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Enumeration of Phytoplankton, Bacteria, and Viruses in Marine Samples

U1WT11.11

The first applications of flow cytometry to the analysis of phytoplankton, in the mid-1980s, revolutionized the study of the smallest organisms in this community—those small enough to pass through 2- μm -pore-size filters, called picophytoplankton. These tiny organisms are generally unicellular and are naturally quite concentrated in seawater, so that they can be analyzed without prior concentration or sonication. Picophytoplankton are present in all aquatic environments, although their relative contribution to the photosynthetic biomass is greatest in the central regions of oceans (90% of the total surface), which are nutrient depleted and relatively poor in chlorophyll (0.2 mg/m³). Data obtained by flow cytometry have helped confirm that picophytoplankton constitute the bulk of the photosynthetic biomass in subtropical waters. This unit presents a method for enumerating phytoplanktonic cells on the basis of their natural parameters (see Basic Protocol 1). This protocol can be performed either on board ship or in the laboratory, and does not require any pretreatment of samples. If samples cannot be tested when freshly obtained, they can be preserved with formaldehyde or glutaraldehyde (see Support Protocol 1) and assayed later.

Highly sensitive nucleic acid-specific stains such as TOTO-1, YOYO-1, and the SYBR Green family (all available from Molecular Probes) have also made it possible to detect and enumerate heterotrophic bacteria and, very recently, viruses in marine samples. Two further protocols detail the enumeration of bacteria (see Basic Protocol 2) and viruses (see Basic Protocol 3) in culture and in natural seawater samples. Both require fixation (see Support Protocol 3) and the use of nucleic acid-specific stains.

Also included is a procedure for calibrating cytometer flow rates (see Support Protocol 2), replacing the standard approach using fluorescent microsphere standards, which is less suitable when working with seawater samples.

FLOW CYTOMETRIC ENUMERATION OF PICOPHYTOPLANKTON BASED ON SCATTER AND AUTOFLUORESCENCE

BASIC PROTOCOL 1

The different populations present in a natural sample are discriminated on the basis of their scatter signals and the fluorescence of their natural phytoplanktonic pigments (see Fig. 11.11.1), which can vary throughout the water column (see Fig. 11.11.2).

Flow cytometry is particularly suited to the analysis of picophytoplankton, which are difficult to study with traditional methods. Generally three major groups of these organisms, two cyanobacteria and a range of picoeukaryotes (algae), can be distinguished; see Anticipated Results for details.

Marine samples may be obtained, for example, from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), McKown Point, West Boothbay Harbor, Maine 04975, USA; <http://CCMP.bigelow.org>. Samples can be used fresh within 12 hr of being obtained (they should be stored at 4°C, but need not be fixed) or can be fixed and frozen (see Support Protocol 1), then thawed before being analyzed.

Materials

- Natural marine samples or cultures, either fresh or frozen (see Support Protocol 1 for freezing procedure)
- 0.95- μm fluorescent microspheres (Polysciences) diluted to $\sim 10^6$ beads/ml (as assessed by epifluorescence microscopy) in distilled water
- Seawater

Microbiological Applications

0.2- μm -pore-size cartridge filter units

Flow cytometer equipped with a 48-nm argon laser (e.g., FACSort, Becton Dickinson)

Additional reagents and solutions for flow cytometer calibration (see Support Protocol 2)

1. If sample has been frozen, thaw at 37°C. Transfer 1 ml of sample to a suitable flow cytometer tube.

If the cell suspensions are too concentrated (as may be the case with culture samples, for example), they can be diluted in seawater previously filtered through a 0.2- μm -pore-size filter:

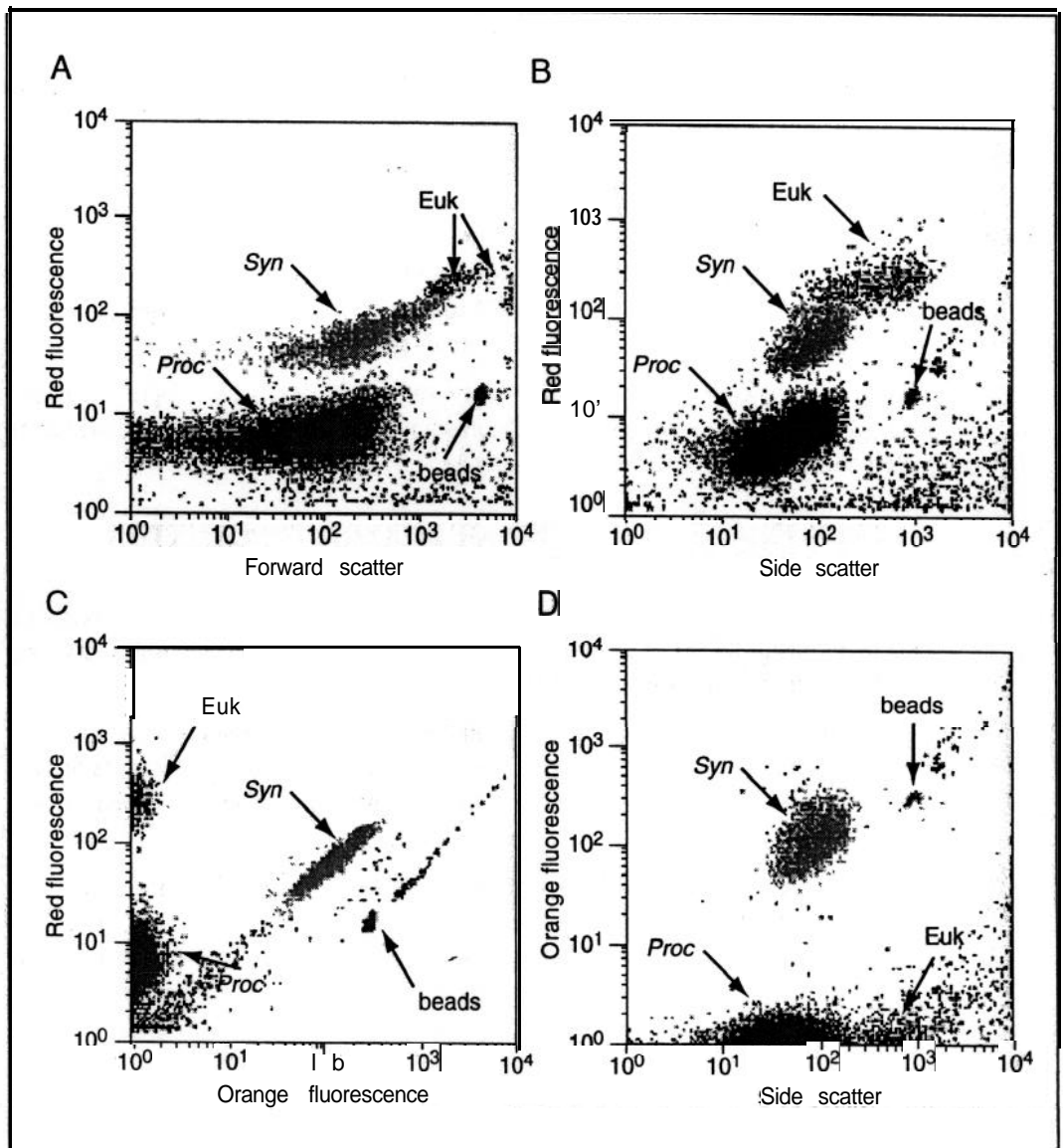


Figure 11.11.1 Cytograms of scatter and fluorescence obtained for a marine sample collected in the Pacific Ocean at a depth of 65 m (OLIPAC cruise, Cast 94, 5° S to 150° W). *Prochlorococcus*, *Synechococcus* (Syn), and picoeukaryotes (Euk) are discriminated on the basis of the fluorescence of their natural pigments, chlorophyll (red) or phycoerythrin (orange). 0.95- μm beads were added as internal reference.

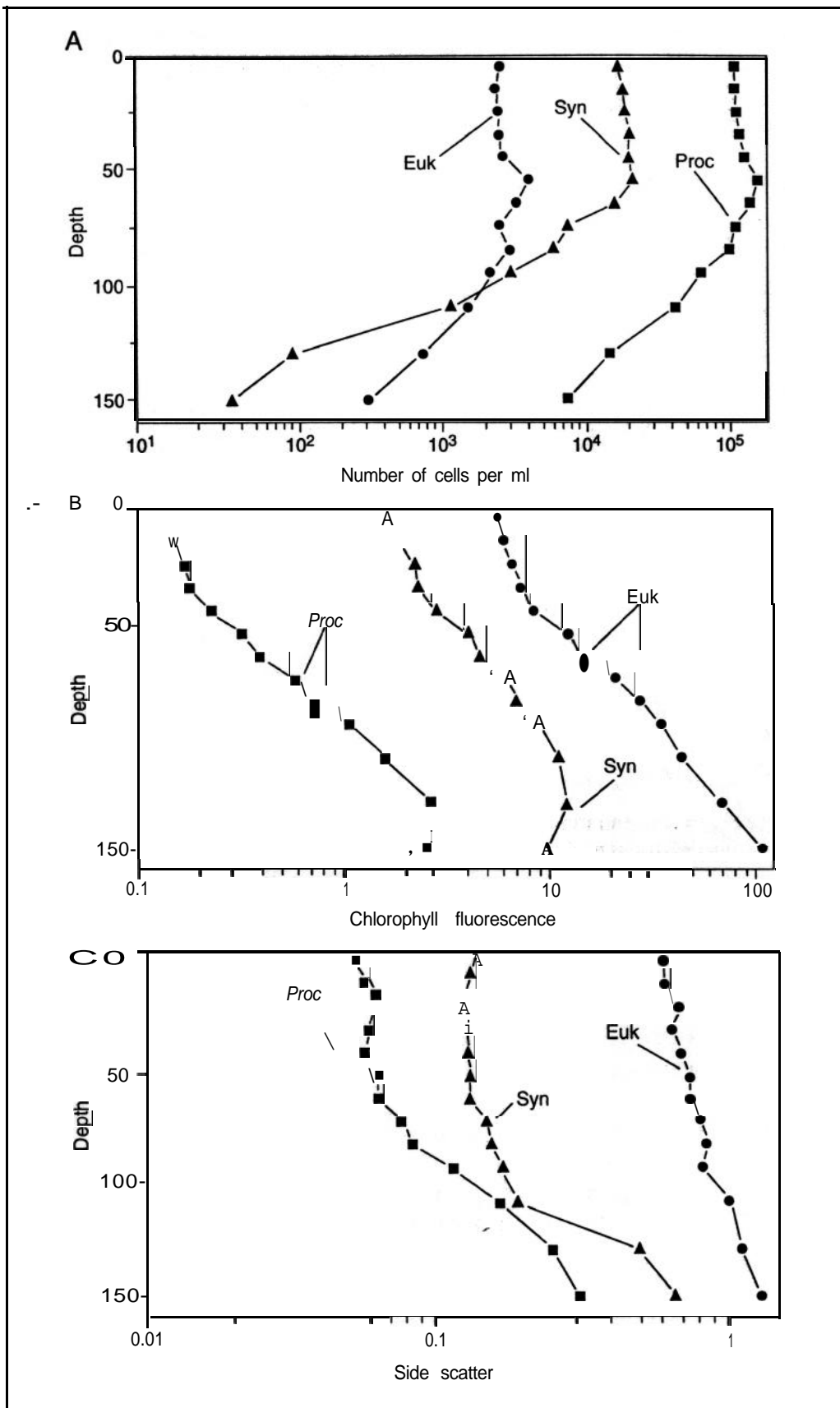


Figure 11.11.2 Vertical profiles obtained for samples collected in the Pacific Ocean and analyzed fresh on board the N.O. *Atalante* during the OLIPAC cruise (Cast 94,5° S to 150° W). Phytoplanktonic cell abundance (A), chlorophyll fluorescence (B), and side scatter (C) per cell normalized to 0.9- μ m beads versus depth. Proc, *Prochlorococcus*; Syn, *Synechococcus*; Euk, picoeukaryotes.

