Reply to the review by Anonymous Referee #1 for the manuscript, "Merging holography, fluorescence, and machine learning for in situ, continuous characterization and classification of airborne microplastics" by N. D. Beres et al.

We thank the anonymous reviewer for their thorough and thoughtful responses and recommendations to improve the manuscript. Below, questions and comments by the reviewer are in blue and the responses by the manuscript authors are in black.

Page 3, line 75 on statement "Some commercially available polymers have previously been examined for their autofluorescence": It might be helpful for the readership to add a few sentences already in the introduction on the molecular motifs that are responsible for the fluorescent emission. Especially for the non-aromatic polymers, which one typically would not consider efficient fluorophores, the observed strong emission reported already in previous studies is an interesting phenomenon.

The authors agree that this could be helpful for readers. We've modified the introduction (Section 1) to include some information about the reasons for fluorescence in polymers:

"An often-overlooked material property of airborne microplastics that has the potential to specify particle type is their natural ability to fluoresce, or autofluoresce, which results from the spontaneous emission of light at one wavelength by the fluorophores (molecule or compound capable of fluorescence) of the polymers from excited electromagnetic states when exposed to higher-energy, lower-wavelength light (Lakowicz, 2006). For polymers, this can be due strictly from their molecular structure containing contain aromatic rings, conjugated double bonds, or other fluorophores, from stabilizers, additives, or impurities unintentionally added to the substance during the polymerization process or after production, or by some combination thereof."

We've also included some context to fluorescence by non-aromatic polymers by modifying the discussion (Section 4): "Additionally, polymers which lack aromatic or highly conjugated double bond structures (i.e., PA, PMMA, PP, and PE) are not traditionally associated with strong autofluorescence (Shadpour et al., 2006); nonetheless, PA, PMMA, PP, and PE microplastics used in this study displayed fluorescence intensities on the same order as the primary biological particles tested. These results may suggest the presence of other factors that contribute to their measured fluorescence, such as the unintended presence of impurities or additives (i.e., non-intentionally added substances; (Bridson et al., 2023)). Additionally, while polyolefins like polyethylene (PE) and polypropylene (PP) do not contain fluorophores in their chemical structure, photo- or thermal-oxidation (Allen et al., 1977; Zhao et al., 2022), impurities (Bridson et al., 2023; Laatsch et al., 2023), fibers structural defects (Poszwa et al., 2016), or formation of high molecular weight (HMW) clusters (Laatsch et al., 2023) can cause PE and PP to become fluorescent. For example, during the photo-

oxidation process, enones and dienones can be formed (Allen et al., 1977), which makes those polymers gain fluorescent properties."

Page 3, line 82 on statement "No study has used the intrinsic fluorescence of polymers for airborne particle identification and characterization in situ." I think the preprint by Gratzl et al., 10.26434/chemrxiv-2023-qzhr8 could/should be cited in this context.

The authors thank the reviewer for suggesting this preprint. We have removed the statement on Line 82 ("No study has used the intrinsic fluorescence…") and added the following to the Introduction (Section 1) to acknowledge the work by Gratzl et al.:

"One recent work has shown the promising ability to classify airborne MPs using their autofluorescence (Gratzl et al., 2024). Here, Gratzl et al. (2024) leverage the Wideband Integrated Bioaerosol Sensor (WIBS; Droplet Measurement Technologies, Longmont, CO, USA) to detect microplastics based on specific fluorescence signatures excited at two wavelengths and detected in two emission wavelength bands."

Page 3, line 90 on statement " … assess the fluorescence response of various common microplastics": I wonder why polystyrol was not included here. It is widely used probably also shows a characteristic fluorescence due to the aromatic structure. Along these lines and in more general terms, according to which criteria were the five polymers selected.

Polystyrol (or "polystyrene") was unfortunately unavailable to the authors during data collection, and it was chosen to work with the five polymers we had available to us at the time rather than increase the complexity of this feasibility study. We agree with the reviewer that polystyrene is likely to have a strong fluorescence signal due to it's aromatic nature, prevalence as an environmental pollutant, and is an important component to include in future studies.

The five polymers tested in our study were chosen based on a combination of commercial availability and those which are likely to be found in the environment. We agree that more testing is needed, expanding the breadth of polymer types as well as non-polymer types. We have addressed this need for more testing in the discussion (Section 4).

