1) Reading the comments from Reviewer #2 again, s/he raises some fundamentally important concerns in the overview of "major points of criticism" starting half-way down the first page. At that point s/he listed four major bullets to concerning the experimental assumptions of the study. S/he also listed four overall comments related to the organization and writing quality in the paragraph before. Lastly, s/he listed several pages worth of detailed comments, some of which are quite significant in themselves. I think all of these comments are relevant and worth carefully considering. I think it may indeed be possible to revise the manuscript sufficiently to raise it to a publishable quality, but that will depend on the nature of the revisions.

Response: We appreciated the editor to summary the comments. We carefully considered all the comments and revised manuscript. We almost rewrote the manuscript marked by red color.

Besides we considered the comments from the two referee's comments, one additional experiment which concerns the bacteria and fungi cultivated in the laboratory was added. During the revision period, we contacted several professors who are experts on bacteria molecular, ecology, and biology. Indeed, the classification on fine bacteria and fungi could be problem because some of fungi have almost same morphology with bacteria. Although bacteria normally have size at <2 μm than fungi with size at 1-10 μm, the particle size could not be used to classify bacteria and fungi. Therefore, we carefully revised the particle classification here to guarantee the right contents for the potential readers.

As the reviewer's comments and literatures, fungal spores have typical morphology and larger size (> 2μm). These particles easily have been identified from TEM and SEM images. Some previous studies well documented the fungal spores (Valsan et al., 2015; Wittmaack et al., 2005).

2) Most fundamentally, Reviewer #2 raised a number of concerns about the methods by which individual particles were classified. The lack of transparency on this issue indeed is one of the major areas of required improvement. After carefully adding specific details about how particles were assigned and categorized, it will be easier to evaluate the observations and conclusions. Without knowing more about the process by which particles were investigated and assigned, it is hard to know if the method itself was sufficient to support the conclusions. The question here is not just about clarifying wording, but that the clarified wording will help evaluate whether the method was sufficient or not. In particular, the question of whether the particle assignments were correct is not sufficiently addressed. Just because a systematic method is established is not a sufficient criterion to determine whether the method leads to correct assignment. For example, consider a skeptical scientist reader. Convince them that your method led to detection and consistent, correct categorization of the particle types you report.

Response: In the revised manuscript, we mainly revised the classification part. We carefully revised the method part and added more details. We tried to clearly make methods of particle classification. After we seriously considered their comments, we decided to prepare the standard samples of bacteria and fungi on the TEM grids. The laboratory samples can further confirm our mythology that TEM and SEM can well observe the bacteria or fungi. Moreover, the TEM and SEM observations of the standard bacteria and fungi are helpful to further

classify the ambient PBAPs collected in the boreal forest air. We found that bacteria and fungi have almost same morphology and composition. Although bacteria normally have smaller size than fungi, particle size could not be acted as one key element to classify the bacteria and fungi. We also noticed that most of bacteria and fungi have rod-like shape in our samples. As the reason, we named the rod-like PBAPs for this type of particles. Furthermore, we classified fungal spores and brochosomes based on the unique morphology of individual PBAPs. Here we added Figure 2 and Figure 6 and revised Figure 5.

As the revised part, we made major revisions on the particle classification. We precisely delivered the information for the potential reader. We had the detailed responses of two referee's comments.

3) - The observations and atmospheric implications are relatively similar to works that have used similar techniques in both boreal and tropical areas, but these are not well cited in the manuscript. At a minimum I suggest you to consider additional literature, and make sure to at least briefly compare results with these in mind. I suggest doing a good literature survey of PBAP observations from forests, as well as a search related to ambient studies related to microscopy of PBAP (i.e. SEM and TEM, as you use). Then make sure that the observations you present and the conclusions you draw are put into context of these previous measurements.

Response: We appreciated the editor's suggestion. Indeed, we missed some important literatures. In the revised manuscript, we added 23 papers to support our results. Please see our revised manuscript with red word part.

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4) - The statement in the manuscript that the work can be used as a "full database" to "be used to identify primary biological particles using single particle techniques" is overstated in my opinion. I think that that study can be revised to show an overview of observations of (bio)-particles in this region, but using the results as a database for future reference is entirely different. This would require a substantially higher threshold of demonstrated quality, which may or may not have been achieved. To claim these data as basis for a database of atmospheric particles implies that a sufficiently systematic representation of bioparticles has been sampled and correctly analyzed. Further, the database would need to show some sort of independent verification that particles were assigned correctly, similar to above comments. Since most methods of independent identification are well beyond the scope of the work you reported, I do not expect that you would want to argue that the assignments have necessarily been verified as correct. They are merely suggestions, with uncertainties and potential assignment errors to be at least briefly discussed in the revised manuscript. So in that case I would suggest that at a minimum you scale back the conclusions to remove the concept of 'database,' and report in the context of 'observations'. This does not get around the first concerns that Reviewer #2 raised about categorization of particle type, but it will help to re-frame the conclusions a bit. I strongly suggest keeping all these comments, including those from the two Reviewers, closely in mind as you revise and respond to all comments line-by-line. I look forward to reviewing the revised manuscript when available.
Response: We noticed that we made overstate. In the revised manuscript, we deleted such words and carefully gave the conclusions. About the classification part, we made one major revision. We also modified the title. We all carefully response the comments raised by the two referees.

Overview of primary biological aerosol particles from a Chinese boreal forest: insight into morphology, size, and mixing state at microscopic scale

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Abstract:

Biological aerosols play an important role in atmospheric chemistry, clouds, climate, and public health. Here, we studied the morphology and composition of primary biological aerosol particles (PBAPs) collected in the Lesser Khingan Mountain boreal forest of China in summertime using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). C, N, O, P, K, and Si were detected in most of the PBAPs, and P represented a major marker to discriminate the PBAPs and non-PBAPs. Of all detected particles > 100 nm in diameter, 13% by number were identified as PBAPs. We found that one type of PBAPs mostly appeared as similar rod-like shapes with an aspect ratio > 1.5 and the dominant sizes ranged from 1 μm to 5 μm. The size distribution of the rod-like PBAPs displays two typical peaks at 1.4 μm and 3.5 μm, which likely are bacteria and fungal particles in the forest air. The second most PBAPs were identified as fungal spores with ovoid, sub-globular or elongated shapes with a smooth surface and small protuberances with their dominant size range of 2 - 5 μm. Moreover, we found some large brochosomal clusters containing hundreds of brochosomes with a size range of 200-700 nm and a shape like a truncated icosahedron. The number size distribution of PBAPs coupled with $PM_{2.5}$ and PM_{10} concentrations were used to estimate the total mass concentration of PBAPs, which is approximately 1.9 μg m^{-3} and accounts for 47% of the in situ $PM_{2.5-10}$ mass. Moreover, there is a higher frequency and concentration of PBAPs at night compared with day, suggesting that the relative humidity dramatically enhances the PBAPs emissions in the boreal forest. Our study also showed that the fresh PBAPs displayed weak hygroscopicity with a growth factor of ~1.09 at RH=94%. TEM revealed that about 20% of the rod-like PBAPs were internally mixed with metal, mineral dust, and inorganic salts in the boreal forest air. This work for the first time provides the overview of individual PBAPs from nanoscale to microscale in Chinese boreal forest air.

Key points

- Based on morphology, composition, and size of individual PBAPs, rod-like PBAPs (e.g., bacteria and fungi), fungal spores, and brochosomes were identified.
- PBAPs emissions tend to occur with high humidity at night rather than during the day.
- Hygroscopic experiments show that most of the PBAPs displayed weak hygroscopicity, and their growth factor was ~1.09 at RH=94%.

1. Introduction

Primary biological aerosol particles (PBAPs) (e.g., bacteria, spores, fungi, viruses, algae, and pollen) are ubiquitous in the Earth's atmosphere and important elements in the life cycle of many organisms and ecosystems (Poschl, 2005;Tunved et al., 2006;Smith et al., 2018). PBAPs are airborne biological materials that are transported from the biosphere to the atmosphere (Huffman et al., 2010), and they can account for a large proportion (25-45%) of the aerosol particle mass in pristine forest air and certain amounts in some rural and marine air (Elbert et al., 2007;Bauer et al., 2008;Hu et al., 2017;May et al., 2018). The growing research interest in PBAPs has one of its goals to better understand how PBAPs or their cell fragments influence cloud condensation nuclei (CCN) and ice nuclei (IN) (Morris et al., 2004;Huffman et al., 2013;Ling et al., 2018). Furthermore, field campaigns have found that abundant biological aerosols occur in cloud ice-crystals, fog/cloud, rain, and snowfall (Amato et al., 2005;Möhler et al., 2007;Christner et al., 2008;Pratt et al., 2009;Prenni et al., 2009;Tobo et al., 2013;Morris et al., 2014;Wilson et al., 2015;Twohy et al., 2016;Hu et al., 2018). These studies addressed the hypothesis that PBAPs indeed influence the hydrological cycle and climate by initiating the formation of clouds and precipitation as CCN and IN or by their bioprecipitation feedbacks.

Previous studies have investigated particle number concentration, size, and composition of primary biological aerosols using online measurement techniques and advanced molecular biological analyses (Wittmaack et al., 2005;Elbert et al., 2007;Fröhlich-Nowoisky et al., 2009;Huffman et al., 2010;Després et al., 2012;Crawford et al., 2015;Hu et al., 2017;Therkorn et al., 2017;Zhang et al., 2017;Chen and Yao, 2018). For example, the contribution of fungal spores to total organic carbon was estimated to be approximately 10% in clean and polluted periods in Beijing

using an online wideband integrated bioaerosol sensor (WIBS) (Yue et al., 2017); To obtain the organisms of PBAPs in the atmosphere, many studies tend to detect biochemical markers (e.g., proteins, fatty acids, sugars) and nucleic acids (i.e., DNA and RNA) to determine their origins such as plant or animal debris, bacteria, fungi, or viruses (Georgakopoulos et al., 2009;Chen and Yao, 2018;Hu et al., 2018;Ling et al., 2018). Although these previous studies provided comprehensive species or detailed molecular compositions of PBAPs, they still could not reflect the physical properties of individual PBAPs in the atmosphere, such as morphology, size, phase, hygroscopicity, and mixing state. Besides particle composition, the previous studies have proved that the morphology, size, and mixing state of individual particles more or less influence their CCN and IN activities and optical properties (Spracklen et al., 2008;Fröhlich-Nowoisky et al., 2009;Wilson et al., 2015;Li et al., 2016;Ault and Axson, 2017;Riemer et al., 2019). Therefore, it is critical to characterize detailed information of different types of individual PBAPs from their natural sources.

