Fungi Diversity in PM_1 and $PM_{2.5}$ at the summit of Mt. Tai: Abundance, Size Distribution, and Seasonal Variation

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Response to Reviewer 1

The study reported in this discussion paper describes fungal compositions and diversities in airborne PM_1 and $PM_{2.5}$ fractions that were collected at the summit of Mt. Tai, China. The study used quantitative PCR and high-throughput sequencing for the analyses of airborne fungal communities. I have several technical concerns, which are described as follows:

We thank the reviewer for the beneficial comments on our manuscript. We respond to the reviewer comments in detail below. The responses to reviewer are in red.

Major comments

Page 3 Line 21 More detailed information of the air samplers used in this study should be reported. Are they inertial impactors? If so, what is the sharpness of cutoff diameters for each stage? Also, how was particle bounces were prevented from the upper stages? Particle bounce can significantly distort the measured particle size distributions (e.g., Dzubay et al. (1976) Atmospheric Environment 10(3), 229-234). In particular, large particles can bounce from upper stages, because of their large inertia, and can penetrate through impactors and reach to an after filter even though they are not in fine fractions. If impactors were used, please state how particle bounces were prevented.

Response of the authors: The samplers used in this study were inertial impactors. Sampling were conducted by two middle volume air samplers (TH-100A, Wuhan Tianhong Instruments Co., Ltd., China), One of which was equipped with $PM_{2.5}$ fractionating inlet, the second one was equipped with a PM_1 fractionating inlet. Every sampler have only one stage, with the sharpness of cutoff diameters of them were 2.5 μm and 1 μm, respectively. Hence there are no issues for the particle bounces during the sampling periods. We have revises as in Page 3 in Line 21-25:

Two middle volume inertial impactors (TH-100A, Wuhan Tianhong Instruments Co., Ltd., China, flow rate: 100 L min-1) equipped with $PM_{2.5}$ fractionating inlet and PM_1 fractionating inlet were used to collect $PM_{2.5}$ and PM_1, respectively. Sixty samples were obtained on the quartz membrane filters (PALL, NY, U.S., 88mm) for 23 h (9:00 am to 8:00 am next day) over 8-13 days during each season from 2014 to 2015 at the summit of Mt. Tai as shown in Table 1.

Page 4 Line 24 Were the chimeric sequences removed? The researchers reported more than 10% of the ITS sequences submitted to the public archives contained chimeric reads (Nilsson et al., (2015) Microbes and Environments 30(2): 145-150). This might affect the alpha diversity analyses, so it may be better to check.

Response of the authors: The chimeric sequences were removed before alpha-diversity analysis. The sequences submitted to the public archives were the raw fastq files. But before the alpha diversity analysis and statistic analysis, we have trimmed the raw sequences by removing the chimeric sequences, low-quality sequences (length < 200 bp and Q value < 20), and chimera by FASTX-ToolKit (http://hannonlab.cshl.edu/fastx_toolkit) and Usearch (version 7.1 http://drive5.com/uparse/). The raw sequences and valid sequences were shown below.

Table 1 Raw sequences and valid sequences number of samples.

No.	RS	VS	No.	RS	VS	No.	RS	VS
A1	16770	14551	A21	58617	51755	B12	43968	39322
A2	38089	32550	A22	45199	40554	B13	34925	31512
A3	100967	79898	A23	57862	46376	B14	50917	44886
A4	12236	9109	A24	63683	50015	B15	72251	63627
A5	35098	20950	A25	16412	13938	B16	15817	13991
A6	99119	51335	A26	43746	38228	B17	65677	57616
A7	82450	66653	A27	43877	38571	B18	57527	52063
A8	27325	24609	A28	45251	38686	B19	77755	70479
A9	100807	47939	A30	180380	164935	B20	56931	48796
A10	48298	44184	B1	27627	24078	B21	45951	38094
A11	144435	137037	B2	42178	38007	B22	60784	50330
A12	73806	65545	B3	76494	53373	B23	10202	8717

