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APPLICATION OF FLOW CYTOMETRY FOR MICROBIOLOGICAL MONITORING OF PHARMACEUTICAL GRADE WATER

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Online Water Bioburden Analysis

As the main excipient for the pharmaceutical industry, water is integral to patient safety and quality of pharmaceutical products (ISPE 2011). 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 three to five-day process with clear limitations for production timelines and recovery rate for living organisms. Rapid Microbiological Methods (RMM) assess microbiological quality faster and, in some cases, continuously. Rapid Microbiological Methods have been in use for more than three decades in other fields. It is, however, an up-and-comer in the pharmaceutical industry and companies are catching on to the added value of continuous contamination control (Miller 2017).

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" (OWBA 2013). 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" (EU-GMP 2020).

Furthermore, the latest release of the GMP ANNEX 1 clearly demands for extended process monitoring which is hardly achievable with the traditional plate count method.

Automated At-line Flow Cytometry

One of the most viable alternatives to traditional compendial methods is flow cytometry, which first gained recognition in the late '90s. Initially used to measure mammalian cells, it has broad applications for medicine, particularly in the context of immunological analysis (Adan 2016). As technology has advanced and novel stains of specific bacterial properties have become available (Hammes 2010), flow cytometry has been applied to numerous industrial microbiological processes (Diaz 2010). Notably, flow cytometry was introduced in the drinking water industry a decade ago and is establishing itself as a standard microbiological tool to enumerate the bacteria present in water, to monitor treatment processes such as disinfection or ultrafiltration and to assess the general microbiological quality of raw and treated water (Van Nevel 2017). As it is already featured 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.

The basic principle of flow cytometry measurement is the single 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 on a narrow stream and illuminated by a tightly focused laser beam. Optical detectors record the light scattering and emitted fluorescence. This reveals 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 at-line bioburden analysis of pharmaceutical grade water (Figure1). The integrated microfluidic sample preparation unit fully automates both water sampling and staining through a continuous batch process. Moreover, reagents sufficient for up to 1,000 measurements are packed in a hermetically sealed and recyclable cartridge that can be replaced without chemical handling.

***Figure 1**: AQU@Sense MB*

With the measurement interval of 30 minutes to 6 hours selected or triggered by the control unit, the instrument can operate autonomously for several months.

The flow cytometer rapidly and directly quantifies Intact Cell Count (ICC) which, in selected lab-cultured bacteria, has demonstrated a linear relationship with CFU (Ou 2017). This has also been proven in the primary validation performed for this instrument (to be published soon). ICC is quantified using an already established double staining procedure. This procedure uses the fluorescent DNA stains SYBR Green I (SGI) and Propidium Iodide (PI) (Nescerecka 2016). Bacterial membrane integrity assessment is possible because of 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 (e.g., 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 (Oliver 2005).

Typical Data of the Flow Cytometer

According to guidance documents such as the pharmacopoeias and the PDA TR-33, alternative microbiological methods 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 as well as cover a variety of bacteria commonly found in pharmaceutical water systems, some of which may not be detectable with standard plate counting methods (Sandle 2018). As shown in Figure 3 below, the flow cytometer detects the relevant bacteria in an inoculated suspension.

The greatest challenge for OWBA application relying on intrinsic fluorescence is the tendency to incorrectly identify particles as bacteria (Martindale et.al. 2020). False positives are often caused by Teflon and roughing particles from the water system. Because flow cytometry uses a DNA-specific stain, it can discriminate between the signals of particles and stained cells (see Figure 3 below). Furthermore, the continuous batch process described above ensures that fluctuations in temperature and flow rate of the medium do not influence the signal.

Continuous Monitoring of Purified Water

Continuous monitoring has clear operational advantages, as demonstrated by customer testing. On Oct. 22, 2019, a customer from the pharmaceutical industry implemented the flow cytometer 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 Oct 27, 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 05.11.2019, 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, assessing correctly that a microbiological problem was developing in the water distribution system. When implemented in a loop or generator, the instrument can prevent further system and product contamination.