In the past decades, several studies have used scanning electron microscopy (SEM) to characterize the morphology and size of individual PBAPs (Nikkels et al., 1996;Wittmaack et al., 2005;Coz et al., 2010;Tamer Vestlund et al., 2014;Valsan et al., 2015;China et al., 2018). They identified fungal spores, brochosome, pollen, and plant or insect debris larger than 2 μm in the atmosphere. Although the SEM observations adequately characterized the coarse fungal spores, pollen, and plant or insect debris particles, comparable results have not been obtained for fine bacteria and fungal particles, which together account for a large number of suspended particles in ambient air detected by online instruments (Tong and Lighthart, 2000;Després et al., 2012;Afanou et al., 2014;Valsan et al., 2016;Priyamvada et al., 2017;Hu et al., 2018). The reason for this shortfall is likely that SEM could not clearly observe carbonaceous bioaerosols smaller than 1 μm (Li et al.,

2016;Ault and Axson, 2017). Posfai et al. (2003) and Patterson et al. (2016) used transmission electron microscopy (TEM) to detect some fine bacteria in marine air. However, there is no study to characterize the morphology, size, and mixing state of individual PBAPs from nanoscale to microscale. For example, many studies directly used SEM images showing the coarse PBAPs (e.g., fungal spores) in support of their conclusions, but missed large numbers of fine PBAPs (e.g., bacteria) (Shi et al., 2003;Wittmaack et al., 2005;Coz et al., 2009;Shi et al., 2009;Martin et al., 2010;Huffman et al., 2012;Tamer Vestlund et al., 2014;Afanou et al., 2015;Valsan et al., 2015;Valsan et al., 2016;Priyamvada et al., 2017;Wu et al., 2019). The result might discourage people considering fine bacteria and fungal particles for their atmospheric effects or for their examination of data from some online instruments. Therefore, it is necessary to integrate SEM and TEM to characterize the morphology, size, and mixing state of individual PBAPs from nanoscale to microscale.

Forests are important contributors of primary biological aerosols in the atmosphere (Tunved et al., 2006;Spracklen et al., 2008;Després et al., 2012;Whitehead et al., 2016). Aerosols in large forests contain abundant biological particles from plants emitted locally and lesser amounts of anthropogenic pollutants from long-range transport (Tong and Lighthart, 2000;Tunved et al., 2006;Gabey et al., 2010;Martin et al., 2010). We chose the Lesser Khingan Mountains in northeast China, which is its second largest boreal forest. In this study, TEM and SEM both have been employed to characterize the morphology, size, and mixing state of various PBAPs collected over the boreal forest. Furthermore, hygroscopic experiments on the primary biological particles have been conducted.

2. Methods

2.1 Sampling site and sample collection

The sampling site is at the Heilongjiang Liangshui National Nature Reserve (47.32°N, 128.54°E; 350m above sea level) in the center of the Lesser Khingan Mountains of northeast China (Figure 1). The boreal region is characterized by large seasonal variations in temperature, and the flora is dominated by Korean pine and spruce species. There are no anthropogenic sources of pollutants, such as villages, industries and vehicles within 80 km of the sampling site. Boreal forests have the highest emissions of biological aerosols during summer. Because there is less rain in late Auguest, we selected 14-21 August, 2016 to collect the bioaerosol samples.

Individual particle samples were collected both on copper (Cu) TEM grids coated with carbon film (carbon type-B, 300-mesh copper; Tianld Co., China) and on silicon membranes (thickness: 500±10 μm, size: 3×3 mm; LIJINGKEJI, China) by a single-stage cascade impactor called the DKL-2 sampler (Genstar Electronic Technology, China). The collection efficiency of the impactor is 50% for particles with an aerodynamic diameter of 0.1 μm when we assume an aerosol particle density of 2 g cm^{-3}. We collected individual particles four times each day at 9:00, 15:00, 21:00, and 02:00 local time. At each sampling event, we first collected TEM grids and then changed to silicon wafers in the sampler. The sampling duration at each time varied from 10 min to 25 min depending on the particle distribution on the substrate. The substrates of the carbon film and silicon wafer both have smooth surfaces with no contamination before we use them to collect aerosol particles. After sample collection, we immediately performed optical microscopy (BST60-100, China) at 100X magnification to determine whether the aerosol distribution on the substrate was suitable for electron microscopy analysis. The distribution of aerosol particles on TEM grids was not uniform, with

coarser particles occurring near the center and finer particles on the periphery. The quick check by the optical microscopy enabled us to tell whether individual particles were well distributed and whether there was any overlap on the substrate. Whenever the distribution was not even enough or when substantial overlap occurred, we had to discard it and re-collect individual particle samples through adjusting the sampling duration. In a word, this sampling procedure guarantees that the collected particles were adequately separated and did not overlap each other on the substrate (Li et al., 2016). The Cu grids and silicon wafers were placed in a dry, clean, and airtight container with 25 ℃ and 20±3% RH which minimizes exposure to ambient air and preserves them for subsequent analysis. The detailed sampling and storage procedures are summarized in Figure S1.

The daily $PM_{2.5}$ and PM_{10} samples were collected on quartz-fiber filters with a diameter of 90 mm through two medium-volume samplers (TH-150, Wuhan Tianhong, China) at a constant flow rate of 100 L min^{-1}. The samples were changed at 08:00 a.m. each day. The DKL-2 and TH-150 samplers and other monitoring instruments in the field experiment were installed on a building roof 15 m above ground. The quartz filters (Whatman, UK) were put in polyethylene boxes immediately after sampling and stored at −5 °C. They were equilibrated at a constant temperature (20 ± 0.5 ℃) and humidity (50 ± 2%) for over 24 h before being weighed with an electronic microbalance (Sartorius-ME5, Germany). This gravimetric procedure provides the mass concentration of $PM_{2.5}$ and PM_{10}.

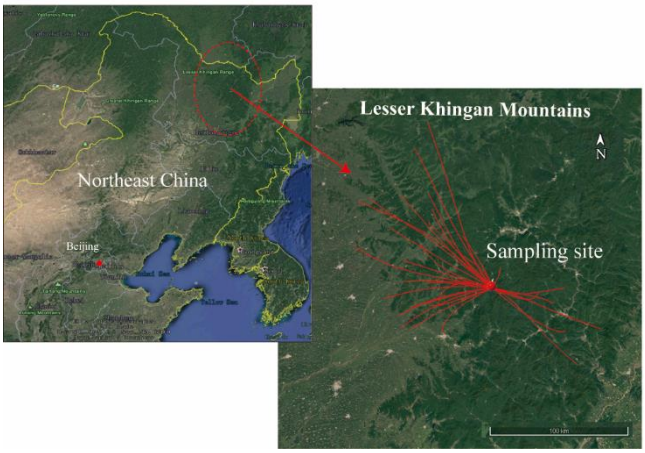


Figure 1 Location of the sampling site and 6-h air mass back trajectories arriving at each sampling time from 14-21 August, 2016 in a boreal forest of the Lesser Khingan Mountain in Northeast China. The map source is Google Earth.

2.2 Transmission electron microscopy analysis

Individual aerosol particles collected on Cu grids were analyzed via transmission electron microscopy (TEM, JEM-2100, JEOL Ltd., Japan) at a 200 kV accelerating voltage. TEM with a beam of electrons is transmitted through a specimen to form an image. An image is formed from the interaction of the electrons with the sample as the beam is transmitted through the specimen. Therefore, TEM images display the inner physical structure of individual particles and the mixing state of different components. The TEM system is equipped with an energy-dispersive X-ray spectrometer (EDS, INCA X-Max^N 80T, Oxford Instruments, UK). EDS is an analytical technique used for the elemental analysis or chemical characterization of a sample. It relies on an interaction between X-rays and a sample. EDS spectra show the peaks of different elements and the contribution of each element in the total. EDS semiquantitatively detects the elemental composition of individual particles with an atomic number greater than six ($Z > 6$). However, Cu peaks in the EDS spectra were not considered because of interference from the copper substrate of TEM grids. We determined the morphology, composition, and mixing state of individual particles through the combination of TEM and EDS. To reduce the damage to particles under the electron beam, the EDS collection duration was limited to 15 s. Individual particles are distributed on TEM grids, with the coarser particles in the center of sampling spot and with the finer particles on the periphery. Therefore, to guarantee that the analyzed particles are representative, five areas are selected from

the sampling center to the periphery on each TEM grid. After a labor-intensive operation, we analyzed 150-250 individual particles with diameters of 100 nm-10 μm in each sample. Finally, we successfully analyzed 20 TEM grids in the study. TEM/EDS can determine the internal mixing structure of different aerosol components in fine particles and their specific composition. TEM clearly shows the morphology of particles smaller than 2 μm. For some larger particles, we might further carry the scanning electron microscopy (SEM) experiments to determine their morphology. In this study, we did observe one fungi (*Yeast)* and one bacteria (*colibacillus)* sample through TEM, which were prepared in biological laboratories (Figure S2). Microscopic observations from the bacteria and fungi samples prepared in the laboratory were helpful to classify PBAPs emitted from the forest.

Once we clearly obtained electron images of different particles, we could then measure particle size and shape factors. In this study, the area, perimeter, shape factor, and equivalent circle diameter (ECD) of individual particles in TEM images are manually or automatically obtained through an image analysis software (RADUS, EMSIS GmbH, Germany). Based on these measurements, we can classify particle types and determine the diameter and shape factor of individual particles among different particle types. Moreover, we statistically analyze the number fractions in different size bins.

