Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities*

JOSEP M. GASOL¹ and PAUL A. DEL GIORGIO²

¹ Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, CSIC. Passeig Joan de Borbó s/n. E08039 Barcelona, Catalunya. Spain. Phone: (3493) 2216416, Fax: (3493) 2217340. E-mail: pepgasol@icm.csic.es
² Horn Point Laboratory. University of Maryland Center for Environmental Science PO. Box 775 Cambridge, MD 21613 USA

SUMMARY: Flow cytometry is rapidly becoming a routine methodology in aquatic microbial ecology. The combination of simple to use bench-top flow cytometers and highly fluorescent nucleic acid stains allows fast and easy determination of microbe abundance in the plankton of lakes and oceans. The different dyes and protocols used to stain and count planktonic bacteria as well as the equipment in use are reviewed, with special attention to some of the problems encountered in daily routine practice such as fixation, staining and absolute counting. One of the main advantages of flow cytometry over epi-fluorescence microscopy is the ability to obtain cell-specific measurements in large numbers of cells with limited effort. We discuss how this characteristic has been used for differentiating photosynthetic from non-photosynthetic prokaryotes, for measuring bacterial cell size and nucleic acid content, and for estimating the relative activity and physiological state of each cell. We also describe how some of the flow cytometrically obtained data can be used to characterize the role of microbes on carbon cycling in the aquatic environment and we prospect the likely avenues of progress in the study of planktonic prokaryotes through the use of flow cytometry.

Key words: heterotrophic and phototrophic bacteria, flow cytometry, counting bacteria, active bacteria.

INTRODUCTION

Bacteria are important components of planktonic food webs: autotrophic bacteria can dominate the primary producers’ compartment in oligotrophic waters (Li et al., 1992; Campbell et al., 1994; Buck et al., 1996) and contribute a large percentage to total primary production (Li, 1994; Vaulot et al., 1995). Chemoheterotrophic bacteria participate with an important share of total plankton biomass (Gasol et al., 1997) that at times can be higher than, or at least similar to, that of primary producers (Li et al., 1992; Fuhrman et al., 1989; Simon et al., 1992). Bacterial contribution to planktonic heterotrophic activity is known to be very large (Azam and Hodson, 1977), and bacterial production has been estimated to be in the order of 30% of primary production (Cole et al., 1988; Ducklow and Carlson, 1992). Altogether, bacterial activity can have a large impact on ecosystem metabolism either in the ecosystem balance between production and respiration (del Giorgio et al., 1997a), the turnover of organic car-
bon and the global carbon cycle (Cho and Azam, 1988), or the back- and forward transformations between POC and DOC (Azam, 1998).

Bacterial abundance and biomass are thus key parameters in aquatic ecosystems, and constitute the most essential measurements made in virtually all studies of planktonic systems. Until recently, most determinations of bacterial biomass were done via a two-step process in which abundance is first determined, usually by epifluorescence microscopy of DAPI- or Acridine Orange-stained samples (e.g. Kepner and Pratt, 1994) and later bacterial biomass is derived from measurements of cell size, usually with image-analysis (e.g. Blackburn et al., 1998). The whole procedure may easily take many person-hours per sample analyzed, thus making real-time analysis of microbial abundance impossible at a rate commensurate with the acquisition of physical measurements of the water masses (Yentsch et al., 1983). The traditional microscopic techniques do not lend themselves to large-scale studies, such as oceanographic cruises, which easily generate thousands of samples for which we would like to have bacterial abundance and biomass estimates.

Microbial ecologists also seek to measure properties other than density and size of bacterioplankton, such as the composition and relative activity of individual cells and their phylogenetic affiliation. Incubations with radioactive tracers (i.e. Kirchman, 1993) or microscopic assessment using a variety of cellular probes (Sherr et al., 1999) are the means employed to obtain estimates of relative activity. These procedures are even more time-consuming and force a sampling frequency further away from the ideal. Thus, all of the methods that offer information on the heterogeneity of the bacterial community (i.e. different sizes, different cell-specific activities, etc.) are very time-consuming, lack resolution and often lack precision because a relatively modest number of cells can be examined. There is now a new generation of methods, including flow cytometry, that are significantly reducing the time employed in each of these determinations, increasing the level of resolution and in addition, providing new insights into the structure and functioning of plankton communities that simply can not be obtained with conventional epifluorescence microscopy.

The suite of techniques based on the analysis of microscope images has been labeled Image Cytometry in opposition to Flow Cytometry (FC). FC allows the examination of a large number of cells at a time, recording for each cell several different parameters that can later be linked to a wide variety of cellular characteristics (Shapiro, 1995). In a flow cytometer, typically on the order of 200 to over 2000 cells per second circulate through the beam of a laser or an arc-lamp, and the electronic circuitry captures the light scattered by each of the particles and the fluorescence emission at different wavelengths generated by the excitation of each particle. This multivariate information is then processed and combined as desired by a computer. Because tens of thousands of cells can be analyzed in a few minutes, and as long as the system is able to operate with particles in the bacterial size range, FC can really reduce the time needed for the determination of bacterial abundance, size and activity, offering simultaneous information on the structure (heterogeneity) of the bacterial assemblage with a large statistical significance. While the cytometer is based on the measure of scattered light, it is the additional capability of resolving natural or induced particle-associated fluorescence that makes the technique particularly useful. Fluorescent DNA stains, activity probes, nucleic acid probes and immunofluorescence probes extend the capabilities of the technique making it able to discriminate cells on the basis of amount and type of nucleic acids, amount of respiratory enzymes, or many other characteristics. In this respect, a FC is conceptually an image cytometry system that can operate at great speed and that can be almost fully automated (e.g. Jacquet et al., 1998a).

