

## Multiparametric Analysis of Aquatic Organisms Using Flow Cytometry

# White Paper

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### Abstract

Environmental research on aquatic microorganisms, such as phytoplankton, aquatic bacteria, and aquatic viruses, often takes advantage of their natural chlorophyll, phycobilins, and other intrinsic fluorescent pigments. Although epifluorescence microscopy is still used frequently, flow cytometry has become a key research tool due to its ability to provide multiparametric analysis at the single-cell level.

Along with the analytical opportunities flow cytometry has brought to the field, aquatic microorganism research poses certain technical challenges as well. These include a large variation in organism size, analysis of unfiltered samples, the generation of complex multiparametric data across a wide dynamic fluorescence range, the need for quantification, and a difficult operational environment. This white paper explores both the opportunities and challenges of aquatic research, and examines how the BD Accuri™ C6 flow cytometer is well suited for this work.

The final section of this paper is a “field guide” to using the BD Accuri C6 in aquatic microorganism research. It describes how to detect natural fluorophores such as chlorophyll, phycocyanins, and phycoerythrin (PE), and how to use fluorescence profiles to identify cyanobacteria, other autotrophic phytoplankton, and heterotrophic bacteria and viruses. Several examples illustrate the BD Accuri C6 cytometer’s role in ongoing field research in the Great Lakes, the Gulf of Mexico, the Netherlands, and Antarctica.





The first personal flow cytometer: the BD Accuri C6.

## Introduction

### The aquatic microbiome

Environmental research on marine and freshwater ecosystems is predominantly focused on their microbiomes, including **phytoplankton** (such as diatoms, cyanobacteria, and dinoflagellates), **aquatic bacteria**, and **viruses**. Critical concerns of aquatic environmental science include the primary productivity of phytoplankton, which forms the basis of the aquatic food chain, and the spatial and temporal distribution of cyanobacteria and other phytoplankton species responsible for harmful algal blooms (HABs).

Aquatic bacteria are ubiquitous ( $10^4$ – $10^8$  per mL) in aquatic environments. Researchers study their roles in the aquatic food web and their effects on other microorganisms, and use them to monitor ecosystems. Changes in the composition of a bacterial community can signal environmental changes that might otherwise be more costly and difficult to detect, such as modest temperature fluctuations or transient increases in industrial or agricultural chemical concentrations. It is also crucial to monitor the health of aquatic bacterial communities that degrade detergents and other synthetic materials discharged into rivers or estuaries.

Aquatic viruses of scientific interest include marine phages that infect phytoplankton, bacteria, and other organisms. The role of these abundant viruses in aquatic environments is not yet well understood. As the principal cause of aquatic bacterial mortality, aquatic viruses have a powerful ecological influence, with largely unexplored effects on the food web, carbon and nitrogen cycles, and genetic diversity.

### Visualizing aquatic organisms using intrinsic fluorophores

Chlorophyll (found naturally in all phytoplankton) and phycobilins (found in cyanobacteria) are natural fluorophores with characteristic optical wavelength excitation and emission profiles. (See Table 2 on page 7 for examples.) These fluorophores are routinely exploited in aquatic microbiologic research, since they enable direct detection, discrimination, morphological analysis, and quantification of these organisms using fluorescence-detection methods, without the addition of extraneous dyes or probes.

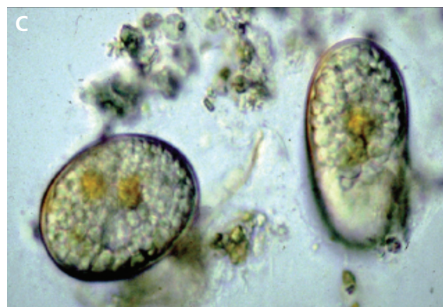
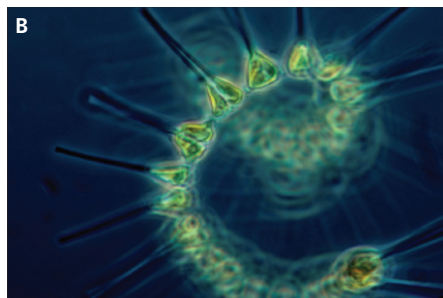
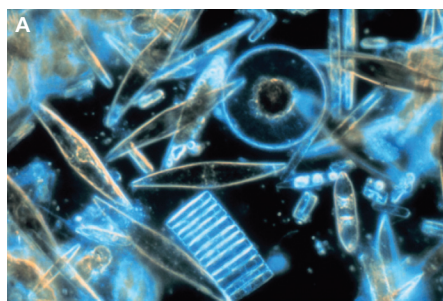
Epifluorescence microscopy has been valuable in advancing our knowledge of aquatic microorganisms, and is still used frequently. The organisms' natural fluorophores can be supplemented with DNA-specific dyes, fluorescence in situ hybridization (FISH), and fluorophore-labeled monoclonal antibodies. When adding probes, investigators usually favor those that emit in the yellow-green band, to avoid overlap with chlorophyll or phycobilin emissions.