Aspect Ratio is the maximum ratio between the length and width of a bounding box for the measured object. An aspect ratio of 1 (the lowest value) indicates that a particle is not elongated in any direction. The aspect ratio is defined as

$$AR = \frac{L_{max}}{W_{max}}$$

2.3 Scanning electron microscopy analysis

SEM is performed using a type of electron microscope that can determine the particle surface by scanning it with a high-energy beam of electrons in a raster scan pattern. An SEM system (Zeiss Ultra 55) equipped with a field emission gun operating at 5–20 kV was used to obtain detailed information on the surfaces of individual aerosol particles. Moreover, the SEMx was equipped with an energy-dispersive X-ray spectrometry (EDS), which can analyze the chemical composition of

individual particles. The SEM/EDS can efficiently obtain the surface morphology, size, and composition of coarse particles without any coating process on the substrate. Finally, we selected six silicon wafers for SEM/EDS analysis (Figure S1). In this study, we used SEM/EDS to observe surface morphology of the coarse particles on silicon wafers and to confirm particle types which cannot be clearly shown in TEM images.

2.4 Hygroscopic experiments

A custom-made individual particle hygroscopic (IPH) system was used to observe the hygroscopic properties of individual biological particles at different relative humidity (RH) values (Figure 2). After the hygroscopic experiment, an SEM analysis of the sample was employed to primarily check particle types. This allowed us to further understand how PBAPs particles grow at different RH values ranging from 5% to 94%.

The scheme of the IPH system is shown in Figure 2, which consisted of four steps;

(1) Introducing N_2 gas with a mass flow controller into a chamber;

(2) Setting a TEM grid or silicon wafer on the bottom of an environmental microscopic cell (Gen-RH Mcell, UK), which can change the RH and maintain the temperature at 20 ℃;

(3) Taking images at incremental RH values using an optical microscope (Olympus BX51M, Japan) with a camera (Canon 650D);

(4) Obtaining through the RADUS software the PBAPs sizes (i.e., D(RH) and D_0) in the images taken from the optical microscopy manually or automatically.. The images can be taken at different RHs during hygroscopic experiments and then are input into the RADUS software for size measurement.

This IPH system has been tested and has successfully captured the hygroscopic growth of

individual aerosol particles collected on either a silicon wafer or TEM grid in our laboratory (Sun et al., 2018). Before the IPH system is used for ambient samples, it must be checked through standard NaCl particles on a silicon wafer made in the laboratory. Figure S3 shows that the delinquence relitive humidity (DRH) of individual NaCl particles on this silicon wafer is at 76%, similar to the standard DRH at 75±1%. After the procedure, we can replace our collected samples into the IPH system.

The particle growth factor (GF), an important parameter used to describe the hygroscopic growth of individual particles, is defined as follows:

$$GF(RH)=\frac{D(RH)}{D_0}$$

where D(RH) and D_0 are the diameters of particles at a given RH and at 5% RH, respectively.

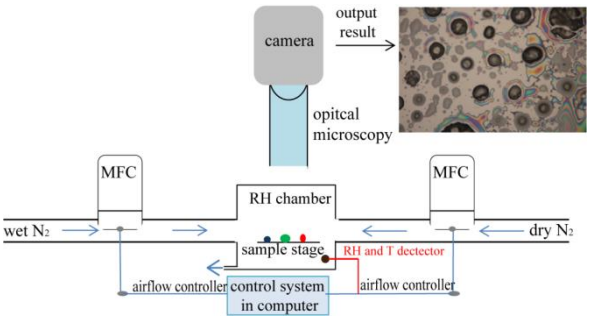


Figure 2 Scheme of a custom-made individual particle hygroscopic system to observe hygroscopic growth of individual particles

2.5 **Meteorological data and back trajectories**

Meteorological data, including the relative humidity (RH), temperature, wind speed, and wind direction, were measured and recorded every 5 min by an automated weather meter (Kestrel 5500, USA). During the sampling period, the relative humidity (RH) and temperature varied from 40-70% and 22-28 ℃ during the day and 90-100% and 10-15 ℃ during the night,

252 respectively. The wind speed was 1.5-7.6 m s^{-1} during the day and 0-1 m s^{-1} at night (Figure
253 S4).

254 To determine the regional transport of air masses, 6-h back trajectories of air masses were
255 generated using a Hybrid Single Particle Lagrangian Integrated Trajectory (HYSPLIT) model
256 at the forest sampling station during 14-21 August, 2016. Based on the sampling times of each
257 day at 09:00, 15:00, 21:00, and 02:00 (midnight) local time, we performed 31 air mass back
258 trajectories. Here we selected an altitude of 500 m as the end point of each back trajectory
259 (Figure 1). Figure 1 shows that all the back trajectories in the past 6-h had been transported
260 over the Lesser Khingan Mountain forest.

261 **3. Results and Discussion**

262 **3.1 Morphology and elemental composition of PBAPs**

263 Among the 4,122 analyzed aerosol particles with diameters of 100 nm-10 μm analyzed by
264 TEM/EDS, individual particles are classified into five groups based on their morphology and
265 composition: S-OM (mixture of sulfate (S), organics (OM)), OM, mineral dust, and PBAPs (Figure
266 3). S can be used to indicate secondary sulfates; abundant C and minor O with transparent color
267 constitute the coating of the sulfate core and represent secondary organic matter; and irregular
268 particles containing Si, Al, Ca, minor Fe, and Ti normally indicate mineral dust particles.
269 Moreover, previous studies have found that elemental P in individual particles and their associated
270 unique morphologies can be used to identify PBAPs by electron microscopy (Poschl,
271 2005;Wittmaack et al., 2005). Thirteen percent of particles were PBAPs, and low magnification
272 TEM and SEM images both revealed that abundant PBAPs occurred in the samples (e.g., Figure 3a-
273 b).

The number fractions of size-resolved aerosol particles show that secondary S-OM and OM particles were the dominant particle groups in the fine mode (< 1 μm) while PBAPs and mineral particles dominated the coarse mode (≥ 1 μm) (Figure 4a). Moreover, we noticed that the number fractions of PBAPs in each sample collected at night were much higher than those collected during the day. Abundant fine secondary sulfate and organic particles from photochemical formation were observed during the day. Figure 4b shows that the average number fraction of PBAPs was 2.5% in the samples collected during the day and as high as 30.0% at night. If we further calculated the number concentration of PBAPs in Figure 4b, the PBAPs concentration significantly increased by approximately seven times from daytime to nighttime, although the non-PBAPs concentration decreased.

Based on the morphology and size of the PBAPs, we definitely identified fungal spores and brochosomes, and plant or insect debris, all of which have been widely reported before (Wittmaack et al., 2005;Huffman et al., 2012;Afanou et al., 2014;Valsan et al., 2015;Priyamvada et al., 2017). Besides these PBAPs, we also found many special rod-like PBAPs with a dominant size range of 1 - 5 μm. Pollen was not found in our samples, which may be because large pollen emissions occur in spring and early summer instead of late summer (August) in boreal forests (Manninen et al., 2014).

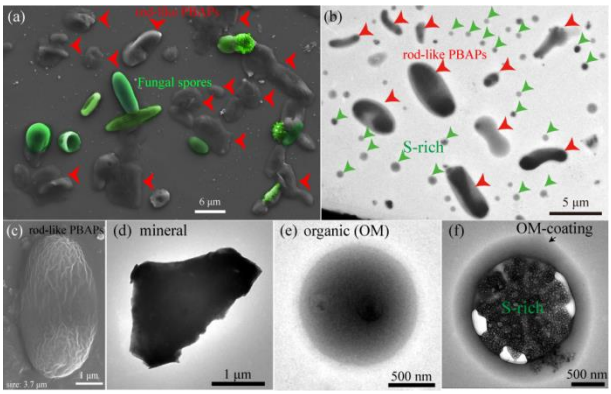


Figure 3 Low magnification SEM and TEM images of individual particles collected from the forest air.

(a) low magnification SEM image of rod-like PBAPs (red arrows) and fungal spores (green); (b) low magnification TEM image of rod-like PBAPs particles and secondary sulfate (S-rich) particles; (c) SEM image of a rod-like particle; (d) TEM image of a mineral dust particle (e) TEM image of an organic matter (OM) particle; and (f) TEM image of OM coating on S-rich particles. The color in (a) was artificially painted on the original SEM images.

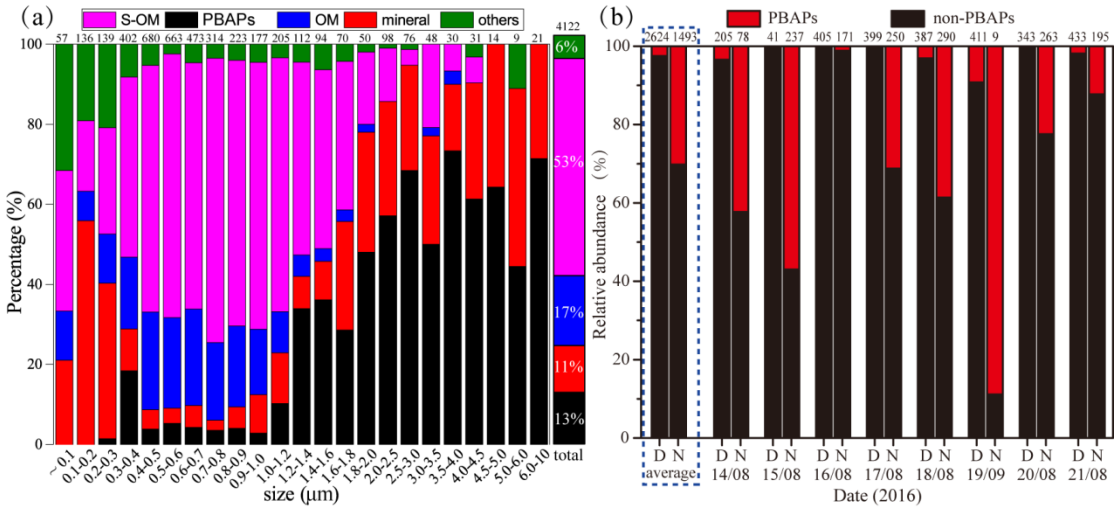


Figure 4 Number fractions of different types of particles in different size bins and their total number fraction (a); and number fractions of primary biological aerosol particles (PBAPs) and non-PBAPs during the day and night (b). The number of analyzed particles is listed above each column. D and N are daytime and nighttime.

