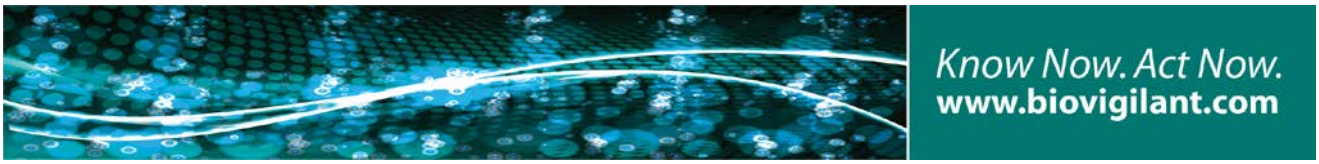

BioVigilant®
Instantaneous Microbial Detection

IMD-W™ Microbial Challenge Test Results

Introduction

This fact sheet provides a summary of microbial test results obtained with the IMD-W™-series instantaneous microbial detection™ system for water. The IMD-W system is a pharmaceutical water quality monitoring and risk management tool. This system has been designed for the routine monitoring of Purified Water (PW) and Water for Injection (WFI) systems, including distribution loops, storage tanks and points-of-use (POU). The IMD-W system can be used to continuously monitor a pharmaceutical water loop and in conventional point sampling applications. Challenge organisms were chosen based on guidance provided in the United States and European Pharmacopeias, and by the Online Water Bioburden Analyzer (OWBA) working group. [A] Testing was designed to show the capability and sensitivity of the IMD-W system in detecting planktonic (single, free-floating) microorganisms through the assessment of ten industry relevant organisms.

Background

Water plays a predominant role in the formulation and manufacture of pharmaceutical products. The traditional culture-based methods commonly used to ensure water quality, however, provide an episodic view of quality at best. Other process monitoring tools such as those for Total Organic Carbon (TOC) and conductivity also play a predominant role, but sensitivity on the order of a single microorganism is outside of the target range for such systems. The pharmaceutical industry continues to recognize a need to implement modern technologies to drive risk reduction and process control, as evident in guidance such as the FDA's 2004 Guidance for Industry document on Process Analytical Technology (PAT), ICH Guidelines Q8, Q9 and Q10, and the FDA's Pharmaceutical cGMPs for the 21st Century, which encourage the adoption of Quality by Design (QbD) principles and new technologies. Recently, the OWBA working group, composed of representatives from key pharmaceutical companies, articulated the industry's need for a real-time system for water quality assessment, with an overall goal of encouraging the development and use of such new technologies. [A]

The fundamental method of microbial detection is different between traditional culture-based methods and light induced fluorescence (LIF)-based technologies like the IMD-W system. Traditional culture-based methods require cell proliferation, leading to the formation of a visually detectable colony-forming unit (CFU) to indicate microorganism presence. Yet, with media, commonly Tryptic Soy Agar (TSA), and incubation parameters typically utilized in industry, not all organisms are culturable. POU sampling with traditional culture-based methods is a currently accepted and primarily practiced method for assessing pharmaceutical water quality. POU testing may be performed as infrequently as once every two weeks at each sample point, however, resulting in a limited sampling frequency and retrospective culture-based results. A LIF-based detection method does not require cell growth and is not restricted by limitations such as an incompatible medium type or incubation conditions. The IMD-W system offers the ability to perform POU testing and continuous monitoring, making the system an excellent tool for use in trending, risk reduction and process control. To further evaluate the system's microbial detection capability, its sensitivity was assessed.

Test Parameters

I. Microbial Species Tested

Ten industry relevant organisms were utilized to challenge the IMD-W system and assess its detection performance. These organisms include *Aspergillus brasiliensis* (ATCC 16404), *Bacillus subtilis* (ATCC 6633), *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 8739), *Methylobacterium extorquens* (NBRC 15911), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* (NCTC 6017), *and Staphlococcus aureus* (ATCC 6538). *A. brasiliensis*, *C. albicans*, *E. coli*, M. *extorquens*, *P. aeruginosa*, *S. enterica*, and *S. aureus* were purchased from the National Institute of Technology and Evaluation (NITE) biological resource center. *B. diminuta* and *P. putida* were purchased from the RIKEN BioResource Center, and the *B. subtilis* spore suspension was obtained from MesaLabs (Ref: SUS-1A-6). *A. brasiliensis* and *B. subtilis* were in the spore state, while all other microorganisms were tested as vegetative cells.

