Application Note

Bacterial count of disinfected drinking water

Quantify bacteria in minutes instead of days

Introduction

When talking about water treatment, one can define two different strategies working together on improving microbial water quality.

The first strategy is removing microorganisms from water, using chemical, physically, or biological processes. Classical water treatment often starts with a chemical coagulation-flocculation step. This process leads to the formation of bigger flocs which can be easily removed by secondary sedimentation and/or filtration. Filtration can be a simple physical treatment like rapid sand, or membrane filtration or a biological process like slow sand filtration.

The second strategy is disinfection. Disinfection means to kill or at least inactivate microorganisms. Chlorination and ozonation are the most common disinfection methods.

Both disinfectants kill via oxidization. Ozone shows the strongest and fastest disinfection effect, it destroys the cell wall of microorganisms via a so called oxidative burst. However, the effect is limited to the point of application and only lasts a few minutes.

The usage of chlorine as disinfectant started in the 1880s. Contrary to ozone, once added, chlorine stays in the water for a longer time (residual chlorine). Dependent on pH, starting concentration and temperature, the disinfecting effect persists also in the distribution network.

In recent years, chlorine has been increasingly replaced by chlorine dioxide CLO_2. The disinfectinction with CLO_2 is stronger, lasts longer, is pH-independent and it reduces the investment costs compared to classic chlorination.

Nevertheless, optimization of the consumptions of CLO_2 saves money and prevents side effects like unwanted smell and taste of the water. Therefore it is essential to check for disinfection efficiency. Bacteria with disrupted membranes are dead. CyStain™ BacCount Viable is the most standardized method to reliably check for the membrane integrity of bacteria.

The amount of living bacteria before and after disinfection, at different CLO_2 concentrations as well as in different distances from the treatment plant, or in storage tanks, can be checked easily. In Combination with online monitoring the CyStain™ Kits offer the possibility to get real-time information on the amount of living bacteria from different places in a water distribution network.

Instrument requirements

The CyStain BacCount reagents use the CyFlow™ Cube 6 V2m Flow Cytometer (Ref. No. CY-S-3061R) – an easy-to-use and cost effective test system. The optional CyFlow™ Robby 6 Autoloading Station (Ref. No. CY-S-3083) allows a semi-automated enumeration of bacteria in water. The Robby is directly attached to the Cube 6 V2m Flow Cytometer and is able to pipette and to shake the samples using a 96 Well Plate.

Fig. 1: The CyFlow Cube 6 V2m Flow Cytometer with the CyFlow Robby 6 V2m Autoloading Station and the BacCount Viable kit.

Material and methods

Sysmex offers two different BacCount reagents: The CyStain BacCount Total kit enables detecting the total amount of bacteria – this is crucial to assess the microbial status of water. The CyStain BacCount Viable kit discriminates between live and dead bacteria, which is important to assess their physiological state. Both kits are designed for the quality control of water-based samples including drinking water.

Kit components

CyStain BacCount Total kit:

- 5 Aliquots x 40 µL CyStain™ Green
- 29 mL CyStain™ Dilution Buffer

Fig. 2: CyStain BacCount Total kit (Ref. No. 05-5008).

CyStain BacCount Viable kit:

- 5 Aliquots x 40 µL CyStain Green
- 5 Aliquots x 400 µL CyStain™ Red
- 29 mL CyStain Dilution Buffer

Fig. 3: CyStain BacCount Viable kit (Ref. No. 05-5028).

Additional required equipment

- 96 Well Plate (Ref. No. 04-2020)
- Heating block/water bath set to 37 °C ± 0.5 °C
- Vortex mixer

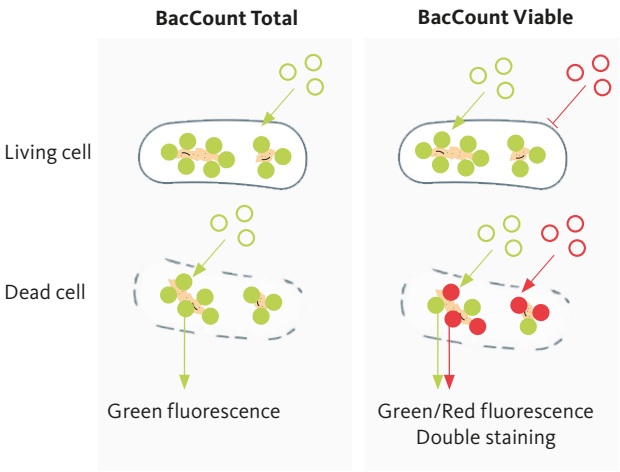


Fig. 4: Staining principle of BacCount Total (left) and BacCount Viable (right).

