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Total Colony Counts (TCC) By Flow Cytometry (FCM) Should Replace the Heterotrophic Plate Count (HPC) Test for Bacteriological Enumeration of Water - Some Recent Developments in Flow Cytometry: A Review

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Abstract

We currently use the gold standard HPC test method for bacterial enumeration of our raw, drinking and waste water; analysis times are relatively long: 1-3 d, as per the international standard reference method. Our tests are accredited to ISO/IEC 17025 requirements. The literature has indicated international use of the current HPC method for monitoring routine microbiological quality of drinking water; current tests for pathogen monitoring have poor sensitivity, accuracy and long analysis times, in general. The aim of this current review is to: Consider shortfalls of the HPC method, Discuss alternative methods for bacterial enumeration, Discuss features and applications of the automated -sample preparation flow cytometer, the BactoSense model, Highlight advantages of flow cytometry, which makes this technique the choice for the bacteriological enumeration in water, and Propose options for standardization of flow cytometry TCC test methods.

Findings indicate that HPC is very inaccurate (~ -99% bias), and time-consuming (1-3 d), compared to flow cytometry (~ 99% accurate; ± 15-20 min analysis time). The evidence confirms a strong preference for the use of flow cytometry for Total Cell Counts/mL; the BactoSense is one viable instrument due to its automated sample preparation, speed of analysis, accuracy and precision. There was a significant gap regarding the standardization of current flow cytometry test methods. Options for standardization are ISO/IEC 17025 accreditation, the US EPA, ASTM, AOAC, and Standard Methods. Some key method validation parameters are: accuracy, bias, precision, specificity, LOD, LOQ, linearity, and the Uncertainty of measurement. The review has highlighted the potential of flow cytometry for the screening of Corona virus SARS-CoV-2 for COVID-19. The BactoSense FCM appears to be a viable option for bacterial enumeration, as a TCC/mL measure, for drinking water, due to its sample preparation automation, speed of analysis, accuracy and precision. The current, and new, FCM test methods should be validated, standardized, or accredited for adoption for bacterial enumeration of water. Flow cytometry is the preferred test method for bacterial enumeration of water - it should replace the HPC method.

Keywords: Drinking water • Heterotrophic plate count • Accuracy • Flow cytometry • Standardization • Method validation

Introduction

The production of drinking water of a suitable quality is critical for ensuring the public health of the consumer.

Umgeni Water (UW), in KwaZulu-Natal, South Africa, provides potable bulk water services, and sanitation to water services institutions in its service area. It treats raw water from its catchments to potable standards, the South African National Standard [1], which is aligned to the World Health Organization guidelines [2]. Water quality is assessed at the Head Office laboratories, which are ISO 17025-accredited [3], by the South African National Accreditation System (SANAS) [4].

Microbial pathogens in water and wastewater are one of the major health risks. Suitable test methods for assessing pathogens are essential for protecting human health.

Literature Review

Culture-based methods are simple, low cost, but are limited by low sensitivity, high labour and time requirements; they can yield false negatives [5]. Molecular methods are generally faster and more sensitive, and can be highly target-specific. However, some of these methods are particularly susceptible to interference from inhibitory compounds [5]. PCR may also have limited ability to distinguish between viable and non-viable organisms.

One of the routine microbiological water quality tests performed on drinking water is the culture-based Heterotrophic Plate Count (HPC) method [6]. One great challenge that faces the water sector is that current test methods for pathogens have poor sensitivity, accuracy and long turn-around times (24 hr., up to 7d). The rapid, accurate identification of pathogens is critical to ensure the production of good water quality and subsequent consumer safety.

Flow cytometry (FCM) is one alternative approach [7,8]. Until recently, all traditional FCMs required manual sample preparation. Recently, researchers at the Swiss Water Research Institute (EAWAG) developed a fast (<15 min.) microbial total cell-counting (TCC) method based on flow cytometry (FCM).

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The SLMB Method 333.1 was standardized, validated and was accepted in Switzerland [9]. Unlike HPC, the TCC-FCM allows – for the first time – a quick, realistic assessment of the general microbiological state of a water sample. This method is already used routinely in several Swiss and Dutch DW works to monitor the performance of treatment processes and distribution networks. This small, low cost, robust FCM, BactoSense [10], developed by SIGRIST [11], also allows for automatic, continuous online monitoring of TCC in water in 30 min (or longer) intervals. The key feature of automated sample preparation, within the same instrument, makes the BactoSense, the only FCM to date with such a feature.

The aim of this current review is to:

- Consider shortfalls of the HPC method.
- Discuss alternative methods for bacterial enumeration.
- Discuss features of the BactoSense.
- Highlight key advantages of flow cytometry which makes this technique the preferred choice for the bacteriological enumeration in water and
- Propose options for the standardization of flow cytometry test methods, like TCC.

The Technical information on the BactoSense and testing results, unless otherwise stated, was provided by Rhine Ruhr, agents in South Africa and the SIGRIST-PHOTOMETER web site [11]. Key reference documents were obtained from the web sites for ISO/IEC 17025 [3], SABS, U.S. EPA, ASTM, AOAC, the Standard Methods for the Examination of Water & Wastewater textbook [12], and ScienceDirect. All S-labelled Figures and Tables are Supplementary Information.

Results and Discussion

Overview of methods used for bacterial quantification in drinking water

Up to about 2003, the available methods used for detecting microbial cells were: cultivation, plating, flow cytometry, microscopy, particle counting, biochemical component-based, molecular methods (e.g., PCR, etc.), and immunology-based (Table S1).

The HPC test method and relevance: Heterotrophs are broadly defined as microorganisms; they include bacteria, yeasts and molds. A variety of simple culture- based techniques that are intended to recover a wide range of microorganisms from water are collectively referred to as "Heterotrophic Plate Count". The HPC provides a method for monitoring changes in the bacteriological quality of drinking water, to give, amongst others, an indication of the effectiveness of chlorine in the water system.

The method enumerates bacteria that are cultivable on semi-solid nutrient-rich agar under defined incubation conditions [6]. In the United States, drinking water must have an HPC of no more than 500 colonies per mL to maintain compliance with the National Primary Drinking Water Regulations (NPDWR) [13]. In South Africa, the limit is ≤ 1000 [1]. Because HPC is so widely employed, HPC results provide a common basis of comparison across different laboratories, settings, and times.

During the 1980s and 1990s, it was decided that HPC measurements have no hygienic relevance [6,14]; rather it can be used as an indicator of process performance. These methods and concepts, and HPC, are still used, even up to ~ 2017, for routine testing of microbiological quality and water safety, as a process variable all over the world [14]. Monitoring of *E. coli* is still a good indicator of faecal contamination [1]. However, it is known to fail to indicate absence, or disinfection, of "hardy" microbes and viruses.

General water quality monitoring requirements: Microbiological test methods used for water quality should ideally meet the requirements for: being relevant, simple, reliable, accurate, and cost-effective. Analytical test methods that are method-validated to meet the full ISO/IEC 17025-accredited, technical competency requirements [3] are deemed to be "fit- for-purpose".

Accuracy is the closeness of the agreement between a test result and the reference value [13,15]. The recovery of a target analyte from a matrix is generally affected by the extraction technique. Ideally, one aims to use an optimized method that will result in maximum (100%) recovery of the target analyte. In the water sector, many decisions are based on the results of test measurements. It is critical that such results are accurate. The required measurement quality [16] can be achieved by method validation, by establishing traceability of the measured test results to stated references and an estimate of the measurement uncertainty (MU) (Uncertainty of Measurement) [17].

It is very difficult to determine the trueness of a microbiological method, especially on a naturally contaminated sample. The most appropriate way is to conduct tests within several laboratories and then determine the mean of the group result. Trueness can be determined by the use of certified reference materials or artificially contaminated samples. These tests can also be performed in a single laboratory using different analysts. The methods must be able to detect organisms at the correct concentrations [13].

The Uncertainty of Measurement (UOM) is the parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand [15,17,18]. For ISO/IEC 17025 accreditation of test methods, UOM is a requirement [3].

Biological assays and test method results generally have larger % RSD (± 30%), or variation, between and within the same sample, compared to chemical tests (≤ 10%). Chemical analytes tend to be more homogeneously distributed in the sample matrix, in the case of water. Some contributory sources of uncertainty for quantitative microbiological test methods have been reported [15].