2. Add 10 μl of an $\sim 10^5$ beads/ml suspension of 0.95- μm fluorescent microspheres (as an internal reference).
3. Filter 5 to 10 liters of seawater through 0.2- μm -pore-size cartridge filter for use as sheath fluid.

Because cell scatter (especially forward scatter) is dependent on the nature of the sheath fluid, the use of filtered seawater as sheath fluid is recommended. If the fluidics system of the flow cytometer is equipped with an in-line filter; this should be removed, because it is likely to become contaminated quickly and will thereafter release particles.

4. Calibrate the flow rate of the cytometer (see Support Protocol 2).
5. Set the discriminator to red fluorescence and set all parameters on logarithmic amplification.

For a surface sample from a moderately oligotrophic area, typical settings on a FACSort flow cytometer are forward scatter (FS) = EOI, side scatter (SS) = 450, green fluorescence (FL1) = 650, orange fluorescence (FL2) = 650, and red fluorescence (FW) = 650.

6. *Insert* the sample, allow -15 sec for the flow rate to stabilize, and then begin data acquisition.

Data for natural samples are typically collected in listmode files for 2 to 4 min with a flow rate of 50 to 100 $\mu\text{l}/\text{min}$.

7. Record the time of analysis to determine precisely the cell concentrations of each population.
8. Compute the absolute cell concentration for each population in a given sample as follows:

$$C_{\text{pop}} = T \times N_{\text{pop}} / R \times (V_{\text{total}} / V_{\text{sample}})$$

where C_{pop} = concentration of population in cells/ μl , N_{pop} = number of cells acquired, T = acquisition time (min), R = sample flow rate ($\mu\text{l}/\text{min}$) as determined for the sample series, V_{total} = volume (μl) of sample plus additions (fixatives, beads, etc.), and V_{sample} = volume of sample (μl).

9. Report parameters relative to the beads added to the samples:

$$X_{\text{rel}} = X_{\text{pop}} / X_{\text{beads}}$$

where X_{pop} is the average value of a cell parameter (scatter or fluorescence) for a given population and X_{beads} the same parameter for the beads.

Before calculation of the ratio, X_{pop} and X_{beads} must be expressed as linear values (not numbers of channels) after conversion from the logarithmic recording scale.

BASIC PROTOCOL 2

FLOW CYTOMETRIC ENUMERATION OF BACTERIOPLANKTON BY DNA STAINING AND FLUORESCENT DETECTION

Because of its accuracy, its speed, and the lack of interference from dissolved organic matter, flow cytometry has been increasingly used to analyze heterotrophic bacteria (Shapiro, 1988; Robertson et al., 1998). In contrast to the photosynthetic prokaryotes *Prochlorococcus* and *Synechococcus*, bacteria do not contain any pigments and cannot be counted based on autofluorescence. Staining of cell DNA has been used as a means to discriminate and enumerate bacteria in natural seawater samples by epifluorescence microscopy (EFM; Hobbie et al., 1977) or flow cytometry (Button and Robertson, 1989; Monger and Landry, 1993; Li et al., 1995; Marie et al., 1997). The combination of DNA and chlorophyll fluorescence allows discrimination of autotrophic from heterotrophic

picoplankton (Monger and Landry, 1993; Campbell et al., 1994). For details of the bacterial populations generally observed using flow cytometry, see Anticipated Results.

Older W-excited dyes, such as DAPI or Hoechst 33342, that require expensive flow cytometric equipment, are currently being superseded by a wide and continually expanding range of nucleic acid-specific dyes synthesized and manufactured by Molecular Probes. These novel dyes are excited at 488 nm, which means they are usable on small, low-cost flow cytometers equipped with air-cooled single-line argon lasers. The affinity of the cyanine dyes TOTO- 1 and YOYO- 1, and their monomeric equivalents YO-PRO- 1 and TO-PRO-1 (all available from Molecular Probes), decreases significantly with increasing ionic strength, so they are inappropriate for direct analysis of seawater samples (Marie et al., 1996). Other dyes such as SYBR Greens I and II, SYTOX Green, and the SYTO family (all available from Molecular Probes) are less dependent on medium composition and can be used for enumerating bacteria in marine environments (Marie et al., 1997; Lebaron et al., 1998). Because SYBR Green I (SYBR-I) has a very high fluorescence yield, the authors strongly recommend the use of this dye to enumerate bacteria in marine samples.

Samples must be fixed before bacterial enumeration can be performed, since fixation allows the nucleic acid-specific stain to penetrate into the cell. A 10,000-fold dilution of the commercial SYBR-I stock solution is used..

NOTE: All stock solutions except the dye must be prefiltered through a 0.2- μm - (or smaller) pore-size filter to avoid contamination.

Materials

Natural marine samples or cultures, either fresh or frozen (see Support Protocol 1 for freezing procedure)

10% paraformaldehyde (see Support Protocol 3) and/or 25% glutaraldehyde (Sigma)

DNA-specific stain such as SYBR Green I, YOYO- 1, TOTO- 1, or TO-PRO- 1 (Molecular Probes)

0.95- μm fluorescent microspheres (Polysciences) diluted to $\sim 10^5$ beads/ml (as assessed by epifluorescence microscopy) in distilled water

Seawater

0.2- μm -pore-size cartridge filter units

Flow cytometer equipped with a 488-nm argon laser (e.g., FACSort, Becton Dickinson)

Additional reagents and solutions for flow cytometer calibration (see Support Protocol 2)

- 1a. *If samples are live:* Add 1% paraformaldehyde or 0.1% glutaraldehyde (final concentrations) and let stand 20 min.

Paraformaldehyde and glutaraldehyde give equivalent results.

- 1b. *If samples have been preserved and frozen:* Thaw samples at 37°C.