In Process Control

Using RMM with connection to several sampling points at the same time with sequential measurement allows for fast and easy process monitoring. Trend analysis and early detection of potential risks lead to significant improvement of process reliability and reduces the risk of out of spec production and cumbersome and costly investigations.

Release Testing

Quality Control of the final water after the water generation system and in the return line of the distribution loop is the most critical application. For this

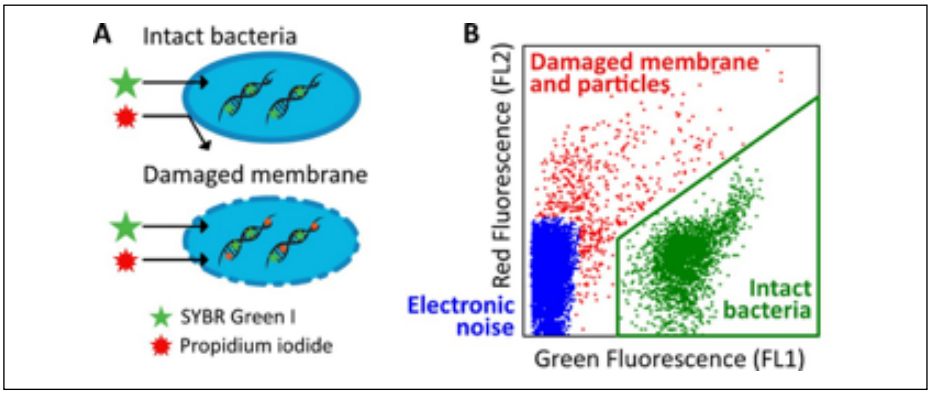

***Figure 2**: (A) ICC enumeration is based on the assessment of bacterial membrane integrity with DNA stains SGI (green fluorescence) and PI (red fluorescence). SGI stains all nucleic acid-containing bacteria, while impermeant PI can only cross damaged membranes. (B) Typical results of flow cytometry 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.*

application a complete validation needs to be performed to prove the suitability for the intended use. To support the user on that topic, a primary validation has been performed for the AQU@ Sense MB.

Validation of Alternative Microbiological Methods

The biggest challenge for establishing the RMM in the market is certainly the validation of the analyzers by the user. Currently, the plate test is still the pharmacopoeia method, and the limits are defined in "CFU/ml" and "CFU/100 ml" for purified water and water for injection. On the other hand, there are the results of the RMM, which usually differ from the results of the plate tests. Both chapter 5.1.6 of the Ph. Eur., as well as chapter <1223> of the USP and the document TR-33 of the Parental Drug Association (PDA) describe the possibilities of validation of alternative methods [8][9][16].

For example, in chapter 5.1.6 of the Ph. Eur., an RMM can be validated by comparing it with the plate tests using various parameters. Previous tests at BWT have shown that flow cytometry detects approximately 10 to 100 times more bacteria than CFU were detected by the plate test [17] [18]*. This statement is in line with the current literature on the use of flow cytometry in the drinking water sector, although in some cases even higher factors are given [12][13]. The USP also confirms this comparison with the statement that plate tests reflect only 0.1 1 % of the bacteria present when comparing the results with those of flow cytometry [9]. A direct correlation between the results of the RMM and the plate tests could not be shown so far.

The described "equivalence" of the results can also be related to the quality of the basis for decision-making instead of the numerical values. In this case, the alternative method must allow an assessment at least equivalent to that of the pharmacopoeia method [8].