The application of flow cytometry to aquatic microbial ecology has been slow relative to other fields. The basic elements of flow cytometry were developed over three decades ago (Shapiro, 1995), and a variety of commercial and in house instruments have been available since then and used in many clinical and research applications. But until recently, flow cytometers remained very expensive and out of the reach of most ecological laboratories, and until a few years ago they needed the work of a dedicated technician which made operating costs even higher. Furthermore, commercial flow cytometers were designed for the analysis of cells that are larger than bacteria (namely blood cells). Extension of their operation to microbiological research was possible but not without difficulties. This was particularly true for natural aquatic bacteria, which are extremely small, have relatively low amounts of cell constituents, and external cell structures that might hamper or obstruct the access of fluorochromes,
antibodies or nucleic acid probes to the cells. Thus, the statement by Jernae and Steen (1994): “Flow cytometry of bacteria is still in its infancy”. There has been much progress since this statement was made, to the point that we can now state that the enumeration and analysis of natural planktonic bacteria by flow cytometry is routine in many laboratories and is becoming an essential technique in aquatic microbial ecology studies. The current explosion in the use of FC in ecological studies has been in part fueled by the availability of new nuclear acid stains together with powerful, sensitive and relatively cheap benchtop flow cytometers. Few areas of research or few techniques have had such an amount of review papers and books in relatively few years: Darzynkiewicz and Crissman (1990), Ormerod (1994), Lloyd (1993), Fouchet et al. (1993), Troussellier et al. (1993), Methods in Cell Biology (1994), Shapiro (1995), Davey and Kell (1996), Porter et al. (1997), Davey et al. (1999), Collier and Campbell (1999), etc. However, and with the exception of the Troussellier et al. (1993) paper, and small sections in the complete reviews of Davey and Kell (1996) and Collier and Campbell (1999), little has been published on the application of flow cytometry to natural planktonic bacteria, an area that has flourished after the papers of Li et al. (1995), del Giorgio et al. (1996) and Marie et al. (1997) that independently realized the potential of the blue-light excitable stains marketed by Molecular Probes.

This paper will not review extensively the methods associated with flow cytometry of bacteria nor will it focus on the work that has been done to date with bacterial cultures in the laboratory. Instead, we intend to provide an introduction to researchers interested in the routine estimation of bacterial abundance, biomass and activity in natural planktonic ecosystems. Some revision of the work done with bacterial cultures is unavoidable as most of the work carried out in ecosystems starts with work at the culture level, but we will try to focus on the applications to natural planktonic bacteria.

BACTERIA AND FLOW CYTOMETRY

Bacteria are small, and planktonic bacteria are usually much smaller than their laboratory relatives. This has been the main limitation preventing the development of applications using flow cytometry to their study. As cultured bacteria (> 2 µm³ cell⁻¹) are many times larger than planktonic or soil bacteria (typically <0.2 µm³ cell⁻¹, and often < 0.06 µm³ cell⁻¹), analyses involving bacterial cultures were possible well before any attempts of studying natural populations of bacteria. As early as 1977 flow cytometry was used to study bacterial cultures (Bailey et al., 1977, Paau et al., 1977). Somehow surprisingly, what researchers were worried about 20 years ago was quite similar to the problems we are interested with natural bacteria today: determination of DNA (Paau et al., 1977) and protein (Hutter and Eipel, 1978), differentiation of “live” and “dead” cells (Hutter and Eipel, 1978) and separation of microorganisms on the basis of DNA content and the presence of chlorophyll (Paau et al., 1979). The first studies applying flow cytometry to bacteria dealt with the description of the macromolecular composition of bacterial cells during the growth cycle: changes in DNA were found to correlate with the fluorescence of the probes used (see below), changes in protein content with changes in bacterial size (Allman et al., 1990) and changes in the scatter of light by the cells have been found to reflect changes in bacterial size (Allman et al., 1990) or the accumulation of reserve polymers such as poly-β-hydroxybutirate (e.g. Sienec et al., 1984).

The ability to characterize the macromolecular changes during bacterial growth has been applied to monitoring the effects of antibiotics on bacteria (e.g. Crissman et al., 1978; Steen et al., 1982, 1986) and other stress-producing substances (e.g. Comas and Vives-Rego, 1997), and characterizing the starvation-survival response of selected bacterial species, usually those with pathogenic relevance (Thorsen et al., 1992; Lebaron and Joux, 1994). The development of fluorescent probes that indicate various aspects of cell metabolism has further stimulated this area of research (McFeters et al., 1995; Porter et al., 1996; Davey et al., 1999).

Most of the early FC work was done with arc-lamp cytofluorometers, and even today these are more sensitive for the analysis of small particles than the commercial flow cytometers (e.g. Bernard et al., 1998). The arc-lamp cytometer is an instrument in which a flat, laminar stream of water, containing the stained cells in a narrow central sector, is formed on a microscope cover slip by a pressurized jet of water directed onto the glass at a low angle. The stream of cells is viewed by means of a fluorescence microscope with incident illumination and one or several photomultipliers (Steen and Lindmo, 1979). These instruments were commercialized under different brand names (Skatron...
light scatter to detect cells. Researchers have turned to fluorescently labeled specific antibodies (Tyndall et al., 1985) or genetic probes (Amann et al., 1990).

