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Atmospheric Chemistry and Physics Discussions

Interactive comment on "Annual distribution of allergenic fungal spores in atmospheric particulate matter in the eastern mediterranean; a comparative study between ergosterol and quantitative PCR analysis" *by* N. Lang-Yona et al.

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General Comment: The paper is exploiting molecular analytical methods to quantify fungal (general and species specific) prevalence in atmospheric environment. In the analysis, the results of the molecular approach is benchmarking with the 'ergosterol' marker resulted from analytical approach (GC-MS) published by the same group. While the intention to exploit more 'precise' methodologies in quantifying the fungal prevalence is appreciated, the current method comparison seems inadequate. Overall, quality assurance is needed to build confidence on the results reported.

We thank the Reviewer for the comment. Below we address all the comments made and describe the quality control measures taken in the study.

Specific comment: 1. Quality control in interpreting qPCR is very important. Yamamoto et al. (2011) study provided a rather solid and concrete quality control (MDL, COV, etc) studies on house dust. What will be the corresponding MDL, COV for the present study?

Thank you for the comment. We agree with the reviewer that there is a need to detail these important validation parameters in the manuscript. The reproducibility (defined as the overall precision) includes all errors due to sampling, sample preparation, and errors inherent to qPCR. Our previous studies found that qPCR reproducibility for measurements of airborne fungi collected on quartz fiber filters ranged from 61% to 67%.1 Similar reproducibility is expected in this study because identical procedures and methods were used in both studies. For instrument repeatability (precision associated with the analytical instrument), only the error in qPCR is included, and defined as the technical triplicate's median. In accordance with the comment, we have included these values in addition to the calculated method detection limits (MDLs) in the text as follows: P. 8, lines 162-173 in the method section of the revised manuscript: "Quantitative-PCR analyses of the sampled filters were tested for their accuracy, precision, and method detection limits (MDL) as described in Hospodsky et al. (2010) and Yamamoto et al.(2011).1,2 Briefly, accuracy was determined by efficiencies in DNA extraction from sampling filters, and 10% of DNA extraction efficiency was used for fungal cells collected on quartz fiber filters.1 Precision was defined as instrument repeatability as coefficients of variations (COVs) based on triplicate measurements for the study samples by qPCR. The qPCR MDL was defined as the smallest fungal concentration where seven successful PCR amplifications out of seven trials (p = 1/128) could be observed to detect a microorganism concentration with 99% confidence.2 These values were converted into corresponding MDLs in air concentration (cell m-3) with information of the DNA extraction efficiency (= 10%) and the collected air volume,

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i.e. 4881.6 m3 (72-hr sampling with 67.8 m3 h−1)." P. 9, lines 181-191, in the result section: "For quality control, analyses were performed to each of the genomic assays, precision was defined as instrument repeatability based on triplicate qPCR measurements of the samples. Median values of instrument repeatability also taken as COVs, were 59%, 19%, 31%, 21%, 33%, 34%, and 13% for FF2/FR1, Aaltr, Afumi, Cclad2, Enigr, PenGrp3, and PenAsp1mgb, respectively. The qPCR MDLs for FF2/FR1, Aaltr, Afumi, Cclad2, Enigr, PenGrp3, and PenAsp1mgb assays were 0.05, 0.28, 0.05, 0.07, 0.07, 2.8, and 0.05 cells per reaction, respectively.2 With information of the DNA extraction efficiency from fungal cells of 10%,1 a sampling volume of 4881.6 m3 (72-hr sampling with 67.8 m3 h−1), and fractions of the DNA extracts and the filter area used for the qPCR analysis, the overall MDLs in air concentration were 8.6, 4.9, 0.9, 1.2, 1.2, 49.3, and 0.9 cell/m3 for FF2/FR1, Aaltr, Afumi, Cclad2, Enigr, PenGrp3, and PenAsp1mgb, respectively." P. 11-12, lines 246-258, in the discussion section: "In quality control analysis1,2 we ensured that our genomic measurements were accurate due to both the correction performed for the DNA extraction efficiency (10%), and the lack of PCR inhibition in our samples. The precision of the measurements was ensured by performing technical triplicates for each of the reactions in the qPCR analysis. While field replicates would enable the definition of the overall measurement precision (including variations associated with air sampling and DNA extraction),2 the variations associated with DNA extraction and air sampling are typically much smaller than those associated with qPCR. Therefore, the qPCR COVs are likely to be good approximations for the overall measurement COVs, including air sampling, DNA extraction, and qPCR. PenGrp3 was the only qPCR assay where many of the sample values were less than the MDL. This is probably due to the relatively low PCR efficiency compared to the other assays. Because this fungal group is scarce, the impact on the overall species distribution in each season is minimal".