[A] Cundell, A., Gordon, O., Haycocks, N., Johnston, J., Luebke, M., Lewis, N., et al. (2013, May/June). *Novel Concept for Online Water Bioburden Analysis: Key Considerations, Applications, and Business Benefits for Microbiological Risk Reduction*. American Pharmaceutical Review, 26-31.

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Organisms were chosen based on guidance set forth in USP <61>, USP <62>, USP <71>, EP 2.16.12, EP 2.6.13, and by the OWBA working group. [A] All seven compendial microorganisms listed in the OWBA testing protocol were tested with the IMD-W system and the traditional culture based method for recovery comparison.[B] According to the OWBA testing protocol, the purpose of testing these seven organisms is to verify that an online water bioburden analyzer system is capable of enumerating the indicator aerobic QC microorganisms listed in USP <61>, USP <62>, and USP <71>.[B] The three remaining microorganisms, *B. diminuta*, *M. extorquen*s, and *P. putida* were added to the test as waterborne organisms of interest.

Table 1 shows the ten microorganisms tested and whether or not each organism is specified in the USP, EP and OWBA documents referenced. A check mark indicates that the tested organism is listed in the referenced document, while dash marks indicate that the organism is not. All organisms listed in USP <61> were tested. For USP <62>, EP 2.16.12 and EP 2.6.13, the only organisms listed in the documents that have not yet been tested on the IMD-W system are one of two listed *S. enterica* strains and the anaerobe *Clostridium sporogenes*. With regards to USP <71>, all listed organisms have been tested except for the anaerobe *C. sporogenes* and its alternate *Bacteroides vulgatus*, and *Micrococcus luteus,* the alternate for the tested *P. aeruginosa*.

Microorganism Tested	USP <61>	USP <62>	USP <71>	EP 2.16.12	EP 2.6.13	**OWBA**
A. brasiliensis	✓	--	✓	--	--	✓
B. diminuta	--	--	--	--	--	--
B. subtilis	✓	--	✓	--	--	✓
C. albicans	✓	✓	✓	✓	✓	✓
E. coli	--	✓	--	✓	✓	✓
M. extorquens	--	--	--	--	--	--
P. aeruginosa	✓	✓	✓	--	✓	✓
P. putida	--	--	--	--	--	--
S. enterica	--	✓	--	✓	✓	✓
S. aureus	✓	✓	✓	✓	✓	✓

Table 1: Microorganisms tested with the IMD-W and traditional culture based method, and their mention in regulatory and guidance documents. Additional organisms listed in USP <62>, USP <71>, EP 2.16.12 and EP 2.6.13 have not yet been tested with the IMD-W system. These include the anaerobe C. sporogenes, a second S. enterica strain, and alternates listed for C. sporogenes and the tested P. aeruginosa.

II. Microbe Preparation

Vegetative organisms, including *B. diminuta*, *E.* coli, *P. aeruginosa*, *P. putida*, *S. aureus*, and *S. enterica* were inoculated from their glycerol stock in Tryptic Soy Broth (TSB) and cultured aerobically overnight at 32°C. The bacteria were then streaked onto TSA and incubated at approximately 32°C for 20 to 30 hours to achieve the stationary phase. *M. extorquens* was cultured in a laboratory prepared liquid medium for four days at 32°C, streaked onto R2A, and incubated at approximately 32°C for four days. *C. albicans* was cultured in Sabouraud glucose broth for 40 to 48 hours at 25°C, streaked onto Sabouraud glucose agar, and incubated at approximately 25°C for 40 to 48 hours. The bacteria were then harvested in sterile distilled water (DW) and washed through centrifugation at 2,100*g* for three minutes. The supernatant was removed and the pellet was resuspended in filtered DW. Optical density measurements at 600 nm (OD_{600}) were then utilized to estimate concentration, followed by dilution with filtered DW to reach the target microbial concentration. This dilution was utilized in the system testing and plated on TSA to perform a final titer check.

