Response to referee comments and suggestions on amt-2017-407 by Könemann et al.

Manuscript format description:

Black text shows the original referee comment, blue text shows the authors response, and red text shows quoted manuscript text. Changes to the manuscript text are shown as *italicized and underlined*. We used bracketed comment numbers for referee comments (e.g., [R1.1]) and author's responses (e.g., [A1.1]). Line numbers refer to the discussion/review manuscript.

Anonymous Referee #2

Received: 16 April 2018

General comment:

The detailed analysis of steady-state fluorescence properties of PSL particles presented in this article will be useful to researchers using LIF-based measurement technologies. This article does make a useful practical contribution to the field. However, the discussion has the opportunity to go further in presenting and discussing results on single-particle fluorescence, which is likely to be what the majority of researchers reading the article will be interested in.

The Excitation-emission matrices obtained by the bulk analyses and the associated discussion are informative with respect to PSL characteristics and excitation/emission bands and as a guide for selection of optimal PSL spheres for a particular instrument/experiment. It is also important to highlight the influence of the detergents and additives as has been done in this work.

Author response: We want to thank Referee #2 for his/her overall positive assessment. Changes and additions are described in the following paragraphs.

Specific/technical comments:

[R2.1] However, the reported results are difficult to translate into practically useful information about how the PSL spheres would appear in a LIF-based single-particle instrument like the WIBS-4A, which is

the one used in this study. The extensive measurements of bulk solutions that are discussed in the article are accompanied by measurements carried out with the WIBS-4A on a select sample of PSL spheres, but the article in its current form has missed the opportunity to extend the discussion on how the intensities of PSL spheres measured by a single particle LIF instrument compare to those of real-world bioaerosols.

[A2.1] We appreciate the referee's comment and clarified the text accordingly. Generally, we would like to point out that quantitative comparisons of fluorescence intensities – particularly between different techniques/instruments (e.g., fluorescence spectrometer vs. WIBS-4A) – are a complex task. To state this clearly, we added the following statement to (P4, L10-17):

"Since the size-dependence of fluorescence intensity on single particle scale is crucially important for LIF-based PBAP detection (Hill et al., 2015; Sivaprakasam et al., 2011; Swanson & Huffman, 2018), we further address selected aspects of the PSL size-intensity relationship. However, it is important to note that a comparison of fluorescence intensities from different instruments (e.g., offline spectroscopy and microscopy as well as online WIBS-4A measurements) is not trivial as it depends on the properties of the fluorescent particles, on one hand, and on the optical design and detector settings of the instruments, on the other hand. Therefore, we discuss certain intensity-related aspects here semi-quantitatively, whereas an in-depth analysis of single particle fluorescence intensities is beyond the scope of this work."

In this context we also refer to the following section in (P15, L13-19), which emphasizes that comparison of intensity levels should be made with great caution:

„Summarizing the fluorescence distributions in this way can enable a comparison of similar PSL fluorophores to be compared across instruments as a very rough intensity check. These data may be used not only by WIBS users, but also by users of other UV-LIF instrumentation who may use such data for fluorescence calibration, instrument alignment, and excitation pulsing. Nevertheless, comparing the specifics of the intensity values (e.g., Table 3) across different studies should be treated with extreme caution. A number of instrumental factors, including gain settings of detectors used

for fluorescence detection, can significantly influence observed fluorescence intensities, making direct comparisons, even within similar instrumentation, challenging at best."

In order to circumvent this intrinsic complexity in terms of comparability of fluorescence intensities, we conducted WIBS measurements of selected PSLs samples, which actually is an experimental "translation" of the offline bulk into online single particle data. Accordingly, for the selected PSL samples, Table 3 shows what the referee requests, namely "how the PSL spheres would appear in a LIF based single-particle instrument" as suggested by the Referee. To work towards a more general "translation" of the offline bulk into online single particle properties, we added further data on the PSL size vs. fluorescence intensity relationship as outlined in detail in [A2.6] below. By combining the WIBS-derived data on the selected PSL samples in Table 3, the EEMs in Fig.1-3 and the size-intensity relationship in Fig. 5 and S3, WIBS-derived intensities can at least be estimated.

