Assessing Water Quality with the BD Accuri™ C6 Flow Cytometer

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White Paper

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Abstract

Rapid and accurate quantitation of bacteria in drinking water is essential to monitor, control, and optimize water treatment processes, and to illuminate the biology of low nutrient water systems. Historically, laboratories have relied on heterotrophic plate counts (HPCs) to monitor water quality, but this method is unreliable and time intensive.

Flow cytometry can rapidly quantitate bacteria and discriminate them from debris. Researchers at the Swiss Federal Institute of Aquatic Science and Technology (Eawag, Switzerland) have developed a standard flow cytometric staining protocol and a corresponding BD Accuri™ C6 software analysis template to discriminate bacteria from debris in drinking water samples. The template employs a single, fixed gate that contains all bacteria when stained with SYBR® Green I, or only viable bacteria when co-stained with SYBR® Green I and propidium iodide.

The BD Accuri™ C6 flow cytometer is ideal for monitoring drinking water because of its transportability, open fluidics system, and ability to determine sample volume and calculate cell concentrations directly. This white paper highlights the importance of standardized bacterial staining and demonstrates that the BD Accuri C6 can reliably and reproducibly quantitate bacteria and evaluate their viability in drinking water samples using the Eawag method. It also shows how the BD Accuri C6 can be used for real-time measurements of dynamic changes in water samples. To help water quality researchers standardize their analyses, the Eawag template for the BD Accuri C6 is available at **bdbiosciences.com**.

Introduction

Drinking water is a unique, low-nutrient environment in which diverse, indigenous microbial communities proliferate.[1] Water treatment systems make use of multiple hygienic barriers, such as ozonation, prechlorination, membrane filtration, and UV disinfection. Many also include a disinfection step such as chlorination to ensure the absence of viable bacteria in distributed water.[2] Despite these efforts, it is common for a variety of bacteria to regrow during the process or after distribution, and concentrations of 10^4–10^5 cells/mL in drinking water are typical.[3,4]

Uncontrolled and excessive regrowth can lead to a deterioration of aesthetic water quality such as undesirable tastes or odors, the growth of opportunistic pathogens, and treatment process malfunctions such as clogging of filters, biofouling, and biocorrosion.[5] The acceptable bacterial level in a water sample depends on the treatment process and local water quality standards. Rapid and accurate quantitation of bacteria in drinking water is essential to monitor, control, and optimize treatment processes, and to allow insight into the biology of low nutrient water systems.[6]

Overview of water quality monitoring methods

Methods to monitor microbiological changes during water treatment and to enumerate bacterial concentrations in drinking water were first established over 100 years ago.[7,8,9] Despite considerable changes in drinking water treatment processes and water quality concerns in the last century, variations of the same methods are still applied worldwide by both water utilities and researchers.[6] The methods require cultivation and rely heavily on conventional plating methods such as heterotrophic plate counts (HPCs) and the detection of index organisms such as *E. coli* after selective plating. However, while specific detection of index organisms is important to determine the hygienic quality of the water, such organisms are often completely absent or are only present at minute concentrations.[10] Further, we now know that <1% of autochthonous drinking water bacteria grow on conventional agar plates, so HPC results underestimate the actual total bacterial cell concentration by several orders of magnitude.[11,12] Finally, HPCs are time consuming and labor intensive, returning results days or even weeks after sampling.

Microscopy allows direct visualization of bacterial cells and enumeration of total bacterial cell counts without cultivation,[4,13] but is not routinely used because it is labor intensive and prone to high variability, including user bias.[14] DNA-based quantification methods such as qPCR or sequencing can also estimate the total bacterial component in water and allow examination of phylogeny without cultivation, but these methods do not characterize single cells, require DNA extraction, and are subject to bias intrinsic to PCR-based methods.[14]

Because these traditional methods typically yield data too slowly to allow in-process changes, most monitoring has been restricted to evaluation of water hygiene as opposed to process control. These methods cannot provide information at the single-cell level or analyze multiple parameters in a single assay to gain a better understanding of cell health. Ensuring drinking water quality requires reliable, rapid, culture-independent techniques for monitoring treatment processes and distribution systems in real time.

Impact of flow cytometry

Flow cytometry offers a powerful and effective methodology for assessing bacteria in water samples. In flow cytometry, particles or cells suspended in a hydrodynamically focused liquid stream pass through a pulsed beam of laser light (Figure 1). Optical detectors collect scattered laser light and fluorescent emissions, and electronics digitize these signals for computational analysis. The light scatter data provides basic information about the cells, such as relative size, shape, and surface features. The fluorescence data reveals the cells' autofluorescence and/or labeling with fluorescent dyes, which can help characterize bacteria, resolve them from electronic noise and debris, and indicate cell viability and vitality. Flow cytometry eliminates manual counting errors and allows simultaneous quantitative assessment of multiple cellular parameters at the single-cell level within minutes of water sampling.