Most of the early published studies focused on the detection of a defined bacterial species either in culture, or in the environment (released, surviving, or in full growth). It was not until the early 90’s that researchers started to use FC to examine and enumerate all bacterial cells in mixed natural assemblages (e.g. Pinder et al., 1990).

**Bacterial detection by light scattering**

Detection of natural planktonic bacteria can not usually be accomplished only on the basis of their light scattering properties, in part because bacterial sizes are in the range of the wavelengths used to “see” them. In addition, natural water samples usually have large numbers of detrital submicrometric particles and colloids that scatter light similarly to live bacteria. Light scattering is used in conjunction with fluorescence to discriminate bacteria, and in itself may provide important information about the cells. The amount of bacterial scattered light is a complex function of cell size, internal structure, particle orientation, refractive indexes of the particle and of the medium, etc. Cellular inclusions like PHB (Srienc et al., 1984), sulfur (E.O. Casamayor and J.M. Gasol, unpubl. obs.), proteins (Wittrup et al., 1988), magnetosomes (Wallner et al., 1997) and cyanobacterial vacuoles (Dubelaar et al., 1987) change the amount of scattered light without associated changes in cell size. To make things more difficult, fixation and dye annexation can also change the scatter properties of the cells (see below). Some authors have relied on scattered light to discriminate among bacterial isolates (e.g. Allman et al., 1993) but most often this has been achieved in combination with some type of nucleic acid staining (Allman et al., 1992). Photosynthetic bacteria of sizes < 1 µm (0.2 – 0.6 µm³) are easily discriminated on the basis of light scattered and chlorophyll content (Chisholm et al., 1988; Cristina X.P., pers. com.) producing usually a quite sharp peak in the light scatter channels despite their small size.

As a general rule, small angle (2-15°) light scattering (FSC or FALS) has been found to be related to cell mass or cell volume while wide angle (15-90°) light scattering (SSC or WALS) is related to the refractive index of the cellular content (e.g. Wittrup et al., 1988). Forward scatter might work well at differentiating organisms larger than bacteria (algae and heterotrophic protists). However, differences exist between machine types (i.e. the arc-lamp based machines are better than most laser flow cytometers, Bernard et al., 1998) and even between the newest machines (i.e. Coulter XL and FACSCalibur, Jiménez-Gómez F., pers. com.).

**Bacterial detection after staining**

Because light scattering does not in itself allow discrimination of bacterial cells from other particles, it is necessary to use fluorescence in addition to light scatter to detect cells. Researchers have turned to fluorescent products that are excitable by blue and UV radiation of the argon lasers and mercury lamps and make bacteria fluorescent with an intensity that can be recorded with the aid of photomultipliers (Haugland, 1999). With a strong enough binding of stain to the cellular product, and large molar extinction coefficients, these stains have fostered the expansion of flow cytometry of bacteria.

The ideal fluorochrome to stain bacteria for FC detection should have an excitation maximum in the regions of the spectrum that match the emission of
available lasers and lamps, should have a high quantum yield (the amount of photons produced per photon absorbed), should be specific for the target compound, its binding characteristics and affinities should be well known, should be soluble in water, should easily penetrate cells, and should not be toxic if cell sorting is desired. However, no single fluorochrome that is currently available fulfills all these requisites. The choice of stains is constrained because most flow cytometers either have argon lasers which emit in the blue (488 nm, usually at low powers, i.e. 15 mW), or UV lasers (usually at high powers, i.e. 5 W), although red excitation is also a possibility in some instruments. Most of the fluorochromes available are membrane impermeant, which requires a step of fixation and/or permeabilization (see below), and although the mechanism of staining of some of the stains in vitro is known, experiments with bacteria, specially those living in the plankton, suggest that the behavior of the stains is quite different in vivo (e.g. Guindulain et al., 1997; Lebaron et al., 1998).

The stains that have most frequently been used to view bacteria by flow cytometry are listed in Table 1. They are mostly specific for nucleic acids, with a few noteworthy exceptions: FITC and SYPRO (see spelling of the abbreviations in Table 1) stain proteins. FITC was used as a protein stain since the beginning of flow cytometry of bacteria (Bailey et al., 1977), but its use is problematic in plankton samples, where there may be many protein-rich particles in the bacterial size range (Long and Azam, 1996). BVC-kanamycin stains all cellular surfaces, and has the interesting property of emitting in the far red, which allows combination to green dyes (Depierreux et al., 1990).

Those stains that bind to DNA and RNA either intercalate into the double-stranded helical structure (EthBr, PI), with a considerable increase in fluorescence emission over that of the free dye, or are nonintercalating and fit specific regions of the DNA (like DAPI and HOECHST). Depending on their molecular size and ionic characteristics, some of these stains are cell-permeant and can

Table 1. – Some characteristics of the stains that have been employed to detect bacteria with flow cytometry (dyce characteristics adapted from Haugland 1996 and Dovey and Kell 1996).