Staining principle

The convenient staining principle allows a fast detection of bacteria in less than 15 minutes. The total cell count is based on the labelling of all bacteria with the membrane-permeant nucleic acid-binding dye CyStain Green. After excitation the fluorescence signal is counted for each individual microorganism. On the other hand BacCount Viable contains the additional CyStain Red dye. This dye is not membrane permeable and stains only bacteria with a damaged cell membrane. This is an indication of dead or dying cells.

Sample preparation: Automated mode

NOTE: There are two options for sample preparation: Manual and automated sample analysis. In this Application Note all measurement were performed in the automated mode. For a detailed overview for manual measurements, please have a look at the Application Notes Total cell count (TCC) - [1] or Viable cell count (VCC) [2] of bacteria in water.

1a. Only for BacCount Total
Prepare a 10X working solution by diluting the 1000X stock solution of CyStain Green 1:100 with CyStain Dilution Buffer.

1b. Only for BacCount Viable
Prepare a working solution for both stains by diluting 20 µL stock solution of CyStain Green and 200 µL stock solution of CyStain Red with 1780 µL CyStain Dilution Buffer.

2. Mix 20 µL CyStain™ working solution with 180 µL water sample in a 96 Well Plate.

3. Mix gently by pipetting up and down the solution.

4. Incubate sample for 13 minutes at 37 °C ± 0.5 °C, protected from light.

Analysis range

The maximum cell number for an accurate data acquisition is 2 x 10^5 cells/mL. It is recommended to dilute a water sample if the cell number is higher. Dilute the water sample with ultrapure water or 0.1 µm filtered water before staining with the CyStain BacCount reagents.

Tab. 1: Analysis range of the CyStain BacCount reagents.

Detection Limit	Reportable Range
200 cells/mL*	1 x 10^3 – 2 x 10^5 cells/mL

*A specific treatment of the CyFlow Cube 6 V2m Flow Cytometer is required if the expected total cell count is lower than 1 x 10^4 cells/mL. Please refer to section General hints in the Application Notes Total cell count (TCC) - [1] or Viable cell count (VCC) [2] of bacteria in water for further information.

Data acquisition

NOTE: For a detailed data acquisition please refer to the Quality Check Manual [3].

1. Switch on the CyFlow Cube 6 V2m Flow Cytometer.
2. Enter your user name and passwort to start CyView™ software.
3. Load the script for automated analysis
4. Start the Priming procedure for an initial cleaning step of the device
5. Start the Quality Check (QC) procedure and follow the instructions on the display
6. Enter QC material information for LOT N° and expiry date on the automated FCS Express report
7. The analysis of stained water samples can be started after a “valid” QC procedure.
8. Insert a 96 Well Plate with your samples into the CyFlow Robby Autoloading Station and start measurement.
9. After the measurement FCS Express automatically starts and opens analysis report.
10. Start the Shutdown procedure after the sample analysis is completed and follow the instructions on the display.

Results

In cooperation with a North Rhine-Westphalian waterworks, water samples were analysed on site by flow cytometry in addition to the daily routine tests. The water samples were taken from two waterworks from different water treatment stations (Fig. 5). For analysis the water samples were stained with the CyStain BacCount Total or the CyStain BacCount Viable kit and measured automatically using the CyFlow Cube 6 V2m Flow Cytometer and the CyFlow Robby V2m Autoloading Station.