### Enhanced analysis with flow cytometry

Once researchers have determined basic information about a microorganism's size, location, and endogenous fluorophores, flow cytometry can greatly accelerate and enhance analysis. In addition to higher sample throughput, flow cytometry adds the advantages of multiparametric analysis at the single-cell level.

In most cases, flow cytometry can analyze phytoplankton directly from freshly collected environmental water samples. By evaluating relative cell size and intrinsic fluorescence profiles, researchers can determine the general taxonomic classification of individual microorganisms. From there, they can study population growth rates, species succession, infection, competition for resources, and other aspects of aquatic ecology.

Along with the analytical opportunities flow cytometry has brought to the field, aquatic microorganism research poses certain technical challenges as well. These include a large variation in organism size, analysis of unfiltered samples, the



Diatoms (A) and other phytoplankton (B) form the basis of the marine food chain and are the primary food source for zooplankton. They convert carbon dioxide into complex organic matter and are responsible for half the oxygen on earth produced by plants. Phytoplankton are also the primary source of food for zooplankton. The dinoflagellate *Alexandrium fundyense* (C) is one of several aquatic microorganisms associated with HABs, which can poison shellfish and humans and cause delicate fish gill tissues to clog with mucus.

generation of complex multiparametric data across a wide dynamic fluorescence range, the need for quantification, and a difficult operational environment. This white paper explores these opportunities and challenges, and discusses techniques and guidelines for detecting common pigments and identifying both autotrophs and heterotrophs in field research. All research examples in this paper were analyzed on the BD Accuri C6 flow cytometer, the first personal flow cytometer, which is especially well suited for aquatic microbiology research.

### The personal flow cytometer

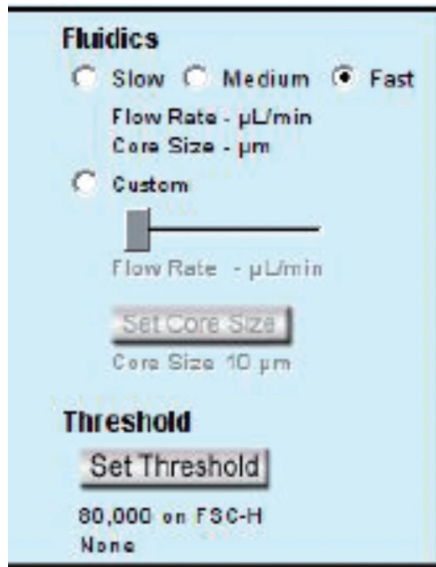
The BD Accuri C6 is a four-color, dual-laser flow cytometer that offers performance, simplicity, and affordability. Easy to learn and use, it is about the size of a microwave oven, allowing it to be set up in even the most limited lab space. It is also rugged enough to be transported, not only into labs and classrooms but also onto research vessels.



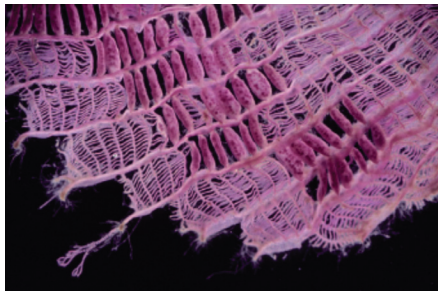
The BD Accuri C6 cytometer system.

A state-of-the-art digital signal processing (DSP) system gives the BD Accuri C6 a dynamic range of 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 microorganisms' intrinsic fluorescence in a single run. The instrument detects this broad dynamic range using standard factory detector settings, without the need for optimization or tuning.

Operation is menu-driven and intuitive. BD Accuri™ C6 software was designed with speed of learning and ease of use in mind, based on hundreds of hours observing researchers using flow cytometers. As a result, BD Accuri C6 software is truly intuitive to use, accessible to novice and proficient users alike. Usability testing shows that new users become fluent with BD Accuri C6 software in less than 30 minutes, assisted only by a 3-page pictorial *Quick Start Guide*.



The BD Accuri C6 cytometer's preset sample flow speeds (Fast, Medium, or Slow) automatically adjust the core diameter to 22 µm, 16 µm, or 10 µm respectively. Alternatively, to optimize detection of very small or large microorganisms, BD Accuri C6 software provides custom user-variable flow rate and core size control from 5 to 40 µm in diameter.



