





Detecting Low Concentrations of Microbiological Contamination in Pharmaceutical Grade Water Using Millipore’s MicropreSure® On-line Filtration Samplers

Objective

Most bacteria in pharmaceutical water systems are likely to exist as biofilm attached to internal surfaces of the system. As such, the contaminants are not likely to be uniformly distributed throughout the system. As a consequence, a QC (quality control) sample taken at any given time may not be representative of the real level of contamination.

One of the most important parameters of pharmaceutical water for injection (WFI) quality is low to no bioburden. Developing QC processes to ensure low to no bioburden is key to a company’s manufacturing success.

This study demonstrates the importance of increased sample volumes and sampling times using an on-line filtration sampler for the detection of low level microbial contamination and/or random biofilm detachment events in a water system.

-  No risk of external contamination
-  Saves steps and speeds collection
-  No bulky samples to carry
-  Batch or continuous on-line sampling



Materials

Hardware

A 20 L pilot size pharmaceutical water system (shown below) was constructed using:

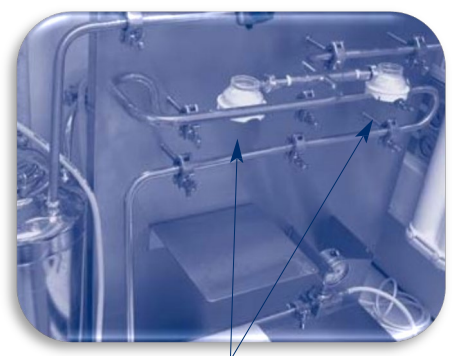
- Masterflex® pump, and a 20 L water storage container with tubing
- Millipore’s MSOpener™ manifold (MSOPENR 01) used to access the MicropreSure on-line filtration membraneSterile sampling tube with Luer fitting (M000 000 01)

Consumables

- Millipore’s MicropreSure on-line filtration samplers, 0.45 µm (MSHA WGS 48)
- Millipore Milli-Q® sterile water

Strains

- *Pseudomonas aeruginosa* ATCC 9027
- *Staphylococcus aureus* ATCC 6538
- *Pseudomonas fluorescens* ATCC 13525



MicropreSure on- line filtration samplers

Methods and Results

For this study, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Staphylococcus aureus* were used to test for microbial contamination. They are common pioneer bacteria and are frequently used in biofilm research. For instance, in one experiment⁷, researchers found that *Pseudomonas* cells adhere to stainless steel, even to electropolished surfaces, within 30 seconds of exposure. The system was filled with sterile Milli-Q water. The Masterflex pump circulates water at a flow rate of 150 mL/min. To ensure sterility, a 100 mL and a 1 L sample were taken after 10 minutes using a MicropreSure on-line filtration sampler, which allows aseptic on-line sampling. Samples were tested for bacterial growth and none was found.

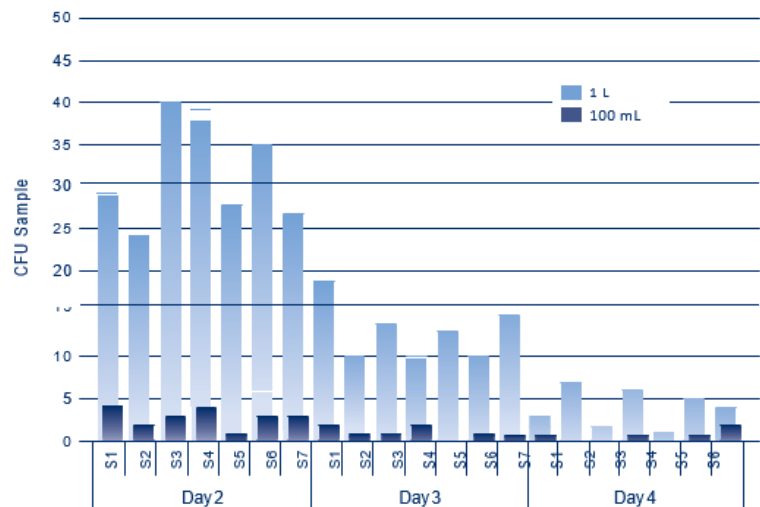
The storage vessel was then inoculated with *Pseudomonas aeruginosa* at a concentration range between 10 and 100 CFU/L. One liter and 100 mL volume samples were taken at the same time at adjacent points on the system during the course of the day. During the sampling, the flow rate was increased from 150 mL/min to 300 mL/min. The contamination levels of the system were monitored for 5 days, when contamination was no longer detected in the 1 L sample.

Figure 1 represents the number of *P. aeruginosa* colonies detected after sampling 1 L (light blue) or 100 mL (dark blue) from the system. As seen in figure 1, the counts are higher in 1 L samples than in 100 mL samples, but not strictly proportional. Increased sample volume provides more accurate and statistically significant counts by improving the variability observed with low microbiological counts.

The pilot system was then inoculated with *P. aeruginosa* and the contamination level was followed. One liter and 100 mL samples were taken at the same time on adjacent points for several days.

Figure 1.