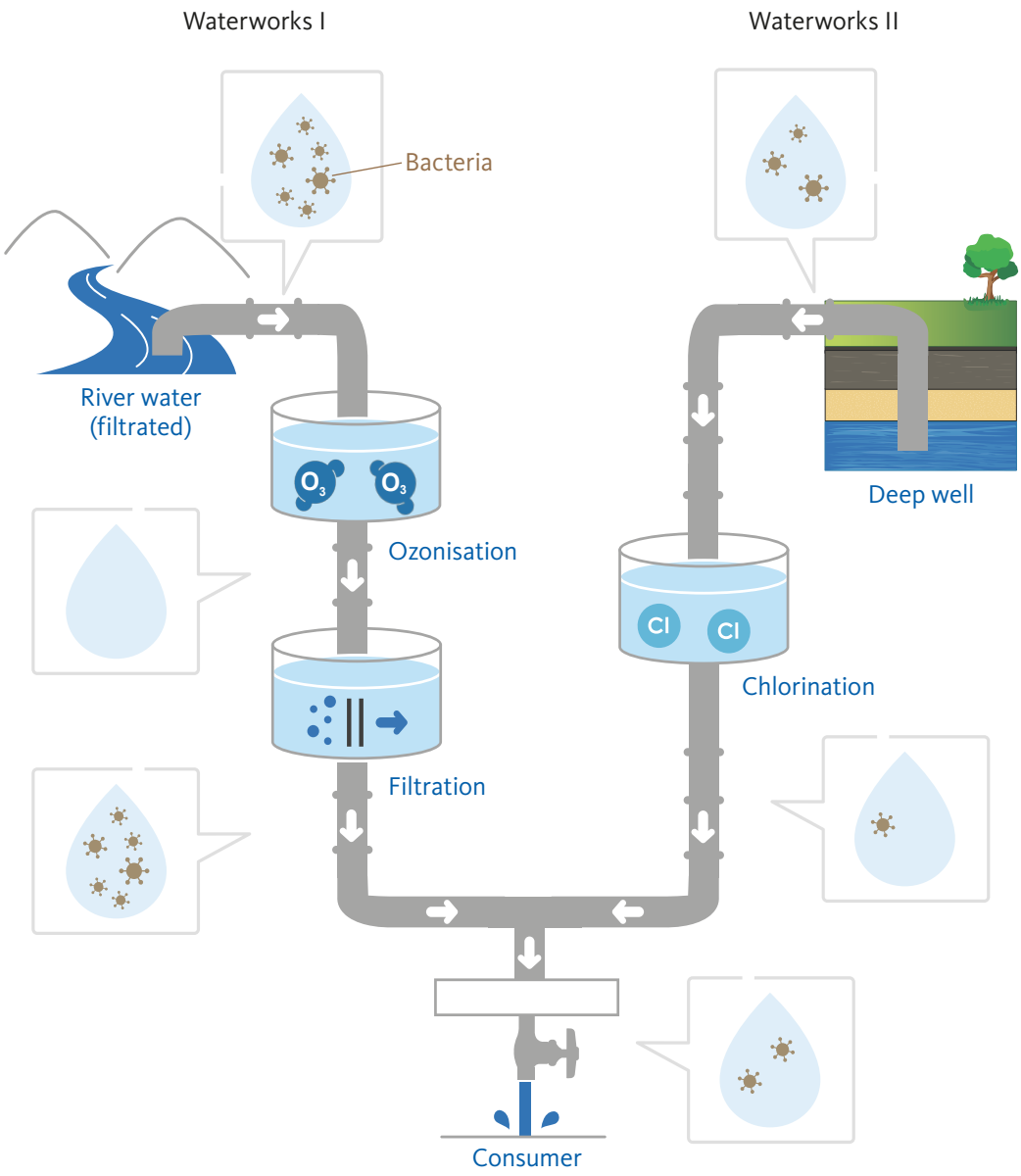


Fig. 5: Illustration of the process of water treatment of waterworks I and II (not all steps shown detail). The bacteria icons in the water droplets symbolize the approximate number of bacteria measured with CyStain BacCount Viable.

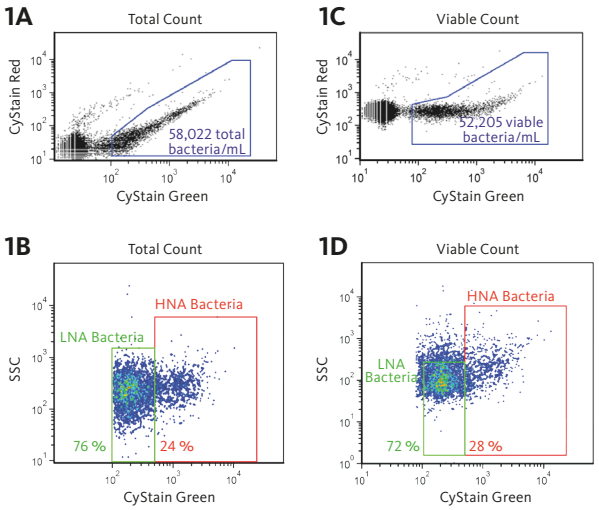
Exemplary results of the CyStain BacCount Total and the CyStain BacCount Viable measurements for the respective station in the waterworks are summarized in Tab. 2 and are shown in Fig. 6.

