

### Instantaneous Microbial Detection Systems for Water Monitoring

New Rapid Microbiological Method (RMM) instruments permitting the instantaneous detection of microbes in water are emerging for use in the assessment of pharmaceutical water quality. Online and offline systems provide an excellent risk management tool, permitting the routine analysis of pharmaceutical grade waters throughout distribution loops, storage tanks, and at points of use via conventional point sampling or continuous monitoring. These Laser Induced Fluorescence (LIF)-based RMM instruments utilize the simultaneous detection of Mie scatter for particle presence, and intrinsic fluorescence for biologic status, to provide results in real time, without the need for staining, reagents or sample manipulation.

### System Validation and End-User Testing

Customer assessment of system performance and appropriate methods for system validation are an important topic for new technologies. Commercial products such as bioMérieux's BioBall® and Microbiologics EZ-Accu Shot™ offer very precise and easy-to-use microbial inoculations in the form of a lyophilized pellet. These products are excellent options for use in growth promotion testing, sterility assurance testing, antimicrobial effectiveness testing, and other applications where a precise microbial concentration is desired. Yet, these products are not well suited for use in the validation or assessment of LIF-based RMM instruments due to significant background particulate and fluorescence present in the lyophilized pellet matrix.

Off-the-shelf lyophilized pellets contain the microorganism of interest in addition to other materials required for the freeze-drying process, such as cryoprotectants and lyoprotectants like serum. Such materials cause background particulate interference when analyzed using a flow cytometer and also fluoresce at excitation wavelengths, such as 405nm, used with LIF-based RMM systems for water. If water-for-injection (WFI) or ultrapure water (UPW) is utilized to rehydrate these pellets (as opposed to the manufacturer's rehydration fluid), background particulate and fluorescence may be reduced but the pellet matrix itself still has a significant particulate load.

### Particulate and Fluorescence Analysis of Lyophilized Microbial Pellets

An investigation was performed to evaluate the suitability of lyophilized microbial pellets (i.e. BioBall

and EZ-Accu Shot), for validating or accurately assessing LIF-based RMM instruments. A Rion KS-42B liquid particle counter (LPC) measured the particle levels present in a blank BioBall (a BioBall with all components except microorganisms), *B. subtilis* BioBall (NCTC 10400), *P. aeruginosa* EZ-Accu Shot (ATCC 9027), and two pure spore samples (*B. subtilis* from MesaLabs, SUS 1A-6, and *B. atrophaeus* from NAMS, SUN-07). The pure spore samples (*B. subtilis* and *B. atrophaeus*) were suspended in deionized (DI) water to minimize background particulate. Figure 1 shows results for the total liquid particle count  $\geq 0.3\mu\text{m}$  and culture results in colony-forming units (CFU) for these samples. The CFU results were obtained using manufacturer's recommendations for incubation. The BioBall samples were rehydrated in WFI, and the EZ-Accu Shot in 0.22  $\mu\text{m}$ -filtered DI water. Even without any microorganism present, the BioBall blank contained a significant number of particles, and no CFU as expected. Particle counts are similarly high for the *B. subtilis* BioBall and *P. aeruginosa* EZ-Accu Shot with levels at least three orders of magnitude greater than the number of CFU making up these pellets. The pure *B. subtilis* and *B. atrophaeus* samples, by comparison, were found to contain CFU counts on the same order of magnitude as the particle counts indicating minimal background material.

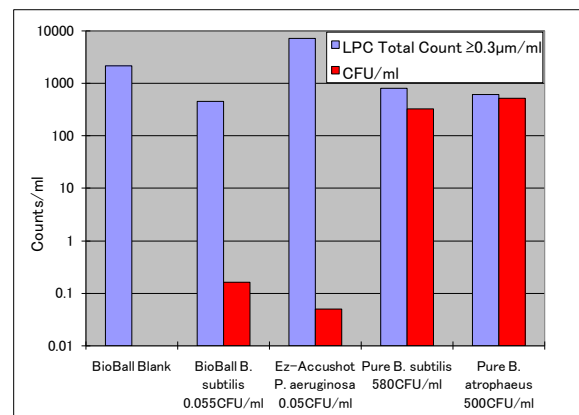


Figure 1: Test comparing the total liquid particle count  $\geq 0.3\mu\text{m}$  to culture results in CFU for a BioBall blank, *B. subtilis* BioBall sample, *P. aeruginosa* EZ-Accu Shot sample, and pure (spores in DI water only) suspensions of *B. subtilis* and *B. atrophaeus*. Only the pure spore samples exhibited CFU concentrations on the same order of magnitude as particles, indicating minimal extraneous material. The high number of particles in the BioBall blank, and low CFU to particulate ratio for BioBall and EZ-Accu Shot, indicated a significant level of background particles in lyophilized microbial pellets.