If the cell suspensions are too concentrated (as may be the case with culture samples, for example), they can be diluted in seawater previously filtered through a 0.2- μm -pore-size filter:

2. Add SYBR-I at a final concentration of 1 part in 10,000 and incubate 15 min at room temperature in the dark.

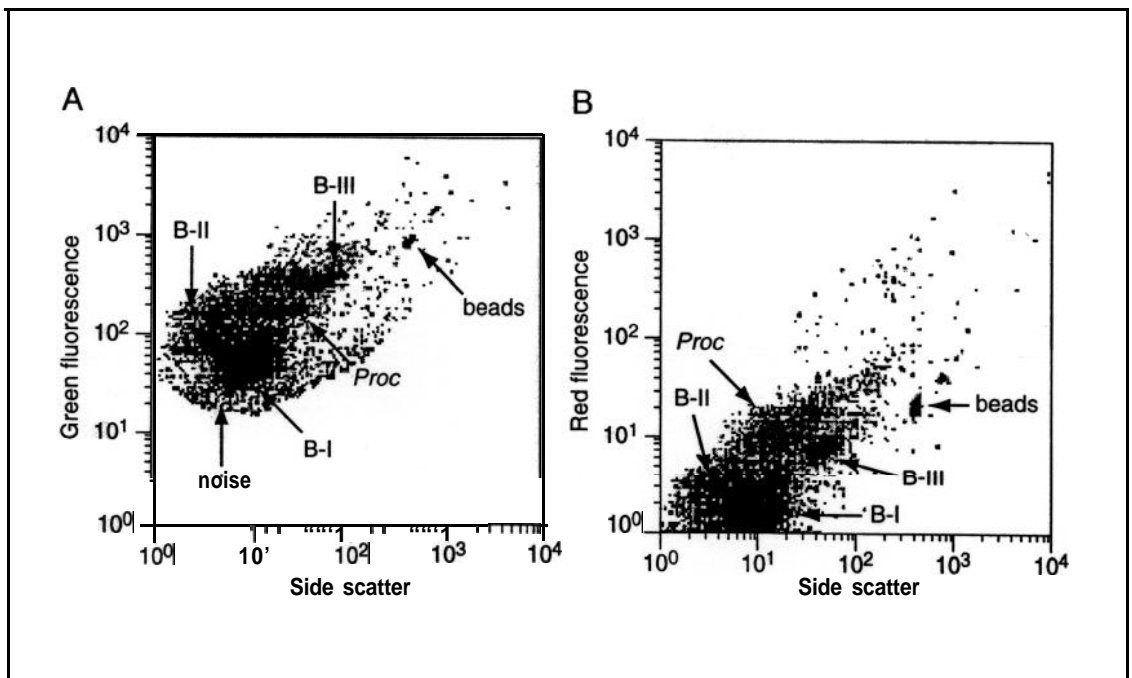


Figure 11.11.3 Cytograms of side scatter and fluorescence obtained for a natural sample collected in the Pacific Ocean at a depth of 65 m (OLIPAC cruise, Cast 94,5° S to 150° W) and stained with SYBR-I. Three different bacterial populations (B-I, B-II, and B-III) can be discriminated from *Prochlorococcus* by the combination of the different parameters recorded. 0.9- μ m fluorescent beads were added as internal reference.

3. To 1 ml of sample, add 10 μ l of an $\sim 10^5$ bead/ml suspension of 0.95- μ m fluorescent microspheres (as an internal reference).
4. Filter 5 to 10 liters of seawater through 0.2- μ m-pore-size cartridge filter for use as sheath fluid.

Distilled water can be used as sheath fluid, but for natural seawater samples, 0.2- μ m-pore-size-filtered seawater is preferable, since cell scatter (especially forward scatter) is dependent on the nature of the sheath fluid. If the fluidics system of the flow cytometer is equipped with an in-line filter, this should be removed, because it is likely to become contaminated quickly and will thereafter release particles.

5. Set the discriminator to green fluorescence.
6. Calibrate the flow rate of the cytometer (see Support Protocol 2).
7. Set all parameters on logarithmic amplification.

It is recommended that no more than 80,000 events be acquired in listmode, in order to avoid very large files.

Typical settings on a FACSort flow cytometer are FS = E01. SS = 450, FLZ = 650, FL2 = 650, and FL3 = 650.

8. Run the sample, adjusting the flow rate and cell concentration to avoid coincidence.

Typically, the authors run samples for 2 to 2 min at a flow rate of 30 to 50 μ l/min and keep the number of events below 1000 per sec (by diluting samples that are too concentrated).

Some samples, particularly those obtained in coastal areas, contain copious quantities of small particles and debris that will increase the level of background noise. This can induce

coincidence or lead to the generation of large listmodefiles. In such cases, the threshold can be increased to reduce the number of events seen by the flow instrument, and/or a "bitmap" window (non-regular region) can be defined that includes the population of bacteria so that only the events belonging to this area will be recorded (see Fig. 11.11.3).

FLOW CYTOMETRIC ENUMERATION OF VIROPLANKTON BY DNA STAINING AND FLUORESCENT DETECTION

BASIC
PROTOCOL 3

The existence of bacteriophages in marine environments has been known for many years (Kriss and Rukina, 1947; Spencer, 1955, 1960), but they were not really investigated until fairly recently (Bergh et al., 1989; Bratbak et al., 1990; Proctor et al., 1990). Viroplankton clearly constitute the most abundant population of biological particles in the ocean and their ecological role has only recently been investigated. These studies initially required techniques such as transmission electron microscopy (TEM) that are time consuming and allow only limited numbers of samples to be analyzed. During the past decade, investigations using epifluorescence microscopy (EFM) in conjunction with nucleic acid-specific dyes such as DAPI (Hara et al., 1991) or with cyanine dyes (Hennes and Suttle, 1995; Weinbauer and Suttle, 1997) have considerably improved knowledge of marine viruses.

Very recently, flow cytometry has been successfully applied to the analysis of viruses in solution, using the nucleic acid-specific dye SYBR Green I (Marie et al., 1999). This has permitted the analysis of viruses with reduced DNA content, down to 40 Kbp (Brussaard et al., unpub. observ.). Other dyes, such as SYTOX, PicoGreen, OliGreen, SYBR Green II, SYBR Gold, or RiboGreen (all from Molecular Probes), can be used with the same efficiency as SYBR-I (Brussaard et al., unpub. observ.). For details of the viroplankton populations generally observed using flow cytometry, see Anticipated Results.