The study by Ornik et al. should be cited (and probably also discussed) somewhere https://doi.org/10.1007/s00340-019-7360-3

We thank the reviewer for suggesting this study be included in our manuscript. We have included the following information in our manuscript's introduction (Section 1):

"Ornik et al., (2020) examined the fluorescence spectra of eight, large commercially obtained polymer samples – including polypropylene, polyethylene, polyethylene terephthalate, and two polyamides – and demonstrated that their emission spectra is generally distinguishable from non-polymer samples. They acknowledged that these same

principles can be applied to microplastics of various sizes and shapes, while leveraging advanced analysis methods, such as machine learning, for high accuracy classification."

Table 1: Did the authors receive any information on the age of the pollen samples? Such commercially available biological reference substances are not necessarily freshly collected, which brings up the question on how atmospherically representative the derived fluorescence signals are.

The authors acknowledge the spectrum of atmospheric relevancy of particles used in this study (Section 2.2, Lines 154-157; Section 4). The primary focus of the current manuscript is to gauge the SwisensPoleno's response to various microplastics as a promising first step towards identifying and characterizing these particles in near real-time.

While commercially available biological reference substances, like pollen, are widely used in laboratory settings, factors such as age and storage conditions (e.g., humidity) may influence their fluorescence properties. For example, Pöhlker et al. (2013) state (Page 3373), "…the fluorescence properties of commercially obtained and freshly harvested pollen samples are overall similar, except for increasing intensity with age, and that all samples are generally comparable." Understanding the effect of the age of the pollen samples used in the present study is outside the scope of this work; rather, the usage of pollen was to (Line 191), "assess the ability for the instrument to distinguish aerosol particle types beyond those previously analyzed with the SwisensPoleno."

However, while not addressed in this study, the authors agree that future work should assess how the SwisensPoleno's fluorescence response, specifically, is affected by different variables, such as the source and age of various pollen taxa, including both commercially available reference pollens and freshly collected samples. We have added an additional statement in the discussion that includes the recommendation that future studies should consider age of pollen as a variable in fluorescence response in the SwisensPoleno: "While not addressed in this study, future work should assess how the SwisensPoleno's fluorescence response is affected by different variables, such as the source and age of various pollen taxa, of both commercially available reference pollens and freshly collected samples".

Page 8, line 235 on statement "Further details about the SwisensPoleno fluorescence measurement system can be found in (Graf et al., 2023).": It is a pity that the Graf et al. reference is not available yet. It is cited few times and could be quite useful for a better understanding of the fluorescence response of the instrument. If the publication of this study still needs some time, it might be worth to put some relevant information still in this study to provide the reader a more comprehensive understanding of the technique.

The authors agree that, without the Graf et al. reference, a more comprehensive explanation of the fluorescence technique used in the SwisensPoleno instrument is needed. We will add a section to the Supplemental Information that provides an overview of the fluorescence

system within the SwisensPoleno instrument. In addition, we have changed the statement on Line 235 to account for this: “Further details about the SwisensPoleno fluorescence measurement system can be found in the supplemental information.”

Page 11, line 296 on statement “Here, the water dataset is used as a proxy for the baseline fluorescence response of the instrument”: Is this the standard procedure to determine the background or is there a force trigger function as commonly used for the WIBS?

The SwisensPoleno data processing algorithm uses an ultrapure water dataset as a thresholding dataset; it does not use a force trigger function as is commonly used for the Wideband Integrated Bioaerosol Sensor (WIBS). This water dataset is then used to threshold the baseline fluorescence response during the routine processing of the measured data, because, as was stated (Line 297), “…ultrapure water is expected to have no detectible autofluorescence beyond instrument background signal”. As stated in the previous reviewer response, further explanation will be included regarding the fluorescence system of the SwisensPoleno in the Supplemental Information, which will also elucidate this point. The statement on Line 296 “Here, the water dataset is used as a proxy for the baseline fluorescence response of the instrument…” will be removed for clarity.

Page 14, line 345 on statement “The relative fluorescence spectra for MPs exhibit a noticeably higher response in the λex/λem=280/357 nm channel compared to other particles tested”: What is the molecular explanation for this spectral feature?