<table>
<thead>
<tr>
<th>Stain</th>
<th>Binds to:</th>
<th>Exc. / Em. (nm)</th>
<th>Type of sample</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium bromide (EthBr)</td>
<td>DNA and RNA</td>
<td>518 / 605</td>
<td>cultures</td>
<td>Paau et al., 1977, Pinder et al., 1990</td>
</tr>
<tr>
<td>Propidium iodide (PI)</td>
<td>DNA and RNA</td>
<td>535 / 617</td>
<td>cultures</td>
<td>Bailey et al., 1977, Hutter and Eipel, 1979, Miller and Quarles, 1990</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate (FITC)</td>
<td>protein</td>
<td>495 / 520</td>
<td>cultures</td>
<td>Bailey et al., 1977, Miller and Quarles, 1990, Allman et al., 1990</td>
</tr>
<tr>
<td>Chromomycyn A3</td>
<td>DNA (GC)</td>
<td>340 / 470</td>
<td>cultures</td>
<td>Boye et al., 1983, van Dilla et al. 1983</td>
</tr>
<tr>
<td>Acridine orange (AO)</td>
<td>RNA*</td>
<td>460 / 650*</td>
<td>cultures</td>
<td>Nishimura et al., 1995</td>
</tr>
<tr>
<td>DAPI</td>
<td>DNA</td>
<td>358 / 461</td>
<td>cultures</td>
<td>van Dilla et al. 1983</td>
</tr>
<tr>
<td>HOECHST 33342</td>
<td>DNA (AT)</td>
<td>350 / 461</td>
<td>cultures</td>
<td>van Dilla et al. 1983</td>
</tr>
<tr>
<td>Benzoazinone-kanamycin (BVC kanamycin)</td>
<td>cell surfaces</td>
<td>495 / 616</td>
<td>cultures</td>
<td>Robertson and Button 1989</td>
</tr>
<tr>
<td>TO-PRO-1</td>
<td>DNA and RNA**</td>
<td>515 / 531</td>
<td>marine</td>
<td>Li et al., 1995</td>
</tr>
<tr>
<td>TOTO-1</td>
<td>DNA and RNA**</td>
<td>514 / 533</td>
<td>marine</td>
<td>Li et al., 1995, Zubkov et al., 1998</td>
</tr>
<tr>
<td>SYTO-13</td>
<td>DNA and RNA**</td>
<td>488 / 514</td>
<td>freshwater</td>
<td>del Giorgio et al., 1996</td>
</tr>
<tr>
<td>TOYO-1</td>
<td>DNA and RNA</td>
<td>491 / 509</td>
<td>cultures</td>
<td>Marie et al., 1996</td>
</tr>
<tr>
<td>YO-PRO-1</td>
<td>DNA and RNA</td>
<td>491 / 509</td>
<td>cultures</td>
<td>Marie et al., 1996</td>
</tr>
<tr>
<td>PicoGreen</td>
<td>dsDNA</td>
<td>480 / 520</td>
<td>cultures</td>
<td>Marie et al., 1996, Veldhuis et al., 1997</td>
</tr>
<tr>
<td>SYBRGreen I</td>
<td>DNA and RNA</td>
<td>494 / 521</td>
<td>marine</td>
<td>Sieracki et al., 1999</td>
</tr>
<tr>
<td>SYTOX</td>
<td>dsDNA</td>
<td>504 / 523</td>
<td>cultures</td>
<td>Veldhuis et al., 1997</td>
</tr>
<tr>
<td>SYTO-9, 11, BC</td>
<td>DNA (and RNA)</td>
<td>480-510 / 500-520</td>
<td>freshwater and marine</td>
<td>Veldhuis et al., 1997</td>
</tr>
<tr>
<td>SYBRGreen II</td>
<td>RNA (and DNA)</td>
<td>492 / 521</td>
<td>freshwater and marine</td>
<td>Lebaron et al., 1998</td>
</tr>
<tr>
<td>SYTO-17</td>
<td>DNA and RNA</td>
<td>633 / 675</td>
<td>cultures</td>
<td>Comas and Vives-Rego, 1997</td>
</tr>
<tr>
<td>SYTO-16</td>
<td>DNA and RNA</td>
<td>488 / 518</td>
<td>cultures</td>
<td>Ibrahim et al., 1997</td>
</tr>
<tr>
<td>SYPRO</td>
<td>Protein</td>
<td>550 / 630</td>
<td>cultures</td>
<td>Zubkov et al., 1999</td>
</tr>
</tbody>
</table>

** AO also stains DNA with excitation / emission maxima at 500 and 526 nm

** Only DNA in plankton samples (see Li et al., 1995 and Guindulain et al., 1997)

COUNTING PLANKTONIC BACTERIA WITH FLOW CITOMETRY 201
easily cross bacterial membranes, or are essentially impermeant to live or intact membranes. In some cases, the stain can cross the cellular membrane but is then actively pumped out from the live cell. DAPI, one of the most commonly used stains for bacteria enumeration on filters by epifluorescence (Kepner and Pratt, 1994), tends to bind nonspecifically to cell membranes (Coleman et al., 1981; Zweifel and Hagström, 1995) and it is likely that most other nucleic acid stains do so to some degree. Most of the stains have selective binding to DNA and to RNA, with different fluorescence yields, at least \textit{in vitro}. However, even those that are marketed as preferentially staining RNA tend to also stain DNA (see the comparison of Sybr-Green I and II in Lebaron et al., 1998). Acridine orange is an intercalating dye that emits orange/red fluorescence when interacting with single-stranded nucleic acids, and fluoresces green when interacting with double-stranded nucleic acids. Even though this characteristic has been used to measure RNA in bacterial samples (Nishimura et al., 1995), the stain is very sensitive to the conditions of use, and tends to bind nonspecifically (Petit et al., 1993). Some of the DNA stains have shown differential binding to AT (DAPI, HOECHST) or to GC (chromomycin, mithramycin and olivomycin) base pairs, and combination of both types of dyes has been used to measure the %GC content of bacterial cultures and as an additional characteristic for classification (van Dilla et al., 1983). The company Molecular Probes started a few years ago to market families of nucleic-acid cyanide dyes with a range of excitation maxima, including many that could be excited with the blue light of the Argon laser that most commercial flow cytometers have. They marketed the TOTO and the TO-PRO series, which are cell-impermeant; the SYTO series, with lower affinity for nucleic acids, but cell-permeant and useful for work with live cells; the SybrGreen series, with very high affinities for RNA and DNA; the SYTOX green stain which is also cell impermeant and is marketed as an exclusion stain; and others such as the PicoGreen, also with high affinity for DNA. A whole variety of these products, with different yields and different excitation and emission wavelengths is available (Haugland, 1999) and some of them have successfully been used to stain bacteria (Table 1). In fact, Molecular Probes is marketing a SYTO Bacterial counting kit to be used with flow cytometry.