Online instruments indicate that large number of fine PBAPs are bacteria and fungi in the forest air (Tong and Lighthart, 2000;Elbert et al., 2007;Huffman et al., 2010;Despr és et al., 2012;Hu et al., 2018). Although many previous studies reported PBAPs through the SEM, no observations of fine bacteria and fungal particles in forest air were reported (Wittmaack et al., 2005;Shi et al., 2009;Martin et al., 2010;Huffman et al., 2012;Tamer Vestlund et al., 2014;Valsan et al., 2015;Valsan et al., 2016;Priyamvada et al., 2017;Wu et al., 2019). Posfai et al. (2003) found a few rod-like

bacterial particles in marine air using TEM. In this study, we found that the rod-like PBAPs (Figure 5a-e) have a morphology similar to bacteria reported by Posfai et al. (2003). These rod-like PBAPs were stable under the electron beam during the TEM analysis, and they contained C, N, O, P, and K with minor Mg, Si, S, Ca and Fe (Figure 5a). These rod-like PBAPs have a size range of 300 nm-7 μm with the dominant size range of 1-5 μm with two typical peaks at 1.4 μm and 3.5 μm (Figure 6a). Figure 6b further shows that the aspect ratio of 85% of these particles is larger than 1.5.

In nature, many fine fungi normally displayed similar composition and rod-like shape. To better compare and confirm differences of bacteria and fungi observed in TEM, we cultured *Colibacillus* and *Yeast* in the laboratory to represent bacteria and fungi. Then we sprayed the solution of *Colibacillus* and *Yeast* onto TEM grids. After drying these samples, we observed the morphology and size of *Colibacillus* and *Yeast* through the TEM (Figure 5f-g and Figure S5). Indeed, TEM/EDS show very similar rod-like shape and composition between *Colibacillus* and *Yeast* particles on the substrate, although the *Yeast* particles with a size range at 1-8 μm with a mean diameter at 4.3 μm are larger than *Colibacillus* (300 nm-2.5 μm with mean diameter of 1.3 μm) (Figure S5). It is interesting that the size distribution of the rod-like PBAPs collected in the forest air displays two typical peaks at 1.4 μm and 3.5 μm, which probably represent bacteria and fungi. Despr és et al. (2012) stated that bacteria mostly have diameters of 1-2 μm and fungi of 1–10 μm in the atmosphere. Although we can indicate the bacteria and fungi based on their sizes, the clue could not be used to precisely identify bacteria and fungi through electron microscopy due to their overlapped size range. Figure 6b shows 85% of particles with larger aspect ratios (> 1.5), suggesting most of these PBAPs particles have typical rod-like shape. Although their identification is tentative, we called all these similar rod-like bacteria and fungal particles “rod-like PBAPs” here.

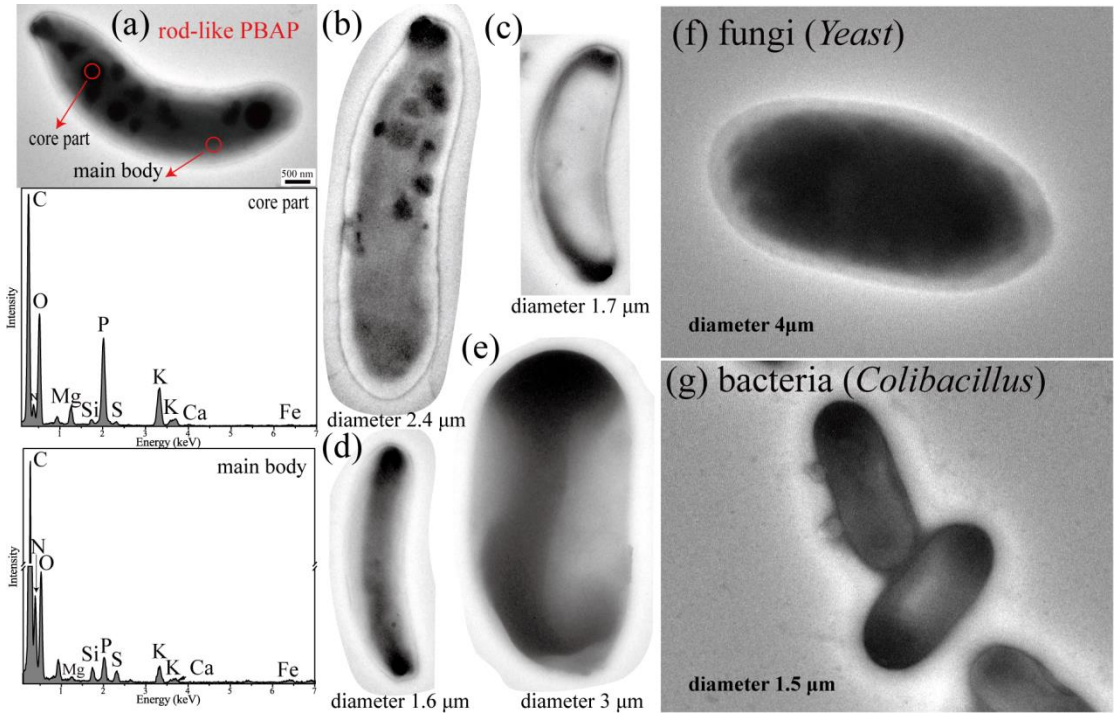


Figure 5 TEM image of the rod-like PBAPs collected in forest air and the fungi and bacteria cultivated in laboratory. (a) Morphology of a rod-like PBAP and EDS spectra of its core and main part. The red circles indicate where EDS impacted the rod-like PBAP. (b-e) Various rod-like PBAPs collected in forest air. (f) One *Yeast* particle cultivated in laboratory (e) One *colibacillus* particle cultivated in laboratory.

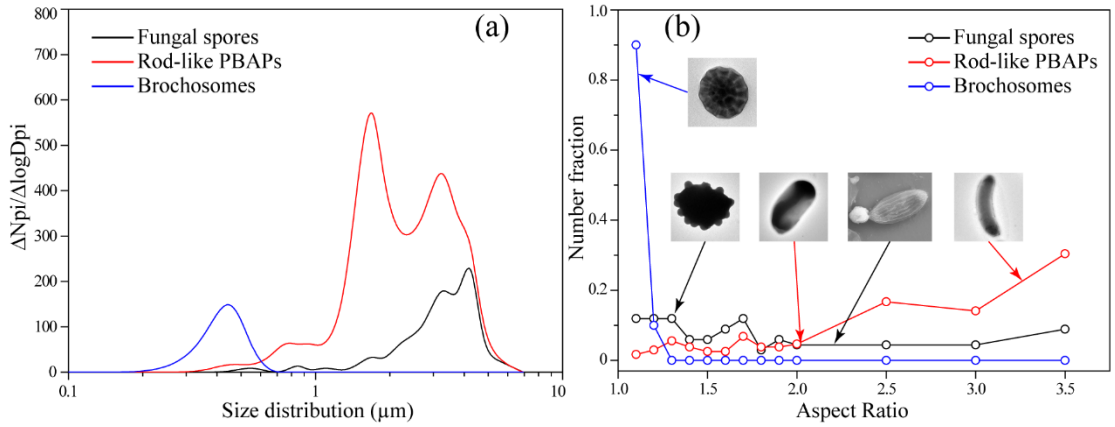


Figure 6 Size distribution and aspect ratios of rod-like PBAPs, fungal spores, and brochosomes collected in boreal forest air.

Fungal spores are microscopic biological particles that allow fungi to reproduce, serving a similar purpose to that of seeds in the plant world (Lacey and West, 2006). Spores can be released as a part of the sexual and/or asexual morph (stage) of the lifecycle of a fungus, and many species are able to produce spores from both stages (Després et al., 2012). Fungal spores have been reported in many places in the global air and their morphologies have been well documented (Shi et al., 2003;Wittmaack et al., 2005;Coz et al., 2009;Shi et al., 2009;Martin et al., 2010;Huffman et al., 2012;Tamer Vestlund et al., 2014;Afanou et al., 2015;Valsan et al., 2015;Valsan et al., 2016;Priyamvada et al., 2017;Wu et al., 2019). In this study, the fungal spores generally appeared as ovoid (Figure 7a), sub-globular (Figure 7b-c) or elongated shapes with a smooth surface and small protuberances (apiculus) (Figures 8a-f). Figure 7d shows that their composition mainly consists of C, O and Si, followed by minor N, Mg, P, S, K and Fe. The size range of the observed fungal spores varied roughly between 400 nm and 7 μm (Figure 6a). The size distribution of fungal spores further showed a dominant size range of 2 - 5 μm and one peak at 4 μm. The number fraction of fungal spores at all aspect ratios is generally lower than 0.15, suggesting that there is no typical shape from either roundness or elongation for fungal spores in the boreal forest. SEM images clearly display that several typical fungal spores with diameters of 3.7-6.5 μm do not have well-defined shapes and that their surfaces have regular strips or regular protuberances (Figure 8). Similar fungal spores have been reported in forest air (Wittmaack et al., 2005;Valsan et al., 2015). Compared with the rod-like PBAPs, fungal spores normally have a rougher surface (Figures 6-7), larger size, and much higher Si and lower N. Therefore, the fungal spores can easily be identified based on their morphology among the PBAPs through the TEM and SEM analysis.

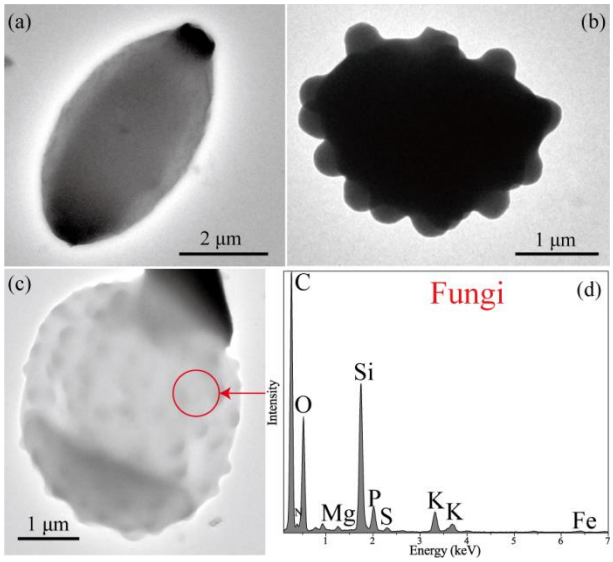


Figure 7 TEM/EDS showing the morphology and composition of various fungal spores. (a) a spindle fungal spore; (b) a fungal spore with protuberances; (c) a fungal spore with protuberances; and (d) EDS spectrum showing the composition of fungal spore. The red circle indicates where EDS impacted the particle.