Some major shortcomings of the HPC test method

Whilst the HPC test method is still used to assess treatment efficiency in water and food industry, partly because *E. coli*, other pathogens, etc., are either absent or present at too low concentration to be detected, it has several shortcomings relative to FCM. The most commonly cited [8] are:

- High time requirements: 3-7 days to deliver results, making it less able to detect temporal changes in microbial water quality;
- Limited detection: About 0.01-1% of bacteria in drinking water are cultivable [20], while FCM analyses usually detects 50-20 000 "events" [21]. This difference is illustrated in the Supplementary Information (Figure S1) [21];
- It is inaccurate: With the plate count method, the initial extraction of the bacteria is largely dependent on the culture step. Various media (plate count, yeast extract, low/high nutrient, R2A), temperatures (20-37ºC) and time periods (incubation conditions: 24-120 hr.) are used [12].
- It is selective and biased: Since there is no universal set of conditions that permits the growth of all microorganisms, it is impossible to enumerate all microorganisms by viable plating.
- The method gives an estimate of the actual number of cells present.
- HPC cannot recover viable but non-culturable (VBNC) bacteria [22,23];
- Lack of standardization of media and incubation conditions.
- Variation in upper limits for HPC in drinking water between countries; guideline values range from < 20 to < 1000 cfus/mL;
- Variable results: Hammes et al. [24] reported that HPC-based TCCs of DWTP samples had a standard error of >30%, compared with < 5% for FCM. Prest et al. [25] reported a < 5% standard error for FCM-based TCCs. The observed RSD via HPC analysis is 30-100% [24,26] but for FCM, the inter-laboratory and instrument variability are <7%, or even <2.5% for a single analyst [13].
- Negative bias: HPC detects single cells or bacterial aggregates but does not necessarily discriminate between the two.
- Not amenable to automation: There are no automated HPC test methods.

- Higher cost.
- Provides limited information: Only the equivalent TCC is obtained as the measurement.
- Monitoring: Continuous monitoring is not possible – generally grab samples are taken.

Flow cytometry and principle

Flow cytometry [8] is a technique used to detect and measure the physical and chemical characteristics of a population of suspended cells or particles in a fluid as it passes through at least one laser. It measures close to 99% of all particles that are within the liquid sample matrix. Figure 1 [8] illustrates the basic components of a typical flow cytometer instrument:

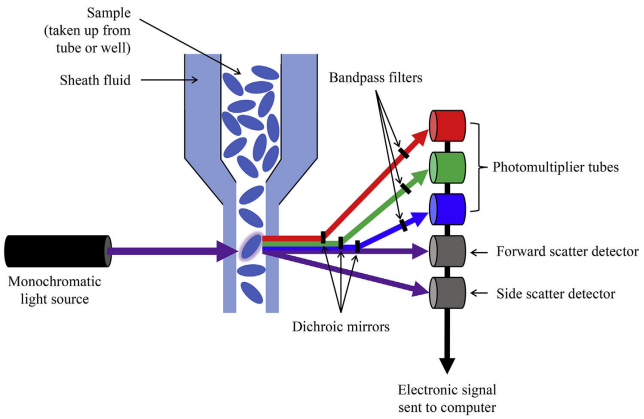

Figure 1. Principle of flow cytometry.

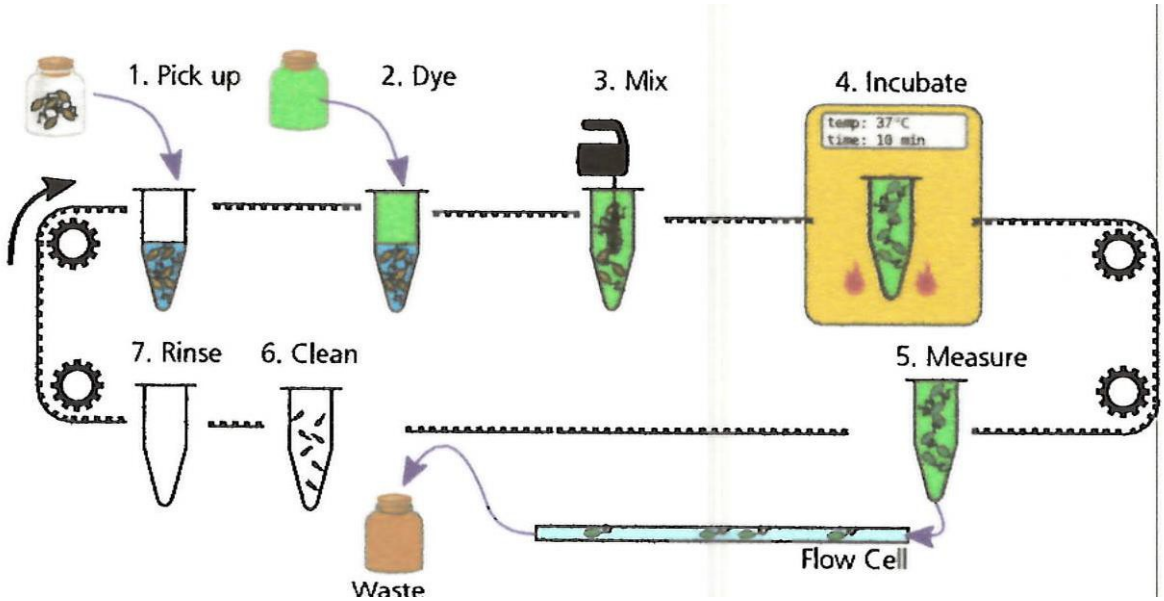

Figure 2. Manual sample preparation and analysis in FCM.

The sample is taken and a dye is added. Most commonly SYBR Green 1 is used. The dye attaches to the DNA of microbial cells. In a next step, the sample is mixed. This distributes the dye evenly throughout the samples and guarantees reproducible results. The sample is then heated (37°C, 10 minutes), (incubation phase), which allows the dye to attach to the DNA. The sample is measured using flow cytometry. The system is cleaned and rinsed. This involves waste generation and removal. This reduces sample contamination and the effect on subsequent measurements.

The instrument draws sample into a focusing chamber that forces suspended particles to align in single file. The focused stream is passed through one or more interrogation points, at which a laser or other monochromatic light beam individually strikes each particle. Detectors measure the extent to which each particle scatters light in the forward and side directions and send these measurements to a computer for display and processing. FCM data is typically presented as histograms or two-dimensional dot plots that visualize the intensity and frequency of signals received on different parameters. In general, forward scatter (FSC) signals are related to particle size, while side scatter (SSC) signals are related to particle complexity and granularity. Fluorescence occurs when certain molecules emit light following excitation by a beam of a compatible wavelength. Most cells have some natural auto fluorescence that can be beneficially exploited for analysis but may also obscure fluorescent signals of interest. In most cases, auto fluorescence alone is not sufficient to conclusively distinguish specific particle populations and/or examine parameters of interest. Researchers therefore apply one or more fluorescent stains prior to FCM.

Sample preparation and measurement of TCC in a traditional, manual FCM

The essential steps are illustrated in Figure 2.

The BactoSense: sample preparation, measurement and features

This is illustrated in **Figure S2**. The technical specifications are summarized in **Table S2** [11]. This is sequentially:

- Sample preparation
- Staining
- Detection
- Analysis
- Reporting
- Waste
- Cleaning (Figure S3)

Sampling can be on-line (flow rate 200-400 mL/min), by connection to the source water, **Figure S4**, or manual, off-line, **Figure S5**. The measurement process is summarized in **Figure 3**, and in **Table S2**. A single laser is used; forward scatter fluorescence is not measured.