NOTE: All stock solutions except the dye must be prefiltered through a 0.2- μ m- (or smaller) pore-size filter to avoid contamination.

Materials

Natural marine samples or cultures, either fresh or frozen (see Support Protocol 1 for freezing procedure)

10% paraformaldehyde (see Support Protocol 3) or 25% glutaraldehyde (Sigma)

TE buffer, pH 7.2 (**APPENDIX @**)

DNA-specific stain(s) such as SYBR Green I or II, OliGreen, or RiboGreen (Molecular Probes)

0.95- μ m fluorescent microspheres (Polysciences) diluted to \sim 16 beads/ml (as assessed by epifluorescence microscopy) in distilled water

Distilled water

0.2- μ m-pore-size filtration units for plastic syringe

Flow cytometer equipped with a 488-nm argon laser (e.g., FACSort, Becton Dickinson)

Additional reagents and solutions for flow cytometer calibration (see Support Protocol 2)

Prepare sample

1a. **For fresh samples:** Add 1% paraformaldehyde or 0.1% to 0.5% glutaraldehyde (final concentrations) and let stand 20 min.

No significant differences have been found between results for virus enumeration performed on samples fixed with paraformaldehyde, glutaraldehyde, or a mixture of both.

1b. **For fixed and frozen samples:** Thaw samples at 37°C.

Microbiological
Applications

11.11.7

2. **Dilute samples** in TF buffer, pH 7.2, to three different concentrations: typically 10-, 50-, and 100-fold for natural seawater samples and 100-, 1,000-, and 10,000-fold for cultured samples.

Preparation of three different dilutions is necessary because the concentration of viruses is not known beforehand. Analysis must be performed with a suspension of 2×10^5 to 2×10^6 viruses/ml final concentration).

To avoid generating large files, samples can be run for 1 or 2 min at a rate ranging from 20 to 50 μ l/s.

Different buffers have been tested for diluting virus samples. Tris-based buffers give the best result.

3. Add SYBR-I at a final concentration of 5 parts in 100,000 and incubate 15 min at room temperature in the dark.
4. To 1 ml of sample, add 10 μ l of an $\sim 10^5$ beads/ml suspension of 0.95- μ m fluorescent microspheres (as an internal reference).

For virus samples that are freshly fixed (i.e., have not been frozen), or for hard-to-stain material, it is necessary to heat the samples 10 min at 80°C in the presence of detergent (e.g., Triton X-100 at 0.1% final) to improve dye uptake.

Acquire data

- 5: Using distilled water as sheath fluid (even for marine samples), begin the cytometric procedure by calibrating the flow rate (see Support Protocol 2).

Since samples are diluted in TE, use of seawater is not necessary.

6. Turn the discriminator to green fluorescence (FL1).

Typical settings on a FACSort flow cytometer are FS = E03, SS = 600, FL1 = 600, FL2 = 650, and FL3 = 650.

7. Before starting data acquisition, wait for the sample flow rate to stabilize (this can take up to 20 sec).
8. Run the sample at a rate allowing <1000 events/sec (to avoid coincidence; see Basic Protocol 2, step 8, for discussion of this problem).

SUPPORT PROTOCOL I

PRESERVATION AND STORAGE OF PICOPHYTOPLANKTON

If samples cannot be run immediately, they may be kept up to 12 hr at 4°C without significant change in abundance or optical parameters. If they cannot be analyzed within that time interval, they must be fixed for 15 to 20 min with formaldehyde or glutaraldehyde, then deep frozen in liquid nitrogen and stored at -80°C until analysis. Frozen samples can be kept for at least 1 year.

NOTE: All stock solutions except the dye must be prefiltered through a 0.2- μ m- (or smaller) pore-size filter to avoid contamination.

Materials

10% paraformaldehyde (see Support Protocol 3) and/or 25% glutaraldehyde (Sigma)

1. Add paraformaldehyde or glutaraldehyde, or both, to freshly obtained water samples at final concentrations of 1% and 0.1%, respectively.
2. Wait 15 min.
3. Freeze the samples in liquid nitrogen.
4. Store at -20°C for a few weeks or at -80°C for longer periods.

CALIBRATION OF THE CYTOMETER FLOW RATE

Accurate calibration of the sample flow rate is essential for obtaining reliable cell counts. As most flow cytometers do not allow delivery of defined volumes of samples, fluorescent microspheres with a known concentration are often used to determine the flow rate. The authors do not use this method, because (1) microspheres are electrostatic, and seawater makes them stick on plastic tube walls, changing their initial concentration, and (2) the usual method of determining microsphere concentration, epifluorescence microscopy, generates large counting errors. The authors present below a method that is usable for flow cytometers such as the FACScan, FACSort, or FACScalibur (Becton Dickinson), but can be extended to most existing flow instruments.

NOTE: All stock solutions except the dye must be prefiltered through a 0.2- μm - (or smaller) pore-size filter to avoid contamination.

1. Select a rate (Low, Medium, or High).
2. Fill a tube with the same liquid as the one containing samples (i.e., seawater for marine samples).
3. Measure the volume of sample (or weigh precisely the tube containing the sample).
4. Remove the outer sleeve of the injection system.

The sheath fluid will drop down the sample needle.

5. Wait until a droplet just falls. Before the next one forms, put on the sample tube and close the sample arm in the running position. Simultaneously, start the chronometer running.
6. Run the sample for at least 10 min.
7. Remove the sample tube and simultaneously stop the chronometer.
8. Measure (or weigh) the remaining volume.
9. Calculate the rate (R), expressed in microliters per minute, by one of the following two methods.

Volume measurement:

$$R = (V_i - V_f)/T$$

where V_i = initial volume (μl), V_f = final volume (μl), and T = time (min).