B. subtilis and *A. brasiliensis* spore suspensions were prepared following a different procedure. *B. subtilis* spore suspensions were *diluted* with filtered DW directly from the stock suspension. *A. brasiliensis* spores were inoculated from the stock culture onto Sabouraud glucose agar at 25°C for approximately seven days. Phosphate buffer supplemented with 0.05% Tween 80 was utilized to recover *A. brasiliensis* spores from the culture plate. The spore suspension was then filtered through eight layers of sterile gauze to remove hyphae and centrifuged at 1,600*g* for ten minutes to wash the spores. The supernatant was removed, the pellet was resuspended in filtered DW, and the suspension was centrifuged at 1,400*g* for ten minutes to continue washing. The second centrifugation step was repeated three times. The supernatant was again removed and the pellet was resuspended in filtered DW. Microscopy was utilized to confirm the absence of hyphae and determine the suspension concentration. Dilution to the desired concentration was performed with filtered DW and a final titer check was performed.

Five target concentrations were tested for each microorganism including 0.1 CFU/mL, 1 CFU/mL, 5 CFU/mL, 10 CFU/mL and 50 CFU/mL. A minimum of three replicates were performed at each concentration, with nine replicates performed for the minimum concentration of 0.1 CFU/mL. Concentrations were chosen to ensure that the IMD-W system has an appropriate sensitivity to assess the current compendial limit of 10 CFU/mL for WFI.

III. Test Systems and Apparatus

Testing was completed with an IMD-W system, a Rion KS-42B liquid particle counter, and a Pall MicroFunnel manifold with 0.45µm disposable filter for water sampling and sample culture on TSA plates.

[B] Cundell, A., Luebke, M., Gordon, O., Mateffy, J., Haycocks, N., Weber, J. W., et al. (2013, April 24). On-Line Water Bioburden Analyzer Testing Protocol. Document ID OWBA-TP-2013-v1.5.

A water loop, shown in **Figure 1**, was specifically designed for this testing in order to obtain a very clean background adequate for low level microbial injections. An ultrapure water supply and inline 0.05µm filter permitted extremely low background particulate counts in the loop such that microorganism testing down to 0.1 CFU/mL was possible. A sample injector was utilized to introduce small microbial samples into the loop. The Rion liquid particle counter was utilized as a reference system to confirm particulate counts within the loop during microbe sampling. The sample preparation and static mixer were used to create a homogeneously-dispersed, planktonic sample within the water loop before concurrent sampling by the three instruments. Water samples were filtered and plated on TSA to obtain traditional culture-based results for comparison to IMD-W data.

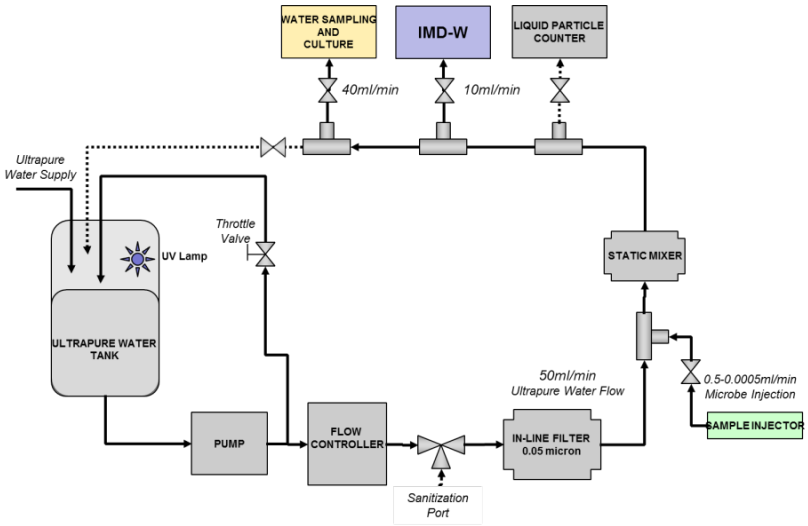

Figure 1: Custom-built water loop for microbial testing.