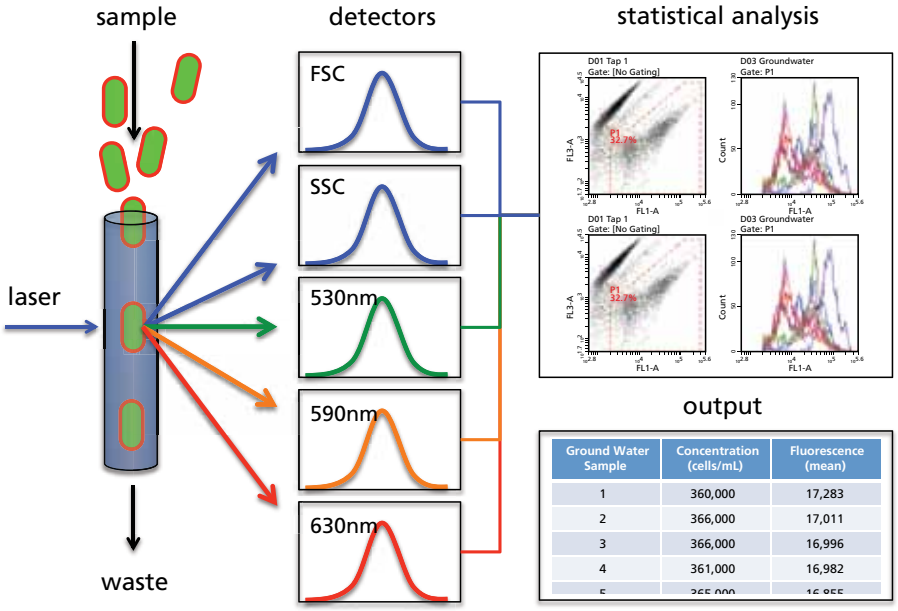

Figure 1. How flow cytometry works.

The BD Accuri C6, the first personal flow cytometer, is a promising candidate for monitoring drinking water because of its portability, open fluidics system, and ability to determine sample volume and calculate cell concentrations directly. This white paper explores the methods, reliability, and reproducibility of using the BD Accuri C6 to monitor drinking water quality. It illustrates the Eawag method for quantitating bacteria and evaluating their viability, and shows how the BD Accuri C6 might be used for real-time measurements of dynamic changes in water samples.

Technical considerations of the BD Accuri C6

Operating environment

The BD Accuri C6 flow cytometer (Figure 2) offers performance, simplicity, and affordability. Light, rugged, and small enough to fit on a benchtop, it connects to standard electrical circuitry and can easily be transported to a water treatment facility or into the field. Fixed optics and capillary sheath-flow fluidics make the BD Accuri C6 portable without the need for realignment. (The instrument has even proved its mettle on an arduous, months-long research voyage to Antarctica.[15])

Figure 2. The BD Accuri C6 personal flow cytometer.
The instrument weighs just 13.6 kg (30 lb). Exterior dimensions (H x W x D) are 27.9 x 54.6 x 41.9 cm (11 x 21.5 x 16.5 in.) with fluid tanks in place.

BD Accuri™ C6 software makes operation intuitive for novice and proficient users alike. Most new users become fluent with the software in less than 60 minutes, assisted only by a 3-page pictorial *Quick Start Guide*. Data files can be analyzed within the program, aided by special tools such as Zoom and VirtualGain™, or exported in FCS 3.0 format into FCS Express™, FlowJo™, and other flow cytometry analysis programs.

Routine instrument cleaning and priming are automated. Maintenance is easy, even for novice users, and requires no tools. The recommended sheath fluid is 0.22-µm filtered, deionized water. These attributes effectively expand flow cytometry beyond the core facility and onto the laboratory bench or into the field.

Optical and analytical modalities

The fully digital BD Accuri C6 features two lasers, two scatter detectors, and four fluorescence detectors. A state-of-the-art digital signal processing (DSP) system gives the BD Accuri C6 a dynamic range of greater than six full decades. This means that it can finely resolve both faint and bright signals at once and analyze a wide span of variation in fluorescence in a single run. The instrument detects this broad dynamic range using standard factory detector settings, without the need for optimization or tuning.

If additional flexibility is needed, the optical configuration is flexible. The Selectable Lasers Module (Cat. No. 653126) allows reassignment of the standard laser-detector associations, and optional filters can modify the effective detector characteristics.