Seaweed or other large-diameter objects might clog a fluidics system when unfiltered environmental samples are acquired.

## Challenges and opportunities

### Microorganism size

Aquatic microorganisms of interest vary in size over a vast range, from viruses too small to be detected by conventional microscopy to multicellular colonies that can be examined without magnification.<sup>1</sup> The first technical concern, therefore, is whether a flow cytometer can process and detect the size range of organisms selected for analysis, as well as other particulate material likely to be present in samples.

The BD Accuri C6 flow cytometer's fluidics system employs a "push-pull" peristaltic pump that enables independent control of both the sheath and sample flow rates. Users can quickly adjust the sample core diameter from 5 to 40 µm to match the anticipated size range of sample microorganisms.

Some aquatic viruses are too small to produce detectable light-scatter signals. Instead, researchers can use fluorescent dyes or probes to label, detect, and identify these microorganisms. As an aid to small particle detection, the software also allows the use of a fluorescence signal as a primary trigger for data collection.

### Unfiltered samples

Introducing impure or unfiltered samples into a fluidics system can clog the fluidics pathways, resulting in project downtime. However, researchers may not want to filter samples before processing, either to maintain sample integrity or to achieve desired throughput.

A benefit of the BD Accuri C6 cytometer's direct-drive fluidics design is that any clogs that arise during sampling are easy to remove. By design, most clogs will occur in the sample injection probe, not in the interior fluidics lines. The operator can push clogs out of the flow cell with a brief burst of sheath fluid followed by a high-volume flush.

### Wide dynamic range

The chlorophyll and phycobilin pigments in many phytoplankton offer a direct and convenient means of detecting them and analyzing the effects of environmental variation on their physiology. However, analysis based on autofluorescence can be challenging. Concentration of these fluorophores in the organisms can vary greatly, especially when organisms may have ingested a fluorescent meal.<sup>2</sup> The resulting fluorescence signals can range across six decades (orders of magnitude) of brightness.

The BD Accuri C6 captures this broad dynamic range using standard factory detector settings. Users do not need to set voltage and amplifier gains to focus on the high or low end. Data can be collected first and analyzed later, without concern over lost data due to improper settings. This is especially important since environmental sample collection may require months of planning and substantial cost. The data can even be re-analyzed at any time if gating or compensation errors are discovered, or in light of new research findings.

In the rare cases where fluorescence is off scale, it is easy to insert a 90% or 99% attenuation filter to bring the signals back on scale. Use of an attenuation filter (as opposed to reducing voltage) means that the fluorescence reduction is predictable and reproducible, and is not affected by subjective operator decisions about how much signal reduction is "enough."

### Multiparametric data

The ability of flow cytometric analyzers to generate multidimensional data opens up many opportunities for research on aquatic microorganisms.<sup>3</sup> In particular, multiparametric flow cytometry is essential to differentiate between microorganisms that are similar in one or more individual parameters, such as size. While forward and side scatter enable identification of microorganisms on the basis of size and complexity, the addition of fluorescent dyes and probes allows more specific identification, sophisticated cell-state analysis (such as ploidy, cell cycle determination, and viability), and the detection of aquatic viruses and other organisms that are too small to produce light-scattering signals.

The high-performance BD Accuri C6 flow cytometer features two lasers, two scatter detectors, and four fluorescence detectors. If additional flexibility is needed, the optical configuration is flexible, as shown in Table 1. The Selectable Lasers Module (Cat. No. 653126) allows reassignment of the standard laser-detector associations, and optional filters can modify the effective detector characteristics. In fact, researchers have found that the compact, affordable BD Accuri C6 can replace multiple specialized flow cytometry systems.

**Table 1.** Standard and optional optical configuration of the BD Accuri C6 cytometer system.

Laser/Detector Associations	"3 blue 1 red" (standard)	488 nm (emissions read in: FL1, FL2, FL3) 640 nm (emissions read in: FL4)
	"2 blue, 2 red" (653126 Module)	488 nm (emissions read in: FL1, FL2) 640 nm (emissions read in: FL3, FL4)
	"4 blue" (653126 Module)	488 nm (emissions read in: FL1, FL2, FL3, FL4)
Scatter Detection	Forward (0° ±15) Side (90° ±15)	
Emission Detection	Four colors, user-swappable optical filters <b>Standard set:</b> FL1 533 ±15 FL2 585 ±20 FL3 670 LP FL4 675 ±12.5	<b>Optional:</b> 510 ±7.5 (Cat. No. 653184) 540 ±10 (Cat. No. 653528) 565 ±10 (Cat. No. 653185) 610 ±10 (Cat. No. 653186) 780 ±30 (Cat. No. 653187)

### Quantification of microorganisms

Aquatic microbiology research often focuses on the growth and senescence of microorganism populations and competition for resources among species. Frequent sample collection at defined surface coordinates within a study area and along depth transects can produce dozens of samples requiring rapid population counts or density analysis.