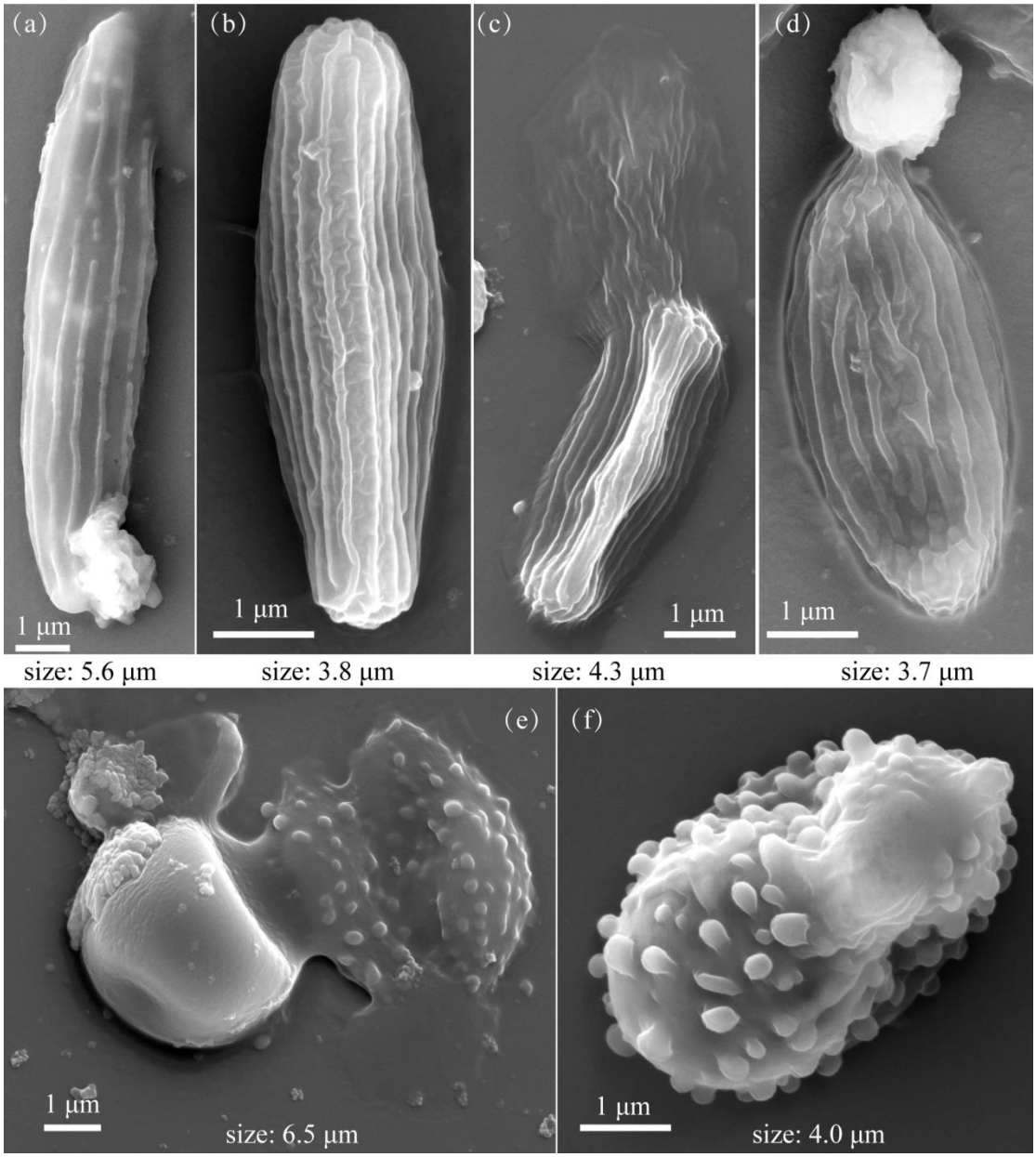


Figure 8 SEM images showing the shape, size, and surface properties of fungal spores. Size represents the diameter of fungal spores. (a-d) Surfaces of three spindle fungal particles with a layer of strips. (e-f) Surfaces of two fungal spores with protuberances.

Brochosomes are hollow spherical particles produced by leafhoppers (Cicadelliae) (Wittmaack, 2005). TEM and SEM observations both found abundant brochosomes in the samples. The low-magnification SEM images showed that there are large brochosomal clusters on the substrate, each containing tens or hundreds of single brochosomes (Figure S6). Wittmaack (2005)

found that most of the brochosomes normally occur as large clusters and reported that each cluster contains up to 100,000 brochosomes. In this study, TEM and SEM both produce clear images showing the structure of the brochosome (Figures 9-10). Interestingly, the outline of each brochosome approximates a truncated icosahedron and the brochosome particles likely have unique inner structures, such as C60 Buckminster fullerenes (Figures 9a-b and 10). Compared with the rod-like PBAPs, chemical composition of the brochosomal particles show extremely high Si and low P in addition to major C and O and minor N, Na, S, K and Fe (Figure 9c). A single brochosome has a size range of 200-700 nm with a mean diameter of 350 nm. The aspect ratio of individual brochosomes is close to 1, suggesting that they are spherical (Figure 6b). Because the brochosomes might be dispersed from their clusters when they impact on the substrate,, it is not meaningful to compare the number fraction of brochosomes with the rod-like PBAPs and fungal spores.

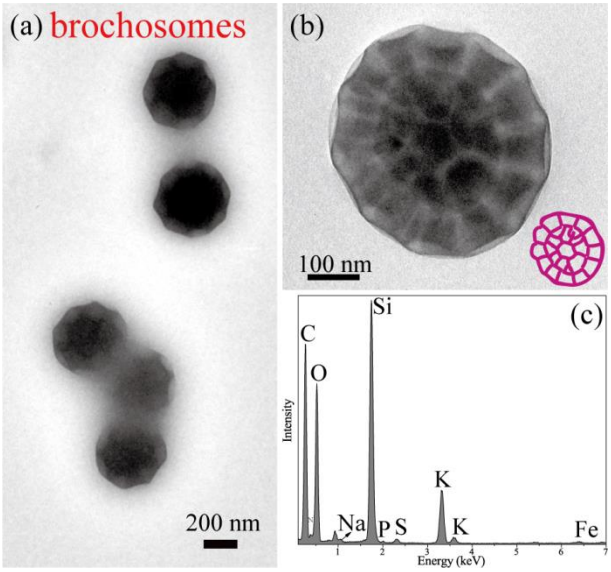


Figure 9 TEM images of brochosomes and the composition of (a) a single brochosome and brochosome aggregations; (b) high-resolution TEM image showing the inner structure of one brochosome; (c) EDS spectrum showing the chemical composition of the brochosomes.

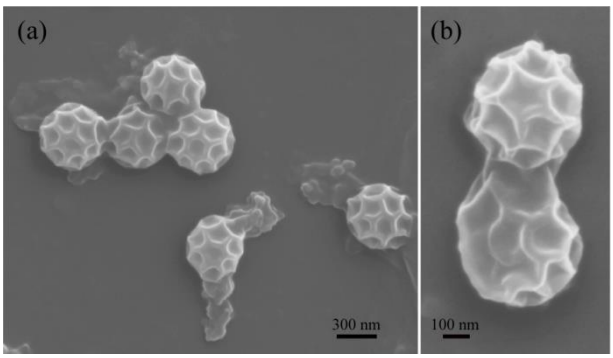
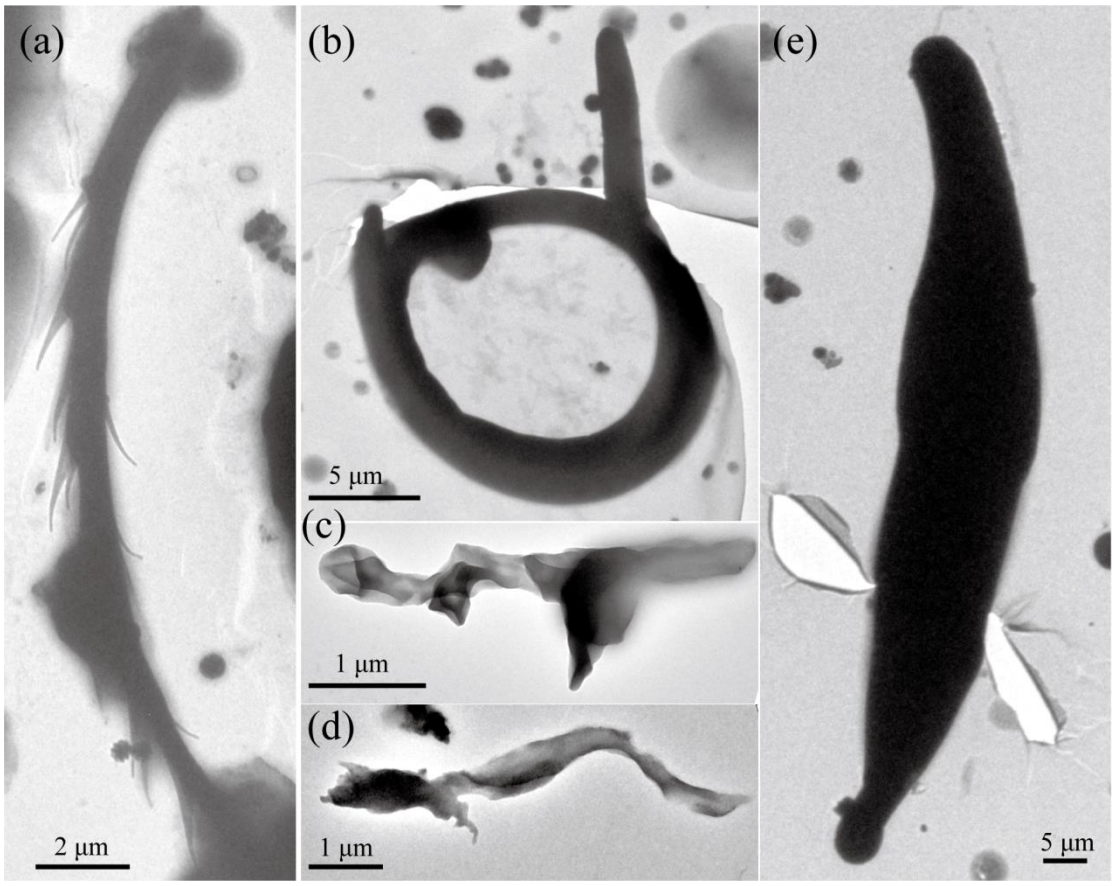


Figure 10 SEM images of brochosomes. (a) Single brochosome and their aggregations. Some brochosomal particles are associated with primary biological species. (b) High-resolution SEM image showing the surface properties of the brochosomal particles.