Detection of planktonic bacteria

Tyndall \textit{et al.} (1985) were probably the first authors to detect the presence of indigenous bacteria using FC, although they were probing for the presence of \textit{Legionella} with FITC-labeled antibodies and propidium iodide. Robertson and Button (1989) reported the use of a more sensitive cytometer in combination with DAPI staining as a means of observing seawater and freshwater planktonic bacteria. They were among the first to report that these natural bacteria were equally stained when treated with RNase (indicating that the stain was attached mostly to DNA), that two subpopulations were at times visible, and that they could differentiate cells according to their chlorophyll content, as the red fluorescence of the chlorophyll appeared in a very different channel than the DAPI blue fluorescence. Similar work, with similar flow cytometers was produced by Monfort and Baleux (1992), Troussellier \textit{et al.} (1993) and Heldal \textit{et al.} (1994), with slight modifications of the Robertson and Button protocol.

Monger and Landry (1993) introduced HOECHST 33342 as a DNA stain because this was superior to DAPI in terms of lower background fluorescence, smaller coefficients of variation of blue fluorescence and better accuracy of abundance estimates. The relative fluorescence quantum yield (relative to unbound dye in solution) is 30% higher for HOECHST than for DAPI and staining was done in less time. Monger and Landry also noticed that UV excitation was not effective to discriminate \textit{Prochlorococcus} in oligotrophic oceanic waters where these organisms make an important contribution to picoplankton abundance, and they suggested the use of double excitation (UV excitation of HOECHST-stained DNA and blue excitation of the chlorophyll). These authors again noted the presence of different planktonic bacterial subpopulations based on light scattering and fluorescence characteristics. Work with this setup allowed Campbell \textit{et al.} (1994) to estimate the (large) contribution of prochlorophytes to planktonic community structure in oligotrophic oceans.