In terms of translating PSL fluorescence intensities into fluorescence intensities of "real-world bioaerosols", we agree with the referee that this aspect was not initially clear enough in the manuscript. In a recent study, Savage et al. (2017) measured a broad extend of both non-biological materials and organisms with the WIBS-4A (actually the same WIBS unit that was used in this work). Therefore, the WIBS-derived fluorescence intensities reported here can be compared to the large data set in Savage et al. (2017), which allows to put the PSL results into the context of the extended library of reference materials, which can be regarded as good approximation of "real-world" bioaerosol particle types. To clarify this aspects, we added the following statement to (P15, L19-25):

"In this context it is worth noting that the data reported here was recorded with the same WIBS unit used by Savage et al. (2017). Moreover, Savage et al. (2017) measured three PSL samples (2.0 µm Green, 2.0 µm Red, 2.1 µm Blue – same type and manufacturer, however different batches as those used in present work) and the obtained results agree with the results in Table 3. This suggests that the WIBS derived PSL fluorescence intensities in Table 3 can be compared to the fluorescence

intensities of the extent library of fluorescent materials and standard organisms in Savage et al. (2017).”

[R2.2] Saturation of the intensity signal is a non-trivial problem. The fluorescence intensities presented in Table 3 indicate that PSL spheres of diameter 4.52 micrometrres that are ostensibly non-fluorescent according to the manufacturer nevertheless causes saturation of the detector in the FL1 channel of the WIBS-4A. Saturation also occurs in channel FL2 with 3.1 micrometres PSL spheres and in channel FL3 with 2.1 micrometres PSL spheres. These observations deserve a lot more comment than what they receive in the article. The implication of the saturation in FL1 is that even non-fluorescent particles are recorded as fluorescent by the WIBS-4A if they are above a certain size and contain even a negligible amount of fluorophore. This suggests that either the PSL spheres were contaminated or the authors have identified an important issue with the WIBS-4A related to the detector calibration settings. This needs some further comment. In the case of the other two channels, the PSL spheres did contain fluorophores, but the particles were an order of magnitude smaller than many important bioaerosols.

[A2.2] Indeed, the observation that the use of 4.52 µm non-fluorescent PSLs results in a WIBS detector saturation is remarkable and important. By using PSLs straight out of stock solutions, a potential contamination is unlikely.

As stated in the manuscript, the manufacturer term “non-fluorescent” is misleading in this case due to the distinct emission of the polystyrene matrix if excited at wavelengths < 300 nm. We use this terminology, even as a misnomer, because of its common use by the manufacturer and thus within the community. Even if the emission signal is considered to be rather low (compared to signals derived from fluorescence dyes), it cannot be ruled out that the polystyrene emission saturates the detector at larger PSLs sizes. The following text was inserted for clarification (P15, L26-31 / P16 L1-9):

“As outlined in Sect. 3.3, the single particle fluorescence intensity increases steeply with particle size. Accordingly, comparatively large PSLs saturate the WIBS detector at some point depending on the detector gain settings. For the WIBS settings used here, saturation occurs for PSL sizes