In practice, it is conceivable that during the qualification phase of an ultrapure water system, in which CFU is determined daily or weekly in the laboratory, measurements are taken in parallel with the alternative microbiological method to be validated. Even if there is no direct

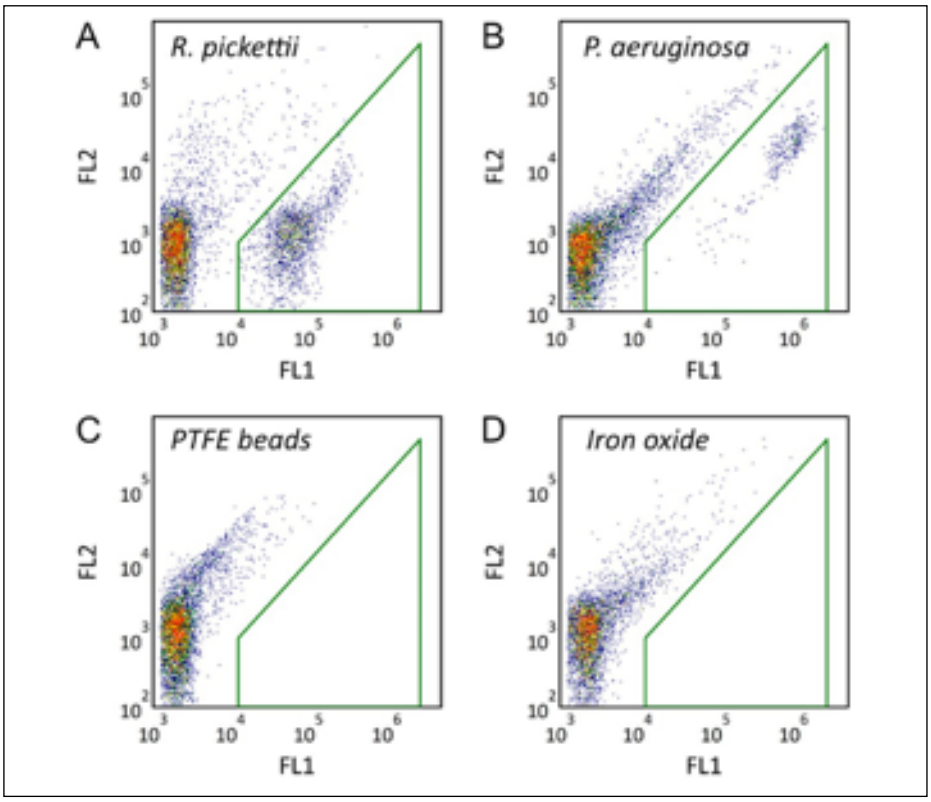

***Figure 3**: Examples of dot plots measured with the AQU@Sense MB. (A) Ralstonia pickettii, (B) Pseudomonas aeruginosa, (C) 1 µm Polytetrafluoroethylene (PTFE) beads at 10 mg/mL in H2O and (D) 5 µm Fe3O4 particles at 10 mg/mL in H2O.*