Weight measurement:

$$R = (W_i - W_f)/(T \times d)$$

where W_i = initial weight (mg), W_f = final weight (mg), T = time (min), and d = density of the liquid used for calibration (distilled water = 1.00, seawater = 1.03).

The weight measurement provides better precision.

PREPARATION OF BUFFERED 10% PARAFORMALDEHYDE STOCK SOLUTION

To preserve marine samples, the authors generally use either 1% paraformaldehyde, 0.1% glutaraldehyde, or a mixture of 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations), with a preference for paraformaldehyde. The solution is buffered so that it will not significantly modify the pH of seawater samples. The following protocol describes the preparation of a 10% paraformaldehyde solution.

CAUTION: Paraformaldehyde is an irritant. **Wear** protective clothing and work in a fume hood.

Materials

Paraformaldehyde (e.g., Sigma)
Distilled water
1 M NaOH
Phosphate-buffered saline (PBS; **APPENDIX 2A**), pH 7.5
1 M HCl
0.2- μ m-pore-size filtration unit for plastic syringe

1. Weigh 10 g paraformaldehyde under a fume hood.
2. Add 85 ml boiling distilled water.
3. Stir vigorously at 70°C for at least 2 hr under a fume hood, until the formaldehyde dissolves and saturates the water.
4. Add small amounts of 1 M NaOH until the solution becomes clear.
5. Add 10 ml of PBS, pH 7.5.
6. Adjust the pH to 7.5 with 1 M HCl, then dilute to 100 ml with distilled water.
7. Filter first through filter paper, then through a 0.2- μ m-pore-size syringe filter.
8. Divide into aliquots in 15-ml tubes and store at -20°C.

Unfrozen aliquots can be stored up to 1 week at 4°C.

Paraformaldehyde is the polymerized form of formaldehyde which, in contrast to formaldehyde, lacks cross-linking properties. When liquid, the solution is unstable over time.

COMMENTARY

Background Information

General considerations

Photoacclimation is widely observed in oceanic samples. Phytoplankton acclimate to changes of photon-flux densities by changing pigment content (Partensky et al., 1996). This leads to variations in the chlorophyll fluorescence per cell along a depth profile (see Fig. 11.11.2) that are extreme for *Prochlorococcus* (up to 50-fold) and less pronounced for picoeukaryotes (5- to 10-fold). The intensity of other cellular parameters, such as scatter and orange fluorescence, also varies throughout the water column. Cell size and chlorophyll fluorescence will also vary with the level of available nutrients, typically decreasing as nutrients become limiting (see Fig. 11.11.2). Thus the photomultiplier (PMT) voltages must be adjusted depending on the size of organisms of interest and on the depth sampled, so that the relative position of the organisms of interest remains approximately the same.

Acquisition and data analysis

Parameters are collected on logarithmic scales in order to obtain the multidecade dynamic range necessary to analyze the majority of the organisms present, which in natural samples can range widely in size and fluorescence properties. Data are always collected as list-mode files; typically 20,000 to 40,000 events are collected for enumeration of phototrophs and up to 80,000 for bacteria or viruses. List-mode files are then analyzed by using the free software CytoWin (Vaulot, 1989), available at <http://www.sb-roscoff/Phyto/cyto.html>, which is very efficient at rapidly processing a large number of files. The different populations are discriminated based on the combination of their scatter signals and the fluorescence of their natural pigments or of the nucleic acid-stain complex. To allow comparison between different samples, the cell parameters for each sample are normalized to those for 0.95 μ m microspheres added as an internal reference, by dividing the mean value of each parameter by the mean value for the beads.

Flow cytometer

The choice of flow cytometer is critical for the analysis of picoplanktonic cells. The chief criterion is sensitivity. *Prochlorococcus* cells are very dim in the upper layer of oligotrophic waters, and can very easily be missed. Natural viroplankton display a wide range of sizes and are often difficult to separate clearly from background noise. The instrument must also be compact enough to be used on board ship during oceanographic cruises. The FACSort and FACScalibur flow cytometers from Becton Dickinson fulfill these criteria and are suitable for the analysis of small picoplanktonic cells. Nevertheless, some populations still may not completely resolved—for instance, *Prochlorococcus* in surface waters of extreme oligotrophic areas. Custom modifications have been proposed to deal with this inadequacy (Dusenberry and Frankel, 1994). Another problem is that the volume of sample typically analyzed by flow cytometers (<1 ml) is too small for accurate enumeration of larger and less concentrated phytoplanktonic populations such as dinoflagellates or diatoms. Custom modifications and even new instruments have been developed for this purpose (Dubelaar et al., 1989; Cavender-Bares et al., 1998).

Sample preservation

Measurements of phytoplankton abundance are best obtained on fresh unfixed seawater samples, which may be kept at 4°C for up to 12 hr. If samples cannot be analyzed within this time interval, it is necessary to preserve them for delayed analysis. Fixation will always result in a loss of cells (Vaulot et al., 1989); the choice of fixative is critical and should be optimized for the species of interest. The fixation procedure must also be simple enough to be usable on board ship. Physical treatments such as centrifugation and classical or tangential filtrations must be avoided because they induce variable losses of cells. Because phytoplanktonic cells are discriminated on the basis of scatter and pigment fluorescence, the fixation procedure must preserve these properties—which rules out such classical methods as formalin and Lugol fixations that modify cell shape or significantly affect fluorescence. Similarly, alcohol fixation will extract lipophilic pigments and lead to a loss of autofluorescence. Natural seawater samples are best preserved with paraformaldehyde (0.5% to 1% final concentration), glutaraldehyde (0.1% to 1%) or a mixture of the two. If careful preparation of paraformaldehyde is not possible, the

use of a commercial 25% glutaraldehyde solution is preferable.

Fixed samples must be stored at -80°C as they degrade within a few months at -20°C. For a mixture of *Prochlorococcus*, *Synechococcus*, and a picoeukaryote, the authors observed no significant loss after 1 month of storage at -20°C, but 50% of *Prochlorococcus* and up to 80% of eukaryote cells were lost after 6 months.

