Microbial activity in waters: comparative study between Solid-Phase Cytometry and Flow Cytometry

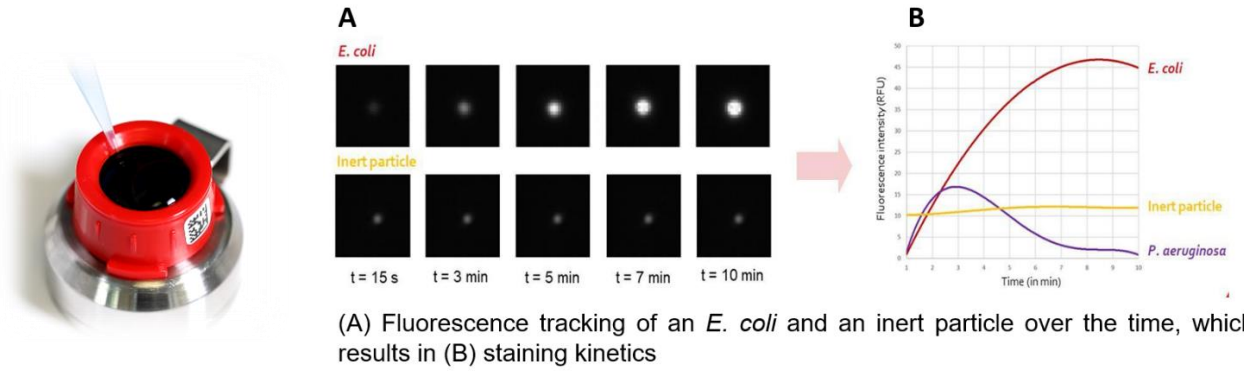
This application note compares Red One™ Solid-Phase Cytometry (SPC) and Sysmex CyFlow™ Cube 6 & 8 Flow Cytometers (FC) for rapid microbial detection in water samples. Key findings indicate that Red One™ is robust for detecting low microbial concentrations and avoids false positives from non-viable particles, unlike flow cytometry. Strong correlations between Red One™ and traditional Heterotrophic Plate Count (HPC) methods suggest its reliability for water quality control.
Additionally, Red One™ offers faster results (within 10 minutes), making it ideal for real-time analysis.

Red One™ SPC patented platform

The Red One™ system is a fully automated rapid microbiology system based on Solid-Phase Cytometry technology **(Figure 1)**. It detects single-cell microorganisms (including biofilms) and delivers instantaneous, quantitative microbiological results. Results are obtained quickly, typically within 10 minutes. The system is user-friendly: the sample is first placed onto a cap and then filtered through a membrane directly on the platform.

Utilizing a fluorescein derivative sensitive to esterase activity, combined with a high-resolution CMOS camera and powerful LED illumination, the Red One™ system captures high-resolution images of microorganisms before, during, and after the application of the viability staining agent. This patented staining kinetics monitoring ensures reliable differentiation between viable cells and non-viable particles, such as those exhibiting auto-fluorescence (**Figure 2**).
The system is able to process sample volumes ranging from 10 µL to 250 mL using single-use caps with track-etched PET membranes (porosity: 0.4 µm). All operations, including filtration and staining, are fully automated, eliminating the need for calibration.

Figure 1: Red One™ - Solid-Phase Cytometry (SPC) System



(A) Fluorescence tracking of an *E. coli* and an inert particle over the time, which results in (B) staining kinetics

Figure 2: Staining kinetics principle on Red One™ (after sample filtration)

1. SPC and FC Cell counting range and correlation with HPC on *E. coli ATCC 8739*.

Objectives

Compare the performances of two alternatives methods, Red One™ and CyFlow™ Cube 6, to enumerate microorganisms over a wide range of concentration. The counting range for each method is determined using serial dilutions of E. coli (ATCC 8739). Correlations with TSYE agar plate culture method are established to assess accuracy and precision of each method.

Method

Counting range and correlation were evaluated on the *E. coli* ATCC 8739 strain.

Two overnight cultures on Tryptic Soy Broth (TSB) (CM0129B, Oxoid) were obtained (considered after as Replicate N°1 and Replicate N°2).