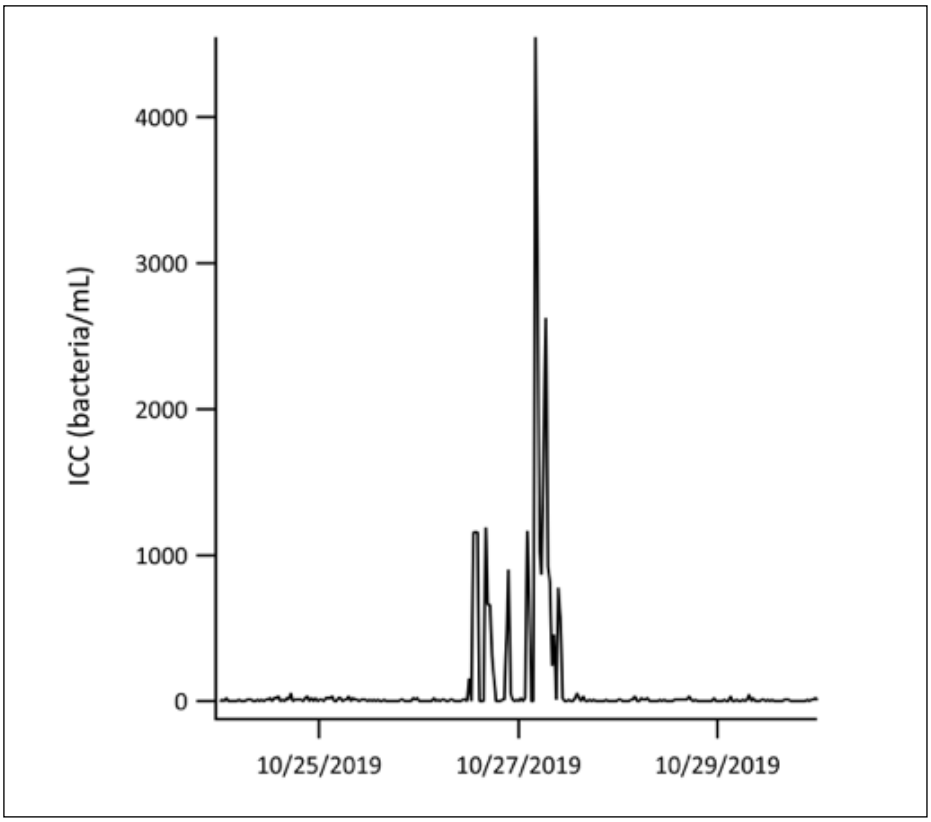

***Figure 4**: Continuous monitoring of Purified Water distribution system.*

correlation, limit values can be defined in this way. If, for example, 2CFU/100 ml is regularly determined in ultrapure water and the results of the alternative method are consistently below 200 cells/ml, a limit of 250 cells/ml could be defined. In the event of an exceedance, an examination with plate tests can be carried out by the microbiological experts. As long as the values of the alternative method are below this limit, only the samples required for release are determined using

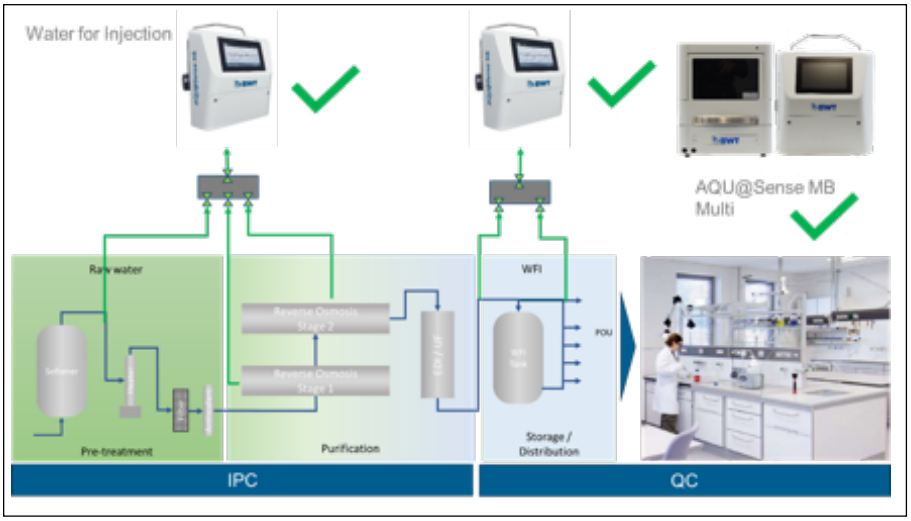

***Figure 5**: Potential applications for In Process control and Quality Control in a pharmaceutical water generation system.*

Activity	Normally carried out by	
	Supplier	User
Primary validation	+	(1)
URS (instrument, application)	-	+
Description of the technique	+	(2)
Risk benefit analysis	(3)	+
Design qualification (DQ)	-	+
Installation qualification (IQ)	(4)	+
Operational qualification (OQ)	(4)	+
Performance qualification (PQ):		
- verification of primary validation data given by the supplier;	-	+
- verification for the intended use (e.g. sterility testing, TAMC/TYMC, ...);	-	+
- method suitability test	-	+

***Table 1** (Left): Validation procedure according to guidelines (Source: Ph. Eur 5.1.6).*

PARAMETER	RESULT	DETAILS
Accuracy	Passed	Non-inferior to HPC
Precision	Passed	RSD < 30%; non-inferior to HPC
Specificity	Passed	All species detected
Limit of quantification	Passed	Non-inferior to HPC
Linearity	Passed	Non-inferior to HPC
Range	Passed	Non-inferior to HPC
Robustness	Passed	Operational and environmental variables have no influence on test results
Ruggedness	Passed	Unaffected by deliberate variations

***Table 2** (Above): Results of the primary validation performed for the flow cytometry*

the plate test.