In Waterworks I, the raw water extracted from a river is constantly treated with ozone so that the original bacterial content is completely reduced. After the subsequent filtration, in this case harmless bacteria are introduced. Based on the LNA/HNA ratio, a different fingerprint than in the raw water is detectable, which indicates that the bacteria are introduced into the water via the filtration step.

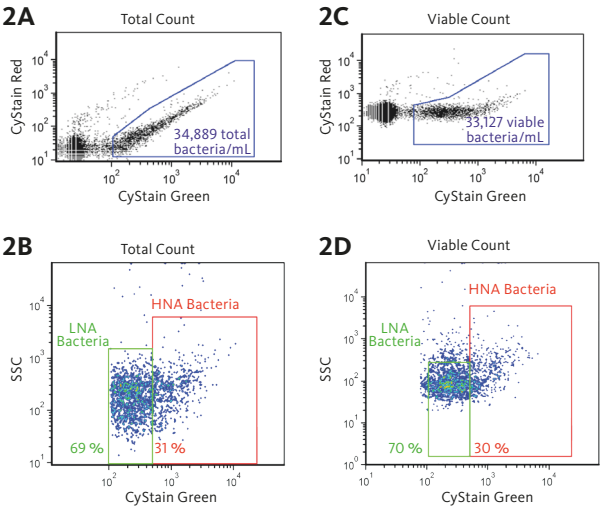
Tab. 2: Bacterial count of water samples at the respective station in the waterworks.

Water sample		Total Count per mL	Viable Count per mL
Waterworks I	River water	58,022 LNA: 76 % HNA: 24 %	52,205 LNA: 72 % HNA: 28 %
	Ozonisation	67 LNA: 33 % HNA: 67 %	22 LNA: 0 % HNA: 100 %
	Filtration	71,355 LNA: 64 % HNA: 36 %	59,858 LNA: 55 % HNA: 45 %
Waterworks II	Deep well water	34,889 LNA: 69 % HNA: 31 %	33,127 LNA: 70 % HNA: 30 %
	Chlorination	27,333 LNA: 43 % HNA: 57 %	4,601 LNA: 85 % HNA: 15 %
Mixed water		88,600 LNA: 65 % HNA: 35 %	5,678 LNA: 60 % HNA: 40 %

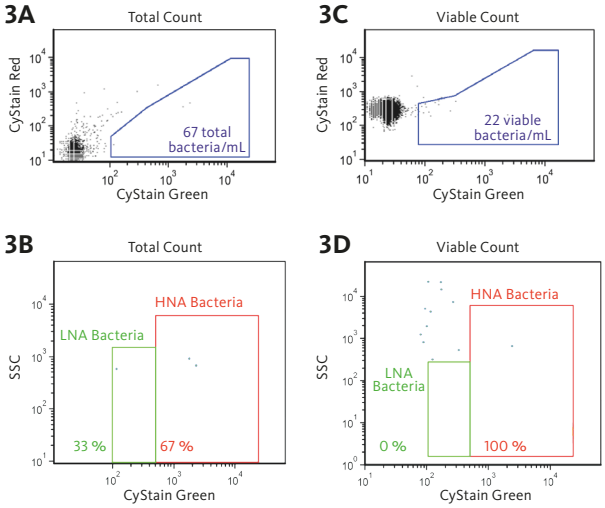
River water (Waterworks I)



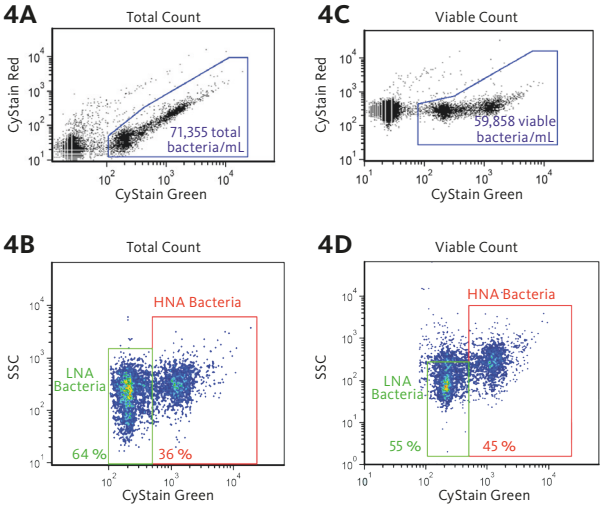
Deep well (Waterworks II)