For each replicate, serial dilutions were obtained using McIlvaine's buffer pH 7.2 (**Figure 3**).

10 concentrations were analyzed on both systems to evaluate the counting range of both technologies.

Staining procedure on Red One™ is fully automated with the Red One™ TVC reagent kit. 1mL of the bacterial suspension is dropped onto a Red Cap for analysis.

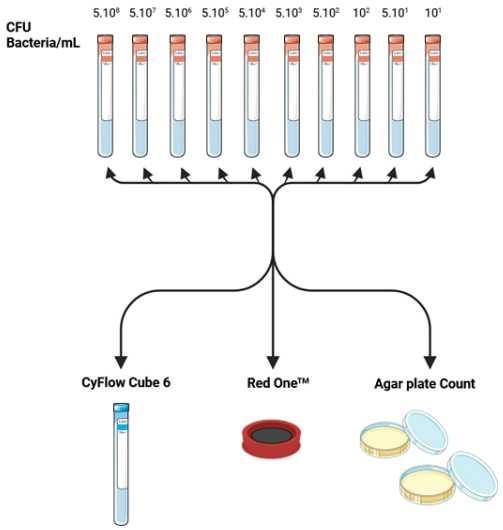


Figure 3: *E. coli* sample preparation

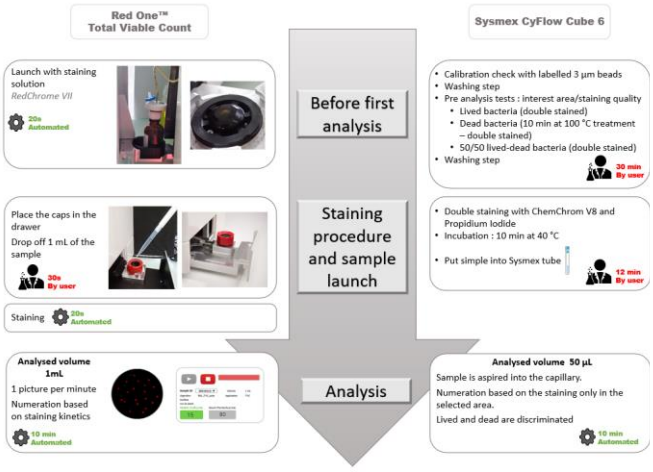


Figure 4: Comparison between Red One™ and CyFlow™ Cube 6 workflows

For more information on both systems:
Red One™ - Rapid Microbiology System:
Click for 2-min video
Sysmex – Cyflow Cube 6 System:
https://www.sysmex.fr/produits/product-list-singleview/cyflow-cube-6-3966.html

Staining procedure for Sysmex CyFlow™ Cube 6 consists in a double staining in 1mL bacterial suspension with 10µL of ChemChrome V8 (306-R1002-01; Biomérieux) and 10 µL of propidium Iodide 1mg/mL (P3566, Invitrogen). Bacterial suspensions were incubated for 10 min at 40°C and 50 µL of stained bacteria are then immediately analyzed on the flow cytometer.

Enumeration of each bacterial suspension was performed on two TSYE agar plate (PO5050A, Oxoid), incubated 24h at 30°C. Correlation between Red One™ and CyFlow™ Cube 6 was established in comparison to TSYE agar plate count.

Results

Both CyFlow™ Cube 6 and Red One™ show strong performance within the bacterial concentration range of $5x10^2$ to $5x10^6$ CFU/mL **(Figure 5),** with high correlation values ($R^2 = 0.99$), indicating that they are equally reliable for bacterial quantification in this range.

However, at lower bacterial concentrations ($5x10^0$ to $5x10^2$ CFU/mL), Red One™ demonstrates greater accuracy and stronger correlation to the traditional TSYE agar plate count method (R^2 > 0.99), even near its detection limit of 10 cells (**Figure 6)**. In contrast, CyFlow™ Cube 6 shows lower correlation values at these concentrations (R^2 = 0.71 and 0.52 for replicates 1 and 2, respectively), suggesting reduced precision for detecting bacteria at the lower end of the concentration range.