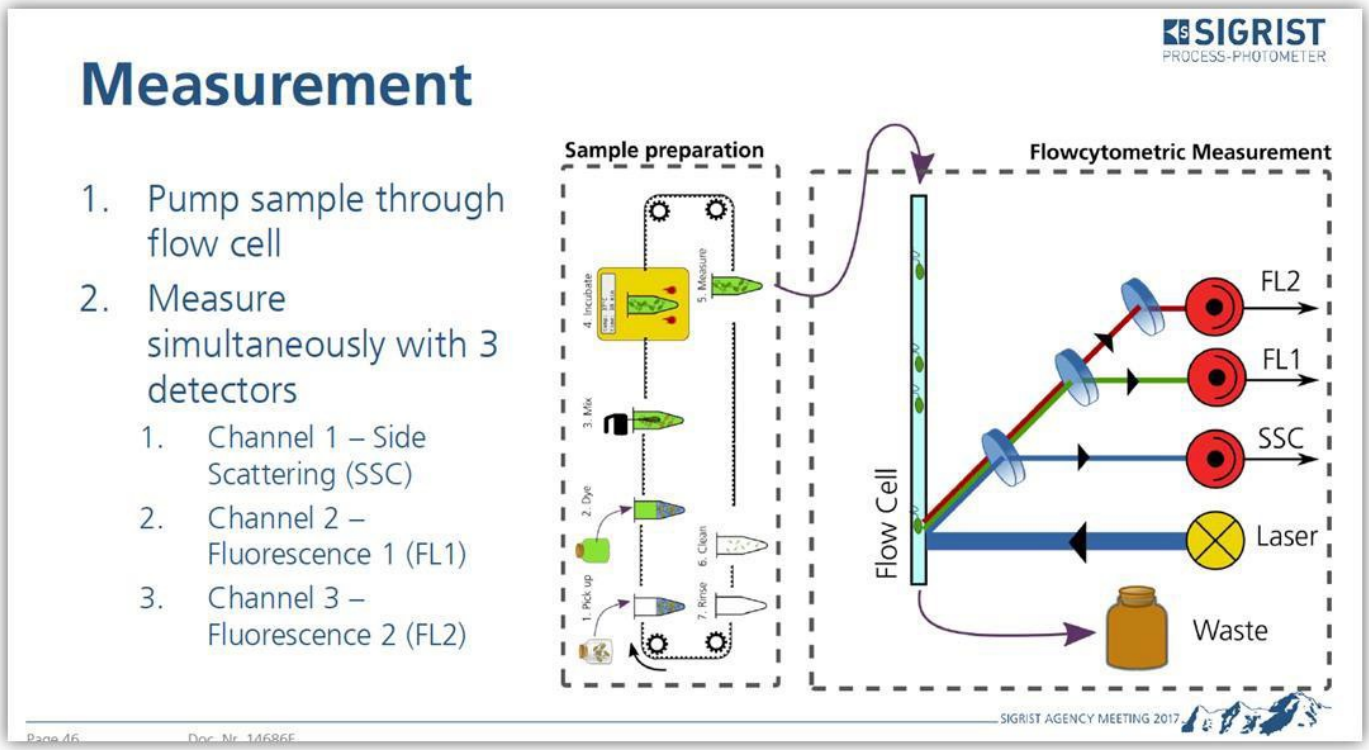

Figure 3. Measurement and the detection principle of the BactoSense.

Footprint: dimensions are 350 mm width × 240 mm depth × 373 mm height; it is fairly compact, weighing 14.5 kg.

Mounting: The BactoSense has a robust compact design for flexibility. It can be mounted directly on the wall or a table in a water supply plant or laboratory bench top.

Sample preparation: This process is fully automated. A sample volume of 2 mL is required, contained in the 2 mL plastic screw cap micro tube; a 260 µL aliquot is taken up and 90 µL analyzed.

Cartridges: The required chemicals and waste products are enclosed in the hermetically sealed cartridge which can be recycled. This allows for safe handling and convenient waste disposal. Depending on the desired application and the sample, there is the option of using either one of the 2 cartridges: one for TCC and one for LDC (Live Dead Count) at any one time. One advantage of the TCC mode (cartridge) is that the instrument measures all microbial cells, plus it shows very well the background noise, which is largely the inorganic and organic matter contribution from the sample. The TCC cartridge provides measures of TCC, HNAC, and HNAP. The LDC is the sum of: ICC (intact cells) + DCC (damaged cells). The use of the LDC cartridge also gives out a "TCC" number, but this is the summation of the ICC +DCC, which is not exactly the same as a true TCC value, as obtained by using the TCC cartridge. The advantage of the use of the LDC mode (cartridge) is that the instrument measures the exact number of intact cells, which is suited for use in disinfected water quality monitoring, but not as good when it comes to network survey, etc. The LDC cartridge provides measures of LDC, ICC, DCC and ICP (Intact Cell Percentage). A single cartridge can do 1000 measurements, with a life span of 9 months. Thereafter, it can be changed within a few minutes. For on-line process monitoring, like disinfection, and other related microbiological applications, one will require the use of 2 BactoSense instruments, one setup for a TCC measurement, and the other containing the LDC cartridge, for the ICC measurement.

Staining: Dyes used are SYBR Green I (SG). The SYBR green in the sample binds to the DNA of a microbial cell and fluoresces, giving off green light when illuminated by blue light. The resulting DNA-dye-complex absorbs at 497 nanometers blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 520 nm). The stain preferentially binds to double-stranded DNA, but will stain single-stranded (ss) DNA with lower performance.

Mixing: This is automated; the dye is distributed evenly throughout the sample.

Incubation: The sample is then heated (37 ºC, 10 minutes); this is also automated and allows the dye to bind to the DNA of each microbial cell.

Measurement: The sample is then measured.

Detection: One laser diode, at 488nm, is used. Fluorescence is measured by 3 detectors, simultaneously, at a wavelength of 525 nm for FL1, at 715 nm for FL2, and at 488 nm for the Side Scatter (SSC). The detection limit is 100 cells/mL- 5000000 cells/mL.

Analysis: A large intuitive touch screen serves as user interface. The measuring interval and further settings can be programmed simply and quickly.

Reporting: The results are available within 20 minutes. The internal data base permanently stores all measured data which can be retrieved and visualized. Each measurement shows the characteristic fingerprint of the sample analyzed. The instrument has extensive communication/connection options:

- A USB port for exporting data,
- An integrated web interface for remote control via Ethernet, and
- An alarm system with a configurable range. In the case of a water system contamination event, the BactoSense can immediately activate an alarm and relay it, through standard transmission interfaces, to a control center display.

Waste: All the generated waste products are enclosed in the sealed cartridge, which allows for safe handling and easy disposal.

Cleaning: A "Cleaning Set" flushes the capillary tubing.

Maintenance: The instrument is designed for an annual maintenance, by a SIGRIST representative. The verification of instrument accuracy can be done by the operator, at any time, using a reference solution.

With the sample analysis, every cell generates signal peaks on the 3 channels: SSc, FL1 and FL2, as shown in **Figure S6**. The signal peaks of FL1 and FL2 are represented by one dot in the dot plot – **Figure S7**. Finally, a cytogram is generated by the data processing, as shown in **Figure 4**. The "Gate", enclosed by the red lines, is the Counted cells. The Dot plot shows

- Noise
- LNA cells
- HNA cells and
- Debris

The TCC (total cell count) value is the count of all points within the gate. The LNAC/mL (low nucleic acid count) is the count of all points in the "left" gate compartment. The HNAC/mL (high nucleic acid count) is the count of all points in the "right" gate compartment. The HNAP (%) is the High nucleic acid percentage = HNAC/TCC × 100%.

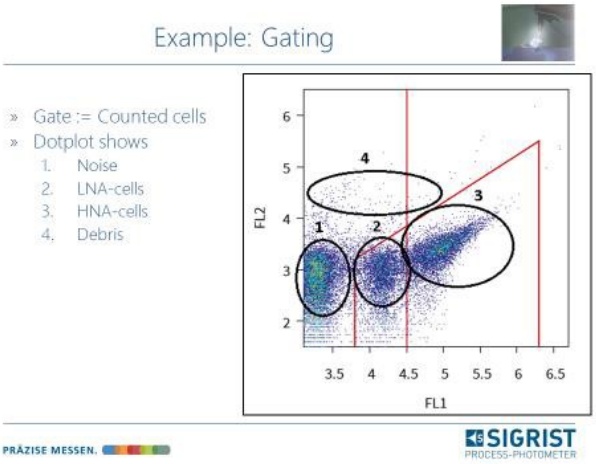

Figure 4. Typical cytogram obtained from the BactoSense flow cytometer.

