cannot rule out the rain events and soil wetness possible role in the enhancement of the HNA population on April-7, April-14 and April-15.*

Table1: 24 hr. relative humidity and temperature average(Avg.), minimum(Min), maximum(Max)

Days	24hr. Avg.Temperature (°C)	Min (°C)	Max (°C)	24 hr. avg. Relative Humidity (%)	Min (%)	Max(%)
7-Apr	21.4	16.7	26.8	70.9	40.0	97.0
8-Apr	24.9	17.9	31.2	53.6	26.0	84.0
9-Apr	25.3	20.4	30.3	53.8	35.0	76.0
14-Apr	22.5	19.1	28.7	76.8	47.0	93.0
15-Apr	18.9	12.8	24.7	83.6	60.0	91.0
16-Apr	12.5	11.3	13.7	86.3	80.0	94.0
21-Apr	16.6	10.4	22.1	43.2	19.0	75.0
22-Apr	18.8	11.6	26.1	38.1	19.0	60.0
23-Apr	16.8	13.9	19.6	48.1	27.0	77.0
28-Apr	17.0	12.8	21.8	45.3	34.0	66.0
29-Apr	14.2	12.0	16.9	79.4	63.0	89.0
30-Apr	17.4	11.3	23.7	57.3	28.0	90.0
13-May	23.5	16.7	30.1	40.1	20.0	63.0
14-May	23.0	18.3	28.0	52.3	39.0	63.0
15-May	23.1	19.8	25.8	64.4	53.0	81.0

Reviewer comment: "This includes but is not limited to the following terms: LNA, bioLNA, HNA. First and foremost, all intact (micro)biological cells contain nucleic acids, and the "bio" subscript prefix is conflicted with the fact that environmental nucleic acids can only be of biological origins, regardless of the "quantity" of nucleic acids inside any give (airborne) microbe. In this context, the authors did not acknowledge the fact that DNA is sequestered differently in

bacteria, fungi, their spores and pollen grains; that this sequestration is sensitive to RH; and, that the configuration of intracellular DNA has tremendous implications for optical recognition methods and quantitation by FCM, regardless of genetic staining."

Response: *The "bioLNA" population highlights the fraction of particles in the LNA population above the autofluorescence threshold value (42k). As a result, we can denote the "bioLNA" population as "LNA-AT" (LNA above threshold) from now on. The DNA sequestration by bacteria, fungal spores and pollen may differ and their cell membrane characteristics will ultimately determine how much stress the cells will sustain before they completely rupture. SYTO-13 is a highly permeable stain and have shown to be effective to detect nucleic acids (DNA and RNA) of bacteria endospores and vegetative cells (Comas Riu et al.,2002). Also, all pure cultures studied during this study are effectively stained by SYTO-13. Fungal spores have also been effectively stained by DNA/RNA probes (Bochdansky et al., 2017; Chen and Li et al.,2005), but we acknowledge in the revised manuscript that some fungal spores might not be equally stained due to their harder cell wall, and chromatin-binding of DNA.*

Pollen can fragment at high RH and possibly be part of the LNA population as we have suggested in the manuscript, but these fragments will likely be below 1µm (Bacsi et al., 2006). Recently Santl-Temkiv et al.2017 observed bacteria cultivability is maintained (80% cultivable based on CFU counts), but leucine uptake rate (to measure metabolic activity) is reduced after 1hr sampling in the Spincon suggesting cells will be in a dormant state after 4hr sampling in the SpinCon II. Airborne microbes may also be stressed upon collection so it is possible that the LNA and HNA populations are two distinctive populations given that no anticorrelation is observed between the geometric mean fluorescence of the two populations. Based on Bouvier et al.2007, cell populations with different metabolic activity (e.g. active and non-active), when detected by FCM, should observe a decrease in fluorescence intensity in consecutive sampling events if transition from the HNA to the LNA population, or vice-versa if transition from LNA to HNA population. The fluorescence intensity of the LNA and HNA populations show small variation throughout the sampling events (BioLNA: $7.38 \times 10^4 \pm 1.39 \times 10^4$; HNA: $6.72 \times 10^5 \pm 2.30 \times 10^5$) and no anticorrelation is observed in the studied parameters (FSC-A, SSC-A, FL1-A), which supports we have in fact two distinctive population of bioaerosols (look Figure 1 below; Also look Figure S15 in the supplemental information).