Thus, while both devices perform similarly at higher concentrations **(Figure 5),** Red One™ provides a clear advantage in accuracy at lower bacterial concentrations **(Figure 6)**.

It makes the method particularly suitable for applications requiring low detection limits along with its ease-of-use (automated staining) and rapidity (results obtained in 10 minutes).

Figure 5: Correlation between Red One™; or CyFlow™ Cube 6 and agar plate counts for dilutions from $5x10^2$ to $5x10^6$ CFU/mL

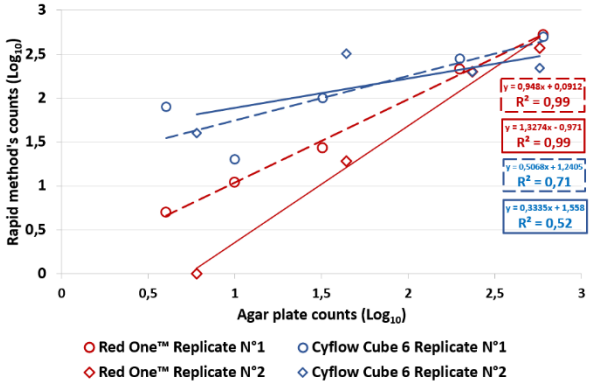


Figure 6: Correlation between Red One™; or CyFlow™ Cube 6 and agar plate counts for dilutions from $5x10^0$ to $5x10^2$ CFU/mL

2. Correlation with R2A agar plate count on *E. coli* environmental strain.

Objectives

Compare correlation of Red One™ and CyFlow™ Cube 6 on an artificially stressed E. coli environmental strain (isolated from Seine River water; FRANCE) with the R2A agar plate culture method. A stress protocol was applied to this strain to simulate a low metabolic activity of E. coli in tap waters from two different origins.

Method

Figure 7 describes the stress protocol applied: 4 dilutions were analyzed with both technologies as follow:

Staining procedure on Red One™ is fully automated with the RedChrome Water reagent kit (adapted to water testing). 1mL of the bacterial suspension is dropped onto a Red Cap for analysis. Two staining procedures were tested for Sysmex CyFlow™ Cube 6:

- a first double staining in 1mL bacterial suspension with 10µL of ChemChrome V8 (306-R1002-01; Biomérieux) and 10 µL of propidium Iodide 1mg/mL (P3566, Invitrogen). Bacterial suspensions were incubated for 10 min at 40°C and 50 µL of stained bacteria are then immediately analyzed on the flow cytometer.

- a second double staining was made with 3µL of Syto9 (Invitrogen) and 10µL of Propidium Iodide 1mg/mL (P3666, Invitrogen) added in 1mL of bacterial suspension. Samples are kept for 15 min at room temperature and 50 µL of stained bacteria were immediately tested on the device. Numeration of each bacterial suspension was made on two R2A agar plate (84671.0500 ;VWR), incubated 24h at 37°C.

Correlation for each technology is established compared to agar plate count.

Results

- Red One™ shows very high correlation with R2A agar plate count: $R^2 = 0.98$
- CyFlow™ Cube 6 has lower correlations $R^2 = 0.3$ for both staining methods used (**Figure 8**).

A low correlation is observed for CyFlow™ Cube 6 counts with two different staining agents and a double staining with Propidium Iodide 1mg/mL. With the exclusion of dead cell an overestimation is visible for low bacterial loads.

This overestimation is probably due to the sensitivity of systems based on viability staining to background noise, assessed section 3.

In conclusion, Red One™ is well adapted to detect low concentration of stressed microorganisms.

Strong correlation (R^2=0.98) between Red One™ and R2A counts suggest that this technology is able to count stressed *E. coli* in autoclaved tap water, included at low concentrations.
The CyFlow™ Cube 6 has low correlation (R^2=0.3) for the two tested staining agents based on different principles: viability staining (ChemChrome V8) and nucleic acid staining (Syto 9). Flowcytometry's performance is probably affected by matrix background noise and cannot detect stressed microorganisms in such matrices.