>2 µm. Specifically, the main mode of 2.1 µm Blue PSLs saturates channel FL3, the minor mode of 3.1 µm Yellow Green PSLs saturates FL2, and 4.52 µm non-fluorescent PSLs saturate FL1. Obviously, PSLs that tend to saturate the WIBS detector are inappropriate for fluorescence intensity checks and spectral validations. For routine performance checks using the WIBS settings outlined in Sect. 2.5, we recommend the use of 1.0 µm Blue, 2.0 µm Green, and 2.0 µm Red PSLs. Crucially important for LIF users is the observation that nominally non-fluorescent PSLs indeed show fluorescence emission in the UV range that can be strong enough to even saturate LIF instrument detectors (i.e., the WIBS FL1 channel). Although weak in comparison to the PSL fluorophore modes (Fig. 3), the responsible polystyrene/detergent signal becomes dominant for large PSL sizes due to the steep intensity increase with particle size. The issue of fluorescence intensity calibration within UV-LIF instruments is sufficiently important and problematic that it has been discussed by a number of authors. In particular, Robinson et al. (2017) developed a fluorescence calibration strategy for WIBS channels FL1 and FL2. The issue requires continued attention, however, from the UV-LIF community."

[R2.3] This observed saturation suggests the question of whether the WIBS-4A is too sensitive for real bioaerosols of size 20-30 micrometres when a 2-micrometre PSL sphere saturates the detector or a 4.5 micrometre particle saturates it even without fluorophore content. When supposedly non-fluorescent particles that are not even big compared to many bioaerosols can saturate the detector, it becomes impossible to distinguish between biological and non-biological aerosols on the basis of fluorescence intensities in real-world environments.

Therefore, the discussion on fluorescence intensity per sphere could be extended, the article has the opportunity to start a discussion on fluorescence intensities and particle size with respect to both PSL spheres and real bioaerosol, which would be useful technical information for researchers employing LIF-based measurements in the study of bioaerosols. Instruments that are calibrated using PSL spheres (that may not even be in the relevant size range) are probably not appropriate for real bioaerosols in all cases.

[A2.3] As stated in [A2.1], a "translation" of PSL fluorescence properties into naturally occurring fluorescence emissions from bioaerosols is not directly implementable. A detector saturation caused

by PSLs, which are fully doped with fluorescent dyes and, therefore, high emission values, does not strictly imply a hypersensitivity for real-world bioaerosols. For example, as shown in Savage et al., 2017 (using the same WIBS-4A unit as in this study), only pure biofluorophores (e.g., Tryptophan and Tyrosine), few pollen (sizes between ~ 15 and 20 µm) and fungal spores show detector saturation effects, while the majority of measured particles (across a broad size distribution) can be displayed within all three detection channels. We have added the following text (P18, L18-23):

"Nevertheless, even if PSLs serve as a simple, but easy to apply approach for routinely sizing, spectral and rough fluorescence intensity validations of LIF instruments, they cannot be compared to the complex nature of spectral information derived from bioaerosols in environmental systems. By comparing bioaerosol data from Hernandez et al., 2016 and Savage et al., 2017, fluorescence intensity values of PSLs, fully doped with fluorescent dyes, will very likely exceed the emission intensity of bioaerosols of equal sizes in most cases."

More importantly, however, the Referee is correct in pointing out the problem in trying to detect fluorescence from a 2 µm and a 20 µm particle with the same instrument. Even this range represents factor of 10 in diameter, translating to a factor of 10^3 in volume and probably fluorescence intensity. This range of fluorescence intensities begins to stretch the dynamic range of detector at a given gain setting. As a result, the current generations of the WIBS are indeed not able to sensitively detect fluorescence for small particles and big particles at the same time, and so a given size range (and matching range of fluorescence intensities) must be pre-determined by manually setting the detector gain using a set screw inside the case of the WIBS. There has been work to utilize detector gain-switching modes (i.e. Healy et al., 2012 who investigated properties of fluorescent PSLs using high and low gain modes of the WIBS), and this is a feature in some other commercially available UV-LIF instruments. A thorough comparison of the specific detector settings of the various versions of the WIBS and other UV-LIF instruments is beyond the scope of the present manuscript, however. We have added text to the manuscript, as mentioned here (P16, L9-12):

"It is also important to mention that previous work using the WIBS-4 utilized detector gain-switching, which allowed detection of highly fluorescent or large (low gain) particles along with weakly fluorescent or small (high gain) particles (Healy et al., 2012). *This feature is not present in the WIBS-4A commercialized by DMT, Inc., but is being explored by more recent generations of various UV-LIF instruments."*

[R2.4] Dependence on particle size in bulk solution is not particularly instructive in this context, because the measurements in that situation relates to the total amount of fluorophore in the cuvette.