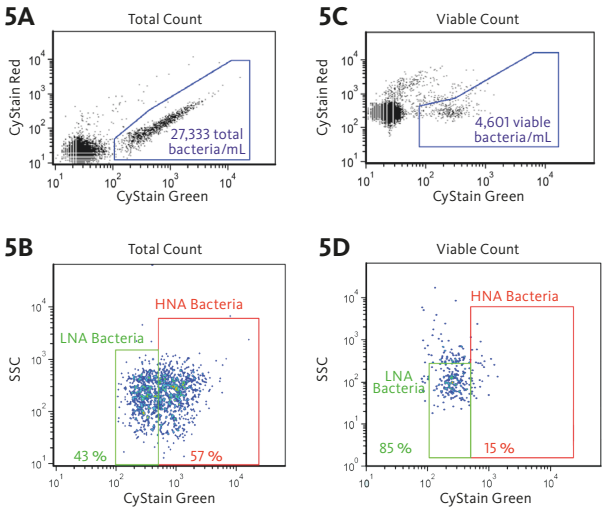
Ozonisation (Waterworks I)



Filtration (Waterworks I)



Chlorination (Waterworks II)



Mixed water

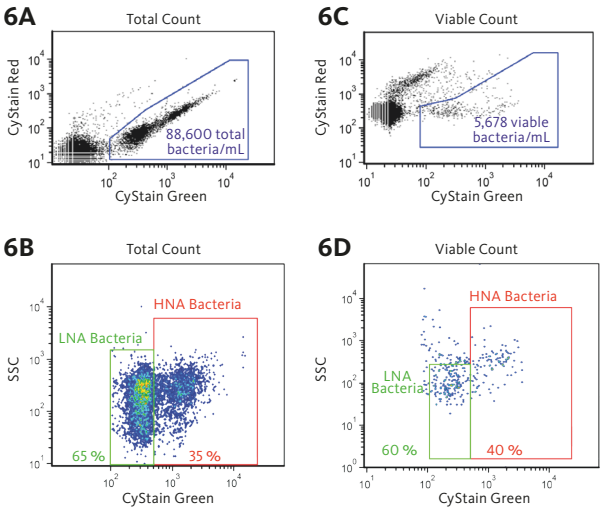


Fig. 6: Water samples were taken from different stations of two waterworks and stained with either the CyStain BacCount Total or the CyStain BacCount Viable kit and automatically analysed using the CyFlow Cube 6 V2m Flow Cytometer and the CyFlow Robby V2m Autoloading Station. (1) River water (Waterworks I), (2) Deep well (Waterworks II), (3) Ozonisation (Waterworks I), (4) Filtration (Waterworks I), (5) Chlorination (Waterworks II), (6) Mixed water. (A) Dot plot showing CyStain Green vs. CyStain Red. All bacteria are located and automatically counted within the predefined blue gate. (B) Dot plot showing the side scatter (SSC) vs. CyStain Green of all bacteria. (C) Dot plot showing CyStain Green vs. CyStain Red. All viable bacteria are located and automatically counted within the predefined blue gate. (D) Dot plot showing the side scatter (SSC) vs. CyStain Green of all viable bacteria.

In Waterworks II, the deep well water is continuously treated with a chlorine concentration of 0.1 mg/L. The original deep well water has a similar fingerprint to river water. After chlorination the deep well water shows a high content of LNA bacteria.

The water of the two waterworks is mixed before it reaches the end customer. Although the mixed water has a total cell count of approximately 89,000 bacteria per mL, more than 90 % of these have been killed. The routinely performed heterotrophic plate count did not reveal any harmful bacteria for all water samples during the process of treatment.

Conclusion

Flow cytometric analyses during the treatment process of waters allows changes in the quality of the water to be monitored in a timely manner. The CyStain BacCount kits are ideally suited for monitoring disinfection processes without the need for special pre-treatment of the water samples. In addition, information about the typical fingerprint of a water sample is obtained and the efficiency of filtration processes can also be checked.

References

1. Sysmex Partec GmbH, 2020. *CyStain BacCount reagents*. Quality Check Manual.
2. Sysmex Partec GmbH, 2020. *Total cell count (TCC) of bacteria in water.* Application Note.
3. Sysmex Partec GmbH, 2020. *Viable cell count (VCC) of bacteria in water.* Application Note.

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