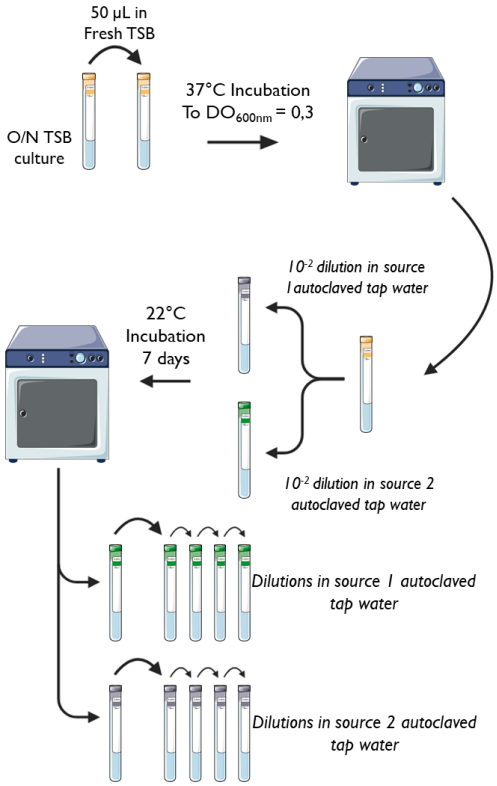


Figure 7: Stress protocol for *E. coli* environmental strain in two originated tap waters.

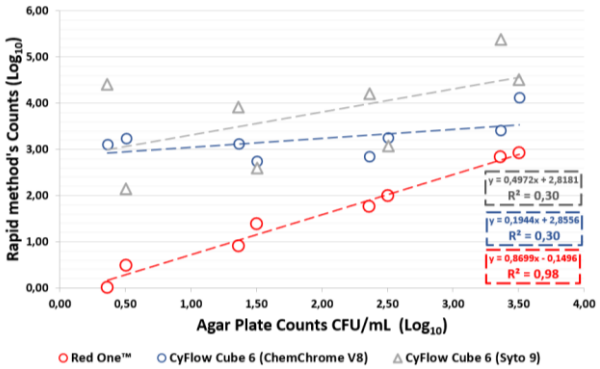


Figure 8: Correlation between Red One™ or CyFlow™ cube 6 and R2A agar plate count

3. Evaluation of sensitivity in SPC and FC methods.

The analytical sensitivity of a system based on viability staining can be determined by the limit of detection estimated by the use of blank measurements (**Figure 9**). Below this limit, any system cannot reliably discriminate the fluorescence emitted by microorganisms from the background noise, leading to a risk of false positives [1].

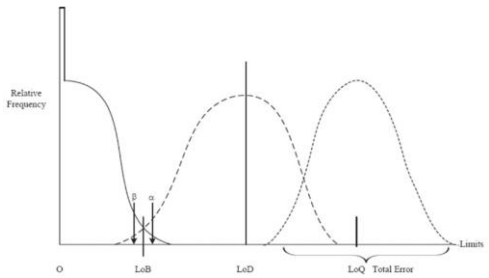


Figure 9: Relationship between LOB, LOD and LOQ

Limit of blank is the **maximal quantity** of microorganisms that can be detected by Red One™ on sample that do not contain any microorganism.

$$(I) - LOB = mean\ blank + 1{,}645 \times SD_{Blank}$$

Limit of detection is the **minimal quantity** of microorganisms that can be detected but not necessarily quantified by Red One™

$$(II) - LOD = mean\ blank + 3 \times SD_{Blank}$$

Limit of quantification is the **minimal quantity** of microorganisms that can be detected by Red One™ with an acceptable level of performance

$$(III) - LOQ = mean\ blank + 10 \times SD_{Blank}$$

Objectives

Compare the limit of detection between Red One™ or CyFlow™ Cube 8 and R2A agar plate count on sterilized tap water samples (1). Determine the reliability of the measure on tap water (2).
A test campaign with 25 samples from several origins was performed to establish the limit of detection of each method. The reliability of measurement was determined on the basis of 110 samples from 31 different tap water sampling points.

Method

(1) To assess the limit of detection, tap water samples from various sampling points were analyzed before and after autoclaving with 1 mL analyzed with Red One™ and 200 µL with CyFlow™ Cube 8.