The TEM and SEM images both show a few elongated large particles at 8-20 μm among the biological particles. EDS shows that these particles mainly contained C, O, and Si but no detectable P in some of these biological particles as shown in Figures 11-12. We speculate that these biological particles were plant or insect debris. For example, Wittmaack et al. (2005) suggested that the spaghetti-type biological particles from Figure 11a-d are likely epicuticlar wax fragments of plants. The SEM images as shown in Figure 12 clearly displayed the surface morphology of the large particles.

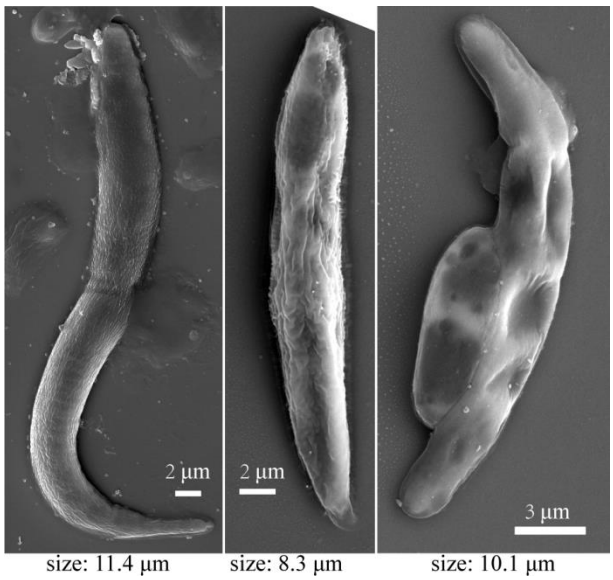


404

405 **Figure 11** TEM images showing the morphology of the primary biological particles. (a) One elongated

406 particle with thorns; (b) one circular particle; (c-d) two elongated particles; and (e) one long spindle

407 particle



408

409 **Figure 12** SEM image showing the morphology and surface properties of three elongated biological

410 particles.

411 **3.2 Relative abundance of PBAPs**

412 In this study, we classified PBAPs but also efficiently obtained the number fraction of rod-like
413 PBAPs and fungal spores in coarse mode particles (> 1 μm). The results from the electron
414 microscopy analysis further estimated that PBAPs, mineral dust, and the remaining particles
415 accounted for 50%, 25%, and 25% of the coarse mode, respectively. Assuming a density of ~1 g
416 cm^{-3} for PBAPs (Elbert et al., 2007), 2 g cm^{-3} for mineral dust particles, and 1.4 g cm^{-3} for the
417 remaining particles (e.g., S-OM, OM, and metal) (Rissler et al., 2006), mass concentrations of the
418 three different types of particles with different size bins can be estimated based on the equation:

419 $$M_i = \frac{\Pi}{6} {D_i}^3 \rho_i N_i$$

420 *i*: particle type (PBAPs, mineral dust, and other remaining particle)

421 D: particle geometrical diameter in a size bin

422 N: particle number in a size bin

423 M: total mass of the analyzed particles in a size bin

424 ρ: particle density (g cm^{-3})

425 In the equation, N_i and D_i both can be obtained through the measurement of individual particles
426 in TEM images. Finally, we estimated that the mass concentration of PBAPs, mineral dust, and
427 remaining particles accounted for 47%, 43%, and 10% of $PM_{2.5-10}$, respectively. The results suggest
428 that PBAPs significantly contributed to mass concentration of $PM_{2.5-10}$ in summertime in the boreal
429 forest air. During the sampling period, we measured the daily mass concentrations of $PM_{2.5}$ of ~6.0
430 μg m^{-3} and PM_{10} of ~10.0 μg m^{-3}. The number size distribution of PBAPs coupled with the mass
431 concentrations of $PM_{2.5}$ and PM_{10} were used to estimate the total mass concentration of PBAPs

using the result from the above equation. We estimated that the PBAPs contributed ~1.9 μg m^{-3} to the concentration of $PM_{2.5-10}$ of 4.0 μg m^{-3}.

Thirteen percent of all detected particles by number collected from the boreal forest air are PBAPs. Such a high fraction of PBAPs has not been reported in urban and rural air in China (Shi et al., 2003;Shi et al., 2009;Li et al., 2016). We noticed that the number concentration of PBAPs was much higher at night than during the day (Figure 3b). A shallow nocturnal boundary layer can lead to a slight increase in the number concentration of coarse particles near the ground (Graham et al., 2003), but this increase cannot explain the large difference in the relative number fraction of PBAPs (12 times larger at night than during the day) (Figure 3b). Alternately, the relative emission strength of PBAPs from the forest between day and night likely induced the difference of the relative number fractions.

It is well documented that meteorological conditions such as RH, wind speed, and temperature can affect PBAPs emission in the forests (Harrison et al., 2005;Whitehead et al., 2016). In particular, the wind speed is especially important in promoting PBAPs emission into air. During the sampling period, the average wind speeds at 5 min intervals had a range from 0 to 7.5 m/s with a mean value of 0.75 m/s. 89% of the measured wind speeds were lower than 2 m/s (Figure S4). Therefore, we conclude that no large consistent wind speeds occurred during the sampling period. Furthermore, we compared all the air mass back trajectories in the past 6-h over the Lesser Khingan Mountain forest at each sampling time (Figure 1). There are similar lengths of these back trajectories, suggesting that wind speeds above the forest canopy had only small changes during the sampling period. Therefore, the result from the ground-based measurements of wind speeds is consistent with air mass back trajectories. Here, we can exclude wind speeds during the sampling period as one

important factor to dominate PBAPs emissions during day and night in the boreal forest. High temperatures normally increase the PBAPs emissions from the plants in the daytime (Harrison et al., 2005). However, we observed contrasting results that more PBAPs occurred in nighttime instead of daytime (Figure S4). Therefore, we also exclude temperatures during the sampling period as a cause of the vastly different PBAPs emissions at day and night in the boreal forest.

Besides wind speed and temperature, RH is an important meteorological variable that influences PBAPs emissions from plants (Harrison et al., 2005;Huffman et al., 2012). In this study, we found large differences of RH between day and night (Figure S4). The elevated RH near 100% at night (Figure S1) appears to be an important factor that increases the emissions of PBAPs. This result is consistent with the conclusion of Elbert et al. (2007), who showed that PBAPs in a boreal forest are generally most abundant in samples collected at night when the RH is close to 100%. A similar phenomenon has been observed in different forests, such as the Amazon rainforest (Huffman et al., 2012;Whitehead et al., 2016), a montane ponderosa pine forest in North American (Crawford et al., 2014), a semi-arid forest in the southern Rocky Mountains of Colorado (Gosselin et al., 2016), and a semi-rural site in southwestern Germany (Toprak and Schnaiter, 2013). These studies above found that a nighttime peak of number concentrations of fluorescent biological aerosol particles is consistent with nocturnal sporulation driven by the increased RH. Moreover, Troutt and Levetin (2001) explained that the increase in PBAP concentrations is caused by the increase in basidiospores concentrations with RH, and they showed that a clear diurnal rhythm occurs and peaks at 04:00-06:00 LT. Furthermore, the number ratio (4.6 at nighttime and 4.0 at daytime) of rod-like PBAPs vs fungal spores and their number concentrations increased from daytime to nighttime (Figure S7). These results all suggest that higher RH can promote the emissions of rod-like PBAPs

476 and fungal spores in the boreal forest.

477

478 **3.3 Mixing state of rod-like PBAPs**

479 Our study shows that rod-like PBAPs contain bacteria and fungi in the boreal forest air.
480 Although approximately 80% of rod-like PBAPs were externally mixed particles in the boreal forest
481 air, we still found that 20% of rod-like PBAPs were internally mixed particles. TEM observations
482 show that the rod-like PBAPs were frequently internally mixed with mineral, metal, organics, and
483 inorganic salts. We noticed that irregular mineral dust particles significantly changed the shape of
484 the rod-like PBAPs (Figure 13a-c). The EDS analysis shows that the internally mixed mineral
485 particles contain certain amounts of C, O, and P in addition to Si, Al, or Ca (Figure 13a-c),
486 suggesting that many rod-like PBAPs were associated with mineral dust particles.

487 In this study, we found that some nanoscale metal particles were internally mixed with rod-like
488 PBAPs. Figure 13d-f further shows that these metals were spherical and contained Mn, Si and/or
489 Fe. As in previous studies, these nanosize metal particles were emitted from industrial activities or
490 power plants instead of natural soil (Li et al., 2017). TEM observations show that these metallic
491 particles were mainly attached to the surface of rod-like PBAPs. Moreover, some rod-like PBAPs
492 were coated by inorganic salts (e.g., K-P in Figure 13g and S-rich in Figure 13i) and organics. The
493 shape of the rod-like PBAPs might change following the aging process during long-range transport
494 (Figure 13), although the elemental P or its associated ionic components ($H_2PO_4^-$ and PO_3^-) did not
495 change (Pratt et al., 2009). Pratt et al. (2009) detected $H_2PO_4^-$ and PO_3^- in individual cloud ice-
496 crystal residues to identify PBAPs using aerosol time-of-flight mass spectrometry. Although one
497 study indicates that a few mineral dust or fly ash particles contain trace inorganic P, these particles

do not contain abundant organics and their number is low in the air (Zawadowicz et al., 2017). Therefore, TEM/EDS is an efficient tool to identify fine bacteria or fungi from non-PBAPs collected in the atmosphere. Moreover, it significantly reveals the mixing state of individual PBAPs, a key to understand their possible CCN and IN activity over the boreal forest air in the future.