Until 1995, however, most DNA stains available required UV excitation and thus high-powered lasers, with sophisticated alignment systems and large refrigeration units, prone to technical problems. These cytometers were expensive, required maintenance, and the dedicated work of a technician. Li \textit{et al.} (1995) and del Giorgio \textit{et al.} (1996) introduced blue-excitable stains for enumerating plankton bacteria. Li \textit{et al.}
### Table 2. Some technical details offered in most of the papers to date where natural planktonic bacteria have been enumerated by FC.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Instrument(s)</th>
<th>Laser (power)</th>
<th>Flow rate (µl min⁻¹)</th>
<th>Sheath fluid</th>
<th>Fixative</th>
<th>Stain</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robertson and Button 1989</td>
<td>Ortho Cytofluorograph IIS</td>
<td>UV 5W</td>
<td>5 dH₂O</td>
<td>75%</td>
<td>DAPI (2.5 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Button and Robertson 1989</td>
<td>Ortho Cytofluorograph IIS</td>
<td>UV 5W</td>
<td>5 dH₂O</td>
<td>75%</td>
<td>DAPI (2.5 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Monfort and Baleux 1992</td>
<td>ACR-1400 SP Bruker*</td>
<td>UV 100W</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Button and Robertson 1993</td>
<td>Ortho Cytofluorograph IIS</td>
<td>UV 5W</td>
<td>5 dH₂O</td>
<td>0.5%</td>
<td>DAPI (2.5 µg ml⁻¹)</td>
<td>Triton X-100 (0.1%)</td>
<td>-</td>
</tr>
<tr>
<td>Troussellier et al. 1993</td>
<td>ACR-1400 SP Bruker*</td>
<td>UV 200 mW</td>
<td>25-40</td>
<td>-</td>
<td>PFA 1%</td>
<td>DAPI (0.5 µg ml⁻¹)</td>
<td>-</td>
</tr>
<tr>
<td>Monger and Landry 1993</td>
<td>Coulter EPICS 753</td>
<td>UV 200 mW</td>
<td>-</td>
<td>-</td>
<td>PFA 0.2%</td>
<td>HOECHST 33342 (0.5 µg ml⁻¹)</td>
<td>-</td>
</tr>
<tr>
<td>Heldal et al. 1994</td>
<td>Argus 100-4</td>
<td>UV 20</td>
<td>-</td>
<td>-</td>
<td>Glut 2%</td>
<td>DAPI (20 µg ml⁻¹)</td>
<td>-</td>
</tr>
<tr>
<td>Campbell et al. 1994</td>
<td>Coulter EPICS 753</td>
<td>UV 200 mW</td>
<td>-</td>
<td>PFA 0.2%</td>
<td>HOECHST 33342 (0.5 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Troussellier et al. 1995</td>
<td>FACSort</td>
<td>Blue 12</td>
<td>-</td>
<td>-</td>
<td>DAPI (2.5 µg ml⁻¹)</td>
<td>-</td>
<td>Triton X-100 (0.1%)</td>
</tr>
<tr>
<td>Li et al. 1995</td>
<td>FACSort</td>
<td>Blue 15 mW</td>
<td>12 Hematall</td>
<td>-</td>
<td>Glut 1%</td>
<td>SYTO 13 (2.5 µM)</td>
<td>RNAse, 30 mM potassium citrate</td>
</tr>
<tr>
<td>del Giorgio et al. 1996</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>25</td>
<td>-</td>
<td>Glut 1%</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
</tr>
<tr>
<td>Binder et al. 1996</td>
<td>Coulter EPICS 753</td>
<td>UV 200 mW</td>
<td>-</td>
<td>-</td>
<td>PFA 0.2%</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
</tr>
<tr>
<td>Landry et al. 1996</td>
<td>Coulter EPICS 753</td>
<td>UV 200 mW</td>
<td>-</td>
<td>-</td>
<td>PFA 0.2%</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
</tr>
<tr>
<td>Buck et al. 1996</td>
<td>Coulter EPICS 753</td>
<td>UV 200 mW</td>
<td>-</td>
<td>PFA 0.2%</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pile et al. 1996</td>
<td>Coulter EPICS 753</td>
<td>UV 225 mW</td>
<td>-</td>
<td>PFA 0.2%</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Campbell et al. 1997</td>
<td>Coulter EPICS 753</td>
<td>UV 200 mW</td>
<td>-</td>
<td>-</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>del Giorgio et al. 1997b</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>12 Hematall</td>
<td>-</td>
<td>SybrGreen I (10⁻⁴)</td>
<td>-</td>
<td>sodium or potassium citrate</td>
</tr>
<tr>
<td>Marie et al. 1997</td>
<td>Coulter EPICS 753</td>
<td>UV 225 mW</td>
<td>-</td>
<td>-</td>
<td>SybrGreen I (10⁻⁴)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wallner et al. 1997</td>
<td>Coulter EPICS Elite</td>
<td>Blue 15 mW</td>
<td>-</td>
<td>-</td>
<td>PFA 3%</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
</tr>
<tr>
<td>Guindulain et al. 1997</td>
<td>Coulter EPICS 753</td>
<td>UV 225 mW</td>
<td>-</td>
<td>-</td>
<td>PFA 2%</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
</tr>
<tr>
<td>Pile 1997</td>
<td>Coulter EPICS 753</td>
<td>UV 225 mW</td>
<td>-</td>
<td>-</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zubkov et al. 1998</td>
<td>FACSort</td>
<td>Blue 15 mW</td>
<td>45</td>
<td>-</td>
<td>Glut 0.2%</td>
<td>HOECHST 33342 (0.5 µg ml⁻¹)</td>
<td>-</td>
</tr>
<tr>
<td>Peters et al. 1998</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>19 dH₂O</td>
<td>PFA 1% + Glut 0.05%</td>
<td>SYTO 13 (2.5-5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Campbell et al. 1998</td>
<td>Coulter EPICS 753</td>
<td>UV 200 mW</td>
<td>-</td>
<td>PFA 0.2%</td>
<td>SYTO 13 (0.125 µM)</td>
<td>-</td>
<td>Triton X-100 (0.1%)</td>
</tr>
<tr>
<td>López-Amorós et al. 1998</td>
<td>Coulter XL</td>
<td>Blue 15 mW</td>
<td>-</td>
<td>-</td>
<td>SybrGreen I (10⁻⁴)</td>
<td>30 mM potassium citrate</td>
<td>(several)</td>
</tr>
<tr>
<td>Jacquet et al. 1998b</td>
<td>FACSort</td>
<td>Blue 15 mW</td>
<td>-</td>
<td>Filt Seawater</td>
<td>PFA 1% + Glut 0.05%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lebaron et al. 1998</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>-</td>
<td>-</td>
<td>Formalin 2%</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
</tr>
<tr>
<td>Ribes et al. 1998a, 1998b</td>
<td>FACSort</td>
<td>Blue 15 mW</td>
<td>50</td>
<td>-</td>
<td>PFA + G / Glut 0.1-0.5%</td>
<td>SybrGreen I (10⁻⁴)</td>
<td>(several)</td>
</tr>
<tr>
<td>Marie et al. 1999</td>
<td>FACSort</td>
<td>Blue 15 mW</td>
<td>19 dH₂O</td>
<td>PFA 1% + Glut 0.05%</td>
<td>SYTO 13 (2.5-5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gasol and Morán 1999</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>19 dH₂O</td>
<td>PFA 1% + Glut 0.05%</td>
<td>SYTO 13 (2.5-5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gasol et al. 1999</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>19 dH₂O</td>
<td>PFA 1% + Glut 0.05%</td>
<td>SYTO 13 (2.5-5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sherr et al. 1999</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>19 dH₂O</td>
<td>PFA 1% + Glut 0.05%</td>
<td>SYTO 13 (2.5-5 µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sienicki et al. 1999</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>50</td>
<td>-</td>
<td>PFA 1%</td>
<td>PicoGreen (10⁻⁴)</td>
<td>-</td>
</tr>
<tr>
<td>Vives-Rego et al. 1999</td>
<td>Coulter XL</td>
<td>Blue 15 mW</td>
<td>-</td>
<td>-</td>
<td>none</td>
<td>SYTO 13 (2.5 µM)</td>
<td>-</td>
</tr>
<tr>
<td>Mostajir et al. 1999</td>
<td>ACR 1400 SP Bruker*</td>
<td>UV 100W</td>
<td>-</td>
<td>-</td>
<td>DAPI (2.5 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chatila et al. 1999</td>
<td>ACR 1400 SP Bruker*</td>
<td>UV 100W</td>
<td>-</td>
<td>Formalin 3.7%</td>
<td>DAPI (2.5 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Karl et al. 1999</td>
<td>Coulter EPICS 753</td>
<td>UV 200 mW</td>
<td>-</td>
<td>PFA 1%</td>
<td>HOECHST 33342 (1 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gin et al. 1999</td>
<td>Coulter EPICS 753</td>
<td>UV 200 mW</td>
<td>-</td>
<td>Glut 0.1%</td>
<td>HOECHST 33342 (0.5 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Troussellier et al. 1999</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>-</td>
<td>-</td>
<td>SYTO 13 (5 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Servais et al. 1999</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>-</td>
<td>-</td>
<td>SYTO 13 (5 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lebaron et al. 1999</td>
<td>FACScan</td>
<td>Blue 15 mW</td>
<td>-</td>
<td>-</td>
<td>SYTO 13 (5 µg ml⁻¹)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Arc-Lamp machines

