Application of flow cytometry for microbiological monitoring of pharmaceutical grade water

Online water bioburden analysis

As the main excipient for the pharmaceutical industry, water is integral to patient safety and the quality of pharmaceutical products.[1] Colony-forming units (CFU) are defined in the pharmacopoeias as the unit for assessing microbiological quality. CFUs are quantified using the traditional heterotrophic plate count (HPC) method; a five-day process with clear limitations for production timelines. Rapid microbiological methods (RMM) assess microbiological quality faster and, in some cases, continuously. RMM have been in use for more than three decades in other fields; however, they are considered up-and-comers in the pharmaceutical industry and companies are catching on to the added value of continuous contamination control.[2]

Online water bioburden analysers (OWBA) have been on the pharmaceutical market for several years and interest is growing, as indicated by the creation of the joint "OWBA workgroup".[3] Guideline documents, including the most recent Annex 1 revision, clearly support RMM implementation and indicate that this technology "should be considered to increase the protection of the product from... microbial contamination".[4]

Automated online flow cytometry

One of the most viable alternatives to traditional compendial methods is flow cytometry. Initially used to measure mammalian cells, it has broad applications for medicine, particularly in the context of immunological analysis.[5]

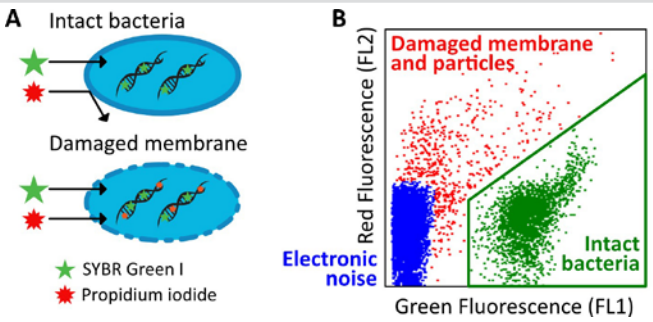
Figure 1



AQU@Sense MB

As technology has advanced and novel stains of specific bacterial properties have become available,[6] flow cytometry has been applied to numerous industrial microbiological processes.[7] Notably, flow cytometry was introduced in the drinking water industry a decade ago and is becoming a standard microbiological tool to enumerate the bacteria present in water, monitor treatment processes such as disinfection or ultrafiltration and assess the general microbiological quality of raw and treated water.[8] Since it already features in the European Pharmacopoeia (Ph. Eur. 5.1.6), flow cytometry is a serious contender in the field of continuous pharmaceutical water monitoring, both in terms of accuracy and time-to-result.

Figure 2



(A) ICC enumeration is based on the assessment of bacterial membrane integrity with DNA stains SGI (green fluorescence) and PI (red fluorescence). SGI stains all nucleic acid-containing bacteria, while impermeant PI can only cross damaged membranes. (B) Typical results of flow cytometry measurement. Each dot represents a particle detected by the flow cytometer. Intact bacteria are depicted in green and damaged bacteria and inorganic particles are in red.

The basic principle of flow cytometry measurement is the detection and quantification of suspended particles present in a water sample by staining cells with fluorescent dyes to distinguish them from inorganic particles. The water sample is focused in a narrow stream and illuminated by a tightly focused laser beam. Optical detectors record the light scattering and emitted fluorescence, revealing multiparametric information, ranging from cell concentration to the viability of the measured cells.