Some recent applications of the BactoSense

The general applications are summarized in **Table S3**. The following examples [11] highlight some of the uses of the automated BactoSense FCM:

Surveillance of total cell count in raw water - sensitivity of the BactoSense: **Figure S8** [11]

Surveillance of groundwater pumps – monitoring water quality: **Figure S9**

Influence of water level of lake on groundwater microbial quality: **Figure S10**

Contamination detection with TCC cartridge: **Figure S11**

Error detection in a disinfection system with LDC cartridge – process enhancement: (**Figure S12**)

Real water samples analysis: bottled water, raw water, tap water: **Table S4**, **Figures S13-Figure S25**. **Figure 5** is that of a cytogram of a raw dam water sample (TCC = 932 430), before the water treatment process.

After treatment, the TCC dropped to 26 459 (Figure S21). Figure 6 is the BactoSense cytogram of a typical tap water sample, in South Africa.

The TCC/mL for this tap water sample is 89655; another tap water sample from the same source gave a TCC/mL of 105483. For various mineral (bottled) water brands analyzed, the TCC/mL was 1368-205055.

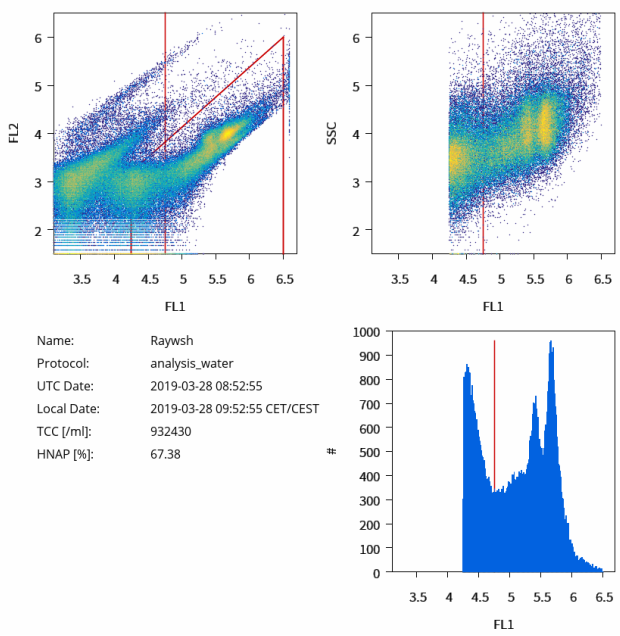

Figure 5. Cytogram for the analysis of a raw, untreated water sample using the BactoSense.

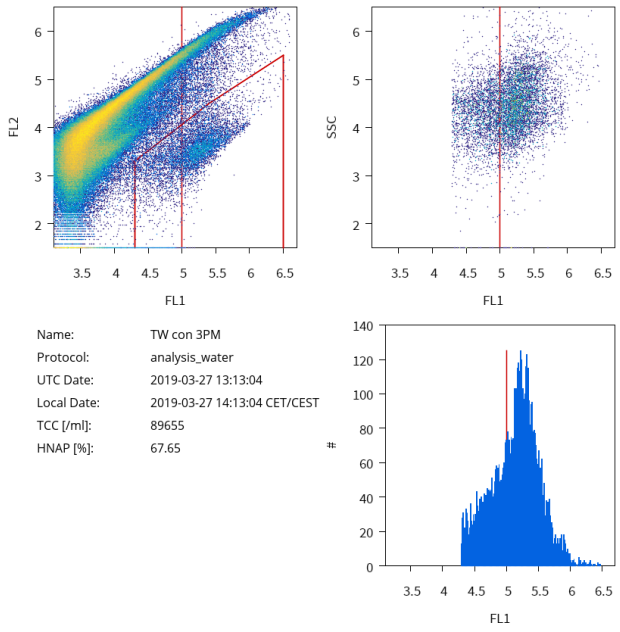

Figure 6. Cytogram for the analysis of a tap water sample using the BactoSense.

These measured values compare fairly well with those ranges reported by Egli and Kotzsch [27] (Figure S26). These data demonstrate the "finger printing" ability of FCM, to identify a specific sample matrix, and within a very short time, unlike the HPC test method.

Accuracy and precision of the BactoSense

Preliminary data (Table 1) are available from the 2018 Ring study [28], conducted in Switzerland- participants received 3 unknown water samples for the analysis of TCC/mL. Thirteen participants used traditional benchtop FCM instruments (A, C, D, E), while three used FCM instrument B/ BactoSense, the only one that is automated for sample preparation. The overall % RSD,

for all the FCM instruments (A, B, C, D, and E) was: 10% for sample 1, 14% for sample 2 and 21% for sample 3. The % RSD for use of the same BactoSense B, was 10% for sample 1, 6% for sample 2 and 12% for sample 3; the average % RSD was the lowest, 9.50% (range = 1.6-12%). This observation is most likely due to the reduced error due to the automated sample preparation, compared to manual, operator sample preparation as required by all the other FCM instruments. The observed RSD via HPC analysis is 30-100% [24,26] but for other reported flow cytometers, the inter-laboratory and instrument variability are <7%, or even <2.5% for a single analyst [13].

Table 1. Ring analysis of 3 unknown water samples by different flow cytometers.

Replicates *n*	FCM Instrument identification/type	Sample 1 TCC/mL	Sample 2 TCC/mL	Sample 3 TCC/mL	Average (Range) % RSD per instrument
1	A traditional benchtop	5226	19036	98467	
2	A	5577	18125	95474	
3	A	6170	22380	89323	
4	A	4920	18823	76177	
5	A	4047	15973	66513	
6	A	4240	17083	73717	
7	A	4293	16867	70913	
	Mean	4925	18327	81512	
	SD	786	2099	12715	
	%RSD	15.96	11.45	15.60	14.34 (11.45-15.96)
	% (Mean/Consensus Mean)	114.7	108.7	114.9	
1	B (BactoSense) Industrial	4874	16422	66189	
2	B	3987	16414	56296	
3	B	4421	18234	72119	
	Mean	4427	17023	64868	
	SD	444	1048	7994	
	%RSD	10.02	6.16	12.32	9.50 (6.12-12.32)
	% (Mean/Consensus Mean)	100.0	101.3	90.7	
1	C traditional benchtop	4020	16435	62748	
2	C	2960	13030	74620	
3	C	5667	17633	79900	
	Mean	4216	15699	72423	
	SD	1364	2388	8785	

	%RSD	32.36	15.21	12.13	19.90 (12.13-32.36)
	% (Mean/Consensus Mean)	95.2	93.4	101.3	
1	D Traditional benchtop	3953	15687	62013	
2	D	2887	13973	58307	
	Mean	3420	14830	60160	
	SD	754	1212	2621	
	RSD	22.04	8.17	4.36	11.52 (4.36-22.04)
	% (Mean/Consensus Mean)	77.3	88.3	84.1	
1	E traditional benchtop	3589	12686	41097	
	% (Mean/Consensus Mean)	81.1	75.5	57.5	
	Consensus Average	4427	16800	71492	
	SD	930	2399	14780	
	%RSD	21	14	21	

Summary of the BactoSense performance

The key feature of the BactoSense is its small footprint, small sample volume required, and the automated sample preparation ability. However, it is not configured to detect Forward Scatter (FSC), which gives information on particle size. For the bacterial enumeration of water, the particle size information is not a top priority. It is suitable for TCC/mL, and ICC/mL, for drinking water, raw water, for contamination detection and for monitoring disinfection systems. The approximate time required for a test result is ± 15 min., which is much faster, compared to the HPC method (1-3 days). The TCC measuring span is: 1 000-2000000 cells/mL, which is ideal for drinking water, where the expected TCC range is ± 1563-1107692 cells/mL. The latter range is within the instrument measuring span and therefore no sample dilution is expected, except for an "over-range" test result (TCC>2000000 cells/mL).

A separate cartridge is required for ICC. For simultaneous determination of TCC and ICC, there is the need for the use of 2 instruments, one with a TCC cartridge and the other with a ICC cartridge, for manual off-line sampling. For on-line sampling, there would be the need for two instruments and separate in-flow streams configurations. Based on the initial proficiency testing study, the BactoSense appears to be fairly accurate and precise (%RSD = 1.6-12%).