Historically, researchers relied on manual counting using grid slide microscopy, but this method can be arduous and imprecise. Flow cytometers can quickly and accurately identify and count microorganisms, but their fluidics systems often cannot measure sample volume. To determine population density (per  $\mu\text{L}$  of sample), researchers sometimes resorted to workarounds such as using a stopwatch to time the flow duration or using an analytical balance to calculate loss of mass during sampling. A more accurate method is to add standard counting beads (of known concentration) to each sample. Counting the beads allows calculation of sample volume and therefore population density.

The BD Accuri C6 cytometer's peristaltic pump system, in contrast, allows precise and direct determination of the sample flow rate. This arrangement combines the advantages of hydrodynamically focused cell sampling, high data acquisition rates, and excellent light-scatter and fluorescence resolution with the ability to measure sample volume and thus calculate absolute counts for any



At only 27.9 x 37.5 x 41.9 cm (H x W x D) in size, and 30 lb in weight (13.6 kg), the robust BD Accuri C6 can be used in a hood or glovebox, in cramped quarters, or aboard ship. Fluid tanks add 6.75 inches (17.1 cm) to the width dimension.



Fixed optics and capillary sheath flow fluidics enable continuous operation even during motion and vibration.

identified population in a sample. The BD Accuri C6 flow cytometer records the sampled volume for each run and can automatically calculate both total and gated events to provide absolute counts and concentrations. For samples in which microorganisms of interest are sparse, researchers can increase sampled volume up to several mL to improve measurement precision.

### Operating environment

Aboard research vessels, instruments must often fit in cramped and makeshift laboratory space. They must be readily transported on- and off-ship, and be able to handle severe pitching, rolling, and engine vibration. Even on land, aquatic scientists must sometimes set up field laboratory operations in temporary or borrowed facilities that lack standard laboratory benchtop space, furnishings, power capacity, or cooling ability.

With a small benchtop footprint and low cubic volume, the rugged BD Accuri C6 is especially suitable for use in the field or aboard ship.

The system is easily connected to standard marine electrical circuitry. Fixed optics and capillary sheath flow fluidics enable continuous operation even during motion and vibration. Indeed, the BD Accuri C6 has proved itself seaworthy under the arduous conditions of a research voyage to Antarctica lasting several months.<sup>4</sup>

The recommended sheath fluid for the BD Accuri C6 is 0.2- $\mu$ m filtered, deionized water, so there is no need to load large volumes of specialized reagents on board. Nor is a technical specialist required, since routine system decontamination, cleaning, and priming are automated. Recommended bimonthly maintenance requires no tools and can be performed easily, even by novice users.

## Analyzing aquatic organisms with the BD Accuri C6 Cytometer

This section is a “field guide” to using the BD Accuri C6 for aquatic microorganism research.

### Detecting pigments

#### Chlorophyll.

Because chlorophyll is present in phytoplankton (and all photoautotrophs), its fluorescence signature is the primary gating factor to discriminate phytoplankton from other particles. Chlorophyll *a* is the dominant photosynthetic pigment in most phytoplankton, although chlorophyll *b* is present in a few species, notably the *Chlorophytes*. Although the 488-nm laser typical on most cytometers (including the BD Accuri C6) does not maximally excite either form of chlorophyll, a significant fluorescence signal generally appears in the long red wavelengths (>640 nm).

#### Accessory pigments.

Particulates—dissolved molecules, dissolved gases, and water itself—unevenly affect the amount and spectral composition of incident light that reaches aquatic microorganisms suspended beneath the water surface. Accessory pigments expand the range of incident light wavelengths that an organism can harvest as energy for photosynthesis. They accomplish this by absorbing the light energy available at the wavelengths to which they are “tuned.” Then, they deliver that energy into a cascade of other accessory pigments and chlorophyll variants, ultimately activating chlorophyll *a*.

The principal accessory pigments of interest in phytoplankton research are the **phycobilins** and the **carotenoids**. Their presence and concentration vary by phytoplankton species and environmental growth conditions such as light and inorganic nutrients.

### Phycobilins.

Phycobilins are water-soluble accessory pigments with absorptions covering the spectral range from approximately 450 to 650 nm. *Phycocyanins* are a class of phycobilins present in all species of phototrophic cyanobacteria. The C-phycocyanin variant is the dominant accessory pigment in blue-green algae and emits fluorescence at 650 nm, while the R-phycocyanin variant is found in red-green algae and emits fluorescence at 646 nm. The phycobilin allophycocyanin is found in both cyanobacteria and red algae and emits fluorescence at 660 nm.

