
# Detection of *Bacillus subtilis* Endospores
## Using an Online Water Bioburden Analyzer

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## Abstract

Bacterial endospores represent a significant challenge to the pharmaceutical industry due to their presence in the environment, resistance to many commonly used inactivation procedures, and difficulty in culturing using traditional plating methods. This may result in the inadvertent release of contaminated products that may present health concerns for the patient. As a result, the use of a Biofluorescent Particle Counter (BFPC) may prove advantageous for the detection of both water-borne and air-borne endospores as their detection is not dependent on traditional culturing methods. In this study, we investigate the ability of an online water bioburden analyzer (OWBA), a specific class of BFPC, to detect *Bacillus subtilis* endospores in pharmaceutical-grade water and present the results as auto-fluorescence units (AFUs) per *B. subtilis* spore. The endospores were a commercial-grade spore preparation and were previously quantified per manufacture recommendations. The results show that the OWBA can detect *B. subtilis* endospores with an accuracy of 1.02 AFU per spore at a target of 100 endospores per mL. Additionally, the limit of detection was determined to be 1 spore/mL with a linearity greater than 0.9025 up to a concentration of 500 endospores/mL. This data shows that OWBAs are a rapid and effective tool for the detection of bacterial endospores in pharmaceutical waters.

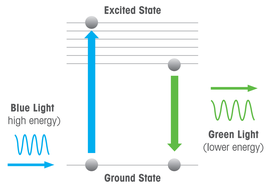
## Learning Objectives

- Bacterial endospores are of high concern in the pharmaceutical industry due in part to their difficulty in detection using traditional plating methods.
- The use of an OWBA overcomes this limitation as it does not rely on the ability to culture the endospore for detection.
- The results show that an OWBA can detect endospores with an accuracy of 1.02 AFU/spore, a limit of detection of 1 spore/mL, and linearity in detection from 1 spore/mL to 500 endospores/mL.

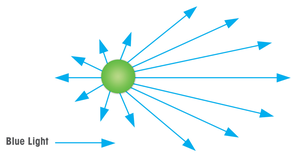
## Introduction

A Biofluorescent Particle Counter (BFPC) is a device that detects and counts microparticles based on their biofluorescent properties. A BFPC that measures biofluorescence of microparticles in an online water source is called an Online Water Bioburden Analyzer (OWBA). The OWBA utilizes two established optical measurement techniques, Laser-Induced Fluorescence (LIF) and Mie scattering and combines them in a unique manner to detect microorganisms in high purity waters.

The Mettler Toledo OWBA uses a 405nm laser to illuminate a flow cell. Microorganisms present in the online water pass through the flow cell and are illuminated by the laser light resulting in both fluorescence emission and Mie scattering. The fluorescence of microorganisms, resulting primarily from Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H) (NADH) and riboflavin, is captured by the fluorescence detector while Mie scattering, which does not result in a change to the light wavelength, is captured by a separate detector. If the fluorescence and Mie scattering signals meet specific criteria at the same it, this is an indication that a microorganism is present and an Auto Fluorescent Unit (AFU) is reported.




**Laser Induced Fluorescence (LIF):** Molecule is excited to a higher energy level laser light source, then releases that energy by emitting fluorescence




**Mie Scattering:** Molecule absorbs light/photons and scatters them at different distances based on their size

The OWBA has a distinct advantage over compendial methods in the monitoring of a pharmaceutical water source:

- Monitoring and reporting is real-time, allowing for a faster response to bioburden excursion.
- Continuous process trending provides the opportunity to reduce sanitization frequency, lowering costs and mitigating wear and tear on water system components.
- Real-time process surveillance ensures water system control, increasing product safety.
- Enabling reduced sampling and lab-based testing.

OWBAs have been shown to be capable of detecting a wide range of microorganisms including Gram-negative and Gram-positive bacteria, yeasts, and molds. However, some concern exists if they can efficiently detect bacteria endospores. The concern is that endospores are not metabolically active and, as such, do not contain the fluorescent metabolites known to be involved in microorganism autofluorescence. Additionally, the primary components of the bacteria endospore are not known to possess fluorescence properties compatible with an OWBA.

The purpose of this study is to investigate the ability of an OWBA to detect a variety of concentrations of bacteria endospores and determine the linearity, accuracy, and limit of their detection.

## Experimental Parameters

A purified, commercial preparation of *Bacillus subtilis* (CrossTex) was used for all the experiments. The spore solution was plated to determine an accurate spore concentration and a spore stain was performed to determine purity. Microscopic images confirm that the spore solution is pure, and free of cellular debris.