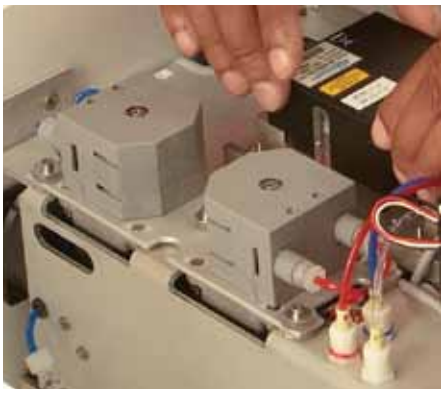

Figure 3. The BD Accuri C6 peristaltic pump system.

The unique laminar-flow fluidics system, driven by peristaltic pumps, combines the advantages of hydrodynamically focused cell sampling with the ability to report absolute cell counts for any identified population in a sample.

High-performance fluidics report volume automatically

The BD Accuri C6 flow cytometer has a unique laminar-flow fluidics system driven by "push-pull" peristaltic pumps (Figure 3). By monitoring the pressure in the Sample Introduction Probe (SIP), a microprocessor can determine the sample flow rate. This arrangement combines the advantages of hydrodynamically focused cell sampling (high data acquisition rates, good light-scatter and fluorescence resolution) with the ability to accurately determine sample volume and automatically report concentrations for any identified population in a sample.

The combination of event counts and metered sample volume ("direct volume counting") eliminates the need to count cells manually or to standardize data using beads. BD Accuri C6 software displays the volume (in µL) as data in the statistics tables and automatically calculates the concentration (count per µL) of any gated population. Users can display counts and concentrations in a data table on the Statistics tab of the software.

The non-pressurized pumps and open fluidics system allow the use of open tubes, such as Eppendorf tubes. This allows convenient addition of test compounds to the cell suspension without interruption during sampling (Figure 4), or continuous sampling from a functioning reactor, which enables real-time measurements of dynamic changes in water samples as described later in this white paper.

Finally, the peristaltic pumps enable independent regulation of both the sheath and sample flow rates. Users can quickly optimize the sample core diameter (adjustable from 5 to 40 µm) based on the anticipated size range of cells or particles within the sample. The design also allows easy removal of clogs (such as cell clumps) from the flow cell.

Applications in water quality monitoring

Figure 4. Continuous sampling with the BD Accuri C6.

Reliable detection of bacterial cells in water samples

Small bacterial cells can be difficult to discriminate from debris particles and background signals in a water sample. However, staining the bacterial DNA with fluorescent dyes and applying acquisition thresholds allow efficient separation of small bacterial cells by flow cytometry.[16]

One common approach is to plot forward- or side-scatter data (FSC or SSC) against the fluorescence signal of the stain, utilizing the size or shape of the bacteria as a selective factor.[17,18] Alternatively, Hammes et al have demonstrated that the combination of two fluorescence signals (green and red fluorescence, for example) originating from the same dye often provides optimal discrimination of bacteria from instrument noise or background in drinking water samples.[2,16,19] This approach is especially beneficial in visualizing and differentiating between bacteria with high and low nucleic acid content (HNA and LNA), which are commonly observed in natural aquatic environments.[20]

SYBR® Green I is an asymmetrical cyanine dye that preferentially stains double-stranded DNA. It results in the formation of a DNA:dye complex that, when excited (λmax = 497 nm) by the 488-nm blue laser of the BD Accuri C6, emits green (λmax = 520 nm) and red light that can be measured in the FL1 and FL3 detectors. The fluorescence intensity, ease of use, reproducibility, and emission spectrum of SYBR® Green I are well suited for staining bacteria in water samples for analysis by flow cytometry.[2,4,18]

Researchers at Eawag (Dübendorf, Switzerland) have developed a standard SYBR® Green I staining protocol to rapidly and reproducibly discriminate stained bacteria from debris in drinking water samples. Briefly, water samples are pre-warmed to 35°C (range: 35–40°C) followed by staining at 35°C (range:

35–40°C) for 10 minutes with SYBR® Green I (Invitrogen AG, Basel, Switzerland) at a concentration of 1X (which is a 10,000X final dilution of DMSO stock solution).[6,21,22] For analysis on the BD Accuri C6, 50 µL of each 500-µL sample is run at medium speed using an FL1-H acquisition threshold of 800 (range: 500–1,000). SYBR® Green I fluorescence is visualized using a two-dimensional FL1-A (emission filter 533/30) vs FL3-A (emission filter 670 LP) log-scale density plot, as shown in Figure 5. Bacteria are gated on the FL1-A vs FL3-A plots using a lower limit of 2,000 on FL1, which was determined based on experience with multiple real samples.[21] If the sample includes unusually large and brightly fluorescent cells, the gate may be extended beyond the indicated upper limit.