The phycobilin phycoerythrin (PE) is the predominant accessory pigment in red algae. PE emits fluorescence at a 575-nm wavelength, and is a common fluorophore in flow cytometry.

### Carotenoids.

Carotenoids are lipid-soluble accessory pigments that absorb light energy with particular efficiency in the blue-to-cyan region from approximately 450 to 490 nm. Fluorescence from carotenoids does not typically contribute significantly to the overall fluorescence profile in comparison to chlorophyll or the phycocyanins.

### Identifying autotrophs

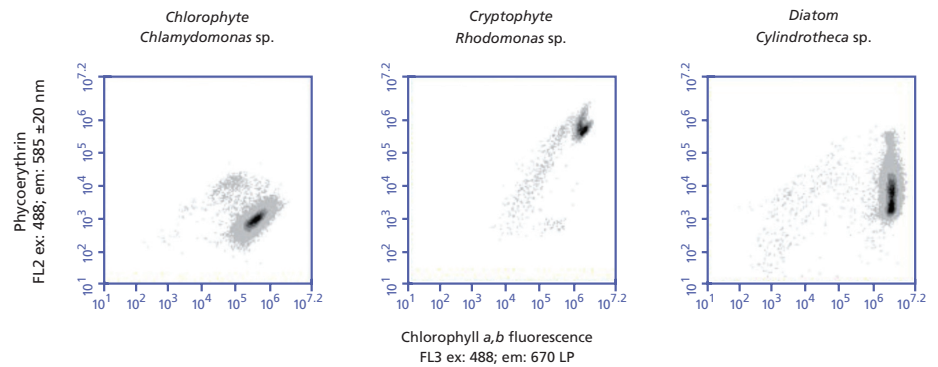
Table 2 indicates which detectors on the BD Accuri C6 flow cytometer, operated in its standard configuration, will receive the most fluorescence signal from each endogenous fluorophore. A given species may contain multiple pigments at the same time. Since their emission profiles may overlap, pigment fluorescence will often be seen in multiple detectors.

**Table 2.** Naturally occurring fluorescent pigments in phytoplankton and the primary BD Accuri C6 cytometer detectors where their fluorescent signals will be detected. Configurations and filters are standard.

Fluorophore	Exciting Laser	Major Emission Wavelength	BD Accuri C6 Cytometer Detector (filter)
Chlorophyll <i>a,b</i>	488	>640 nm	FL3 (670 LP)
Phycoerythrin	488	575 nm	FL2 (585 ±20)
C-phycocyanin	640	650 nm	FL4 (675 ±12.5)
R-phycocyanin	640	646 nm	FL4 (675 ±12.5)
Allophycocyanin	640	660 nm	FL4 (675 ±12.5)

### Phytoplankton.

The native chlorophyll, PE, and/or phycocyanins in many phytoplankton can aid in species identification and enumeration. Because chlorophyll *a* or *b* is the dominant fluorophore in most classes of autofluorescent phytoplankton, the FL3 detector will pick up the strongest signals (488-nm laser excitation, 670 LP filter emission). If PE is present, FL2 will also detect a strong signal (488 nm, 585  $\pm$ 20 filter). Figure 1 shows examples of three cultured phytoplankton species analyzed on the BD Accuri C6 flow cytometer. All three species are high in primary chlorophyll fluorescence (x-axis), but can be distinguished by their secondary PE fluorescence.



**Figure 1.** Chlorophyll and PE fluorescence profiles for three species of phytoplankton cultures, analyzed on the BD Accuri C6.