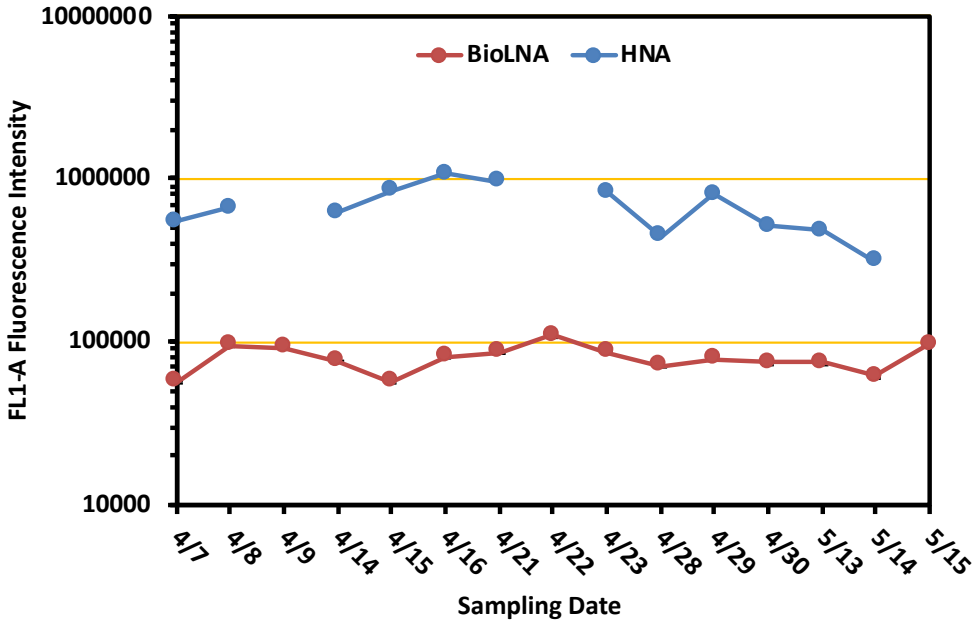

Figure 1: FL1-A fluorescence intensity of the BioLNA and HNA populations during the 15 sampling events. No HNA population identified on 4/9, 4/22, 5/15. Standard deviation of the fluorescence intensity is negligible for both populations throughout all sampling events.

Reviewer comment: "To support their "low/high" DNA (genome) assignments, and associated microbial classifications, the authors should have, at a minimum, executed some (simple and inexpensive) DNA extractions on at least a subset of their aerosol samples, characterized sentinel sequences (basic qPCR) and juxtaposed this to their optical/cytometry data. In addition to the length/copy number variability presented above, in this midrange of relative humidity spores is held in a constant conformation regardless of RH…"

Response: *Certainly, DNA extraction and sequencing of the atmospheric samples would allow the identification of specific bacteria, fungi taxonomical groups in the samples and their respective relative abundances. They are less effective when compared against FCM results, as it is unclear how the DNA is sufficiently different between the HNA and LNA populations. Sorting and subsequent DNA extraction of the sorted populations could be the path to determine the composition of the HNA and LNA population, but we could have limited biomass content to perform DNA sequencing of each population. In addition, qPCR quantification would not be directly comparable to FCM concentrations because bacteria (1 to 15 gene copies per cell) and fungi (30 to 100 gene copies per cell) ribosomal RNA gene copies vary depending the species considered (The ribosomal RNA operon copy number database, https://rrndb.umms.med.umich.edu/about/ ; DeLeon-Rodriguez et al., 2013). Then, to perform quantification an average copy number per cell has to be assumed, which can affect qPCR quantification by up to two orders of magnitude. The main point is that the corroboration of the HNA and LNA population through DNA extraction and sequencing would need to include effective sorting of the populations to be conclusive and thus, should be the subject of a future study/manuscript.*

Reviewer comment: "While the specific characterization work of SpinCon II by Kesavan and coworkers is appreciated, this does not mean the authors can simply dismiss collection stress and sampling efficiency differences, where it cannot be dismissed (Page 7) and the qualification of on (page 22) is convoluted for a reviewer skilled in this art; indeed the SpinCon II correction factors presented on page 22 are at odds with the statement on page 7."

Response: *We understand the point raised – and have also been considering the effects of long sampling times on the integrity of the cell membrane. The correction factor derived by comparing the WIBS and FCM size distributions is consistent with the Kesavan et al.2015 results, whom conducted shorter time sampling (< 30 min) than ours (4 hours). The estimated overall sampling efficiency is lower than Kesavan et al.2015, which means additional particle losses mechanisms are important during long sampling events (look Figure S12b in the supplemental information).*

Reviewer comment: "While the impingement, flow cytometry methods and DNA intercalating agents used are widely accepted, their simple extension from aquatic environments (pages 3 and 7)) to generically understanding the "stresses" airborne microbes experience in aerosol environments does not directly support the authors analytical arguments or conclusions"