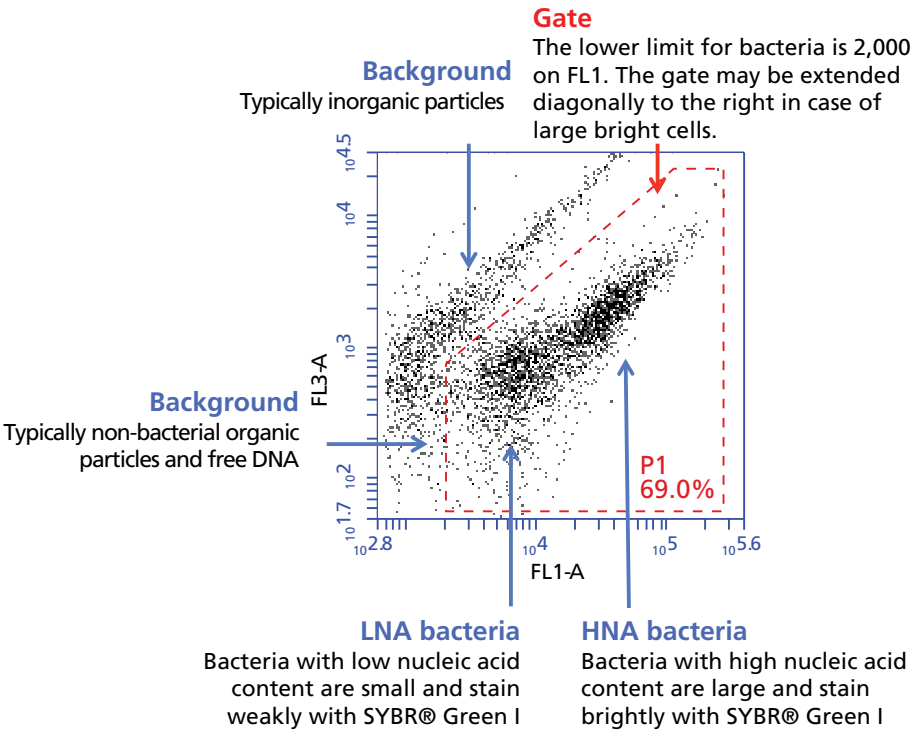

Figure 5. The Eawag bacterial cell analysis template.

SYBR® Green I staining of bacterial DNA can be visualized on a 2-dimensional, log-scale FL1-A vs FL3-A density plot on the BD Accuri C6. The bacterial cell gate includes both smaller (LNA) and larger (HNA) bacteria that stain weakly and brightly with SYBR® Green I, respectively, and excludes non-fluorescent debris and autofluorescent background particles. Although SYBR® Green I is primarily detected in FL1, the red fluorescence signals detected in FL3 represent the tail of its emission spectrum.

The Eawag method works well for bacterial concentrations ranging from about 10^2–10^7 cells/mL. Dilution is required for higher cell concentrations, and accurate detection of low cell concentrations requires a well cleaned instrument and an increase in analysis volume.

The use of standard staining and analysis methods, in which the instrument settings and electronic gates are held constant across all samples, allows direct comparison of sample data, both within and across experiments. A BD Accuri C6 software template, available on bdbiosciences.com, makes it easy to collect and compare data.[23]

Direct volume counting of bacterial cells

The ability to count cells quickly, accurately, and automatically is a key advantage of flow cytometry over traditional techniques such as epifluorescence microscopy for quantifying bacterial cells in water samples. Digital flow cytometers with laminar-flow fluidics allow fast, phenotypic data collection (up to 10,000 events per second) on a wide range of cell types (submicron-sized bacteria through large mammalian cell lines), counting the cells as they are acquired. However, because these instruments cannot usually determine sample volume directly, counting beads must be added to each sample to determine cell concentrations (cells per unit volume). Since the bead concentration is known, the cell concentration can be calculated based on the ratio of beads to cells.

On the other hand, flow cytometers with syringe-driven fluidics can determine cell concentrations directly, without counting beads. However, they are often limited by lower data acquisition rates (<1,000 events per second), diminished fluorescence and light-scatter resolution, and a propensity for clogs in the flow cell.

The unique fluidics system of the BD Accuri C6 (see Figure 3) combines the advantages of these two types of instruments, allowing it to both count cells and accurately determine sample volume while maintaining high sampling rates and resolution. Thus, it can calculate cell concentrations without the use of counting beads.