Correlation between HPC and FCM

Some data has been reported [29]. The dataset showed an extremely weak correlation between FCM-TCC and HPC (Kendall correlation test: R^2< 0.1; p< 0.001). On average, less than 1 out of 3 000 cells (or 0.032%) detected with FCM was detected by HPC.

TCC determined by FCM in different water samples

A shown in Figure S1, these values (cells/mL), as determined by flow cytometry [27], tend to be higher in general, compared to the corresponding HPC values (cfus/mL) using HPC. From this Figure S14 [27], the approximate, estimated TCC ranges are: (cells/mL): ± 1000-100000 for groundwater and spring water, ± 1563-1107692 for drinking water, ± 36563-309375 for mineral water (bottled), ± 365625-6187500 for surface water, and ± 5062500-67500000 for wastewater. These values are in agreement with earlier reports [24], who reported bacterial concentrations of 1000 cells/mL (groundwater) to 1000000 cells/mL for untreated surface water, and 50000-500000 cells/mL for biofiltration processed water. For SANS 241: 2005, the HPC limit is <1000 counts/mL. Based on ± 1% recovery by HPC, we can estimate, after the negative bias correction, that the corresponding TCC value, when determined by flow cytometry, would be a figure of < ± 99000 cells/mL for drinking water. Other researchers have noted similar TCC values for drinking water, determined by flow cytometry (89655 cells/mL) using the BactoSense.

Some advantages of FCM over HPC

Some of the advantages of FCM [8,21,30,31] are the following:

- FCM detects virtually all (± 99%) bacteria that are present water.
- provides meaningful process- variable information on various drinking water treatment processes and microbiological water quality changes.
- 1-20 min per sample.
- high number of cells can be analyzed per sample: 10000- 50000 cells/s.
- from 5 µL.
- 100 cells/mL.
- multi-parameter analysis.
- single cell detection; no culture, or amplification is required.

- with a variety of staining and labelling methods.
- less than 5% instrumentation error.
- no DNA extraction is required.
- Inter-laboratory FCM comparisons (e.g., Ring Study in 2018 (SIGRIST)) have RSD < 10%; single operator measurements have RSD < 3%.
- beside TCC, FCM can provide information on bacterial viability (ICC) and bacterial identity.
- Multivariate FCM data can be used to create a unique fingerprint of the bacterial community, which improves rapid detection of small changes to that community.
- Depending on labor costs and preferred instruments, FCM costs are equal to or less than those of HPC from about 15 samples per day.
- FCM offers easy automation options, e.g., BactoSense (SIGRIST).
- less space is required compared to HPC method.
- Our HO lab currently does about 800 HPC tests at 37°C and 500 HPC tests at 21°C per month. The SLA is 3 d for HPC 21°C, and 1 d for HPC 37°C. The maximum number of samples that can be analyzed is 20 for HPC 21°C and 30 for HPC 37°C in an 8 hr. working day. With the BactoSense, a cartridge can do 1000 tests; each test takes ± 20 min = 24 tests in 8 hr. and the test result per sample, is available within 20 min, compared to 1-3 d = 24-72 hr., for HPC.

Potential for further FCM applications

Some of these are: Monitoring of bacteria like *E. coli* [1,2], detection of *Cryptosporidium* in water and the screening for SARS-CoV-2 for COVID-19 in clinical samples. For the latter, for FCM, one relevant sample consideration is the target analytes "particle" size and the instrument capability. The virus has also been detected in wastewater, by PCR; there is potential for FCM as a rapid alternative for mapping the spread of infection ("sewage epidemiology"). The use of molecular beacons for DNA multiplexing-flow cytometry detection bioassays is another area. These assays also have potential for bacterial enumeration and for the detection and quantitation of different pathogens, like *E. coli*, in drinking water. Immunomagnetic separation-flow cytometry detection is another promising area for investigation, for both drinking water and body fluid matrix (e.g., for COVID-19), as is Fluorescence Cell Sorting (FACS). The latter is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest.

Due to climate change the pressure on water resources is increasing globally. Concerns on how to find new resources capable to help to reach equilibrium within demand and offer arise. One of the main possibilities to cope with water scarcity is wastewater reclamation and reuse. Umgeni Water is piloting a 2 mL/day reuse plant at its Darvill Wastewater Treatment Works (WWTW) (Umgeni Water Annual Report, 2019). The speed of flow cytometry compared to the much slower HPC method is an attractive option for monitoring the water quality of the influent and effluent (permeate) water at the various treatment stages.

Other flow cytometers for investigation

A large number and variety are on the market that equally deserves an investigation of their suitability for microbiological water quality monitoring, especially as an option to the HPC test. These include: Accuri C6 Plus, NovoCyte 2070V, Attune NxT, MACSQuant 10 and the OnCyt OC-300 automation Add-on [32].

Some research gaps and improvements for FCM

Some gaps have been identified [8]. This review has highlighted the following additional areas:

- Investigation of molecular beacons-flow cytometry based methods.
- Investigation of immunomagnetic separation-flow cytometry.
- Investigation of onCyt Add-On and its claimed compatibility with other FCM models.
- Comprehensive method development and validation for a TCC/mL test method by flow cytometry, for the bacterial enumeration of drinking water (e.g. BactoSense), and other FCMs.
- Standardization and ISO/IEC 17025 accreditation of FCM test methods, e.g., TCC.
- Investigation of related FCM techniques: molecular beacons, immunomagnetic separation, FACS, etc., for pathogen monitoring of drinking water.
- Investigation of FCM for screening of SARS-CoV-2 (virus).

Standardized or accredited test methods by flow cytometry for water analysis

The only known standardized and validated test method, for the determination of total cell counts and distinct bacterial populations in water, is that by the Swiss Health Authority [9]. This was followed by work by Prest et al. [26], partially based on the Swiss guideline [9], who monitored microbiological changes in drinking water systems. While this approval [9] is for TCC in water by flow cytometry, there is no available test method details for use by other water testing laboratories.

Furthermore, in comparison to international test method standardization and accreditation bodies, to date, there are no ISO/IEC 17025-accredited flow cytometry test methods, for bacterial, or viral, enumeration of water matrix. There are also no standardized methods with the U.S. Environmental Protection Agency, the American Society for Testing and Materials (ASTM), Standard Methods for the Examination of Water and Wastewater [12] textbook, the Association of Analytical Chemists, etc.

Towards the development of standard flow cytometry test methods

It is evident that there is a general need for the production of reliable, valid, test results, be it chemical or microbiological, for general drinking water quality monitoring.

The literature has substantial evidence pointing to flow cytometry, using TCC/mL, as a viable option to HPC (cfus/mL) for bacterial enumeration of water [8,10,11,29,32,33]. One of the main issues appears to be a lack of standardization, and ISO/IEC 17025 accreditation, of the reported flow cytometry methods, especially "TCC/mL".

Standards contain technical specifications and criteria designed to be used consistently. One route to standardization of test methods is method validation, defined as a process that demonstrates suitability of an analytical method for its intended purpose [34]. This can be further followed by suitable international accreditation, like ISO/IEC 17025.

Method validation and accreditation

Validation [3,13,15,18] is the process that is followed to demonstrate with the provision of objective evidence, that a specified test method is suitable for the intended purpose.

For the validation of quantitative, microbiological test methods, the minimum validation parameters are: specificity, sensitivity, relative trueness, positive and negative deviation, precision (repeatability, reproducibility), limit of determination within a defined variability, and if necessary, quantitatively determined in assays, and the matrices. The differences due to matrices must be taken into account when testing different types of samples. The raw data should be evaluated with appropriate statistical methods.

Quality control and quality assurance includes proficiency testing refers to interlaboratory comparisons, blind test samples analyzed by the laboratory and proficiency testing schemes. It is an accreditation requirement for laboratories [3]. It also includes internal quality control consists of all the

procedures undertaken by a laboratory for the continuous evaluation of its testing work [3,15,27]. The main objective is to ensure the consistency of test results produced day-to-day and their conformity to defined criteria, or internal limits. A program of periodic checks is necessary to demonstrate that variability (e.g., between analysts and between equipment or materials, etc.) is under control.