A13	123617	111296	B4	15338	13969	B24	152770	127661
A14	47854	38137	B5	56068	50431	B25	48400	43593
A15	38086	32625	B6	13823	9797	B26	47504	41459
A16	100545	83655	B7	70444	61531	B27	63400	56821
A17	25850	9008	B8	58302	51779	B28	50117	43514
A18	35313	31841	B9	17488	12427	B29	81316	73897
A19	61030	55692	B10	41966	37598	B30	34285	29926
A20	21763	18760	B11	29285	26147			

* RS indicates Raw Sequences number

* VS indicates Valid Sequences number

We have revised as in Page 4 in Line 32-37 and Page 5 in Line 6-7:

Page 4 in Line 32-37:

After high-through sequencing, We removed the chimeric and low-quality sequences by FASTX-ToolKit (http://hannonlab.cshl.edu/fastx_toolkit) and UCHIME algorithm (Edge et al., 2011) before diversity analysis and statistic analysis. The remaining high quality sequences were normalized to 7973 reads in order to compare the different samples effectively and then clustered into Operational taxonomic units (OTUs) at 97% similarity cutoff using USEARCH software (version 7.1, http://drive5.com/uparse/).

Page 5 in Line 6-7:

The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database under accession number SRR5146156.

Page 5 Line 5 How were airborne fungal concentrations calculated? Specifically, how did the investigators confirm or assume DNA extraction efficiency from fungal spores from air filters? It can affect final air concentrations reported.

Response of the authors:

We use the real-time quantitative PCR for ITS region to calculate the gene copy numbers. The fungal concentration were estimated, assuming an average gene copy number of 200 per fungal genome (Lee et al., 2010, Science of the Total Environment 408:1349-1357).

To optimized the efficient of DNA extraction, We modified some steps of laboratory experiment (sample pretreatments, DNA extraction and purification) for the further high-throughput sequencing following Jiang (2015, Nature Protocols 10(5): 768-779). Half of filters (about 121.64cm^2) were cut into small pieces; inserted into 50 ml Falcon tubes filled with sterilized 1×PBS buffer; centrifuged at 200g for 3 h at 4^{o}C; collected the resuspension into a 0.2μm Supor 200 PES Membrane Disc Filter. We cut the PES Membrane Disc Filter into small pieces for DNA extraction. The filters were heated to 65^{o}C in PowerBead tubes for 15 min, vortexing for 15 min. The DNA were extracted according to the standard PowerSoil DNA isolation protocol and purified by AMPure XP bead purification. All the above steps

were carried out in a decontaminated biosafety cabinet. We have revised as in Page 4 in Line 5-13:

To optimized the efficient of DNA extraction, We modified some steps of sample pretreatments and DNA extraction experiments for the sufficient DNA yields following Jiang et al., (2015). Half of filters (about 121.64 cm^2) were cut into small pieces, inserted into 50 ml Falcon tubes filled with sterilized 1×PBS buffer, and centrifuged at 200g for 3 h at 4°C. The resuspension were collected into a 0.2μm Supor 200 PES Membrane Disc Filter. We cut the PES Membrane Disc Filter into small pieces. The filters were heated to 65°C in PowerBead tubes for 15 min and then vortexing for 15 min. The DNA were extracted according to the standard PowerSoil DNA isolation protocol and purified by AMPure XP bead purification.

Page 7 Line 39 Fungi in the class Dothideomycetes, including Alternaria, produce large multicellular spores, with reported spore sizes of 18-83μm – 7-18 μm for Alternaria (Cole and Samson (1984) Mould allergy. Lea & Fibiger: Philadelphia, pp 66–104). It is hard to believe Alternaria was found in PM1 fraction, given with their large spore sizes, and I suspect it might be caused by sampling artifacts (e.g., particle bounces).