The Argus-100 (Skatron), the BioRad Bryte and the Bruker ACR 1400-Sp are essentially the same machines.

The FACScan and FACSort evolved to the FACScan, while the FACStar evolved to the FACSVantage
(1995) showed that TOTO-1 and TO-PRO-1 stained preferentially DNA, that they could differentiate two or three bacterial subpopulations, and that these subpopulations appeared to have ecological meaning. del Giorgio et al. (1996) and Guindulain et al. (1997) introduced the use of SYTO-13, and Marie et al. (1997) compared another blue-green stain, SybrGreen favorably to HOECHST 33342 for counting planktonic bacteria. These authors also noticed that the phototrophic prokaryotes Prochlorococcus and Synechococcus could be discriminated from chemotrophic bacteria in a plot of red vs. green fluorescence, except where the phototrophs’ autofluorescence was very weak, as in the surface well-stratified oceanic waters, Olson et al., 1990). SybrGreen I has also been successfully used to enumerate planktonic viruses (Marie et al., 1999) and flagellates (authors’ unpublished results). Sieracki et al. (1999), Veldhuis (pers. com.) and Gregori and Denis (pers. com.) are more comfortable using PicoGreen for staining bacteria in marine samples. This stain has been used to estimate the amount of DNA in planktonic phototrophs and also

Fig. 1. – Flow cytometric analysis of a surface open Atlantic ocean sample after staining with SYTO 13. In the upper panels, 10000 events are displayed after acquisition in log mode. In the left side of the graph, a representation of 90° light scatter (SSC or WALS) vs. green fluorescence (FL1 in our instrument). In the right side, a representation of green fluorescence vs. red fluorescence (FL3 in our instrument). In this specific acquisition, no electronic compensation was applied to the fluorescences, and slightly more voltage was applied to the FL3 photomultipliers than to the FL1 photomultipliers. Central panels: A log-density plot of the same data presented in the upper panels. One step of data smoothing was applied, and a threshold of 1% eliminated isolated data. Denser gray levels identify logarithmically increasing quantities of events. Interpretation of the different subpopulations that appear is as follow: B: Yellow-green 1 µm Polysciences latex beads. N: electronic noise. HDNA: Bacteria with High DNA content (also known as Li et al.’s Group II bacteria, and Marie et al.’s B-III bacteria). LDNA: Bacteria with Low DNA content (also known as Li et al.’s Group I bacteria, and Marie et al.’s B-I bacteria). Syn: Synechococcus. Proc: Prochlorococcus. B-II: Group II bacteria according to Marie et al. (1997). Lower panels: a three-dimensional representation of the same data after 1 smoothing step and removal of 1% of data. Same symbols identify the same populations.
stains bacteria well (Veldhuis et al., 1997). Lebaron et al. (1998) compared most of the above-cited stains and others from the Molecular Probes catalog to find out that most were similarly adequate for staining freshwater and marine planktonic bacteria, although SYTO 9 appeared to perform best in terms of mean cell fluorescence. At this time it seems that the most widely used stains are SYTO 13, SybrGreen I, and PicoGreen, but the reasons for the choice are mainly of personal habit. Table 2 summarizes the different protocols reported in the literature used to determine the abundance of planktonic bacteria with FC. Note the exponentially increasing number of papers using the technique, and the general switch from UV to blue light lasers after 1995.

Figure 1 presents an example of a Mediterranean surface water sample as it appears when stained with SYTO 13. The central and lower panels of this figure help understanding of the raw data presented in the upper panels. The threshold to trigger an event is set in green fluorescence, and that determines the vertical line below which no events appear. The plots of green fluorescence vs. side scatter, orange vs. green fluorescence and red vs. green fluorescence are helpful in discriminating subpopulations. In this example there are at least five subpopulations of bacteria that can be differentiated on the basis of autofluorescence, SYTO staining and light scatter: two of phototrophic and three of chemotrophic prokaryotes. In the following sections we discuss the meaning of the different subpopulations.

Button and Robertson (1993) have emphasized some of the advantages of analyzing bacteria with flow cytometry: large sample size that allows robust statistics; speed, accuracy and reproducibility; minimal interference by noise; resolution of specific subpopulations on the basis of size and DNA contents, estimation of genome size and sorting capabilities. To these, some others should be added: the procedure is fast (~1 minute per sample, 100 samples can be processed -data analysis included- in a morning’s work), allows counting in very small volumes (down to 1 µl, Troussellier et al., 1993), allows physiological probing simultaneously to enumeration (e.g. López-Amorós et al. 1998), and sample processing can be automated (e.g. Jacquet et al., 1998a). We have further determined that counting bacteria by FC in our laboratories saves ~50% of the cost of epifluorescence analysis (including consumables and personnel, but not including machine purchase and maintenance). The unwanted consequence is that, being easier and faster, we are now taking more samples for bacterial counts!