Test Results

The IMD-W system was challenged with ten microorganisms, at five distinct concentrations. Testing was designed such that single cells, as opposed to agglomerates, were sampled by the IMD-W system to ensure sensitivity down to the level of intrinsic fluorescence emitted by planktonic microbes. **Figure 2** shows a summary of microorganism results for the IMD-W system and the traditional culture-based method. Note that the lowest concentration data point is not indicative of the IMD-W system's limit of detection (LOD), but is instead based on the minimum concentration tested in this challenge testing. The testing of very low microbial concentrations is quite difficult due to the necessity for an extremely clean background. As shown in Figure 1, a custom-built water loop with a 0.05µm in-line filter was required for testing down to even 0.1 CFU/mL.

IMD-W biologic count results correlate well with culture-based results for the organisms tested. This is shown by the R^2 values in **Table 2** that are close to a value of one, indicating a high correlation in the results from both techniques. *B. diminuta* and *P. putida* showed a lower level of correlation than the other organisms tested. Furthermore, recoveries for these organisms were lower than expected based on previous testing performed. Additional testing and investigation is underway. Although there is variability in recovery, all ten organisms are detected by the IMD-W system down to very low concentration levels, as shown by the data below.

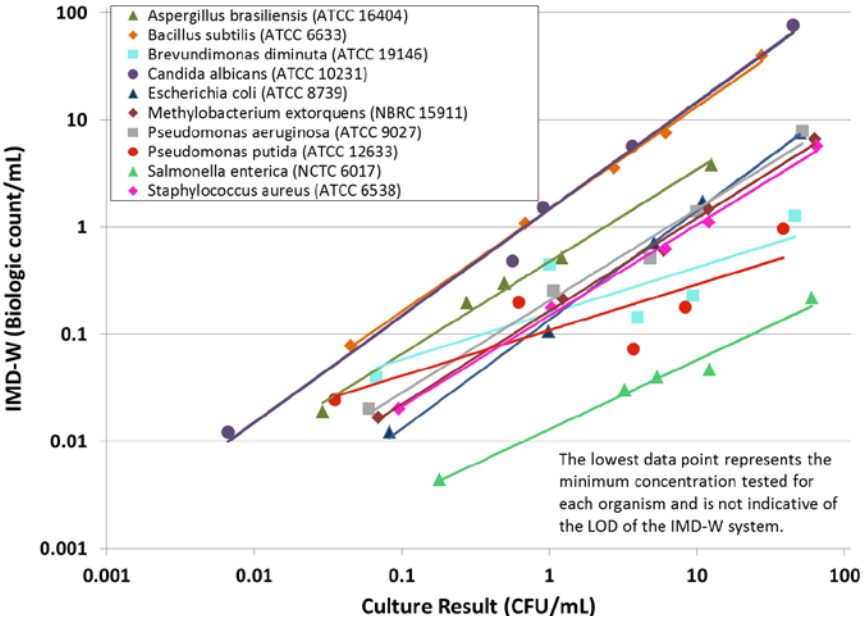

Figure 2: IMD-W system and culture plate results for the ten industry relevant microorganisms tested.

Microorganism Tested	Coefficient of Determination (R^2)
A. brasiliensis	0.992
B. diminuta	0.677
B. subtilis	0.998
C. albicans	0.991
E. coli	0.997
M. extorquens	0.996
P. aeruginosa	0.985
P. putida	0.712
S. enterica	0.980
S. aureus	0.997

Table 2: Coefficient of determination (R^2) values are shown for the relationship between IMD-W biologic counts and culture CFU results. A value close to one shows a high level of correlation in the results from both methods.

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Conclusion

- The IMD-W system is capable of continuous and real-time bioburden monitoring, and offers end users the ability to monitor water system control and react to out-of-specification events in a much timelier manner than with episodic/traditional methods alone.
- This microbial testing challenged the IMD-W system with ten industry relevant microorganisms to determine the system's ability to detect a range of organisms down to the single cell level.
- The IMD-W system is capable of single-cell detection for all organisms tested.
- With a focus on sensitivity and the ability to monitor water systems continuously and in real-time, the IMD-W system is a powerful monitoring and trending tool capable of increasing product quality assurance and process understanding.

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