As outlined in the response to the reviewer’s first comment, fluorescence emission spectral features in the examined MPs, in general, are due to their chemical structure, which may be efficient at absorbing UV wavelength light (Lionetto et al., 2022), such as aromatic functional groups or other conjugated bond systems. The presence of impurities, additives, or other substances within plastic may also alter the fluorescence response (Bridson et al., 2023; Laatsch et al., 2023). External factors, such as photo- or thermal-oxidation may alter the polymer’s chemical structure, leading to the introduction of oxygen-containing groups (i.e., carbonyls) which may change their fluorescence characteristics. This is important, because photooxidation may occur to airborne microplastics through atmospheric aging (Ouyang et al., 2021), for example. Monteleone et al. (2021) showed that thermal treatment also alters the fluorescence characteristics of various polymers, which may modify detection abilities or spectral response in fluorescence spectroscopy applications, such as the SwisensPoleno. While future work can focus on understanding the specific excitation and emission wavelength bands that can be targeted towards environmental MP detection, delving too deep into the molecular explanation of this response is beyond the scope of this feasibility study.

References:

Allen, N. S., Homer, J., and McKellar, J. F.: Origin and role of the luminescent species in the photo-oxidation of commercial polypropylene, J Appl Polym Sci, 21, 2261–2267, https://doi.org/10.1002/app.1977.070210823, 1977.

Bridson, J. H., Abbel, R., Smith, D. A., Northcott, G. L., and Gaw, S.: Release of additives and non-intentionally added substances from microplastics under environmentally relevant conditions, Environmental Advances, 12, 100359, https://doi.org/10.1016/j.envadv.2023.100359, 2023.

Gratzl, J., Seifried, T. M., Stolzenburg, D., and Grothe, H.: A fluorescence approach for an online measurement technique of atmospheric microplastics, Environmental Science: Atmospheres, https://doi.org/10.1039/D4EA00010B, 2024.

Laatsch, B. F., Brandt, M., Finke, B., Fossum, C. J., Wackett, M. J., Lowater, H. R., Narkiewicz-Jodko, A., Le, C. N., Yang, T., Glogowski, E. M., Bailey-Hartsel, S. C., Bhattacharyya, S., and Hati, S.: Polyethylene Glycol 20k. Does It Fluoresce?, ACS Omega, 8, 14208–14218, https://doi.org/10.1021/acsomega.3c01124, 2023.

Lakowicz, J. R.: Principles of Fluorescence Spectroscopy, 3rd ed., edited by: Lakowicz, J. R., Springer US, 954 pp., https://doi.org/10.1007/978-0-387-46312-4, 2006.

Lionetto, F., Lionetto, M. G., Mele, C., Corcione, C. E., Bagheri, S., Udayan, G., and Maffezzoli, A.: Autofluorescence of Model Polyethylene Terephthalate Nanoplastics for Cell Interaction Studies, Nanomaterials, 12, https://doi.org/10.3390/nano12091560, 2022.

Monteleone, A., Brandau, L., Schary, W., and Wenzel, F.: Using autofluorescence for microplastic detection – Heat treatment increases the autofluorescence of microplastics, Clin Hemorheol Microcirc, 76, 473–493, https://doi.org/10.3233/CH-209223, 2021.

Ornik, J., Sommer, S., Gies, S., Weber, M., Lott, C., Balzer, J. C., and Koch, M.: Could photoluminescence spectroscopy be an alternative technique for the detection of microplastics? First experiments using a 405 nm laser for excitation, Applied Physics B, 126, 15, https://doi.org/10.1007/s00340-019-7360-3, 2020.

Ouyang, Z., Zhang, Z., Jing, Y., Bai, L., Zhao, M., Hao, X., Li, X., and Guo, X.: The photo-aging of polyvinyl chloride microplastics under different UV irradiations, Gondwana Research, https://doi.org/10.1016/j.gr.2021.07.010, 2021.

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.

Poszwa, P., Kędzierski, K., Barszcz, B., and Nowicka, A. B.: Fluorescence confocal microscopy as effective testing method of polypropylene fibers and single polymer composites, Polym Test, 53, 174–179, https://doi.org/10.1016/j.polymertesting.2016.05.025, 2016.

Shadpour, H., Musyimi, H., Chen, J., and Soper, S. A.: Physiochemical properties of various polymer substrates and their effects on microchip electrophoresis performance, J Chromatogr A, 1111, 238–251, https://doi.org/10.1016/j.chroma.2005.08.083, 2006.

Zhao, Y., Long, J., Zhuang, P., Ji, Y., He, C., and Wang, H.: Transforming polyethylene and polypropylene into nontraditional fluorescent polymers by thermal oxidation, J Mater Chem C Mater, 10, 1010–1016, https://doi.org/10.1039/D1TC05520H, 2022.