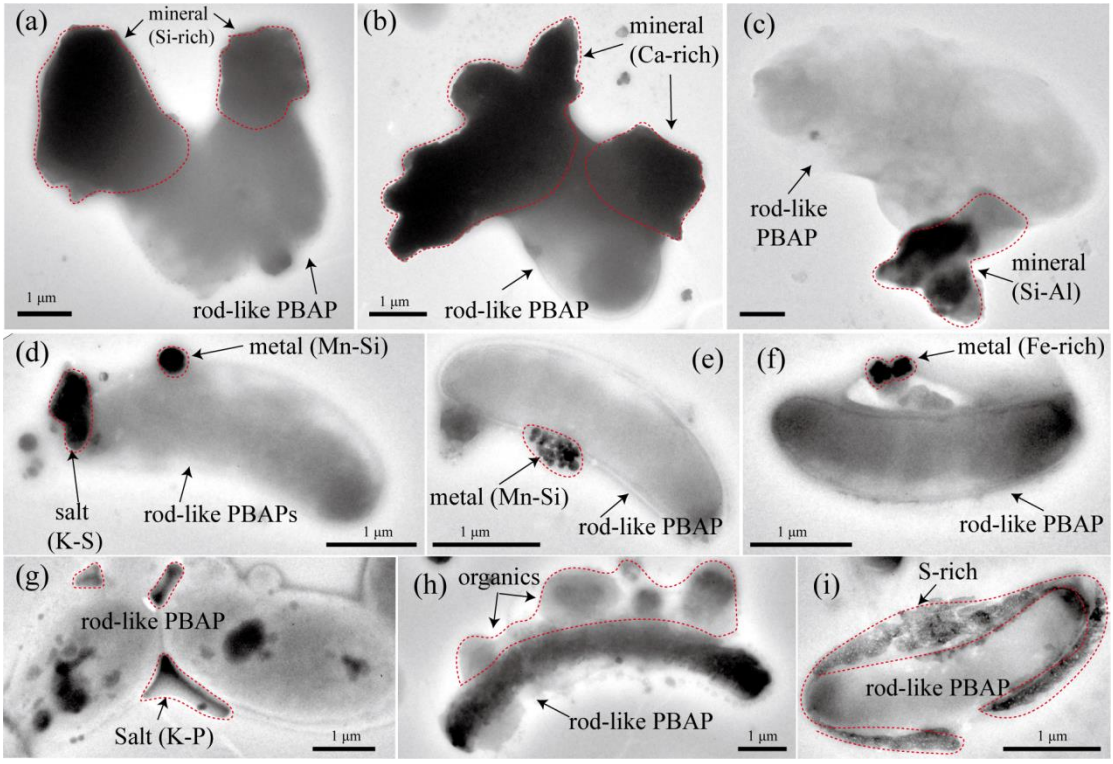


Figure 13 TEM showing the internally mixed rod-like PBAPs. (a-c) Internal mixture of mineral and rod-like PBAP; (d-f) Internal mixture of metal and rod-like PBAP; (g) Internal mixture of inorganic salts and rod-like PBAP; (h) Internal mixture of organics and rod-like PBAP; and (i) Internal mixture of S-rich salts and rod-like PBAP.

3.4 Hygroscopicity of PBAPs

In this study, we conducted an experiment to observe the hygroscopic growth of fresh PBAPs. In the hygroscopic experiment, the PBAPs all take up water and grow by up to 88% during hydration, and they lose water and return to the dry particle size (reduction of 83%) during dehydration (Figure

14). The growth factor of the PBAPs is ~1.09 at RH=94% based on the particle diameter change, which is much lower than growth factor of NaCl at ~2.3 (Figure 14). These results show that the fresh PBAPs have extremely weak hygroscopicity.

Recent studies found that fungal fragments collected in Amazon forests displayed strong hygroscopic properties (China et al., 2016;China et al., 2018) and were internally mixed with certain amounts of sodium salts. However, we found weak hygroscopic growth of 1.09, whereas this value was in the range of 1.05-1.3 for bacteria and fungal spores in previous studies (Reponen et al., 1996;Lee et al., 2002). However, the result is much lower than the value of 2.30 at RH=94% for NaCl (Figure 2a) and 1.60 at RH 94% for ammonium sulfate (Sun et al., 2018). This comparison suggests that fresh PBAPs display extremely weak hygroscopicity and do not contain any sodium salt in the boreal forest (Figure 2a). Overall, our results indicate that PBAPs from the substantial biological emissions from the Khingan Mountain boreal forest are weakly hygroscopic in nature.

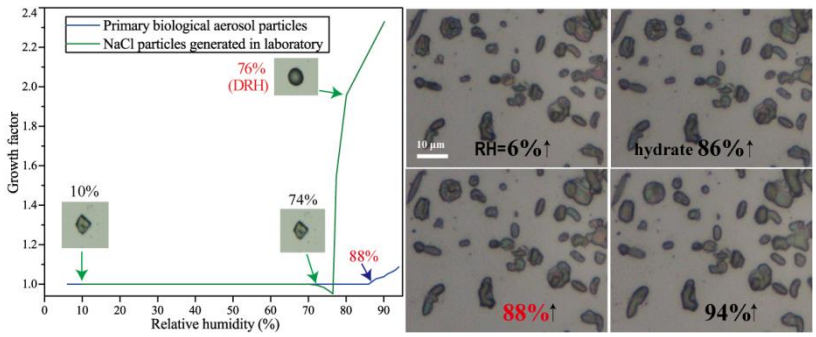


Figure 14 Hygroscopic growth of NaCl prepared in laboratory and primary biological particles collected in boreal forest air. The up arrows (i.e., RH) represent hydration.

4. Conclusions

The TEM and SEM observations both showed that the morphology of PBAPs were unique; they differed markedly from that of the sulfate, mineral, soot, organics, and metal particles in

continental air. Our results indicate that significant amounts of PBAPs are emitted from the Khingan Mountain area. In this study, we establish detailed information that includes the morphology, size, and composition of rod-like PBAPs, fungal spores, and brochosomes. C, N, O, P, K, and Si were detected in most of the PBAPs, and P represented a major marker to discriminate the PBAPs and non-PBAPs. We found that one type of PBAPs mostly appeared as similar rod-like shapes with an aspect ratio > 1.5 and the dominant sizes ranged from 1 μm to 5 μm. The size distribution of the rod-like PBAPs displays two typical peaks at 1.4 μm and 3.5 μm, which likely represent bacteria and fungal particles in the forest air. However, our study shows that there was no clear boundary between bacteria and some fungi from their size because of their size range partly overlapped.

The second most plentiful PBAPs were identified as fungal spores with ovoid, sub-globular or elongated shapes with a smooth surface and small protuberances (apiculus) with size at 400 nm - 7 μm with a mean diameter of 4 μm. Moreover, we found some large brochosomal clusters containing hundreds of brochosomes which have sizes from 200-700 nm and shapes like truncated icosahedrons. We estimated that the mass concentration of PBAPs, mineral dust, and remaining particles accounted for 47%, 43%, and 10% of the $PM_{2.5\text{-}10}$ mass concentration, respectively, indicating that large boreal forests might represent a major source of PBAPs in the atmosphere. Moreover, there is a higher frequency and concentration of PBAPs at night compared with day. This difference could not be explained by wind speed or temperature, but was explicable by RH, which appears to be critical in enhancing PBAPs emissions from plants at night. The hygroscopic experiment shows that the primary bacterial and fungal particles show weak hygroscopicity.

Author Contributions: WL designed the study. WL, LL, QY, LX, and HY collected aerosol particles. WL, QL, LL, LX, YZ, BW, XD, and JZ contributed laboratory experiments and data analysis. WL prepared the manuscript with contributions from all the coauthors. BW, DH, DL, WH, DZ, PF, MY, MH, XZ, and ZS commented and edited the paper.

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Supplemental Materials

Overview of primary biological aerosol particles from a Chinese boreal forest: insight into morphology, size, and mixing state at microscopic scale

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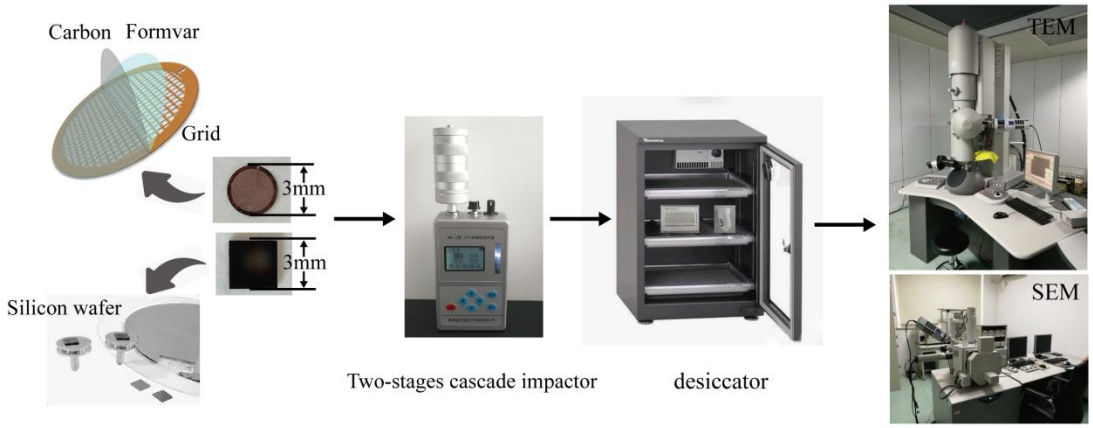

Figure S1 The sampling procedures of substrate, sampler, storage, and analyzed technique.