Staining procedure on Red One™ is fully automated with the RedChrome Water reagent kit, 1mL of sample is dropped onto a Red Cap for analysis.

Staining for CyFlow™ Cube 8 consists of a double staining of 1mL bacterial suspension with 10µL of BacCount Total (ZPS40632; Sysmex). Bacterial suspensions were incubated for 10 min at 40°C and 200 µL of stained bacteria were immediately analyzed on the flow cytometer. Each sample was analyzed once on the flow cytometer. 1mL of each water sample were plated on R2A agar plate and incubated for 7 days at 22°C.

The limit of detection (LOD) is obtained from enumeration of microorganisms detected in autoclaving tap water with the formula: LOD = $\overline{X} + 3 \times \sigma$

(1.1) To test the different water sterilization methods, 5 conditions were tested on the CyFlow™ Cube 8: tap water, 0.2µm filtered tap water, autoclaved tap water, filter after autoclaving or before autoclaving tap water. 12 tap water samples were tested.

(2) To determine the reliability of the measure, 110 samples of tap water were tested on each method and plated on R2A agar plate (1 mL, incubated 7 days at 22°C). Staining procedure on Red One™ and CyFlow™ Cube 8 is the same as described above.

Results

(1) Red One™ counting decreases after autoclaving, whereas the CyFlow™ counting remains unchanged before and after autoclaving (**Figure 10**). The limit of detection is 5 966 cells for the FC and 18 cells for the SPC.

(1.1) With high background noise on the FC, we tested different ways of sterilizing tap water to reduce the number of particles.

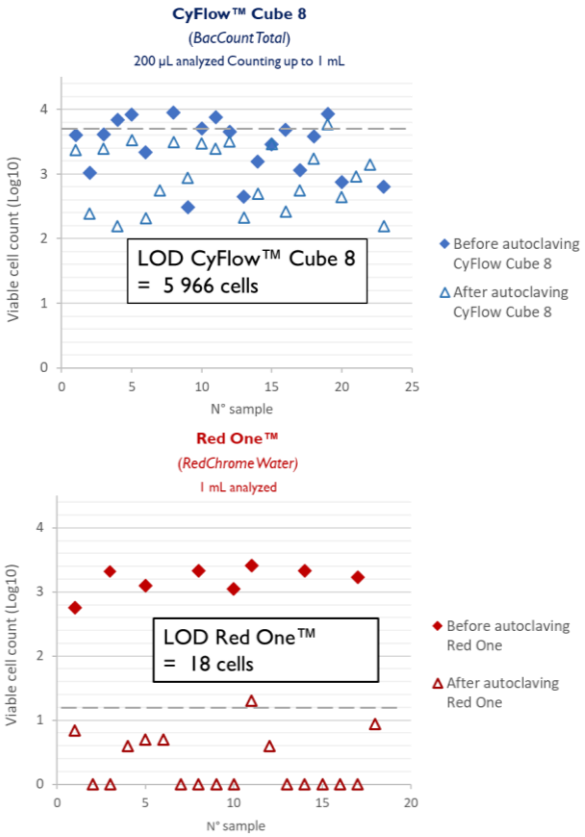


Figure 10: LOD results for Red One™ or CyFlow™ after autoclaving tap water

In the **Table 1**, we observed that autoclaved then filtered tap water is the least noisy. These results demonstrate the sensitivity of CyFlow™ Cube 8 to background noise with the BacCount total staining kit.

Conditions	Tap water	Filtered 0.2 µm Tap water	Autoclaved Tap water	Autoclaved then filtered Tap water	Filtered then Autoclaved Tap water
Average of counts on CyFlow™	2 724	2 353	1 054	645	715

Table 1: Average FC counts the different water sterilization methods

(2) Among the 110 tap water samples tested, 54 were positive on R2A agar plate (count >1 CFU/mL). The reliability of the methods was evaluated using these 54 positive samples. The number of samples under the LOD on each equipment was determined **(Figure 11)**. FC has a measurement of uncertainty of 65 % on real water samples (n=35/54) while Red One™ is only at 8% (n=4/54).