Figure 6A shows the analysis of six water samples from different origins (tap water, surface water, and ground water), along with one pure culture of *E. coli* (included as a control), using the Eawag method and template on the BD Accuri C6. The stained bacteria from all the water samples fall nicely within the standard gate, while background, when present, is excluded from the gate. When the SYBR® Green I fluorescence of the gated bacterial populations is overlaid on a histogram plot (Figure 6B), each water sample has a unique "fingerprint," indicative of the distinct microbial populations present in each sample.[21,24] This information can be used to distinguish minute changes in bacterial populations in water samples. The concentration and fluorescence intensity of gated SYBR® Green I cells in each sample are automatically calculated in BD Accuri C6 software and are reported in Table 1.

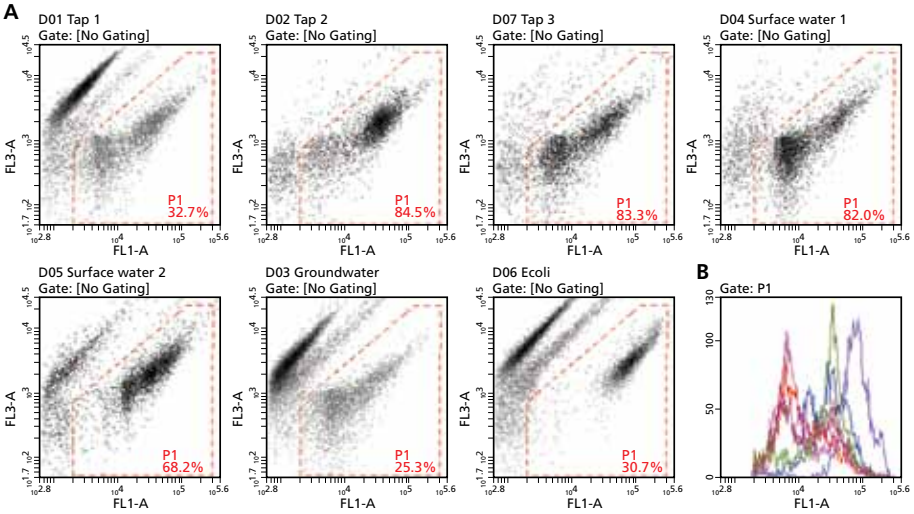

Figure 6. Water samples analyzed using the Eawag method and template on the BD Accuri C6.

A. *Bacterial cells in six water samples from different origins, and one pure E. coli culture (included as a control), were stained with SYBR® Green I and visualized on FL1-A vs FL3-A density plots on the BD Accuri C6. A standard gate (P1) was applied to each sample to exclude debris and background.* ***B.*** *A histogram overlay of gated SYBR® Green I fluorescence (P1) in all seven samples highlights each sample's distinct bacterial fingerprint. Histogram colors and bacterial concentrations within each gate are included in Table 1.*

Table 1. Bacterial cell concentrations obtained from water and bacterial samples.

Sample Source	Concentration (cells/mL)	Fluorescence (Mean FL1-A)	Histogram Color
Tap water 1	60,000	23,498	
Tap water 2	217,000	31,326	
Tap water 3	271,000	21,030	
Surface water 1	611,000	19,313	
Surface water 2	309,000	35,687	
Ground water	37,000	17,123	
E. coli	37,000	83,423	

Monitoring viability of bacterial cells

Disinfection is an essential step in water treatment that safeguards consumers against pathogenic microorganisms. Typically, the water is exposed to a chemical oxidant such as chlorine, monochloramine, chlorine dioxide, or ozone, which specifically disrupts cellular membrane integrity to induce bacterial cell death.[25] However, oxidants vary in their selectivity and reactivity, and their effects depend on the composition and physiological state of the indigenous bacterial community. These factors influence the efficacy of the bacterial disinfection process.

SYBR® Green I staining enables efficient analysis of the total bacterial cell concentration, but does not differentiate between intact and damaged cells. Adding a viability dye in a multiparametric flow cytometric assay can rapidly assess the viability of individual cells after disinfection without culturing.[26] Propidium iodide (PI) is a large, positively charged molecule that binds to DNA and RNA in cells that have lost membrane integrity, but is excluded from cells with intact cellular membranes (Figure 7A).[27] Staining of cells with PI indicates severe membrane damage and is therefore viewed as a conservative indicator of cell death appropriate for oxidative disinfection processes.[25] Together, SYBR® Green I and PI can optimally discriminate bacteria with disrupted vs intact membranes.[26]