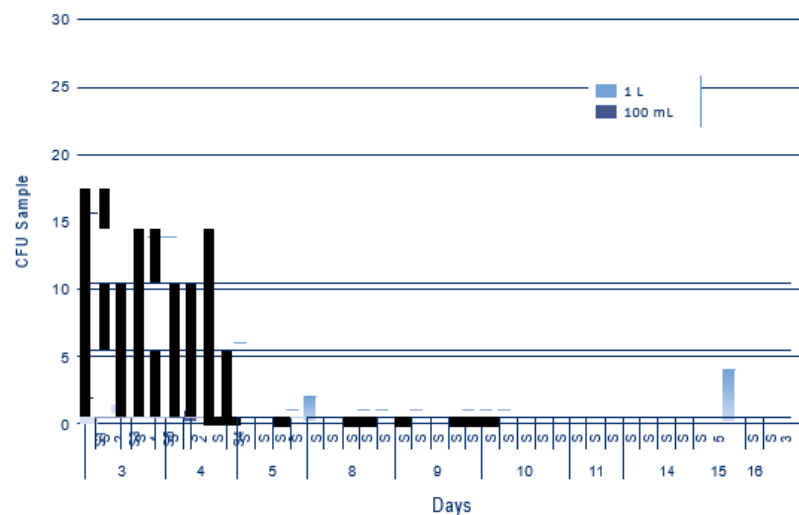
Microbial Monitoring



This figure represents the number of *P. aeruginosa* colonies detected after sampling 1 L (light blue) or 100 mL (dark blue) from the system.

Figure 2.

Microbial Monitoring After Day 4



Positive results observed after day four can be attributed to biofilm detachment.

MicropreSure™, MilliporeSigma, Millipore, and Sigma-Aldrich are trademarks of Merck KGaA, Darmstadt, Germany or its affiliates. Detailed information on trademarks is available via publicly accessible resources.

© 2018 Merck KGaA, Darmstadt, Germany and/or its affiliates. All Rights Reserved.

After 4 days, the contamination level was too low to be detected in a 100 mL sample volume. It was necessary to sample 1 L to detect the presence of microorganisms in the system. The positive results observed after day 4 can be attributed to biofilm detachment. Some bacteria had colonized the system by attachment or entrapment and were randomly released into the water.

The system was then inoculated successively with *P. aeruginosa*, *S. aureus*, or *P. fluorescens*, at low concentration. Sampling 1 L and 100 mL, as described above, over 8 days tracked the contamination level. This test was monitored for the number of times contamination was not observed in a 100 mL sample but detected on the 1 L sample. The final calculated rates are given in table 1. For low contamination levels, testing volumes of 100 mL dramatically increases the risk of concluding that the system is clean when in fact contamination is present. Increasing the sampling volume to 1 L decreases the risk by improving the overall water quality monitoring.

Table 1.

Microbial Monitoring at Low Concentrations

Strain	%
<i>Staphylococcus aureus</i>	47%
<i>Pseudomonas fluorescens</i>	75%
<i>Pseudomonas aeruginosa</i>	32%

Percentage of times when contamination was not observed in a 100 mL sample but was detected with the 1 L sample.

Discussion

One of the most important parameters of WFI quality is low to no bioburden. For this reason, validation engineers and quality control specialists regularly sample the drops and tanks of process water loops to ensure low bacterial levels and to identify bio-contamination problems early. To guarantee the quality of pharmaceutical water, an action level for WFI and highly purified water is set at 10 CFU/100 mL by both USP¹ and European Pharmacopeia². The European Pharmacopeia further stipulates "For aseptic processing, stricter alert limits may need to be applied". Studies performed over one year of daily monitoring of a WFI storage tank showed that the maximum total count of microorganisms was 3 CFU/100 mL³. This means that levels must be set at very low levels of contamination to provide an early warning of water quality deterioration.

The USP recommended method of testing water for injection is membrane filtration of a 100 mL sample. The European Pharmacopeia recommends a sample volume of at least 200 mL. The *Guide to Inspections of High Purity Water Systems* found sample volumes of less than 100 mL to be unacceptable and recommends 100-300 mL⁴. M.K. Patterson et al.⁵ highlighted the importance of adequate sample volume. The volume sampled must be correlated with the projected number of organisms. The authors of this study demonstrated that if the goal is to determine the number of viable cells per liter and the numbers are projected to be in the order of 1 per 100 mL, statistical considerations dictate a minimum sample volume in the range of 3 to 30 L.

As outlined by Potera⁶, microbiologists have traditionally focused on free-floating bacteria growing in laboratory cultures. They have recently come to realize that, in the natural world, most bacteria aggregate as biofilm. It has been estimated that 99% of the bacteria in an automated pharmaceutical water system is likely to exist as biofilm attached to internal surfaces⁷. Biofilm can be defined as a complex and highly diversified community of viable and non-viable microorganisms, including glycocalyx, adsorbed organics, and entrained particles. Basically, biofilm is chronic microbial contamination that is difficult to control using conventional heat, mechanical or chemical treatment procedures.