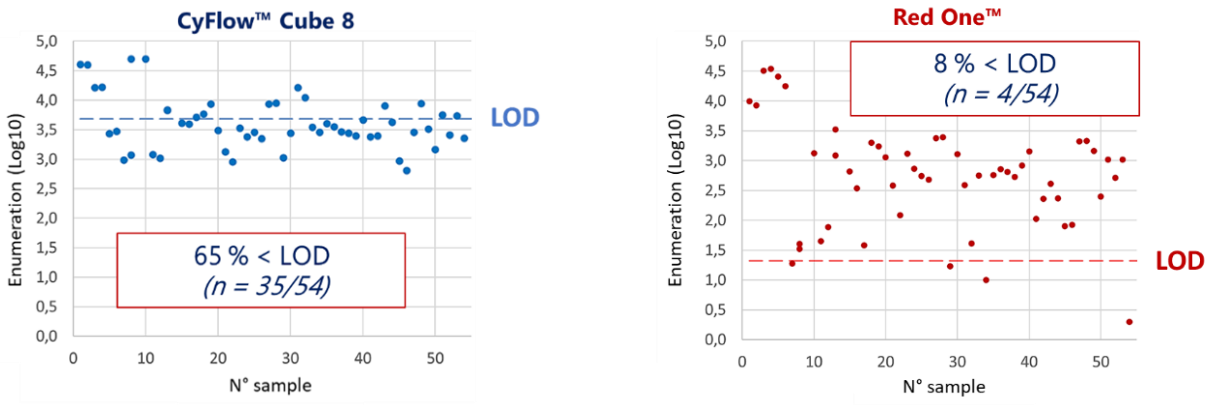


Figure 11: Evaluation of the reliability of measurement for Red One™ and CyFlow™ Cube 8

In conclusion, the CyFlow™ Cube 8 is more sensitive to background noise than Red One™ inducing a higher LOD with the kit BacCount. In addition, the reliability of the measure is lower for CyFlow™ than Red One™.

Due to this LOD, the flow cytometry shows significant limits to provide reliable and actionable results on drinking water quality on this type of sample.

Conclusion of the study

This study demonstrates that Red One™ technology shows stronger correlations with agar plate count than CyFlow™ Cube 6 for microbial detection in water, particularly at lower concentrations. Esterase-sensitive staining, utilized in both technologies, depends on several factors such as the metabolic state and permeability of the cells.

The objective of this study was to evaluate the ability of Red One™ Solid-Phase Cytometer and CyFlow™ Cube 6 and Cube 8 Flow Cytometers to serve as rapid indicators of microbiological water quality, potentially replacing traditional plating methods. Several tests were conducted, including assessments of a laboratory strain of *E. coli*, a stressed strain of *E. coli* isolated from the Seine River, and tap water samples. Initially, both systems demonstrated similar performance at higher microbial loads (>5.10⁶). However, for the stressed *E. coli* strain, Red One™ showed much better correlation with agar counts than CyFlow™ Cube 6. When tested on tap water samples, CyFlow™ Cube 8 showed elevated microbial counts at low actual concentrations, which can be attributed to its heightened sensitivity to background noise [2]. This was further confirmed by the elevated counts (~645 cells/mL) in autoclaved sterile water, then filtered (pore size 0.2 µm). Notably, this aligns with the admitted detection limit of 10^3 to 10^4 cells/mL for many flow cytometry-based methods [3], a threshold at which background noise can interfere with accuracy in low-biomass environments.

In conclusion, this study confirms the robust performance of the Red One™ technology, highlighting its suitability as a rapid indicator of microbiological water quality [4], with clear advantages in sensitivity and reliability over traditional methods and other cytometry technologies.

Partnership: Aerial, Technological Resource Center and Technical Institute for Food Industry (France)

Eau de Paris, Research & Development Biology (DRDQE, France)

Find out more at www.redberry.net, www.aerial-crt.com and www.eaudeparis.fr

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[3] Fontana, C., Ou, X., et al. (2018). Absolute bacterial cell enumeration using flow cytometry. *Journal of Applied Microbiology*.
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