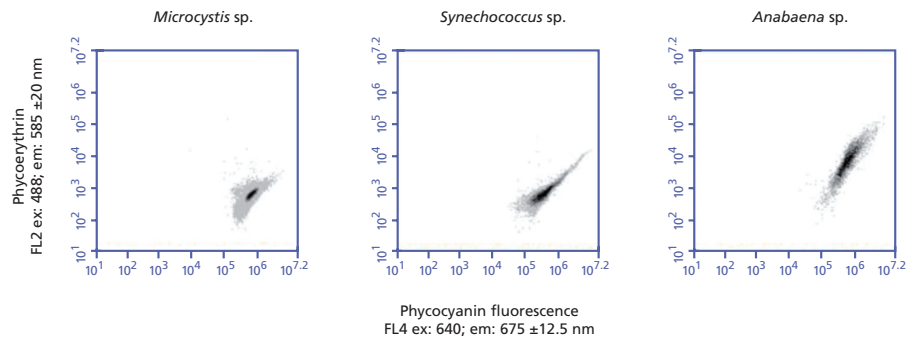
All three species have high-intensity chlorophyll fluorescence (x-axis, FL3 670 LP optical filter), which varies from a median channel value of  $3 \times 10^5$  (*Chlamydomonas*) to  $5 \times 10^6$  (*Cylindrotheca*) on a scale with maximum channel =  $16.7 \times 10^6$ . The PE signals (y-axis, FL2 585  $\pm$ 20 optical filter) vary from a median channel value of 800 (not above background, *Chlamydomonas*) to  $4 \times 10^5$  (*Rhodomonas*). Data courtesy of Jason Adolf, PhD, University of Hawaii, and Juli Dyle Bressie, PhD, NOAA, Seattle, WA.

The fluorescence emissions from these three phytoplankton species are comparable even though they were analyzed on different instruments. That is because each BD Accuri C6 instrument is calibrated for optimal performance to the same bead standard, and detector voltages are locked down at the factory. As long as the instruments are checked daily for fluorescence calibration, their readings are directly comparable even without including a separate, internal negative fluorescence standard. In this case, the chlorophyll fluorescence signal for the *Cylindrotheca* culture (FL3 median channel value =  $5 \times 10^6$ ) is more than ten times that of the *Chlamydomonas* culture (FL3 median channel value =  $3 \times 10^5$ ).



### Cyanobacteria.

A hallmark of cyanobacteria (blue-green algae) is the presence of phycocyanins and PEs in their light-harvesting phycobilisomes. The 640-nm red laser will maximally excite phycocyanins, with emissions detected in FL4 (675 ±12.5 optical filter). A strong, secondary signal will also be detected in FL3 (670 LP), and PE, if present, will be detected in FL2 (585 ±20). Figure 2 shows sample plots for several different cyanobacteria species. Only *Anabaena* sp. shows significant PE signal (y-axis, 488-nm excitation, 585 ±20 filter) while all three cyanobacteria have strong phycocyanin signals in FL4 (x-axis, 640-nm excitation, 675 ±12.5 filter).



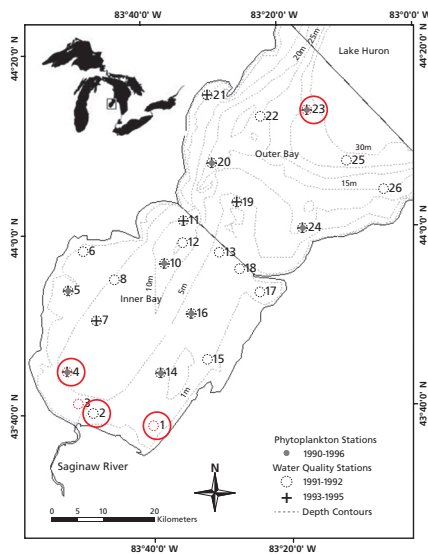
**Figure 2.** Phycocyanin and PE fluorescence profiles for three species of cyanobacteria cultures, analyzed on the BD Accuri C6.

Endogenous phycocyanins in cyanobacteria dominate the red fluorescence range (emissions >660 nm) as indicated by the high FL4 values on the x-axis. Only *Anabaena* sp. shows significant PE emissions (y-axis median channel value of 6,000); the other two species have PE similar to background. Data courtesy of Juli Dyble Bressie, PhD, NOAA, Seattle, WA.

### Samples from the environment.

When analyzing environmental samples on the BD Accuri C6 flow cytometer, researchers can identify the broad categories of phytoplankton using their characteristic fluorescence profiles. As a bonus, they can easily enumerate each identified population because the cytometer measures volume directly and reports counts per  $\mu\text{L}$  as a sample statistic. If desired, they can microscopically examine the water samples to confirm the flow cytometric findings and identify the exact species.

This strategy was used by Juli Dyble Bressie and Gary Fahnensteil of the National Oceanic and Atmospheric Administration's (NOAA) Great Lakes Environmental Research Laboratory (GLERL). As part of their research on the ecology of the Great Lakes region and the impact of aquatic organisms on human health, they sampled water from various locations around Saginaw Bay, Michigan, in June 2009. They used the BD Accuri C6 to analyze samples collected at the locations circled in red in Figure 3.