[A2.4] In terms of fluorescence intensity, this is true. Note that it is not the primary aim of the data in Figure 2 to retrieve size-intensity relationships. Instead, the primary aim for performing those measurements was to ensure that derived fluorescence spectra (i.e., position and shape of fluorescence modes) do not alter as a function of size, disregarding fluorescence intensity properties. More details on this can be found in [A1.1]. We have revised the manuscript as outlined in [A1.1] to clarify this aspect.

[R2.5] The work also involved fluorescence microscopy measurements that do report fluorescence intensity per sphere. However, the intensities in the fluorescence microscopy experiments cannot be compared to the detector responses in a single particle instrument like the WIBS-4A for the same spheres, as units are arbitrary and recorded intensities are dependent on detector settings in the individual instruments.

[A2.5] What the referee states is certainly true. The comparison of measured fluorescence intensities is a complex task, as the details of the optical setup and settings of the detectors etc. must be taken into account. We did not suggest any quantitative relationship between the WIBS and microscopy single particle data. Instead the microscopy data in Fig. 5 fulfills the purpose to verify that size and fluorescence intensity distributions are comparatively narrow. Moreover, the data allow to draw conclusions on size-intensity relationships for the PSL species (see our responses to comments [R2.1]). We have added the following text for clarification (P4, L12-17):

"However, it is important to note that a comparison of fluorescence intensities from different instruments (e.g., offline spectroscopy and microscopy as well as online WIBS-4A measurements) is not trivial as it depends on the properties of the fluorescent particles, on one hand, and on the optical design and detector settings of the instruments, on the other hand. Therefore, we discuss certain intensity-related aspects here semi-quantitatively, whereas an in-depth analysis of single particle fluorescence intensities is beyond the scope of this work."

[R2.6] As shown in figure 5B, the fluorescence intensity of a fluorescent particle increases with size of the particle, and the dependency appears to be linear. If fluorescence intensity in single particles shows a linear dependence on size within a given type of PSL sphere or bioaerosol, then there is valuable information in the size-normalised fluorescence intensity that can aid bioaerosol identification. If, however, the detector is saturated, then the information value in the size-normalised fluorescence intensity is lost. Based on the figures reported in Table 3, it is likely that the majority of bioaerosols would saturate the WIBS-4A detector and the information would be lost.

This may not be the case, but the article has an opportunity to address this question based on the experiments carried out; it would increase the relevance of the article if it included a table or estimate of fluorescence intensities or fluorophore concentrations and comparing to bioaerosols or at least discussing whether the fluorophore content in the PSL spheres is appropriate for real bioaerosols, also with respect to size.

This would enable other researchers in the field to use the data to estimate optimal detector settings in their instruments in order to measure real bioaerosols without saturating. Of course, the fluorophores in the PSL spheres would have different concentrations and fluorescence quantum yields from biological fluorophores like tryptophan/NADH in real bioaerosols, but having the information would nevertheless enable researchers to make a more informed decision than is currently possible, and the article would have greater potential interest than it does in its current form.

[A2.6] Due to the narrow scales in Fig. 5B, the size-intensity erroneously appears to be linear. We extended the microscopy analysis and fitted the resulting scatter plots with a power law relationship.

The results suggest a quadratic to cubic relationship. The corresponding text section in (P14, L23-27) has been revised and extended from:

"The fluorescence intensity increases with increasing PSL size (Fig. 5B), as expected due to the increasing amount of fluorophore being excited, resulting in a higher photon flux (e.g., Hill et al., 2015; Sivaprakasam et al., 2011, Swanson & Huffman, 2018). However, even if fluorescence intensity values are not consistent within one PSL batch, the influence on fluorescence calibration of LIF instrumentation will be minor, since the calibration is usually based on the integration over hundreds or thousands of homogenous PSLs."