Routes to standardization and accreditation of new microbiological test methods: TCC/mL

The following options are proposed as routes to the standardization of flow cytometry test methods, especially for the TCC/mL test on drinking water: ISO/IEC 17025 accreditation, US EPA, ASTM, Standard Methods and the AOAC.

ISO/IEC and 17025 Accreditation: ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies), including the South African Bureau of Standards (SABS). The work of preparing International Standards is normally carried out through ISO technical committees. In the field of conformity assessment, ISO and the International Electrotechnical Commission (IEC) form the specialized system for standardization.

Accreditation is the confirmation by an accreditation body that the organization is competent to conduct its technical activities (tests or calibration) and produce valid reliable results to specified requirements. Implementation of the requirements of a recognized standard as a management system gives confidence that technical activities of an organization will consistently produce valid results. It is competence-based.

The ISO/IEC 17025:2017(E) document titled General requirements for the competence of testing and calibration laboratories, is an international standard that has been developed with the objective of promoting confidence in the operation of laboratories. This standard covers both the technical and management system requirements that need to be fully met, for the accreditation of a test method; the generic requirements are summarized in Table 2. Testing laboratories that are ISO/IEC 17025-accredited are deemed competent to generate valid test results for their accredited test methods. In order for a test method to be ISO/IEC 17025-accredited, there is the prior need for method validation (technical), in addition to the laboratory complying with all the other technical and management requirements as per the ISO/IEC 17025 standard. The generic test method validation and accreditation data requirements [3, 15], are summarized in Table 2.

Table 2. Microbiological test method validation and key accreditation criteria comparison among some reputable, international standard bodies.

Number/ ISO 17025 Clause	Parameter	ISO/IEC 17025	US EPA[a]	US EPA ATP protocol	ASTM	AOAC	Standard Methods
4	General requirements	√					
4.1	Impartiality	√					
4.2	Confidentiality	√					
5.1-5.7	Structural requirements	√					
6	Resource requirements	√					
6.1	General	√					
6.2	Personnel	√					
6.3	Facilities and environmental conditions	√					
6.4	Equipment	√					
6.5	Metrological traceability	√					
6.6	Externally provided products and services	√					
7	Process requirements	√					
7.1	Review of requests, tenders, contracts	√					
7.2	Selection, verification and validation of methods	√					
7.3	Sampling	√					
7.4	Handling of test or calibration items	√					
7.5	Technical records	√					
7.6	Evaluation of measurement uncertainty						

7.7	Ensuring the validity of results	√				
7.8	Reporting of results	√				
7.9	Complaints	√				
7.10	Non-conforming work	√				
7.11	Control of data and information management	√				
8	Management system requirements	√				
8.1	Options	√				
8.2	Management system documentation	√				
8.3	Control of management system documents	√				
8.4	Control of records	√				
8.5	Actions to address risks and opportunities	√				
8.6	Improvement	√				
8.7	Corrective actions	√				
8.8	Internal audits	√				
8.9	Management reviews	√				
	Reason for an ATP	√		√		
1	Method validation	√				√
	Criteria to consider if validation is necessary		√			
	Selection of methods: Standard, rapid, Non-standard	√	√			
	Qualified and competent staff	√	√			
	Procedures for assuring quality of results generated by test methods used for routine/ad hoc/ non routine testing	√				
	Procedure for method validation	√				
	Staff assigned for validation	√				
	Staff training to carry out validation and evaluation of raw validation data	√				
	Appropriateness of equipment used	√				
	Acceptance criteria and basis of acceptance	√				
	Extent of validation	√				
	Standard operating procedure for validation	√				
	Identification of uncertainty sources	√				
	Identification of validation parameters	√				

2	Test method						
	Method development						√
	ATP case number			√			
	Date	√		√	√		
	Revision number	√		√			
	Method summary		√	√	√		
	Introduction/principle	√					
	Scope and application	√	√	√	√		
	Definitions and acronyms	√	√	√	√		
	Interferences/sources of error	√	√	√	√		
	Facilities and environmental conditions	√	√				
	Safety, health and environment	√	√	√	√		
	Sample collection			√	√		
	Sample preservation	√	√	√			
	Sample storage			√			
	Equipment	√	√	√	√		
	Maintenance and services	√					
	Reagents and standard solutions	√	√	√	√		
	Preparation of samples	√					
	Calibration and standardization		√	√	√		
	Analytical procedure	√	√	√	√		
	Quality control	√	√	√			
	Calculation of results	√	√	√	√		
	Data analysis		√	√	√		
	Method performance		√	√			
	Reporting of results	√					
	References	√		√	√		
	Appendix	√			√		
	Document revision and change history	√					
	Pollution prevention		√	√			
	Waste management		√	√			
	Operational limits		√		√		

	Table, diagrams, flow charts, validation data			√	√			
3	Validation Plan	√	√					
	Method title	√						
	Description of measurand/s	√				√		
	Description of matrice/s typically analyzed	√	√	√		√		
	Validation parameter (description of samples, experimental approach and acceptance criterion	√						
	Accuracy/Analysis of unknown samples or Standards (e.g., NIST)	√	√				√	
	Bias or Recovery		√	√			√	
	Selectivity	√	√					
	Limit of detection/method detection limit	√	√	√			√	
	Limit of quantification		√					
	Robustness	√	√					
	Precision: Repeatability	√	√				√	
	Precision: Reproducibility	√	√					
	Uncertainty of Measurement	√						
	Sensitivity		√					
	Calibration curve		√	√				
	Linearity/R^2 statistic		√					
	Range		√					
	Analyte stability/sample holding time/ ruggedness		√	√			√	
	Storage condition of sample		√	√				
	Equivalency testing (samples at 3 different concentrations by the standard and by the new alternate method)						√	
	Multilaboratory validation studies		√					
	Standard Operating procedure						√	
	Tier 1 single laboratory, 1 or more matrix		√					
	Tier 2 multiple laboratory, 1 matrix		√				√	
	Tier 3 nation-wide laboratory, all matrices		√					
	Primary validation/laboratory performance study		√					
	Secondary validation/method performance		√					
	Participating laboratories		√					

	Test materials: CRMs, laboratory prepared spiked materials		√				
	Replication of test materials: ≥ 2		√	√			√
	Precision: 6 analysts, not more than 2 per laboratory						√
	Concentration levels: ≥ 3 over the entire method Range						√
4	Validation Report	√	√				
	Identification of participant laboratory/ laboratories		√	√			
	Description of reagents, spiking materials, reference standards and source/s		√	√			
	Study design summary		√	√		√	
	Test materials used: collection and preparation details		√	√			
	Procedure/s used for analyses of results		√				
	Statistical method/s used for analyses of results		√				
	Title	√					
	Introduction	√					
	Validation parameter/s investigated	√		√			
	Acceptance criteria	√					
	methods		√	√			
	Results and discussion	√	√	√			
	Specifications – method performance summary	√	√	√			
	Development of quantitative QC criteria		√				
	Statement on fitness for purpose	√		√			
	Statistical assessment of method's comparability with any available reference method		√	√			
	Location of raw data	√		√			
	References	√		√			
5	Quality control and quality assurance	√					
	Internal quality control	√					
	Use of spiked samples	√					
	Use of reference materials replicate testing	√					
	Replicate evaluation of test results	√					
	Intralaboratory comparisons	√					

	Proficiency testing	√
	Interlaboratory comparisons	√
	Blind test samples analyzed by the laboratory	√
	Proficiency Testing Schemes (PTS)	√
6	Post-validation activity	
	Evidence that method has been transferred to routine use	√
	Periodic verification that documented performance can be met	√

For internal use only by US EPA personnel

The South African National Accreditation System (SANAS) is the single National ISO/IEC 17025 Accreditation Body that gives formal recognition that Laboratories, Certification Bodies, Inspection Bodies, Proficiency Testing Scheme Providers and Good Laboratory Practice (GLP) test facilities are competent to carry out specific tasks. SANAS is responsible for the accreditation of Certification bodies to ISO/IEC 17021, ISO/IEC 17024 and 65 (and the IAF interpretation thereof), and laboratories (testing and calibration) to ISO/IEC 17025.