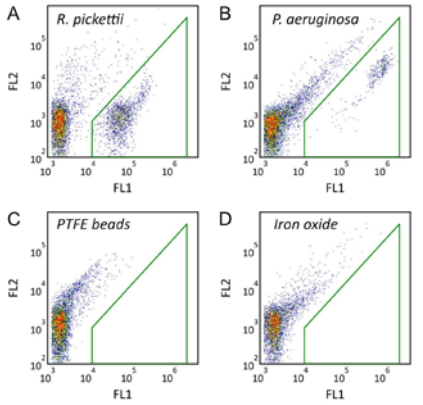
Developed in collaboration with bNovate Technologies, the AQU@Sense MB is a fully automated flow cytometer intended for online bioburden analysis of pharmaceutical grade water (**Figure 1**). The integrated microfluidic sample preparation unit fully automates both water sampling and staining through a continuous batch process. Moreover, sufficient reagents for up to 1,000 measurements are packed in a hermetically sealed and recyclable cartridge that can be replaced without chemical handling. With the measurement interval of 30 minutes to six hours selected, the AQU@Sense MB can operate autonomously for several months.

The AQU@Sense MB rapidly and directly quantifies intact cell count (ICC) which, in selected lab-cultured bacteria, has demonstrated a linear relationship with CFU.[9] ICC is quantified using an established double staining procedure. This procedure uses the fluorescent DNA stains SYBR Green I (SGI) and propidium iodide (PI).[10] Bacterial membrane integrity assessment is possible due to the differing penetration properties of the two stains. While hydrophobic SGI molecules can freely cross bacterial cell membranes, PI is a membrane-impermeant dye that can only penetrate bacteria with compromised membrane integrity;[8] for example, after heat shock or oxidative processes (**Figure 2**). Thus, membrane integrity is a significant criterion of bacterial viability. This method has the added advantage of detecting all viable bacteria present in a sample, including so-called viable but non-culturable (VBNC) cells.[11]

Typical data of the AQU@Sense MB

According to guidance documents such as the pharmacopoeias and the PDA TR-33, alternative microbiological methods

Figure 3

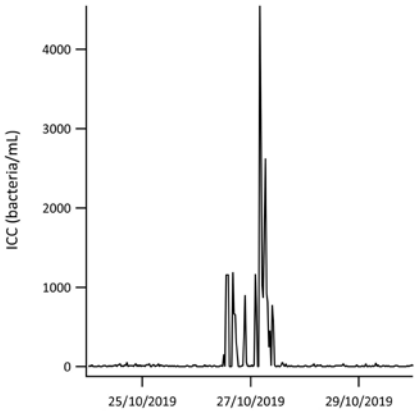


Examples of dot plots measured with the AQU@Sense MB. (A) *Ralstonia pickettii*; (B) *Pseudomonas aeruginosa*; (C) 1µm Polytetrafluoroethylene (PTFE) beads at 10mg/mL in H_2O; and (D) 5µm Fe_3O_4 particles at 10mg/mL in H_2O.

must be able to detect a panel of relevant pharmaceutical bacteria (USP <1223>; Ph. Eur. 5.1.6; PDA TR-33). This panel should include culture collection strains mentioned in the relevant chapters and cover a variety of bacteria commonly found in pharmaceutical water systems, some of which may not be detectable with standard plate counting methods.[12] As shown in **Figure 3**, the AQU@Sense MB detects the relevant bacteria in an inoculated suspension.

The greatest challenge for OWBA relying on intrinsic fluorescence is the tendency to incorrectly identify particles as bacteria.[13] False positives are often caused by Teflon and roughing particles from the water system. Since flow cytometry uses a DNA-specific stain, the AQU@Sense MB can discriminate between the signals of particles and stained cells (**Figure 3**). Furthermore, the continuous batch process described in the previous section ensures that fluctuations in temperature and flow rate of the medium do not influence the signal.

Figure 4



Continuous monitoring of Purified Water distribution system.

Continuous monitoring of Purified Water

Continuous monitoring has clear operational advantages, as demonstrated by customer testing. On 22 October 2019, a customer from the pharmaceutical industry implemented the AQU@Sense MB into a loop of Purified Water and analysed the microbiological quality of the water continuously every 30 minutes, as illustrated in **Figure 4**. The baseline was stable until 27 October 2019, when values of up to 4.500 ICC/ml were detected. Since the customer only performed conventional plate counting on a monthly basis, a direct correlation with the event could not be established. When the routine conventional plate count sample was taken on 5 November 2019, however, unusually high CFU values were detected. Following this, the company conducted an internal investigation and found technical issues with their water distribution system that explained the increase in microbial counts.

