RC1: Anonymous Referee #1, 01 Feb 2026

The study is oriented towards identifying airborne pollen and the authors imply that flow cytometry is an efficient alternative for microscopical identification. It is particularly valuable to see that analysis of flow cytometry measurements seems to enable discrimination between different species of the same plant genus which is usually not possible in routine microscopical analysis. The manuscript is well written, and it clearly describes possibilities of standard flow cytometers for identification pollen.

However, in my opinion the manuscript lacks tests and discussion on the applicability of proposed method (flow cytometry measurement and the developed random forest classification model) for analysing aerosol samples. The authors emphasized importance of adapting the models to real environment samples (lines 259-264). But in my opinion for Atmospheric Measurement Techniques more than just theoretical discussion is needed when linking to atmospheric measurements. Without tests on atmospheric samples, it is just a speculation that proposed approach has a “potential for large-scale urban pollen monitoring”.

We thank the reviewer for this important comment. We acknowledge that the manuscript may overstate the goal of the model developed. We have revised the text to articulate more clearly that this work focuses on developing and validating a robust reference classification framework based on flow cytometry measurements of known pollen taxa. Such controlled conditions are a prerequisite before the method can be applied to atmospheric samples, which are inherently more complex and heterogenous.

L65-67 : We modified to : « Our study aims to develop a classification model capable of identifying pollen from urban environments at species and genus levels. Here we present the necessary first step in the development of a broader methodological pipeline for the analysis of airborne pollen. »

There are several aspects that should be addressed/discussed:

1.How the identification algorithm handles large diversity of aerosols (i.e. fungal spores, dust clusters, starch) which could be in the same size class as pollen. So far, the studies showed that very good classification algorithms in lab settings (as seen in confusion matrix) in real environment tend to fail.

We thank the reviewer for asking this question. We note also that Referee #2 had a similar concern, which further highlights that we need to be clearer on this issue. Prior to model training, we performed a data cleaning step (Figure A2 and Lines 127–131) to separate pollen grains from “debris” classified as non-pollen (other) in our training dataset. To distinguish these two groups, we first used excitation violet laser fluorescence parameters (detectors PB450 and Violet610), while verifying that the distinction held true for the same groups across the other variables. This excitation/emission range is characteristic of sporopollenin which contains fluorophores that in turn are specific to pollen grains [1]. The model is therefore able to identify all particles sharing the fluorescence signature of pollen; everything else is categorised as "other”. Even if some non-pollen aerosols (e.g. fungal spores, dust, starch) may emit autofluorescence in similar wavelengths, the probability that any such particle would simultaneously reproduce the sporopollenin-specific fluorescence signature and all multi-parametric characteristics used by

the model (size, granularity, multi-channel fluorescence) remains very low. The robustness of our approach relies precisely on this combined multi-parametric classification.

[1] Pöhlker, C., Huffman, J. A., Förster, J.-D., and Pöschl, U.: Autofluorescence of atmospheric bioaerosols: spectral fingerprints and taxonomic trends of pollen, Atmos. Meas. Tech., 6, 3369–3392, https://doi.org/10.5194/amt-6-3369-2013, 2013.

In response to the Reviewer's comment, we made the following changes to the manuscript.

L146-147 : We added : « This excitation/emission range is characteristic of sporopollenin which contains the fluorophores specific to pollen grains (Pöhlker et al., 2013). »

L136 we also clarify what we consider as debris : « […] debris, that is non-pollen particles, […] »

The authors claim using flow cytometers could enable near-real-time pollen identification. How the aerosol sample is to be processed to be delivered to the flow cytometer (here bear in mind that sample could contain large particles that might require filtering out to avoid clogging).

We acknowledge that the term "near real-time" may have been an overstatement; we have revised the manuscript to more accurately state that our approach is faster compared to traditional methods, in the lab.

L318 ; 329 : We replaced « near-real-time » by « faster »

Regarding the filtration step, the samples contain a large number of particles, and filtration serves two complementary purposes: first, to retain only particles within the size range of interest (5–100 µm, corresponding to pollen grains), and second, to prevent clogging of the flow cytometer.

In addition to size-based filtering, we took care to minimize pollen grain aggregation by resuspending samples in PBS and stirring them regularly during data acquisition with the cytometer, as pollen grains, particularly the larger ones, sediment rapidly (within less than 30 seconds). This protocol is described in lines 92–94 of the manuscript and in more detail in supplementary material.