To (P14, L28-30 / P15, L1-4):

"*The fluorescence intensity increases with increasing PSL size (Fig. 5B) due to the increasing amount of fluorophore being excited. The relationship of the measured intensity, I, and particle diameter, D, can be described by a power law fit* $I = A + BD^y$*, typically with* $2 \leq y \leq 3$ *(e.g., Hill et al., 2015; Sivaprakasam et al., 2011, Swanson & Huffman, 2018). Hill et al. (2015) reported that small and/or slightly absorbing particles typically show y approaching 3 (i.e., volume-dependence), whereas rather large and/or absorbing particles show y approaching 2 (i.e., surface-dependence). The microscopy-based results obtained here agree well with this y range: For 2.1 µm Blue PSLs we observed* $y = 2.6 \pm 0.5$ *and for 2.0 µm Green PSLs we observed* $y = 2.9 \pm 0.2$ *(Fig. S3).*"

We agree that further analyses of the size-intensity relationship of standard particles as well as authentic bioaerosols will likely help to improve bioaerosol detection. Clearly, detector saturation hampers this approach. Since PLS spheres are highly doped with fluorophore, their fluorescence intensities typically exceed intensities of bioaerosol particles of comparable size. Accordingly, we are confident that most bioaerosol classes range below the detection limit of the WIBS. Evidence is provided by Savage et al., 2017, providing an extended library of standard materials and particles and showing that most of these materials/particles do not saturate the WIBS detector. However, a

certain levels of saturation – particularly for very large particles such as pollen – is unavoidable. Additionally, we believe that the text added in [A2.3] contributes to this topic.

[R2.7] Table 3 also reports standard deviations of the intensities in each channel for different spheres. Presumably, this is related to the Gaussian distribution that is referred to on page 9, line 8, but this should be clarified. Furthermore, standard deviations for the intensities are reported also at saturation levels, but once the detector saturates, one cannot know by how much the signal exceeds saturation and consequently it makes no sense to give a range above the saturation point.

[A2.7] In Table 3, we changed detector saturation events from arbitrary units to the term "Saturation".

References:

Healy, D. A., O'Connor, D. J., & Sodeau, J. R. (2012). Measurement of the particle counting efficiency of the "Waveband Integrated Bioaerosol Sensor" model number 4 (WIBS-4). *Journal of Aerosol Science*, *47*, 94–99. https://doi.org/10.1016/j.jaerosci.2012.01.003.

Hernandez, M., Perring, A. E., McCabe, K., Kok, G., Granger, G., & Baumgardner, D. (2016). Chamber catalogues of optical and fluorescent signatures distinguish bioaerosol classes. Atmospheric Measurement Techniques, 9(7), 3283–3292.

Perring, A. E., Schwarz, J. P., Baumgardner, D., Hernandez, M. T., Spracklen, D. V., Heald, C. L., ... & Fahey, D. W. (2015). Airborne observations of regional variation in fluorescent aerosol across the United States. Journal of Geophysical Research: Atmospheres, 120(3), 1153-1170

Robinson, E. S., Gao, R.-S., Schwarz, J. P., Fahey, D. W., & Perring, A. E. (2017). Fluorescence calibration method for single-particle aerosol fluorescence instruments. *Atmospheric Measurement Techniques*, *10*(5), 1755.

Savage, N. J., Krentz, C. E., Könemann, T., Han, T. T., Mainelis, G., Pöhlker, C., & Huffman, J. A. (2017). Systematic characterization and fluorescence threshold strategies for the wideband integrated bioaerosol sensor (WIBS) using size-resolved biological and interfering particles. Atmospheric Measurement Techniques, 10(11), 4279.