2. The conversion of the qPCR results to spore concentration depends on validated calibration curves. While the standard curves of specific allergenic groups can be prepared by using the known species/or member species, how is the total spores using

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the universal fungal primer was referencing to? What are the precision and accuracy will this standard curve be?

We used Aspergillus fumigatus (ATCC 34506) spores as a reference fungal strain for universal fungal qPCR with primers FF2/FR1. In the discussion we described the assumptions made, and addressed the limitations of calibrating different species with one fungal marker, which has the simplest form of one genome per spore (see P. 28700, lines: 2-21 in the ACPD manuscript). It should be noted that bias is unavoidable in studies using PCR amplification owing to variations in copy numbers of target genes. However, special attention was paid to the selection of primers, and the major advantages of selecting FF2/FR1 primers, which were developed by Zhou et al. (2000)3 for use of fungus-specific PCR assay for detecting low-level fungi in an indoor environment, as follows: 1. Zhou et al. (2000)3 reported that FF2/FR1 was the only primer sets, among others tested, that did not show cross-amplification with non-fungal DNA. 2. Shorter amplicon sizes are generally desirable for efficient qPCR. The amplicon size of FF2/FR1 primers is reasonably short (435 bp), and we confirmed PCR efficiency of >95% with the reported PCR conditions.2 3. This primer set targets 18S rRNA genes, with a repetitive nature of rRNA genes (e.g., 38 to 91 gene copies per A. fumigatus cells4). This enables sensitive fungal detection, ideal for air samples studies, in which the fungal quantities are typically much smaller than in other media such as soil and water. 4. Due to the limited seasonal variability observed in the detected species, we specifically assume that calibrating according to A.fumigatus will have the same error percentage for all the measurements, and therefore can serve well for the comparison to the ergosterol analysis. Further investigation on universal fungi quantification is of course needed, in order to perform proper quantification procedures for cases of environments with varying fungal distribution. Precision and accuracy were calculated for this calibration as well, and are now included in the text: P. 9, lines 183-187, in the result section; FF2/FR1 assay for universal fungi: “Median values of instrument repeatability as COVs were 59%, 19%, 31%, 21%, 33%, 34%, and 13% for FF2/FR1, Aaltr, Afumi, Cclad2, Enigr, PenGrp3, and PenAsp1mgb, respectively. The qPCR MDLs for

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FF2/FR1, Aaltr, Afumi, Cclad2, Enigr, PenGrp3, and PenAsp1mgb assays were 0.05, 0.28, 0.05, 0.07, 0.07, 2.8, and 0.05 cells per reaction, respectively.2".

3. It seems that the authors had used two different thermal cycles in running the PCRs. Pg 28694, line 2: initial 20s denaturation and enzyme activation at 95oC, followed by 45 cycles of 3s denaturation at 95oC, and 30s annealing and extension at 60oC; while in line 25 of the same page: 50oC for 2 min, 95oC for 15 min of initial denaturation and 45 cycles at 95oC for 15s dissociation and 60oC for 1 min of annealing and extension. We know that the PCR products depend much on conditions of the thermal cycle. Why there are two different operations and how will this affect the results and their interpretations?

These two analyses for the same filters served as two biological repeats, and were performed at different laboratories (Yale University and Weizmann Institute of Science) with different instruments. Therefore the thermal conditions are not identical. The calibration procedure serves for the elimination of differences in the qPCR amplification performance. We performed statistical analysis on the calibrated results (both performed with the same protocols, with the same fungal specie), and found no significant difference between them. This statistical analysis is presented in P. 28696, Lines 16-17 in the ACPD manuscript: "The two repeated fungal spore concentrations are statistically similar (tested by independent two sample t-test, P -value=0.713)".

Interactive comment on Atmos. Chem. Phys. Discuss., 11, 28689, 2011.