Accordingly, L94-98 was modified to « Pollen was extracted from the floral units using a filtration system that retained only particles between 5 and 100 µm in diameter, a size range that includes pollen grains but also particles of similar size. Filtration also prevents clogging of the flow cytometer because, as is generally recommended, particles should not exceed one-third to one-fifth of the width of the flow cell, which limits particle size to approximately 100 µm on the CytoFLEX instrument we used. »

We have also added these clarifications to the detailed protocol in supplementary material.

2. Also related to previous item please discuss to what extent the flow cytometry approach could be challenging for quantification. For example, some devices can handle up to 200 microliters of sample for a single analysis which then requires either subsampling from larger volumes in which aerosols are collected or sample should be concentrated.

Instruments such as the CYTOFlex can accommodate volumes up to 5 mL and allow consecutive tube addition.

In our study, samples were centrifuged and resuspended in PBS to limit aggregation. We typically started with approximately 200 µL of resuspended sample and further diluted it when an excessively high particle concentration was observed during sample analysis on the flow cytometer. When necessary, multiple tubes were run per sample, and the resulting counts were summed to obtain the final value for each sample.

These precisions have been added to the detailed protocol in supplementary material.

The approach to rely pollen identification exclusively on flow cytometry measurements that most cytometers routinely used in healthcare is very important. But the use of the same classification algorithm on different devices (even the same model) appeared to be challenging (as authors also clearly noted in lines 273-281). If not possible to test the model on different device measuring same parameters, the authors should at least discuss the measurement uncertainty for each parameter and refer to other studies that observed differences in flow cytometry parameters between different devices.

We agree that absolute signal intensities in flow cytometry are device-dependent and that model transfer across platforms without correction could fail. Our intended operational scenario is deployment of harmonized analyzers of the same model, under bead-based daily QC and targeted detector settings, procedures that are now standard practice in clinical research labs. Under such procedures, studies have demonstrated that comparable readouts can be achieved between instruments, including within a given model/brand and, with specific workflows, even across brands with different optical trains [1].

[1] Solly F, Rigollet L, Baseggio L, et al. Comparable flow cytometry data can be obtained with two types of instruments, Canto II, and Navios. A GEIL study. Cytometry Part A. 2013;83(12):1066-1072.

In response to the comment, we modified L297-307 to add "However, deployment within harmonized analyzers is feasible under standard bead-based daily QC protocols (CS&T/Application Settings for conventional analyzers; SpectroFlo QC beads for spectral systems), which have been shown to control inter-instrument MFI drift within single-digit percentages (Cornel et al., 2020; Omana-Zapata et al., 2019; Solly et al., 2013b). Channel-wise normalization during data processing further reduces residual variability, and a lightweight domain-adaptation step, based on acquiring a small reference pollen set on the target instrument, can re-anchor feature distributions prior to inference. FSC and SSC parameters remain more sensitive to flow-rate and optical alignment and should therefore be monitored carefully. Where direct comparison between instruments is required, ERF/MESF calibration from NIST's Flow Cytometry Standards Consortium allows comparing fluorescence results between different instruments (Wang and Hoffman, 2017)."

In line 133 authors indicated the training dataset for *Thuja* genus was impossible to clean from debris. Is presence of debris confirmed by microscope? If not, how can you be sure it is not a part of the normal pollen variability? The pollen from Thuja (and many other Cupressaceae) tends to break in wet environment resulting in separation of exine from the resto of pollen grain. Could it be that those separated exines are the "debris" you see in the data.

We confirmed by microscopy that intact pollen grains and debris, mainly cone debris, were indeed present in our samples. We observed by microscopy that the majority of pollen grains were not broken. Pollen grains tend to break apart when they have been stored for too long in a humid environment (more than approximately 6 months), which was not the case here. However, regardless of the cause, in our case, we were unable to distinguish the pollen grains from other debris present in our Thuja samples and were therefore unable to include them in the model training.

L273 : We added « we had visually confirmed the presence of intact pollen grains »

In Table A1, authors reference is missing for an accurate scientific name (e.g. *Ambrosia artemisiifolia* L.). Genus should be written in cursive font and also should include author references

Thanks for the comment; it has been updated in the revised manuscript, see Table A1.