Response of the authors: Among all the fungal genus detected, *Alternaria* were common in aerosol particles (e.g. TSP, PM_{10}, $PM_{5.8-9}$, $PM_{4.7-5.8}$, $PM_{3.3-4.7}$, and $PM_{2.5}$) (Yamamoto et al., 2012, The ISME Journal 6: 1801-181; Yan et al., 2016, Frontiers in Microbiology 7: 487; Hwang et al., 2016 Air Quality Atmospheric Health 9: 561-568). In PM_1, Huang (2016, Environmental Pollution 214: 202-210) also reported the existence of *Alternaria* using low-volume samplers (BGI, USA, 16.7 L min^{-1}) in Urumqi, China. In the present study, we collected the PM_1 and $PM_{2.5}$ with two middle-volume air samplers (TH-100A, Wuhan Tianhong Instruments Co., Ltd., China, 100 L min^{-1}). There were no particle bounces during sampling periods. While the existence of *Alternaria* may be influenced by the following reasons:

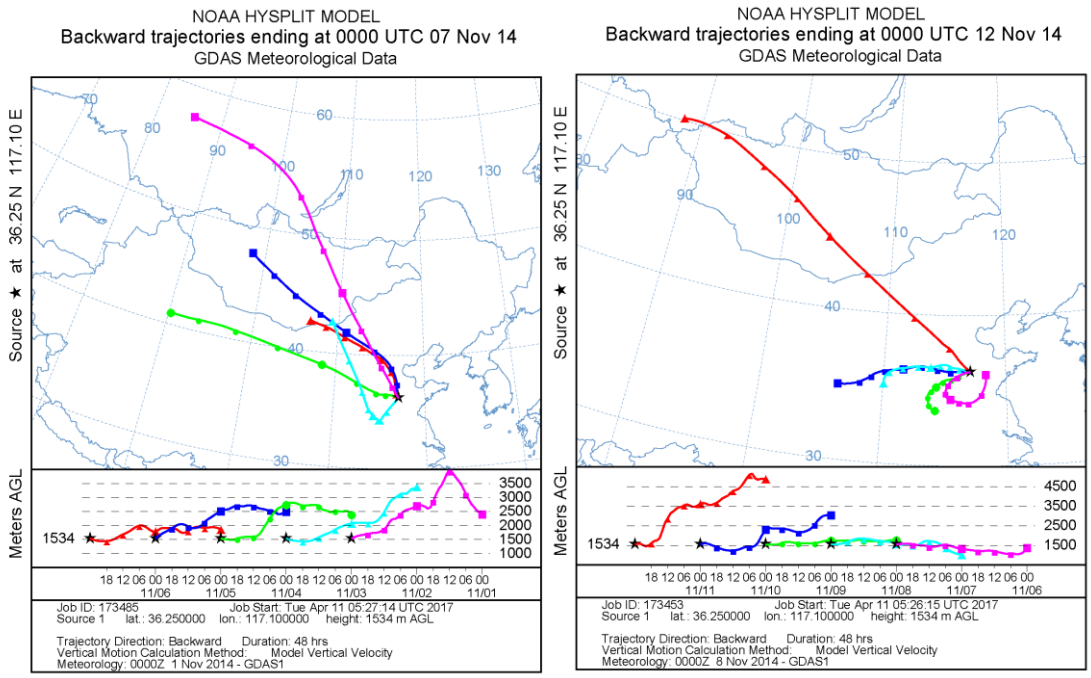
1) The ambient *Alternaria* are ageing and losing their activity following the air parcel movements. The inactive *Alternaria* were broken into small fragments with component size is equal to or lower than 1μm when suspending in the atmosphere. So we obtained the damaging fragments of *Alternaria* in PM_1.
2) Due to the relative higher rate of pump (100 L/min), some intact *Alternaria* were cut into small fragments by sharp PM_1 fractionating inlet before deposited into the quartz membrane filters.

Minor comments

Page 6 Line 25 I could not understand why straw combustion can contribute airborne fungal DNA in PM_1.

Response of the authors: Previously, Huffma (2010, Atmospheric Chemistry and Physics 10(7): 3215-3233) considered the fine fluorescent biological aerosol particle (<1μm) were influenced by long-range transport and anthropogenic sources such as combustion. At the sampling time of Autumn, no obvious straw combustion phenomenon were observed. And we monitored some long-range transported events based on the 48h backward trajectories of air parcels (Figure 1). The long-range transported air parcels mainly derived from outer Mongolia Areas (the famous dustiest place, Nov.6), Siberia regions (Nov 3rd and Nov. 12), and desert region (Nov.5). Influenced by the air movements from desert region, the corresponding fungal abundance in Nov.5 enriched from 6.18 $\times 10^4$ to 103 $\times 10^4$ copy m^{-3} (about 16.7-folds). Similarly, the corresponding fungal abundance influenced by air parcels from Siberia regions enriched from 22.2 $\times 10^4$ and 18.3$\times 10^4$ copy m^{-3} (about 16.7-folds). So we hypothesized that the long-range transport of air parcels from north China may be the possible reason of the fungal enhancement of PM_1.