In addition to the ISO/IEC 17025: 2017 document, supplementary requirements for validation and quality assurance in microbiological testing, are specifically outlined in the SANAS document: Criteria for validation and quality assurance in microbiological testing [15]. The key technical, requirements, by some international scientific bodies, like the US EPA, ASTM, and AOAC, are compared with the ISO/IEC 17025 guide, summarized in Table 2.

Before submission of a new test method for approval to ISO, the country needs to approach their national ISO body. In South Africa, this is the South African Bureau of Standards. The Standards Division of the SABS acts as a facilitator for the South African State in the development and maintenance of South African Standards and acts as a publishing house. The national process for the submission of a new test method, to ISO is firstly via the SABS: a proposal is submitted for the development of the standard in which a detailed justification for the need of the standard has been identified and how this will have an impact on the South African economy (SABS Project proposal and registration Form (AZ 96.22_2019/02/01 sabs pta - Supplementary Information). The proposal will be presented to the Technical Committee SABS/TC 147/SC 04 Water- Microbiological methods, in the form of a ballot. In the event of the proposal being accepted by the committee, it will then be presented to the Standards Approval Committee (SAC) which will accept or decline the project. If accepted, experts (Working Group) will be invited to draft the document. The final working draft will be presented to the TC 147/SC 04 as a committee draft (CD) which, once approved by the committee, will be circulated for public comment. Upon the address of all comments (if any) and finalization of the document, SAC will give approval before the standard is published.

Registration with the U.S. EPA: With specific regard to drinking and source waters, the Agency develops regulations designed to address the issue of microbial contamination. EPA develops methods that are then validated and used to support regulations. EPA publishes microbiological methods used by public water utilities, academia, industries, and other government agencies. Methods used for these purposes therefore must be validated before they are published as an EPA method. The US EPA has a website for their process of test method approval. The Office of Science and Technology (OST) is responsible for developing, reviewing and promulgating these test methods as well as developing alternatives.

EPA periodically combines new methods and modifications to existing methods into a single package – a proposed "Methods Update Rule" (MUR). Once EPA promulgates final rules, it codifies the approved methods at 40 CFR Part 136. For MURs, EPA considers new or revised methods from two major sources: one is the Agency's Alternate Test Procedure (ATP) program: method developers submit an application for a proposed new method or modification to an approved Part 136 method- an "alternative method"– directly to OST for evaluation for nationwide use. For microbiological test methods, the EPA has a specific guideline document: the EPA Microbiological Alternate Test Procedure (ATP) for drinking water, ambient water, wastewater and sewage sludge monitoring methods [12]. Their specific requirements are summarized in Table 3. The 2nd major source is those methods that are adopted by voluntary consensus standards body (VCSB), such as ASTM International and Standard Methods, or another government agency such as the United States Geological Survey.

Table 3. Summary of the US EPA Microbiological Alternative Test Procedure (ATP) protocol for drinking water, ambient water, wastewater and sewage sludge monitoring methods.

Number	Parameters	Required √
1	ATP or new method	
2	Type of application:	√
	Type of application: Limited use: use by a single laboratory, one or more matrix, excludes drinking water	
	Type of application: Nation-wide use: use by all regulated entities and labs, one or more matrix, includes drinking water	

3	Type of study	√
3.1	Type of study: side-by-side method comparison	
3.2	Type of study: QC acceptance criteria-based method comparison study	
4	Scope of alternate test procedure	√
4.1	EPA approved reference method (For HPC, its Pour plate SM 9215B)	√
4.2	Modifications to sample preparation techniques	√
5	**Application**	√
	Limited use:	
	for wastewater, ambient water;	
	to EPA Regional Administrator	
	Approval authority: EPA Regional Administrator	
	Nation-wide use –	
	for drinking, wastewater, ambient water, sewage sludge (biosolids);	
	all applicants:	
	to ATP program coordinator, EPA Headquarters	
	Approval authority; EPA Administrator	
	Application information: name, address, phone, email;	√
	Date of submission	
	Method number	
	Analyte,	
	Matrix,	
	EPA approved reference method,	
	Type of application	
	Level of use desired	
	Applicants NNPEDS details (if applicable)	
6	**Attachments**	√
	Completed Application Form	√
	Reason for ATP	√
	Method in EPA Format:	√
	Scope and Application	√
	Summary of Method	√
	Method definitions	√

	Interferences	√
	Safety	√
	Equipment and Supplies	√
	Reagents and standards	√
	Sample collection, preservation, storage	√
	Quality Control	√
	Calibration and Standardization	√
	procedure	√
	Data analysis and Calculations	√
	Method performance: data on precision, bias, specificity, detection limits, statistical procedures, summary of side-by-side comparison of performance vs Reference Method specifications	√
	Pollution prevention	√
	Waste Management	√
	References	√
	Tables, Diagrams, Flowcharts, and Validation data	√
7	Study Plan	√
	Background	√
	Objectives	√
	Study design	√
	Coordination: Management Technical approach	√
	Data reporting	√
	Comparison of Methods: for: recovery, precision, false positive rate/specificity, False negative rate/sensitivity.	√
	Number of laboratories	√
	Number of samples; number of matrices; number of replicates	√
	Verification of results	√
8	Quality assurance/quality control	√
	Quality assurance	√
	Control	√
9	Sample preparation and analysis	√
	Collection of samples for analysis	√
	Sample spiking and stressing procedures for bacteriological methods	√

	Spiking procedures for virus methods	√
	Spiking methods for “Crypto” and Giardia	√
	Analysis of samples	√
10	Review of study results (done by the EPA)	
	Assessment of compliance with approved study plan	
	Data review	
	Data validation	
	Development of descriptive statistics: Mean, Recovery, Precision, False positive rates, false negative rates, sensitivity, specificity	
	Statistical acceptance of method comparability	
	Method recommendation and approval	
11	Study Report	√
	Background	√
	Method summary	√
	Organization, Method number, Title for new ATP or Method	√
	Method number or title and publication number for the EPA-approved reference method that is being used for demonstrating method comparability (reference method)	√
	Description of nature of ATP alternate media, alternate concentration technique, etc.)	√
	The matrices, matrix types, and/or media of the new ATP or method	√
	Analyte/s measured by the ATP or new method	√
	Study objectives and design	√
	Study implementation	√
	Data reporting and validation	√
	Results	√
	Data analysis and Discussion Eg, recovery, precision, false positive rates, false negative rates, specificity, sensitivity, etc, which includes the Mean, Standard Deviation, and RSD	√
	Conclusions Based on data analysis A statement/s regarding achievement of study objective/s	√
	Appendix A – Method (as per EPA guidelines and format)	√
	Appendix B – Study Plan (that is approved by EPA)	√
	Appendix C – Supporting data (raw data, QC, calculations)	√
	Appendix D – Supporting References	√

Where appropriate, the validation protocols developed by the EPA are referenced, e.g., ISO, American Society for Testing and Materials (ASTM), International Association of Analytical Chemists (AOAC). The US EPA document: FEM Document Number 2009-01 (Revision: December, 2016), key requirements summarized in Table 2, was prepared by the FEM Microbiology Action Team, for general validation of microbiological methods, to solely provide internal guidance to US EPA personnel. The FEM Document Number 2-009-01 and the ISO/IEC 17025 requirements (Table 2) are fairly similar as regards the key technical requirements.

Registration with ASTM: The high quality of ASTM International standards is driven by the expertise and judgment of members who represent industry, governments, academia, trade groups, consumers, and others. For a new ASTM standard (Test Method, Specification, etc.), the required templates are available on the ASTM website, summarized in Table 2. Unlike the rigorous, defined technical requirements of the ISO/IEC and US EPA, there seems to be little focus on method validation requirements.

Development of Standard Methods: The Standard Methods for the Examination of Water & Wastewater textbook is a comprehensive reference that covers all aspects of water and wastewater analysis techniques. Standard Methods is a joint publication of the American Public Health Association (APHA), the American Water Works Association (AWWA), and the Water Environment Federation (WEF). The work of the Standard Methods committees of APHA, AWWH and WEF is coordinated by a Joint Editorial Board (JEB), on which all three are represented.