Identification of phytoplankton population

Before attempting to analyze natural samples, it is critical to analyze laboratory cultures of each cell type likely to be observed (see Anticipated Results). Failure to do so could result in misinterpretation of natural samples.

Analysis of depth profile

For analysis of samples taken at different depths along a vertical profile, it is best to start with a sample collected at a depth corresponding to the chlorophyll concentration maximum as measured remotely. Use the same setup to analyze samples obtained from deeper levels, where cells of interest become too scarce and it is difficult to adjust PMTs precisely. Then continue by going up the water column, progressively increasing the voltage of the PMT as needed to detect the cells of interest. If only surface samples are available, set the threshold at the minimum value and increase the red PMT voltage until some noise appears (-50 events per sec). Then fix the PMT voltage and run the sample.

Bacterial staining

For bacterial enumeration, if samples are in suspension in a nonsaline solution, or can be diluted enough to minimize the effects of seawater or ionic strength, the authors recommend final concentrations of 1 µg/ml for DAPI or Hoechst, 30 nM or TOTO, YOYO, TO-PRO, or YO-PRO, 1 part in 1000 for PicoGreen, and 1 part in 10,000 for SYBR Green I or SYTOX Green.

Viroplankton analysis

viruses are too small to be discriminated solely on the basis of their side- or forward-scatter properties on flow cytometers such as the Becton Dickinson FACS series. Nucleic acid-specific staining is therefore necessary. Because flow cytometry was not designed for the analysis of such small particles, care must be taken in order to obtain reliable data. If samples are too diluted, there will be loss in the emission signal of the nucleic acid-dye com-

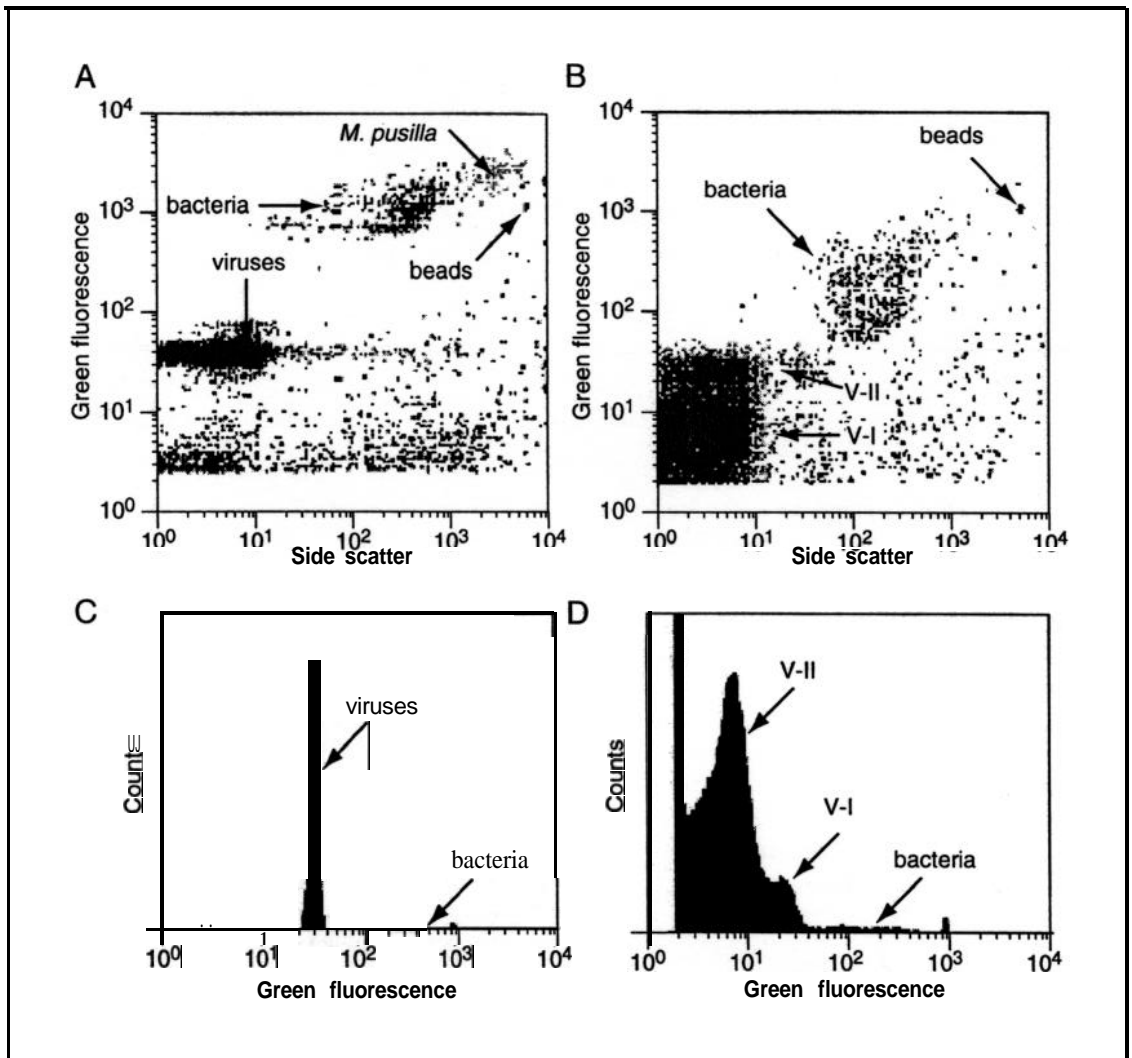


Figure 11.11.4 Side scatter versus green fluorescence and monoparametric DNA distribution obtained for a non-axenic culture of *Micromonas pusilla* infected by the virus MpV-01 (A and C) and for a natural sample (B and D) collected in the Raunefjorden, Western Norway, both stained with SYBR-I. 0.95- μ m fluorescent microspheres were added as internal reference.

plex; if they are insufficiently diluted, coincidence will occur. Analysis of different dilutions of natural seawater samples has shown that coincidence occurs for viruses above 800 events per second. However, on flow cytometers such as the FACSsort, for suspensions of beads, bacteria, or small algae coincidence normally occurs above 2000 events per sec. For concentrated suspensions, above 800 objects per second, more virus doublets are observed, which result in an increase of the fluorescence signal due to viruses passing simultaneously through the laser beam.