Response: *Microbial cells in both environments could be under starvation given the limited amount of nutrients compared to pure culture liquid media. Furthermore, given SYTO-13 fluorescence intensity is directly related to the amount of nucleic acids in cells we performed a direct comparison between the atmospheric sample populations and pure culture populations, but we understand the LNA and HNA may represent a mixture of different types of cells and by no mean we aim to identify a specific microbial population in the atmospheric samples through this comparison. The main goal of the pure culture experiments in this manuscript is to serve as positive controls to ensure SYTO-13 effectively stains bacteria, fungi and pollen, and have reference fluorescence and scattering properties of each population.*

Reviewer comments: "This reviewer finds the qualitative descriptions of fluorescence correlations to different microbial phenotypes, and any suggestion that WIBS can "speciate" different airborne microbes, unsupported and inappropriate (page 21). Clearly, the authors are skilled in descriptive statistics and have executed an exhaustive literature on fluorescence-based optical particle recognition instruments. To suggest ABC and HNA are "highly correlated" based on an R2 = 0.4 (figure 4), and that the AB type is "weakly correlated" with HNA where R2 = 0.17, is a subjective presentation of what should be objective thresholds (otherwise these authors need to present interpretive precedents in context and supported their qualitative arguments on why this is "highly" or "weakly" correlated)."

Response: *First, we do not claim the WIBS-4 can speciate between different airborne microbes; we do however observe similar behavior between the FCM HNA population and ABC type particles, especially during humid and warm days after rain events (4/7, 4/14, 4/15). Also, we observed a moderately strong correlation (R^2 = 0.40; p-value = 0.016) between HNA and ABC type concentrations as well as similar size distributions between both populations. Compared to previous literature our level of correlation is comparable to those observed by Healy et al.2014 between microscopy quantification and WIBS-4 measurements. We also understand Gosselin et*

al.2016 observed stronger correlations between fungal spores (inferred from mannitol and arabitol concentrations) and WIBS-4 concentrations, but that may just be because our studies were carried out in completely different environments (Rocky mountains vs. polluted urban environment). Our results therefore suggest WIBS-4 ABC type and FCM HNA population correspond to wet-ejected fungal spores on humid and warm days after rain events. As additional supporting information, the figure below shows the enhancement in the AB and ABC type concentration right after the beginning of the rain event on 4-13-15 (6pm; not correlated to NON-FBAP concentration), FBAP concentration enhancement previously linked to wet-ejected fungal spores (Huffman et al., 2013; Gosselin et al., 2016). Similar FBAP enhancement is observed during the rain events before sampling on 4/7 and 4/15.

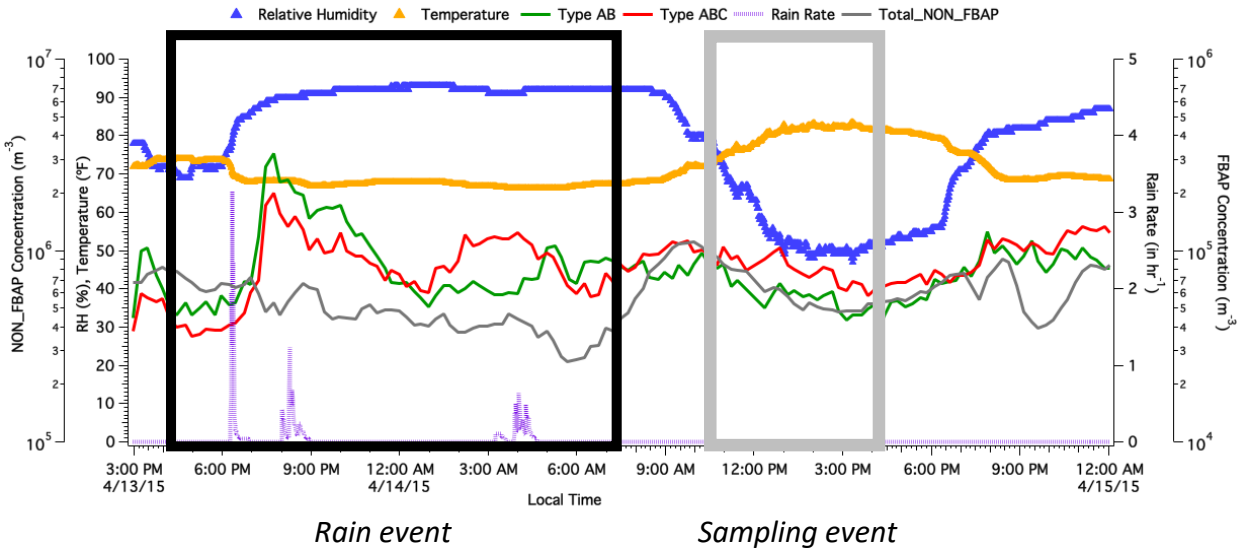

Figure 2: WIBS AB and ABC type concentration enhancement during rain events between 4/13 to 4/14. Includes high resolution temperature(yellow), relative humidity(blue) and rain rate(purple) measurements taken in the ES&T rooftop.

Additional References:

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