Today very little is known about the behavior of mature biofilm, including biofilm detachment and the movement of biofilm over solid surfaces. Many models have been created to try to simulate these

MicropreSure™, MilliporeSigma, Millipore, and Sigma-Aldrich are trademarks of Merck KGaA, Darmstadt, Germany or its affiliates. Detailed information on trademarks is available via publicly accessible resources.

© 2018 Merck KGaA, Darmstadt, Germany and/or its affiliates. All Rights Reserved.

phenomena. Horn et al.⁸ simulated growth and detachment of biofilm and described two distinct phases, exponential and quasi steady-state.

Exponential Phase

- The exponential phase corresponds to a rapid increase of biofilm thickness. During this homogenous growth phase, detachment was modeled proportional to the biofilm growth rate. Ten percent of the biomass production is removed through detachment.

Quasi Steady-state Phase

- In the quasi steady-state phase, detachment is described by random detachment events, depending on different factors such as hydro- dynamic conditions, pipe design and type of bacteria involved.

The two distinct phases highlight an important issue. When microorganisms are attached to the system surfaces (biofilm), the contamination is not uniformly distributed in a system and a sample taken at any defined time may not be representative of the real type and level of contamination in this system.

Conclusion

This study shows that increasing the sample volume and sampling times increases the probability of detecting microorganisms. It also improves the trend analysis of low levels of contamination in water systems. The study has also demonstrated that cells can be detached heterogeneously and randomly from a biofilm. By increasing the sample volume and the frequency of sampling, the probability of detecting random detachment of bacteria from a biofilm increases. To efficiently increase sampling, you need the right tools.

Millipore's MicropreSure on-line filtration samplers are an innovative and convenient tool that allows water samples to be aseptically processed at the collection site. The membrane, enclosed in its own housing, is protected until it is ready to be processed in the laboratory. This saves steps and speeds collection—providing an easy method to secure the samples needed for tracking and trending water purity.

It uses the pressure in tanks or pressurized lines to process and collect samples through a microporous membrane within an enclosed chamber. Because the MicropreSure samplers are securely locked together, sampling pressurized water lines does not create water leaks.

MicropreSure on-line filtration samplers are sterile and ready to use. With these samplers, you sample and process in one step, eliminating the need to sterilize sample containers or transport them back to the laboratory. After processing, microorganisms are contained and protected within the lightweight MicropreSure membranes. The MicropreSure on-line filtration samplers allow for processing liters of water through the enclosed housing. In addition, the strong fit between the Luer fitting and the sanitary sampling valve means you can continuously sample over an extended period of time. Results can then reflect the average contamination over a batch, a shift, or a day, helping to ensure the quality of the water in the system.

References

MicropreSure™, MilliporeSigma, Millipore, and Sigma-Aldrich are trademarks of Merck KGaA, Darmstadt, Germany or its affiliates. Detailed information on trademarks is available via publicly accessible resources.

© 2018 Merck KGaA, Darmstadt, Germany and/or its affiliates. All Rights Reserved.

The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the US and Canada.

1. European Pharmacopeia. Water Highly Purified (01/2005: 1927 Version 5.0); Water for Injections (01/2005: 0169 Version 5.0).
2. USP 28. <1231> Water for Pharmaceutical Purposes.
3. Jahnke, M. Jan/Feb 2001. One-Way Distribution System for Water for Injection: Process Management, Microbiological Quality Control, and Meeting Regulatory Requirements. PDA Journal of Pharmaceutical Science & Technology 55 (1): 3-9.
4. Guide to Inspections of High Purity Water Systems. July, 1993. Division of Field Investigations, Office of Regional Operations, Office of Regulatory Affairs, U.S. Food & Drug Administration (Rockville, MD).
5. Patterson, M.K., G.R. Husted, A. Rutkowski, and Mayette, D.C. May/June 1991. Isolation, Identification, and Microscopic Properties of Biofilms in High-Purity Water Distribution Systems. Ultrapure Water 8 (4): 18-24.
6. Potera, C. Biofilms Invade Microbiology. Sept. 27, 1996. Science 273 (5283): 1795-1797.
7. VanHaecke, E, J-P. Remon, M. Moors, F. Raes, D. DeRudder, and A. VanPeteghem. March 1990. Kinetics of Pseudomonas aeruginosa Adhesion to 304 and 316-L Stainless Steel: Role of Cell Surface Hydrophobicity. Applied and Environmental Microbiology 56 (3): 788-795.
8. Horn, H., Reiff, Helmut, and E. Morgenroth. 2003. Simulation of growth and detachment in biofilm systems under defined hydrodynamic conditions. Biotechnology and Bioengineering 81 (5): 607-617.

MicropreSure™, MilliporeSigma, Millipore, and Sigma-Aldrich are trademarks of Merck KGaA, Darmstadt, Germany or its affiliates. Detailed information on trademarks is available via publicly accessible resources.

© 2018 Merck KGaA, Darmstadt, Germany and/or its affiliates. All Rights Reserved.

The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the US and Canada.