For each new edition, both the technical criteria for selection and the formal procedures for their approval are reviewed critically. A Joint Task Team is established for each section; appointment of an individual to the Team is based on expressed interest or recognized expertise. The task team reviews the pertinent methods, makes recommendations and presents those methods, which is then ratified by vote by the Standards Committee. Issues not resolved are made by the Joint Editorial Board. Generally, the methods presented here are believed to be the best available and generally accepted procedures for the analysis (physical, chemical, microscopic, bacteriological) of water, wastewaters and related materials. They are truly consensus standards, which offer a valid and recognized basis for control and evaluation.

There are 2 classes of Methods: 1) Proposed: this method must undergo development and validation that meets the requirements of Section 1040A of Standard Methods, and 2) Standard: A procedure qualifies as a Standard Method in one of two ways: a) the procedure has undergone development and validation and collaborative testing that meet the requirements set forth in Section 1040B and C of Standard Methods [35]. The key requirements are summarized in Table 2. It is "widely used" by the members of the Standards Committee; or b) the procedure is "widely used" by the members of the Standards Committee and it has appeared in Standard Methods for at least 5 years. A new method may be adopted as Proposed or Standard by the JEB at any time. Such a decision is based on the usual consensus procedure. Such methods are then added to Standard Methods On-line.

The Association of Analytical Chemists (AOAC): The AOAC International provides the international platform, processes and scientific rigor that enable industry and regulators to keep food and environment safe. The AOAC INTERNATIONAL brings together government, industry, and academia ("expert stakeholders") to establish standard methods of analysis. The "expert stakeholders" first define specifically what is needed in a test, then evaluate the reliability and accuracy of testing solutions submitted by scientists. Standards that pass this "consensus" scrutiny are accepted as valid by the global analytical community.

Standard Method Performance Requirements℠ (SMPRs) is voluntary consensus standards, developed by stakeholders that prescribe the minimum analytical performance requirements for classes of analytical methods.

"Analytical Methods" are detailed guidelines and protocols used in performing laboratory chemical or microbiological analyses. AOAC INTERNATIONAL's Official Methods of Analysis℠ program is the organization's premier program for consensus method development. Methods approved in this program have undergone rigorous scientific and systematic scrutiny and are deemed to be highly credible and defensible.

AOAC consensus standards development programs have active science programs. There is currently the Quantitative Microbiology Method Validation Acceptance Criteria – ASF Working Group (Alternative Methods), who aim to develop acceptance criteria in support of validating quantitative microbiology alternative methods.

To submit a test method to the AOAC Official Methods program (OMA), individual method authors first submit the required documentation (Method manuscript, in OMA format, package insert or user manual, Safety checklist, and Validation protocols). ASTM staff will then set up a teleconference to discuss the author's method in detail; the Expert Review Panel (ERP) process and answer questions pertaining to the review of their submitted method. There are two other available guides for method performance and method validation: The Appendix F: Guidelines for Standard Method Performance Requirements document details general method validation. The AOAC document: Appendix J: AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces outlines details for the validation, summarized in Table 2: The latter is dated 2012. A Working Group is currently looking at its update; this is encouraging to note as there appears to be a significant gap when one compares the current criteria with that of ISO/IEC, or the US EPA (Table 2).

Current test methods for bacterial enumeration as TCC using flow cytometry.

Whilst there are various test methods for TCC/mL using flow cytometry [8,29,26], it is clear that, based on the international requirements for standardization, and accreditation, none of them meet all the requirements or the test method performance data are not available: e.g. accuracy, precision, etc.

Conclusion

The present review has highlighted the following:

- The current HPC plate method for bacterial enumeration of water is inaccurate; the bias is approximately -99% .
- The time required to perform an HPC test is very long: 1-3 days.
- The BactoSense FCM: preliminary validation data indicate: Measuring span for TCC = 1000-2000000 cells/mL; Detection limit = 100-5000000 cells/mL; precision: % RSD = 6-12% for the TCC range ± 4427-64868 cells/mL; preliminary accuracy = 91-100%, for the TCC range ± 4427-71492 cells/mL, based on unknown samples analysis. Due to its upper measuring range (at least 2000000 cells/mL), there appears to be no need for drinking water sample dilution as the expected TCC by FCM would be in the range: ± 1600 – 1100000 cells/mL.
- The BactoSense FCM appears to be a viable option for bacterial enumeration, as a TCC/mL measure, for drinking water, due to its sample preparation automation, speed of analysis, accuracy and precision.
- Flow cytometry is the preferred test method for bacterial enumeration of water, due to its relatively higher accuracy (± 99%) and speed of analysis (± 15-20 min.). The real samples analysis with the BactoSense has confirmed that FCM is ideally suited for rapid, accurate "finger-printing".
- Test method standardization promotes availability of relevant technical information (instrument method validation data), e.g., via peer-reviewed publications.
- Current test methods for bacterial enumeration of drinking, using flow cytometry, are not standardized or accredited; test method performance data is not available.

- Possible options towards the standardization of a test method for the bacterial enumeration of water, by flow cytometry, as a TCC/mL measurement are, in decreasing rank, ISO/IEC 17025 accreditation>US EPA>ASTM/Standard Methods>AOAC.
- The degree of technical rigor required for microbiological test method standardization, and accreditation, decreases in the order: ISO/IEC 17025 accreditation>US EPA>ASTM/Standard Methods>AOAC.
- Key microbiological test method validation parameters are: scope (sample matrices), accuracy, precision (repeatability, reproducibility), bias, specificity, LOD, LOQ, relative trueness, positive and negative deviation, measurement range, linearity, and uncertainty of measurement.

The data collection and analysis strategy work by Prest et al. [26]– use of the same gates in the form of a fixed template to allow direct comparisons of data from different sample analyses – is one foundational basis for future method validation proposed here, as is the recent analytical validation of probiotic bacterial count by FCM [36]. The time has come for the optimization, standardization and accreditation of current and new, FCM-based TCC test methods.

Recommendations

The following are proposed:

- Further comprehensive method validation of the BactoSense as per the recommended guidelines (e.g., ISO/IEC 17025, US EPA, etc.); accuracy should be additionally assessed using a reference standard or a Certified Reference Material, like NIST.
- Investigation of the market for other automated FCM instruments and other FCM-FACS technology, and an evaluation of their suitability for microbiological water quality testing, starting with its application to bacterial enumeration (total cell count). In essence, the 3 main FCM hardware components that require consideration and optimization are: the initial fluidics (sample introduction), the optics and detection (detector), and the signal and pulse processing (data).
- Practical evaluation of the BactoSense as a supplement to current microbiological tests, especially the HPC, and its eventual replacement as the goal.
- Evaluation of the BactoSense for important pathogens monitoring, like .
- Addressing the research gaps in flow cytometry.
- Standardization and accreditation of current, and new, optimized flow cytometry test methods, and equipment, for microbiological water quality monitoring, specifically TCC. The preferred route appears to be via the US EPA or ASTM, as ISO/IEC 17025 accreditation requires testing laboratories meeting and complying with the full management requirements of ISO/IEC 17025 as well. As per the US EPA, the Flow Cytometry-based method is not currently an approved method to determine Heterotrophic bacteria under the current regulation, Surface Water Treatment Rule: the reference method is the Standard Methods 9215B [37], using Plate Count Agar.
- Change of current regulations for HPC in drinking water: Once standardisation has been achieved, there will be a need to reconsider the current regulatory limits for HPC (currently <1000 in South Africa, and ≤ 500 cfus/mL in the US). Based on the current base line for total cell counts in drinking water, obtained by flow cytometry, this will be expected to be in the range: ± 1 563-1107692 cells/mL, as a TCC/mL measure.
- Uncertainty of measurement: Inclusion of this method validation parameter needs to be considered by the US EPA, and other standardization bodies, in their current protocols.
- Investigation of molecular beacons DNA multiplexing and immunomagnetic separation-flow cytometry detection for improved selectivity.
- Screening for SARS-CoV-2 virus for COVID-19: Rapid, accurate test methods are required in light of the risk of the global pandemic; flow cytometry needs to be considered due to its speed of analysis.
- Technological developments of a single FCM instrument that has dual channels/sample streams, with a single cartridge, that can measure TCC and ICC without the need to change cartridges - the current BactoSense can be used as the base/platform.

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