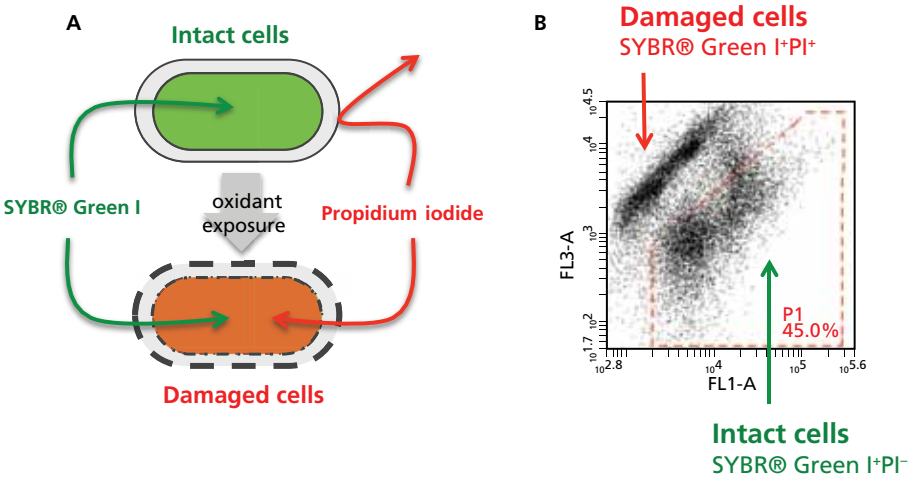

Figure 7. Co-staining with SYBR® Green I and PI discriminates between intact, viable cells and damaged cells.

A. *Schema of SYBR® Green I and PI staining. Exposure to chemical oxidants during disinfection disrupts bacterial cell membrane integrity. Damaged cells stain with both dyes (SYBR Green I+PI+) while intact cells stain with SYBR® Green I but exclude PI (SYBR® Green I+PI−).* ***B.*** *An illustrative water sample co-stained with SYBR® Green I and PI and analyzed using the Eawag template, as described in Figure 5. Viable, intact bacteria are gated in P1.*

Figure 7B shows a water sample co-stained with SYBR® Green I and PI and analyzed using the Eawag template. SYBR® Green I stains all bacterial cells irrespective of membrane damage, and allows discrimination between fluorescent cells and non-fluorescent debris in the FL1 detector of the BD Accuri C6. PI stains only severely damaged and dead cells and is detected primarily in the FL3 detector (emission filter 670 LP). Bacterial cells that co-stain with PI are thereby shifted out of the bacterial cell gate (P1). Thus, in the presence of PI, the same gate that was used to determine the total bacterial cell concentration (P1 in Figure 5) now includes only viable, intact bacteria.

Chlorination is the predominant worldwide method for disinfection of drinking water. Figure 8 shows an example of bacterial analysis after exposure to chlorine disinfection. A non-chlorinated tap water sample was exposed to increasing concentrations of chlorine and dispensed from a concentrated stock solution into a continuously stirred sample, as described by Ramseier et al.[25] Subsamples were taken before chlorination and at 5-minute intervals, and oxidation was quenched with thiosulfate. The subsamples were then stained with SYBR® Green I (10,000X dilution of DMSO stock) and PI (final concentration = 0.3 mM) at 35°C in the dark for 10 minutes and analyzed on the BD Accuri C6. As expected, exposure to increasing chlorine concentrations resulted in increased PI staining and, therefore, decreased concentrations of viable cells (SYBR® Green I^+PI^-) in the P1 gate.

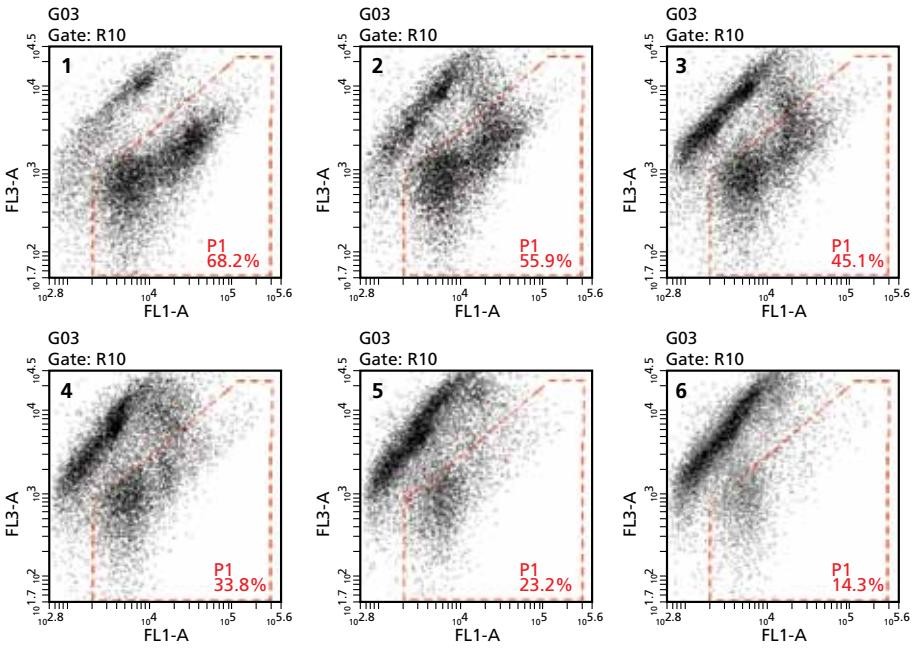

Figure 8. SYBR® Green I and PI staining of bacterial cells after exposure to increasing concentrations of chlorine.