The AQU@Sense MB was able to raise an early red flag, identifying that a microbiological problem was developing in the water distribution system. When implemented in a loop or generator, the AQU@Sense MB can prevent further system and product contamination.

Conclusion

The official guiding authorities for the pharmaceutical industry support the implementation of RMM to improve product safety. Flow cytometry uses a DNA-specific staining agent and thereby avoids the pitfalls of other OWBAs. The AQU@Sense MB is an early warning system that enables users to accurately assess the water quality at any time, ensuring safer and more efficient plant operation.



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References

1. ISPE Baseline Guide Volume 4. Water and Steam Systems. 2nd Edition. December 2011
2. Miller M. The regulatory acceptance of rapid microbiological methods [Internet]. European Pharmaceutical Review. 2020 [cited 12 June 2020]. Available from: https://www.europeanpharmaceuticalreview.com/article/62880/rapid-microbiological-methods-regulatory-acceptance/
3. Cundell, Gordon, Haycocks, Johnston, Luebke, Lewis et al. Novel Concept for Online Water Bioburden Analysis: Key Considerations, Applications, and Business Benefits for Microbiological Risk Reduction [Internet]. Americanpharmaceuticalreview.com. 2020 [cited 12 June 2020]. Available from: https://www.americanpharmaceuticalreview.com/Featured-Articles/140513-Novel-Concept-for-Online-Water-Bioburden-Analysis-Key-Considerations-Applications-and-Business-Benefits-for-Microbiological-Risk-Reduction/
4. EU GMP Annex 1 Revision: Manufacture of Sterile Medicinal Products (Draft) - GMP Navigator [Internet]. Gmp-navigator.com. 2020 [cited 12 June 2020]. Available from: https://www.gmp-navigator.com/guidelines/gmp-guideline/eu-gmp-annex-1-revision-manufacture-of-sterile-medicinal-products-draft
5. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. Critical Reviews in Biotechnology. 2016;37(2):163-176.
6. Hammes F, Egli T. Cytometric methods for measuring bacteria in water: advantages, pitfalls and applications. Analytical and Bioanalytical Chemistry. 2010;397(3):1083-1095.
7. Díaz M, Herrero M, García L, Quirós C. Application of flow cytometry to industrial microbial bioprocesses. Biochemical Engineering Journal. 2010;48(3):385-407.
8. Van Nevel S, Koetzsch S, Proctor C, Besmer M, Prest E, Vrouwenvelder J et al. Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. Water Research. 2017;113:191-206.
9. Ou F, McGoverin C, Swift S, Vanholsbeeck F. Absolute bacterial cell enumeration using flow cytometry. Journal of Applied Microbiology. 2017;123(2):464-477.
10. Nescerecka A, Hammes F, Juhna T. A pipeline for developing and testing staining protocols for flow cytometry, demonstrated with SYBR Green I and propidium iodide viability staining. Journal of Microbiological Methods. 2016;131:172-180.
11. Oliver J. The Viable but Nonculturable State in Bacteria. J Microbiol. 2005;43:Spec No:93-100.
12. Sandle T. Burkholderia cepacia complex: review of origins, risks and test methodologies [Internet]. European Pharmaceutical Review. 2020 [cited 12 June 2020]. Available from: https://www.europeanpharmaceuticalreview.com/article/80557/burkholderia-cepacia-complex-review-of-origins-risks-and-test-methodologies/
13. Experimental Methods For Microorganism Challenges On Online Water Bioburden Analyzers [Internet]. Pharmaceuticalonline.com. 2020 [cited 12 June 2020]. Available from: https://www.pharmaceuticalonline.com/doc/experimental-methods-for-microorganism-challenges-on-online-water-bioburden-analyzers-0001

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