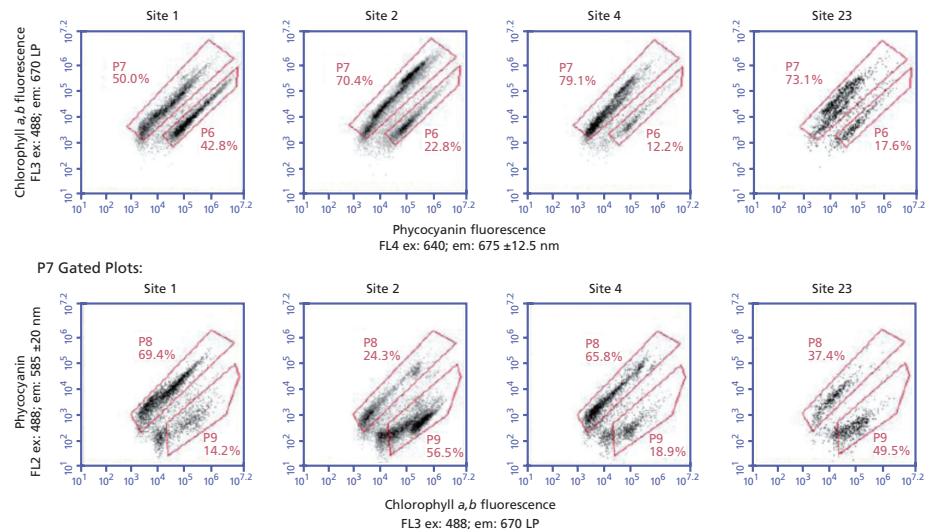


**Figure 3.** Map of sampling stations in Saginaw Bay, MI.

Red circles indicate sites sampled for analysis on the BD Accuri C6. Inset shows approximate location on a map of the Great Lakes. Flow cytometric data are shown in Figure 4 and Table 3.

The researchers ran 200- $\mu$ L samples in duplicate on the BD Accuri C6 flow cytometer, using a dual trigger on FL3 and FL4, set just above background of non-fluorescent events and debris. Results are shown in Figure 4. First, they used plots of phycocyanin vs chlorophyll *a, b* fluorescence (FL4 x FL3, top row) to distinguish cyanobacteria (P6) from other autofluorescent phytoplankton (P7).

Then, gating on the non-cyanobacteria population (P7), they used plots of chlorophyll vs PE fluorescence (FL3 x FL2, bottom row) to further distinguish subpopulations based on their PE signal.



**Figure 4.** BD Accuri C6 cytometer analysis of water samples from Saginaw Bay, MI, collected in June 2009.

**Top row:** Plots of phycocyanin fluorescence (FL4, x-axis) versus chlorophyll fluorescence (FL3, y-axis) were used to separate fluorescent phytoplankton into cyanobacteria (region P6) and other fluorescent phytoplankton (P7). **Bottom row:** The non-cyanin expressing phytoplankton (P7) were further analyzed and enumerated according to the presence (P8) or absence (P9) of PE signal (y-axis). See Figure 3 for site map and Table 3 for enumeration of the three populations identified in this figure. *Data courtesy of Juli Dyble Bressie, PhD, NOAA, Seattle, WA.*

Table 3 shows the enumeration, in events per mL, of the gated populations from the Saginaw Bay samples. Sites 1 and 2, close to the mouth of the Saginaw River (see Figure 3), had the most cyanobacteria, at least six times more than the other two locations. Site 2 also had the most abundant chlorophyll-dominated non-cyanobacteria species, almost 10 times greater than the other three sites. These results were expected, since Sites 1 and 2 see the largest nutrient load deposited by the river, resulting in the largest blue-green and green algal blooms later in the season (J. Dyble Bressie, personal communication). Site 23, the closest to open water (Lake Huron), had the lowest counts for all species.

**Table 3.** Compiled counts per mL of fluorescent populations in water samples collected at four Saginaw Bay locations in June 2009. A total sample of 200  $\mu$ L was run on the BD Accuri C6, employing a back-calculation to events per mL. *Data courtesy of Juli Dyble Bressie, PhD, NOAA, Seattle, WA.*

Site	Population Concentration (per mL)			
Sag. Bay, MI	phycocyanin dominated (P6)	chlorophyll <i>a,b</i> dominated (P9)	chlorophyll <i>a,b</i> and phycocerythrin (P8)	total fluorescent events/mL sample
1	18,730	3,120	15,200	37,050
2	18,635	32,450	13,955	65,040
4	3,145	3,840	13,390	20,375
23	1,550	3,185	2,405	7,140

As these field data show, the BD Accuri C6 flow cytometer's wide dynamic range and multiparametric analysis capability enable ecologists such as Dyble Bressie and Fahnensteil to resolve multiple microorganism populations in aquatic samples. By adding PNA-FISH probes, they can also perform strain-specific analyses of aquatic bacteria. The BD Accuri C6 cytometer's ability to analyze many samples quickly is an important advantage in field studies such as profiling cyanobacteria by distance from shore.