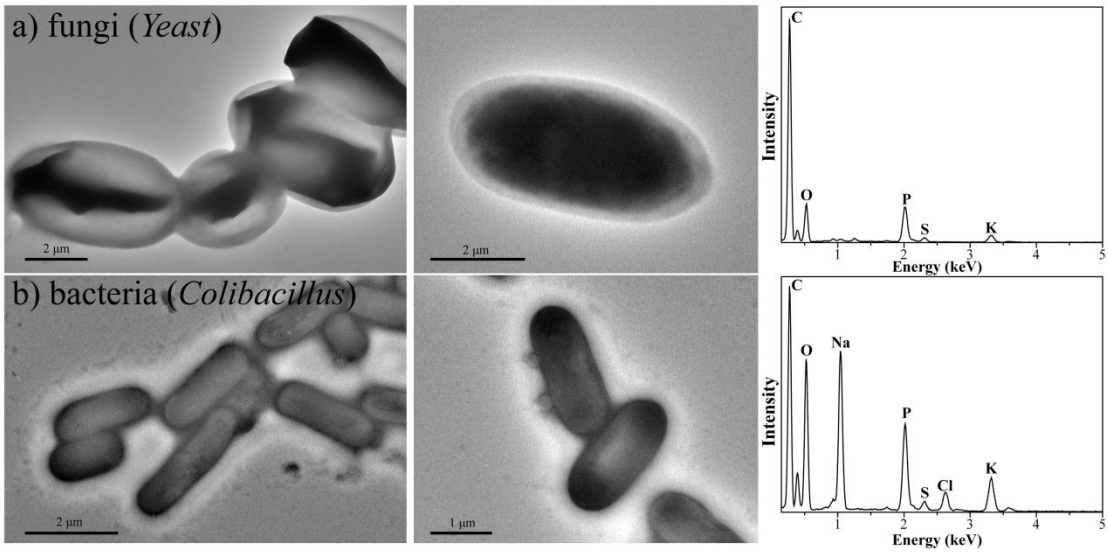

Figure S2 The *Yeast* and the *colibacillus* particles cultivated in laboratory. TEM image showing morphology and EDS showing compositions.

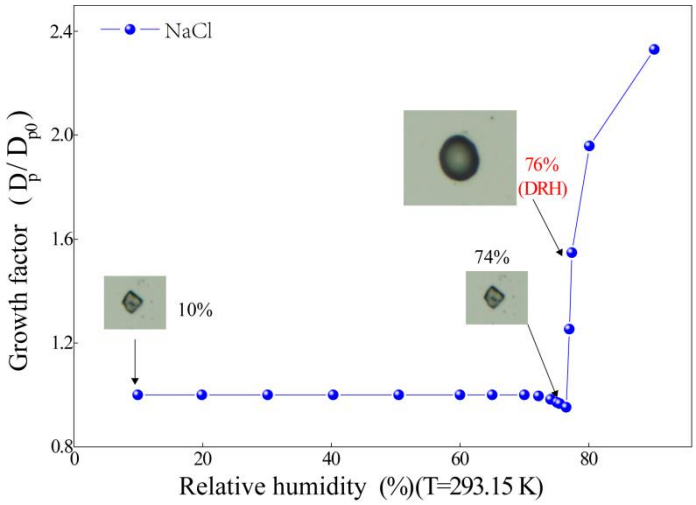

Figure S3 Hygroscopic growth of NaCl generated in laboratory

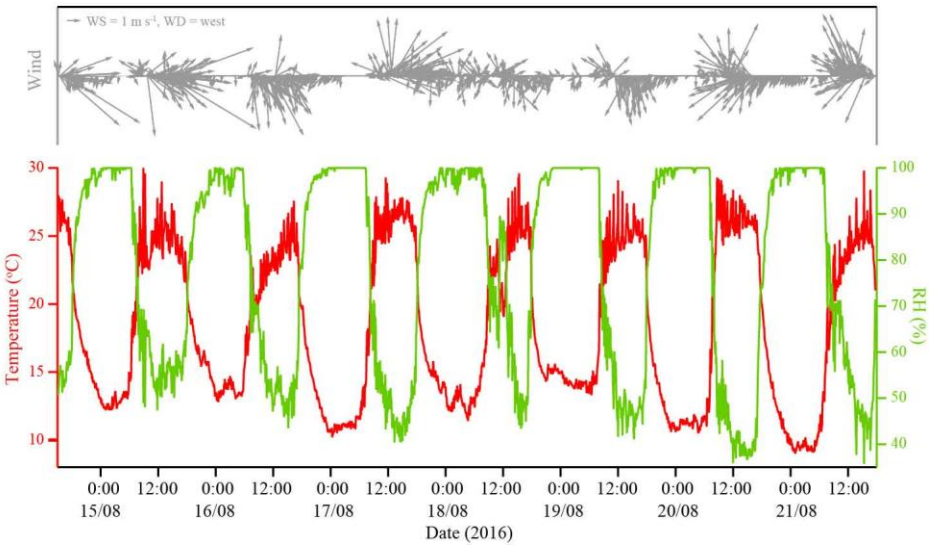

Figure S4 Meteorological data during the sampling including Wind speed and direction, Temperature, and relative humidity (RH).

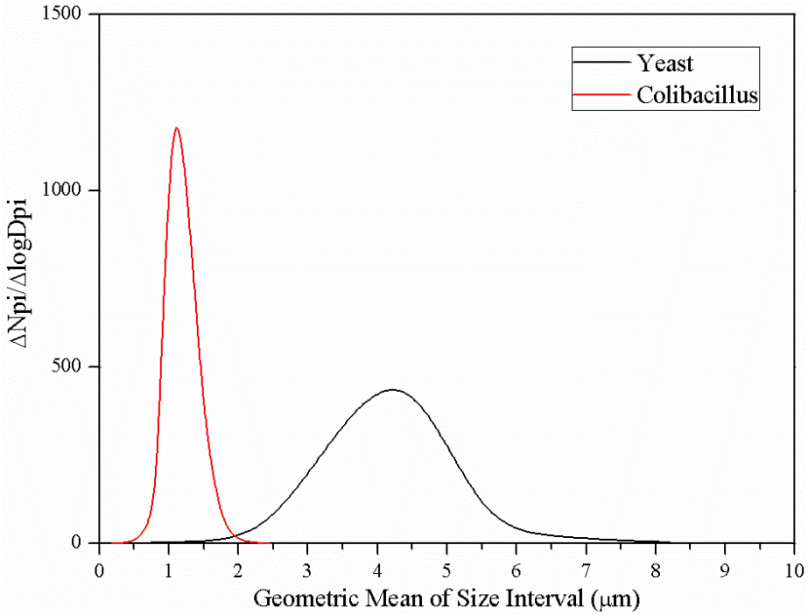

Figure S5 Size distribution of Yeast and Colibacillus cultivated in laboratory.

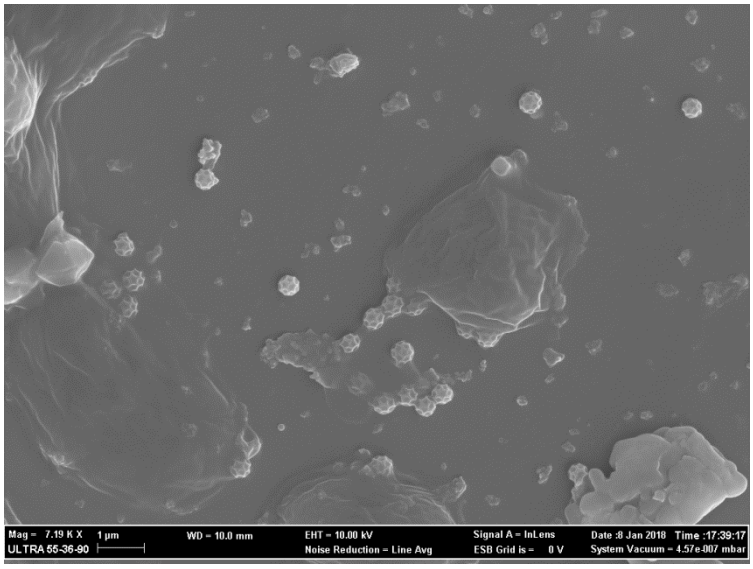

Figure S6 SEM images of brochosomes.

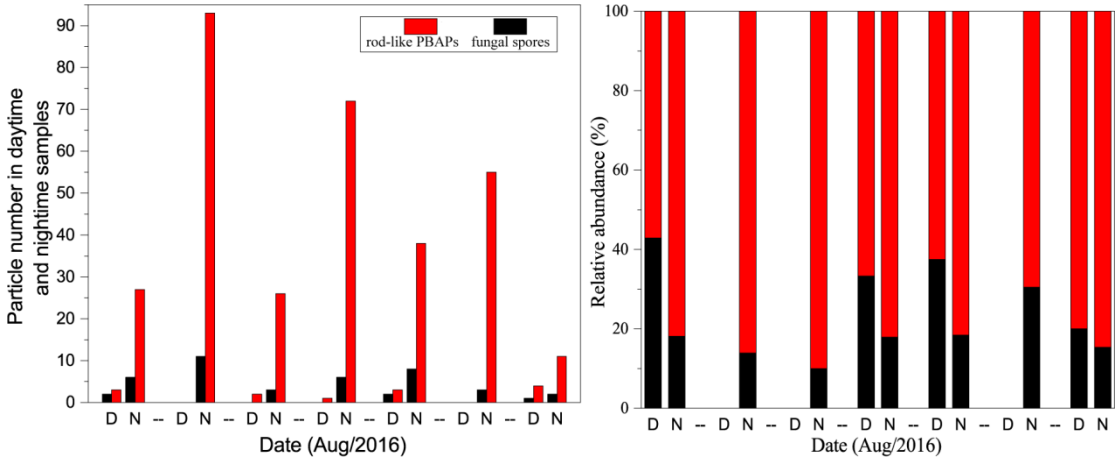

Figure S7 Particle number and relative abundance of rod-like PBAPs and fungal spores in the samples collected in daytime and nighttime.