*Some of the results were presented in a previously published article by A. Minzenmay (see Pharmind issue 01.2018: "Membrane-based WFI generation" A. Minzenmay 2017).

Primary Validation

The AQU@Sense MB is the first at-line bioburden analyser based on flow cytometry that has passed a primary validation in accordance with guidance documents such as the pharmacopoeias and the PDA TR-33. Alternative microbiological methods must be able to detect a panel of relevant pharmaceutical bacteria (USP <1223>; Ph. Eur. 5.1.6; PDA TR-33).

Only RMMs which have successfully completed the tasks required on the Ph. Eur. 5.1.6 are suitable for their use as per the new Annex 1. This compliance requires meeting the different tasks included in the validation process (table 1) and the successful test for the different parameters included in the 5.6.1 (table 2). Any device not having completed this process is therefore not suitable for his use under the Ph. Eur. for the purposes included in the Annex 1 of the GMPs.

BWT has initiated a primary validation study in partnership with the original manufacturer, bNovate, the FHNW (University of Applied Sciences and Arts Northwestern Switzerland) and the Technical University Eindhoven. Data will be published soon.

What was tested?

Multiple bacterial and fungal strains

When the routine conventional plate count sample was taken on 05.11.2019, 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.

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BWT has initiated a primary validation study in partnership with the original manufacturer, bNovate, the FHNW (University of Applied Sciences and Arts Northwestern Switzerland) and the Technical University Eindhoven. Data will be published soon.

What was tested?

Multiple bacterial and fungal strains were tested with this flow cytometer and compared to the official plate count method proposed by the Ph. Eur. (membrane filtration, R2A, 30-35°C for 5 days). Furthermore, the instrument was challenged by artificial particles and by dead cells. The Validation Master Plan was written based on customer and expert input. The data was evaluated against criteria defined in the relevant documents under the lead of a well-known expert for biostatistics.

The outcome of that primary validation was that the AQU@Sense MB is not inferior to the HPC plate count under the conditions used in this study. With this primary validation the first step for the validation procedure

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 only primary validated instrument for at-line bioburden analysis based on the flow cytometry technology, the AQU@Sense MB enables for in process control as well as for release of the final product after complete validation. The primary validation in this case is the basis of the overall validation process. Flow cytometry has been proven to be a reliable and fast alternative for HPC, ensuring safer and more efficient plant operation.

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PHARMIG NEWS CORNER #60

Compiled by Tim Sandle

Welcome to the 60th Pharmig news corner (15 years of bringing news to our members)

ICH Q9R1

ICH Q9 'Quality Risk Management' has been updated. It now becomes "ICH Q9(R1)". ICH Q9 is a GMP requirement, with EU GMP and it is adopted by the FDA.

The changes are orientated towards approach and philosophy rather than process (i.e. no new risk tools have been included).

Structurally, the changes are:

- There is a new section "5.1: Formality in Quality Risk Management".
- There is a new section "5.2: Risk-based Decision Making".
- The title of Annex 1 – 'Risk Management Methods and Tools' – has been renamed 'Quality Risk management Methods and Tools'.
- A new sub-section II.9 has been added into Annex II (Quality Risk Management as part of Integrated Quality Management). The new sub-section is titled 'Quality Risk Management as Part of Supply Chain Control'.

See: https://database.ich.org/sites/default/files/ICH_Q9%28R1%29_Guideline_Step4_2022_1219.pdf

Pyrogen and endotoxin testing

For injectable pharmaceuticals in particular, the presence of pyrogenic substances is a key patient concern. The European Pharmacopeia is undertaking a revision of its general chapter "guidelines for using the bacterial endotoxin test" 5.1.10 (reference note PA/PH/Exp. BET/T (22) 2 ANP). This is part of a broader