A non-chlorinated tap water sample (plot 1) was exposed to increasing concentrations of chlorine. Successive subsamples were taken (plots 2–6), stained with SYBR® Green I and PI using the Eawag protocol and template, and analyzed on the BD Accuri C6. In identical density plots of SYBR® Green I (FL1-A) vs PI (FL3-A) for each sample, a standard gate (P1) was applied to each sample to exclude debris and background as described in Figure 5. Intact cells stain only with SYBR® Green I and thus remain in the gate. Damaged cells, which increase with chlorine exposure, additionally stain with PI (SYBR® Green I^+PI^+) and shift out of the gate.

Table 2. Concentrations and percentages of intact bacterial cells in the P1 gate after increasing chlorine exposure.

Sample	Concentration (cells/mL)	% cells in P1 (relative to Sample 1)
1 (untreated)	220,000	100%
2	190,000	84%
3	170,000	77%
4	120,000	55%
5	77,000	35%
6	51,000	23%

Table 3. Reproducibility of ground water bacterial counts on the BD Accuri C6.

Ground Water Subsample	Concentration (cells/mL)	Fluorescence (mean)
1	360,000	17,283
2	366,000	17,011
3	366,000	16,996
4	361,000	16,982
5	365,000	16,955
Average	**364,000**	**17,045**
%CV	**0.79%**	**0.79%**

Table 4. Reproducibility of tap water bacterial counts on the BD Accuri C6.

Tap Water Subsample	Concentration (cells/mL)	Fluorescence (mean)
1	59,000	23,750
2	58,000	25,066
3	58,000	23,631
4	60,000	24,676
5	57,000	23,662
Average	**58,000**	**24,157**
%CV	**1.95%**	**2.76%**

Reproducibility of bacterial cell counts

To investigate the reproducibility of the Eawag staining method and analysis on the BD Accuri C6, two independent water samples (ground water and tap water) were obtained. Each water sample was divided into five identical subsamples, which were stained with SYBR® Green I and analyzed on the BD Accuri C6 using the methods described.

Gated bacterial cell concentrations and the mean SYBR® Green I fluorescence for each sample are reported in Tables 3 (ground water) and 4 (tap water). For the five ground water subsamples, the %CVs of both direct volume counting and mean SYBR® Green I fluorescence were only 0.8%, signifying very high reproducibility. These measurements were also highly reproducible in the five tap water subsamples, which contained a lower concentration of bacterial cells than the ground water, with %CVs of 2.0% and 2.8% for counts and fluorescence measurement, respectively. The reproducibility of the Eawag method on the BD Accuri C6 is further visualized in Figure 9, in which the fluorescence intensity "fingerprints" of the five subsamples in each group overlay each other closely.

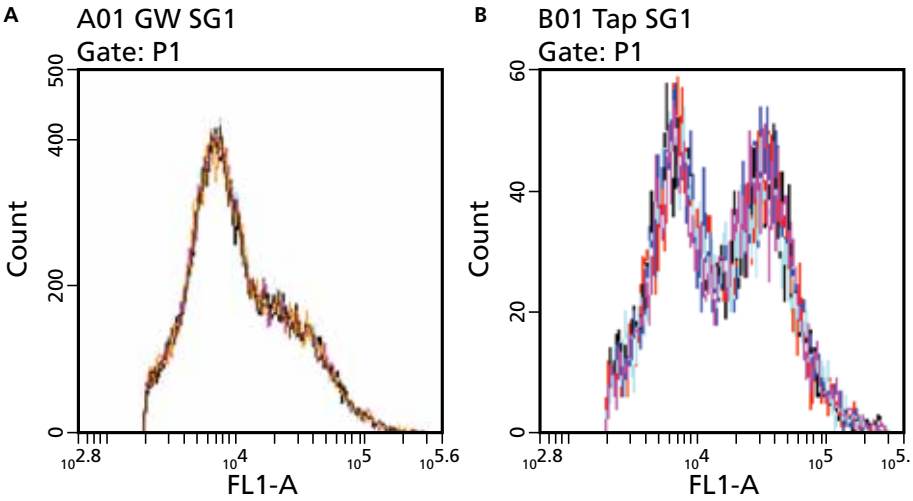

Figure 9. SYBR® Green I fingerprints of bacteria in ground water and tap water subsamples.