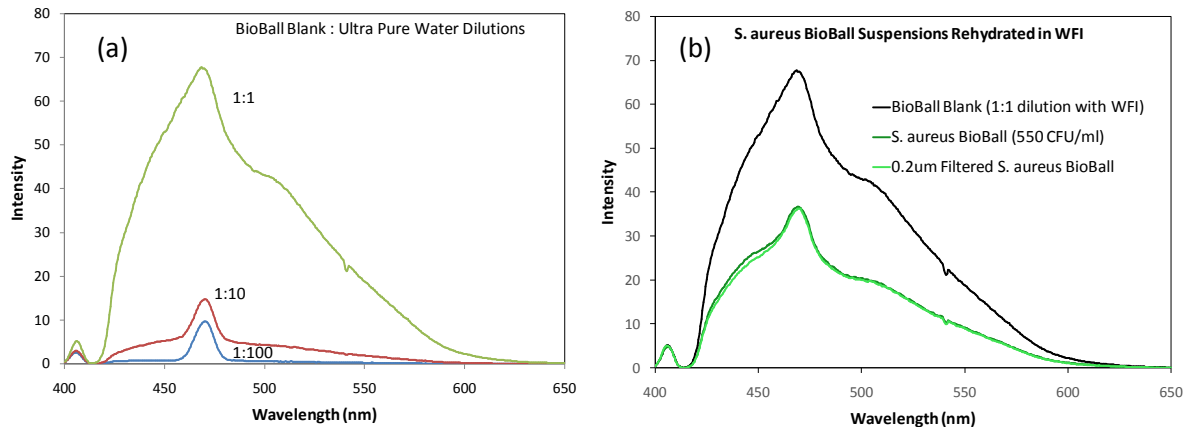
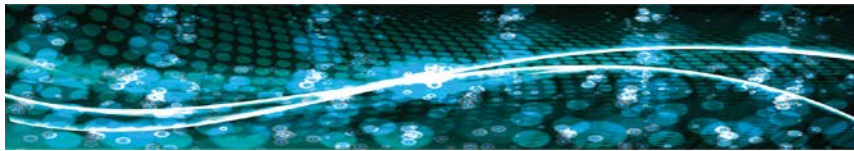


Figure 2. (a) Fluorescence spectra of three BioBall blank dilutions, and (b) the fluorescence spectrum of the 1:1 BioBall blank dilution in WFI compared to a 0.2 $\mu$ m filtered *S. aureus* BioBall suspension and non-filtered *S. aureus* BioBall suspension. Both graphs indicate the BioBall blank matrix has significant bulk fluorescence compared to the fluorescence of the microbes alone.

To assess sample fluorescence, a Jasco spectrofluorometer was utilized to compare the fluorescence of WFI, DI water, the BioBall blank, *B. subtilis* and *S. aureus* BioBall (NCTC 10788) suspensions, and pure *B. subtilis* and *S. aureus* samples. This data compared the lyophilized pellet background fluorescence to that of the microbes themselves. It was also important to investigate whether a decrease in blank pellet concentration could mimic fluorescence decreases from a microbial pellet concentration, which may generate misleading results to the end user about a LIF-based system's sensitivity. Figure 2 (a) shows the fluorescence of three different BioBall blank dilutions, and (b) the fluorescence of the BioBall blank suspension compared to 0.2 $\mu$ m-filtered and unfiltered *S. aureus* BioBall suspensions. In Figure 2 (a), the highest intensity distribution is a 1:1 dilution of the rehydrated BioBall blank suspension with WFI (the dilution recommended by the manufacturer). A 1:10 dilution of the rehydrated BioBall blank suspension with WFI resulted in a significantly decreased fluorescence intensity, followed by the 1:100 dilution. In Figure 2 (b) the 1:1 BioBall blank suspension was compared to a filtered and non-filtered *S. aureus* BioBall suspension, also diluted in WFI. The BioBall blank fluorescence distribution was similar in both spectral shape and magnitude to that of a microbe-containing BioBall, like *S. aureus*. Furthermore, minimal difference was seen between the emission spectra of filtered and unfiltered *S. aureus* BioBall suspensions (filtering should essentially remove all *S. aureus* organisms). This indicated that bulk fluorescence of the microbes is significantly lower than the BioBall matrix. Furthermore, the BioBall blank dilution results mimic what may be expected on a LIF-based RMM instrument from the dilution of a microbial BioBall,

confirming risk to accurate LIF-based RMM system assessment.

Pure *B. subtilis* and *S. aureus* samples were compared to analogous BioBall suspensions. As mentioned above, the pure samples were suspended in DI water only to remove extraneous material. Figure 3 compares the fluorescence spectra of WFI, DI water, the *B. subtilis* BioBall suspension, and pure *B. subtilis* suspension. Note the peak at 405 nm was due to stray excitation light, and had a magnitude that was related to the concentration of the suspension. The Raman water peak has also been labeled in this figure (Raman fluorescence should be excluded in instantaneous detection systems). The *B. subtilis* BioBall fluorescence was significantly higher than the pure *B. subtilis* suspension, as would be expected based on previous data.