### Identifying heterotrophs

Detection of heterotrophic bacteria and viruses in water requires a nucleic acid stain to differentiate DNA/RNA-containing events from debris and electronic noise.<sup>4</sup> Because of their small size (<2  $\mu\text{m}$ ), data collection is best triggered on the positive fluorescence signal after staining, instead of light scatter.

Researchers who want to study both autotrophs and heterotrophs in the same sample should choose a nucleic acid stain that fluoresces in the green wavelengths and emits in FL1 (530  $\pm$ 15 nm filter) on the BD Accuri C6. This reduces interference with endogenous fluorophore signals in the other three detectors.

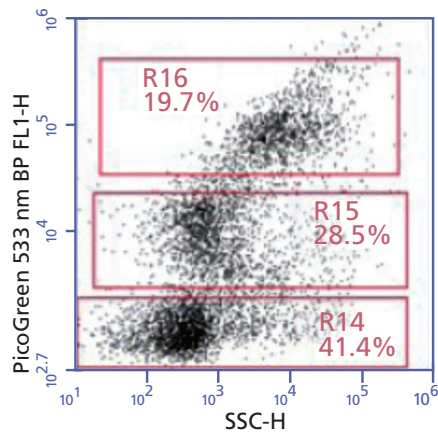
Figure 5 shows an example of the use of nucleic acid staining to identify bacteria and viruses in seawater. Marcel Veldhuis, a flow cytometry expert from the Royal Netherlands Institute for Sea Research (NIOZ), used a BD Accuri C6 to help identify unknown microorganisms in seawater. Seawater samples were stained with PicoGreen® (Invitrogen), a fluorescent dye that selectively binds double-stranded DNA (dsDNA). Positive fluorescence signal was defined by comparison to an equal volume of unstained seawater.

The intensity of PicoGreen fluorescence in FL1 (y-axis) reflected the relative amount of dsDNA in the three identified populations, with R16 >R15 >R14. Pairing the dsDNA data with side scatter (SSC, x-axis) provided information about relative particle size and/or shape differences between the populations. In this case, particle size or shape changed as DNA content increased. Based on known information about dsDNA content and identification by other molecular methods, Dr. Veldhuis was able to identify R14 as an aquatic virus, while R15 and R16 are heterotrophic bacteria. For Dr. Veldhuis, the BD Accuri C6 was able to replace three specialized flow cytometers in analyzing aquatic bacteria, viruses, algae, and cyanobacteria.

### Emerging applications

Access to a low-cost, high-performance flow cytometer such as the BD Accuri C6 is encouraging researchers to develop new applications and protocols for aquatic microorganism research. One such application is nitrogen cycle analysis. Karen Orcutt and Kjell Gundersen of the University of Southern Mississippi have tested the use of quantum dot (Qdot®) antibody conjugates to study the growth physiology of phytoplankton cells using flow cytometry.<sup>5</sup>

As fluorophores, Qdots offer a wide absorption spectrum, a narrow, tunable emission spectrum, and resistance to photobleaching. Orcutt and Gundersen are now using anti-nitrogenase Qdot antibody conjugates with the BD Accuri C6 to develop methods for quantifying single-cell nitrogen-fixing organisms, and measuring their nitrogen-fixation activity, in the Northern Gulf of Mexico.



**Figure 5.** Detection of aquatic bacteria (R15 and R16) and a virus (R14) using a nucleic acid stain (y-axis, PicoGreen) and side scatter (x-axis, SSC). Data courtesy of Marcel Veldhuis, PhD, Royal Netherlands Institute for Sea Research (NIOZ).



Dan Whitley and Maggie Waldron with their BD Accuri C6 outside Palmer Station, Antarctica.

## Conclusions

The BD Accuri C6 flow cytometer system has proven to be a valuable tool for the water science researcher. Its standard laser and detector configuration is well suited to analysis of all three naturally occurring fluorophore categories in common aquatic microorganisms—chlorophyll, phycocyanins, and phycoerythrin. The BD Accuri C6 cytometer's unique peristaltic fluidics system and broad dynamic optical range allow it to analyze a wide range of particle types and sizes, from virus and bacteria to large algal species. Because it can directly measure sample volume, it can determine the concentration of microorganism populations without the addition of counting beads.

A small footprint, low power requirements, ease of use, and ease of transport make the BD Accuri C6 an ideal cytometer for aquatic field work. Indeed, it has already proven its usefulness in field studies in the Great Lakes, the Gulf of Mexico, the Netherlands, and Antarctica.

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