Because all the V-II and 20% of the V-I virus populations can pass through 0.2- μ m-pore-size filters, 0.2- μ m-pore size-filtered seawater cannot be used to dilute the samples. Reasonable alternatives are 0.05 μ m-pore-size-filtered

seawater and buffers such as TE, the use of TE improves the emission signal of stained viruses, making this the best option.

Troubleshooting

Detection of *Prochlorococcus* in surface waters

In highly oligotrophic waters, such as those of the subtropical Pacific Ocean, it is not always possible with unstained samples to detect the whole *Prochlorococcus* population in surface water samples because its chlorophyll fluorescence is too weak. In such cases, after staining with SYBR-I, *Prochlorococcus* cells will be included in the heterotrophic bacteria population, from which they cannot be discriminated

(see Fig. 11.11.3A); this must be taken into consideration in interpreting results.

Preservation of the samples

Preliminary tests must be performed to choose the best fixative for the cells of interest. Seawater is naturally buffered at about pH 8. Glutaraldehyde is acidic, and when dissolved at final concentrations of 0.1%, 0.25%, 0.5%, and 1% in seawater will produce pH values of 7.84, 7.42, 6.85, and 6.35, respectively. pH <7 is particularly damaging to fragile phytoplanktonic cells such as *Prochlorococcus* or small eukaryotes.

Virus staining

Viruses contain small amounts of nucleic acids. The critical point for virus staining resides in the equilibrium between dye concentration and virus abundance. If viruses are too concentrated, a decrease in fluorescence will result. For virus numbers that do not saturate the machine's acquisition capacity, an increase in dye concentration will also result in loss of signal.

Moreover, virus abundance determined by flow cytometry on fixed but unfrozen samples is 3- to 10-fold lower than that found for frozen samples. This suggests that live viruses may have a structure that prevents access of SYBR-I to nucleic acids. Detergent or heat treatment up to 95°C may be needed to denature the virus capsid and allow the stain to penetrate.

For unknown virus material, the concentration of dye as well as the effect of heating must be assessed. In general, the authors observed that using half the concentration of SYBR-I used for bacterial staining (to 5 parts in 100,000) and heating between 70° to 80°C are suitable for the majority of viruses analyzed.

Anticipated Results

Picophytoplankton

Picophytoplankton are categorized into three major groups (see Fig. 11.11.1). *Prochlorococcus*, a cyanobacterium 0.6 µm in size, is a photosynthetic organism that contains divinyl derivatives of chlorophylls *a* and *b*. Its discovery in 1988 was one of the most significant results obtained so far from the application of flow cytometry to aquatic sciences. This organism is undoubtedly the most abundant genus of phytoplankton, reaching >10⁵ cells/ml, and its vertical distribution extends from the surface to depths of 150 m or more within the intertropical belt (see Fig. 11.11.2; Chisholm et al., 1988,

1992; Partensky et al., 1999). *Prochlorococcus* is responsible for about half the biomass and the primary production in warm offshore waters.

Synechococcus, also a cyanobacterium, is 1 µm in size and was discovered in 1979 (Waterbury et al., 1979). It is characterized by the dual fluorescence of its pigments: orange from phycoerythrin and red from chlorophyll. *Synechococcus* is found at low concentrations in oligotrophic waters (Campbell and Vault, 1993) but can be very dense (~10⁵ cells/ml) in mesotrophic and coastal areas (Olson et al., 1988; Partensky et al., 1996).

Picoeukaryotes belong to a variety of algal classes, in particular Prasinophyceae, Pelagophyceae, and Bolidophyceae. Field data indicate a typical abundance of 10³ cells/ml in open ocean waters (Li and Wood, 1988; Campbell and Vault, 1993) and up to ten times higher concentrations in coastal waters (Courties et al., 1994).

Bacterioplankton

Using TOTO-1 or TO-PRO-1, some authors have distinguished two different populations of bacteria (B-I and B-II) in natural seawater samples that differ both in scatter and in DNA content (Li et al., 1995). With SYBR-I two or three different populations can be distinguished (see Fig. 11.11.3). The B-I group seems to dominate in oligotrophic areas, while the B-II group increases in less oligotrophic conditions (Li et al., 1995; Marie et al., 1997). The third group is commonly found in coastal areas.

Viioplankton

Using SYBR-I, the authors have been able to distinguish at least two different populations of viruses (V-I and V-II) in natural seawater samples (see Fig. 11.11.4B and 11.11.4D). Viioplankton belonging to the V-I population have a higher fluorescence similar to that of cultured viruses specific to eukaryotic algae such as *Micromonas pusilla* (see Fig. 11.11.4A and C).

Time Considerations

Phytoplanktonic cells do not require any treatment prior to enumeration, so samples of such cells can be analyzed immediately. When analysis must be delayed or when enumerating bacteria and viruses, where fixation is required, samples must be fixed for 15 min and then frozen. Staining is achieved by incubating samples for 15 min at room temperature in the dark before flow cytometric analysis.

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Internet Resources

<http://www.sb-roscoff.fr/Phyto/cyto.html>

Lists marine applications of flow cytometry and provides a downloadable copy of the Cyto Win software.

<http://carl.im.uib.no/sup>

Provides information on marine viruses.

<http://www.flowcytometry.org>

Contains a wide range of resources for marine applications of flow cytometry.

<http://CCMP.bigelow.org>

Catalogs and maintains algal strains to be used for protocol development.

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The authors wish to acknowledge the support of the European Community MAST III programs (MAS3-CT96-015- MEDEA, MAS3-CT97-0128- PROMOLEC, MAS3-CT96 5033[DG12-ASAL]), the JGOFS-France PROSOPE program, and the Research Council of Norway (project number 121425/420). The FACSort flow cytometer was funded in part by CNRS-INSU and the Région Bretagne.