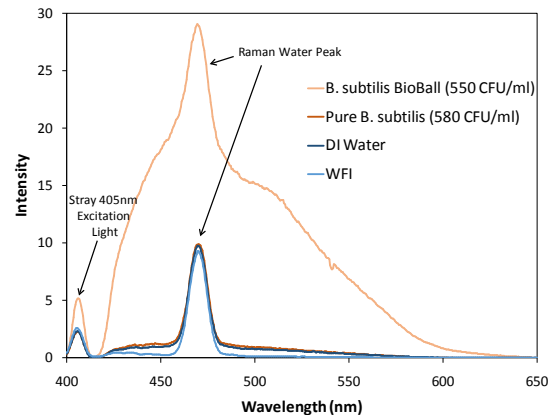
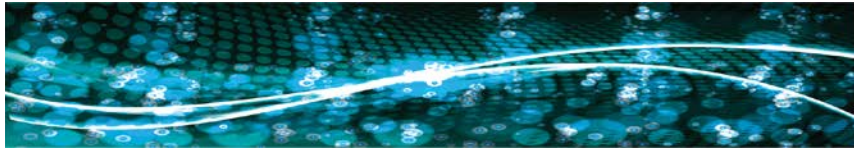


Figure 3: Fluorescence spectra of a *B. subtilis* BioBall suspension, pure *B. subtilis* suspension, WFI and DI water. The pure *B. subtilis* suspension exhibits little fluorescence over the background DI Water, confirming weak bulk fluorescence of the microbes alone.



Furthermore, the pure *B. subtilis* fluorescence intensity and spectrum were very similar to that of the DI water utilized to create the suspension. As corroborated in Figure 2 (b), the fluorescence of the pure *B. subtilis* microbes was very weak, and significantly less intense than the BioBall spectrum.

Figure 4 shows a comparison of two pure *S. aureus* suspensions of different concentration. The 525 CFU/ml *S. aureus* suspension has a similar spectrum to *B. subtilis* (at 580 CFU/ml). When the concentration was increased by approximately seven orders of magnitude to  $2 \times 10^9$  cells/ml, the fluorescence spectrum became more pronounced (500~550 nm), showing the significant microbial concentration required for intensities approaching those of the BioBall blank and BioBall microbial suspensions.

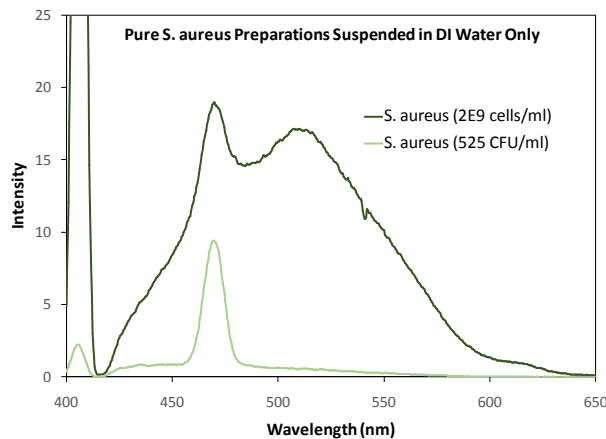


Figure 4: Fluorescence spectra of *S. aureus* suspensions at concentrations of 525 CFU/ml compared to 2E9 cells/ml.

## Conclusions

It has been found that lyophilized microbial pellets such as BioBall and EZ-Accu Shot are inappropriate for use in the validation or performance assessment of an instantaneous microbial detection system for water. Using such materials at different dilutions displayed an expected reduction in particle counts, likely with a low percentage of biologics, due to the presence of background particles in the pellet matrix. The background matrix also is strongly fluorescent in the same spectral region as microbes, which poses a strong interferent risk to the accurate quantification of microbes. While it is important to distinguish bulk fluorescence as measured in this study from individual particle fluorescence as measured by LIF-based RMM systems, a fundamental benchmark for RMM instrument performance should be established without background particles/fluorescence before any such impure assays are evaluated. Washing and preparing bacteria, spores, and other microorganisms in WFI or filtered DI water removes background particulate and fluorescence from materials such as agar, broth, or serum. Companies such as NAMSA and MesaLabs also offer a number of organisms suspended in DI water only. Use of such pure suspensions permits an accurate assessment of system sensitivity, which is not possible with current off-the-shelf lyophilized pellets. For more information and a procedure for such preparation, please contact Azbil BioVigilant.

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<sup>i</sup> C.A. Morgan et. al., "Production of Precise Microbiology Standards Using Flow Cytometry and Freeze Drying," Cytometry Part A, 62A:162-168 (2004).