Figure 1 The 48h backward trajectory of air masses during Nov. 2014.



We have revised as in Page 7 Line 2-14:

In this study, the highest fungal concentration in $PM_{2.5}$ was observed in summer (641 spores m^{-3}), whereas the highest value in PM_1 was found in autumn (1033 spores m^{-3}) indicated different origins of fungal spores. Huffman et al., (2010) found the long-range transport and anthropogenic sources such as combustion have a influence on the fluorescent biological aerosol particle with diameter smaller than 1μm. During sampling time in autumn, no obvious straw combustion phenomenon observed and we detected some long-range transported events in Nov. 2014. The long-range transported air parcels mainly derived from outer Mongolia

Areas (the famous dustiest place, Nov. 6), Siberia regions (Nov 3rd and Nov.12), and desert region (Nov. 5). Influenced by the air movements from desert region, the corresponding fungal abundance enriched from 6.18 × 10^4 to 103 × 10^4 copy m^{-3} (about 16.7-folds). Similarly, the corresponding fungal abundance influenced by air parcels from Siberia regions enriched to 22.2× 10^4 and 18.3× 10^4 copy m^{-3}, respectively. So we hypothesized that the long-range transport of air parcels from north China may contributed to the fungal enhancement of PM_1.

Page 7 Line 5 Do the authors believe 0.067% and 0.096% contributions truly nonnegligible ?

Response of the authors: Previously, the tracer-based methods were used for the fungal OC concentration based on micro-tracer levels such as polysaccharides, phospholipids, mannitol, proteins and ergosterol. Herein we calculated the fungal OC concentration according to the mannitol levels. The comparison with previous investigation used the same tracer were shown as below:

Sampling Site	Sample Type	Fungal OC concentration	Fungal OC contribution to OC	Reference
Rinnbockstrasse, Austria	PM_{10}	0.3μg m^{-3}	8%	Bauer et al., 2008
Schafberg, Austria	PM_{10}	0.35μg m^{-3}	14%	Bauer et al., 2008
Beijing, China	$PM_{2.5}$	0.3 μg C m^{-3}	1.2%	Liang et al., 2017
Beijing, China	PM_{10}	0.8 μg C m^{-3}	3.5%	Liang et al., 2017
Hongkong, China	$PM_{2.5}$	3.7 ng C m^{-3}	0.1%	Cheng et al., 2009
Hongkong, China	PM_{10}	9.7 ng C m^{-3}	0.2%	Cheng et al., 2009
Mt.Tai, China	$PM_{2.5}$	6.1 ng C m^{-3}		In the present study
Mt.Tai, China	PM_1	8.3 ng C m^{-3}		In the present study

Due to the limited filters for estimation of OC mass concentration in the present study, we compare the fungal OC contribution to the total PM concentration. Assuming that the OC contribution to $PM_{2.5}$ were 17.5% in Beijing (Wang et al., 2015 Environmental Monitoring and Assessment 187(3):143) and 17% in Hongkong (Ho et al. 2004 Atmospheric Environment 38(37): 6327-6335), the percentage of fungal OC to total $PM_{2.5}$ in Beijing (0.069%) and Hongkong (0.00588%) were compared with the value in this study (0.067%). Hence I think the remarkably precise number (0.067% and 0.096%) was believable. Although the fungal spores contributed a minor OC source of $PM_{2.5}$ and PM_1, but it is also can not be ignored. The trace fungal OC were also able to participated in the atmospheric process or involved in the human health. We have revised as in Page7 Line 30-33:

The daily averaged concentrations of fungal OC in $PM_{2.5}$ and PM_1 were 6.1 and 8.3 ng C m^{-3} with the corresponding contribution to PM were 0.067% and 0.096%, indicating that

airborne fungal spores acted as a minor source of carbonaceous aerosol can not be ignored at Mt. Tai.