*Five independent subsamples from (**A**) ground water and (**B**) tap water were identically stained and analyzed on the BD Accuri C6. For each set of subsamples, the SYBR® Green I fluorescence "fingerprints" (FL1-A) overlay each other closely, demonstrating a high level of reproducibility for measuring microbial samples on the BD Accuri C6.*

Real-time flow cytometry

Real-time monitoring of drinking water bacteria by flow cytometry can provide high-resolution information not possible with conventional analysis or even with conventional flow cytometry. For researchers studying dynamic biological processes, visualization of bacterial characteristics in real time would accelerate process understanding to a new level.

One application of real-time flow cytometry is disinfection kinetics. For example, Figure 8 showed successive snapshot samples of chlorine damage to bacterial membranes at discrete intervals. However, optimal analysis of kinetics would track the process continuously in a single sample over time.

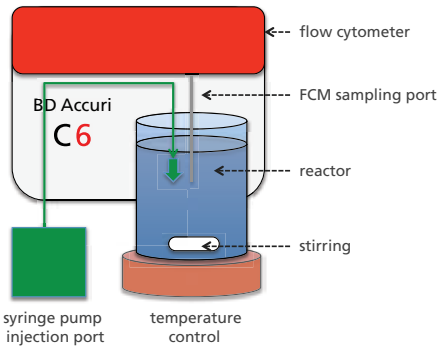

Figure 10. Schematic of real-time flow cytometry on the BD Accuri C6.

A syringe-pump injection port adds reagents or sample to the reactor as desired. The reactor contents are continuously stirred, sampled, and analyzed on the BD Accuri C6.

The non-pressurized fluidics system of the BD Accuri C6 lends itself to real-time analysis, supporting the use of open sample tubes and even sampling from a small reactor (Figure 10). This design feature gives researchers the flexibility to add reagents or sample during data collection, performing kinetic assays without interruption.[28]

Figure 11 shows sample data generated by this approach, measuring the loss of pre-stained SYBR® Green I fluorescence in a pure *E. coli* culture in real time. Three different concentrations of chlorine were added to the reactor, oxidizing the dye and/or its DNA binding site. The plots clearly visualize the kinetic effects of the different chlorination levels on both HNA and LNA bacteria, continuously and precisely over time.

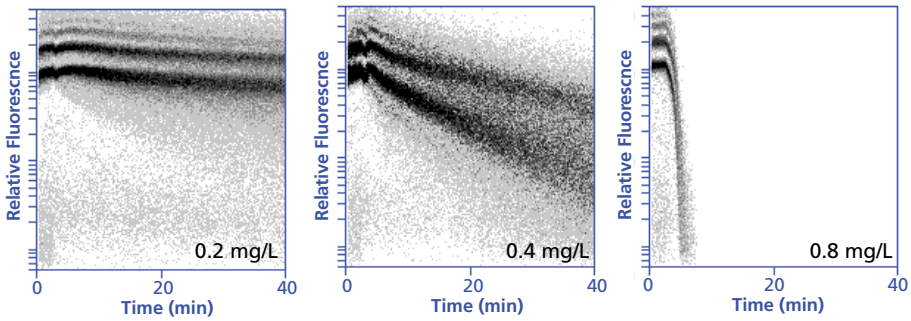

Figure 11. Real-time effects of three chlorination regimes on intact bacterial concentrations.

A pure E. coli culture was pre-stained with SYBR® Green I, and excess dye was washed away. Stained bacteria were maintained at a temperature of 35-40°C in a stirred reactor. Chlorine was added to the reactor using a syringe pump at t = 0 minutes under one of three conditions (final concentrations = 0.2, 0.4, and 0.8 mg/L of hypochlorite). Sample was continuously drawn into the BD Accuri C6 and analyzed for 40 minutes. Plots of relative FL1-A (SYBR® Green I) fluorescence vs time show loss of pre-stained fluorescence during exposure to increasing concentrations of chlorine.

Summary and conclusions

Using flow cytometry, water quality researchers and water utilities can generate statistically strong and reproducible data that supports informed decisions for treatment process control. Researchers at Eawag have developed a standard flow cytometric staining protocol and analysis template to rapidly and reproducibly discriminate stained bacteria from debris in drinking water samples. The method establishes a standard gate that contains all bacteria when stained with SYBR® Green I, or only viable bacteria when co-stained with propidium iodide.

Offering both performance and simplicity, the BD Accuri C6 flow cytometer is particularly well suited to the analysis of drinking water. With its ability to determine sample volume directly, it can calculate cell concentrations automatically, without counting beads. Its open fluidics system allows researchers to track the effects of chlorination and other water treatments in real time. Finally, its compact size and ruggedness make it transportable to water treatment plants and into the field.

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