To investigate the ability of the OWBA to detect *B. subtilis* endospores, solutions of endospores at 1, 10, 100, and 500 CFU/mL in Ultra Pure Water (UPW). The spore solutions were analyzed through the OWBA in sample mode, collecting three 50-mL samples of each concentration to determine an average AFU/mL reading. This experiment was repeated a total of three times.

For each experiment, the spore solutions were quantified by performing filtration plate analysis, in triplicate, for each dilution. The bacteria filters were plated on R2A media and incubated at 30°C for 48 hours.

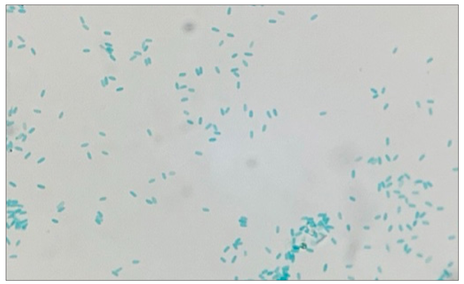
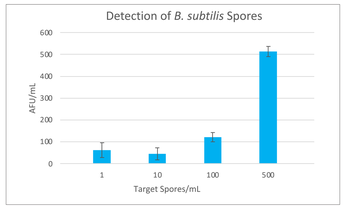
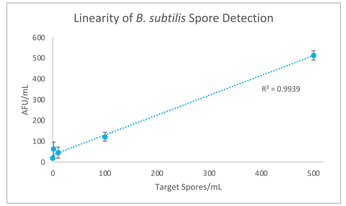


**Image of *B. subtilis* spore stain.**



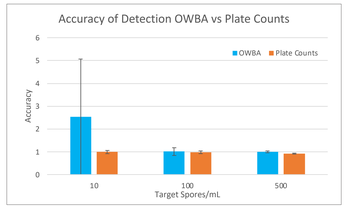

**Detection of *B. subtilis* endospores**

Detection of *B. subtilis* endospores with the Mettler Toledo OWBA. The *B. subtilis* endospores were diluted to 1, 10, 100, and 500 CFU/mL. The target concentrations were verified by membrane filtration plate counts. Results show that detection of endospores at concentrations as low as 1 CFU/mL was observed with increasing detection observed with increasing concentrations. For this testing, two separate OWBAs were used for testing across three separate experiments.




**Linearity of *B. subtilis* Spore Detection**

Linearity of *B. subtilis* spore detection. Results show that the linearity ($R^2$ value) for this is 0.9939. These results show that the OWBA is capable of detecting *B. subtilis* endospores and this detection demonstrates superior linearity across the entire tested range of spore concentrations.




**Accuracy of *B. subtilis* Spore Detection**

Accuracy of *B. subtilis* spore detection comparison between OWBA and the compendial plate count method. Accuracy of the OWBA is defined as AFU/mL to actual CFU/mL. Plate count accuracy was defined as Actual CFU/mL to target CFU/mL. Results show that the accuracy of detection of the OWBA was not inferior to that observed with plate counts at 100 and 500 CFU/mL targets. Interestingly, the accuracy of detection of the OWBA at 10 CFU/mL was much higher than plate counts (~2.5 AFU:CFU). This is an observation that has been made consistently with all spore testing. These results suggest the detection of *B. subtilis* endospores by the OWBA is non-inferior to that of plate counts. The OWBA detection is in real-time, allowing for a more rapid and effective response to any excursions, while the detection of endospores can take up to seven days before any response can be initiated.

## Conclusion

The release of contaminated products due to the presence of bacterial endospores is a significant challenge to the pharmaceutical industry. As endospores can persist in a wide range of environments, early detection is critical. The current compendial method, plate counts, can require up to seven days before results are obtained. Furthermore, there is a significant probability of human contamination occurring from taking grab samples. Additionally, plate counts are often associated with false-positive and false-negative results depending on media type and incubation temperature. Utilizing a specific class of BFPC such as OWBA to detect microorganisms in pharmaceutical-grade water would alleviate these concerns and allow for an immediate response to any excursions. The result of this experiment demonstrates that an OWBA is capable of detecting *B. subtilis* endospores with linearity and accuracy that is non-inferior to the compendial method.

It has been known that microorganism autofluorescence primarily results from metabolites like NADH and Riboflavin. Due to this, the ability of BFPC to detect bacterial endospores has been questioned. Bacterial endospores do not contain large amounts of these metabolites and their primary components do not exhibit fluorescence that is compatible with many BFPCs. These results make clear that bacterial endospores can be detected and exhibit fluorescence, but the exact mechanism of this fluorescence is not known. Further testing is planned to understand the molecules evolved in bacterial endospore fluorescence.