COUNTING BACTERIOPLANKTON, IN PRACTICE

In this section we will explore practical aspects of the flow cytometric enumeration of bacteria, focusing on three key steps of the protocol: Cell fixation, cell staining and data processing and interpretation. In Table 2 we have summarized the different protocols currently used by researchers, to highlight the diversity of approaches that have been taken.

Fixation

Fixation of samples is needed whenever the samples cannot be processed fresh immediately after sampling. But fixation may in addition be required to permeabilize cells and thus facilitate the penetration of certain stains into the cell (Bullock, 1984). The ideal fixation protocol should be fast, should effectively preserve nucleic acids, and protect autofluorescence without altering the size and the light scatter properties of the cells. Fixatives currently used include ethanol (70%), formaldehyde, diluted as formalin or methanol-free as paraformaldehyde (PFA), glutaraldehyde (Glut) and even TCA (Rice et al., 1997) and cold shock followed by metabolic inhibition (to block stain efflux pumps, Wallberg et al., 1998). Paraformaldehyde (the solid form of formaldehyde, as opposite to the commonly used hydrolysed form, which is 40% formaldehyde and has methanol) quickly penetrates the cells and is assumed to be the most effective fixative of nucleic acids and proteins. Glutaraldehyde penetrates slowly and may not permeate all gram negative bacteria (Bullock, 1984). However, 1% Glut was found to protect microbes from cell lysis and loss of autofluorescence upon rapid freezing in liquid nitrogen and long-term cryogenic storage (Vaulot et al., 1989). 1% PFA has been seen to offer similar protection (Monger and Landry, 1993) and fluorescence protection was even better when the samples were frozen after fixation (Hall, 1991; Zubkov et al., 1999). Campbell et al. (1994), however, did not find any differences between fixation with PFA and with Glut. Glutaraldehyde, unless of very good quality, may produce an autofluorescence signal in FC that can be very annoying (Booth, 1987). And formalin is known to negatively affect cell fluorescence (Crisman et al., 1978; Lebaron et al., 1998; Troussellier et al., 1999).

Some degree of post-fixation cell disappearance
and cell alteration has been reported with most common fixatives. Marie et al. (1993) reported that 0.5% PFA produced a 9% loss of Prochlorophyte cells, and Trouessier et al. (1995) reported a similar value. del Giorgio et al. (1996) found that fixation with formalin and glutaraldehyde decreased the forward scatter and green fluorescence and increased side scatter of fixed cells relative to live cells. They assigned those changes to post-fixation cell shrinkage that seemed to be particularly important in the case of formalin.

We tested some of these fixatives in two marine samples, with or without, freezing in liquid nitrogen (Fig. 2). The protocol labeled PFA+G consists in PFA 1% + 0.05% Glut (Marie et al., 1996). The two samples, one from an oligotrophic site and the other from eutrophic waters, responded differently to some of the treatments. There was some degree of cell loss even in the fixed and frozen samples, but loss was greater for formalin and Glut treatments. Side scatter increased in all treatments, especially if no freezing was involved. A 10% decrease in green fluorescence occurred with the PFA+G fixation while a stronger reduction in fluorescence was produced by formalin and freezing. With the present data, and given that PFA is the fixative of choice for fluorescent in situ hybridization (Wallner et al., 1993) and that the PFA+G protocol has been seen to reduce the variability in DNA analyses of the microbes (Jacquet et al., 1998b), we tend to recommend that protocol for cell fixation of prokaryotes.

**Staining**

The length of time of cells incubation with the fluorochromes to attain optimal staining prior to FC analysis varies with each type of compound. The recommended incubation time for DAPI and HOECHST 33342 is at least 1 h (Robertson and Button, 1989) or more (Campbell et al., 1994; and Monfort and Baleux, 1994, stained for 2 h), although one of the advantages of HOECHST over DAPI was its lower staining time. The newer blue stains require much lower times, usually less than 15 min (del Giorgio et al., 1996; Marie et al., 1996; Veldhuis et al., 1997). The behavior of some of these stains is quite interesting. For example, Li et al. (1995) used TO-PRO 1 to stain and count all bacteria, but because this fluorochrome is marketed as cell-impermeant by the manufacturers (Haugland, 1999), these authors used fixed and permeabilized cells which quickly took up the stain. But on fresh samples, TO-PRO 1 stains only a fraction of the cells in the first minute, and this number slowly increases until most of the population has been stained within the next 15-20 min. The mean fluorescence per cell is very high for the cells that have been stained in the first minutes and decreases expo-
nentially afterwards (del Giorgio et al., in press). The interpretation is that only cells with damaged membranes allow the stain to enter the cell and bind to the nucleic acids, while later all cells have their outer membranes stained with less fluorescence.

Staining is sometimes done with the addition of buffers (acting also as cell permeants), such as Triton X-100 (Button and Robertson, 1993; Li et al., 1995), TE buffer (Marie et al., 1996), EDTA or EGTA (Kaprelyants and Kell, 1992; López-Amorós et al., 1995b). The reason would be that some of the dyes are very sensitive to ionic strength (Marie et al., 1997; Veldhuis et al., 1997). Some authors, however, have found these treatments unnecessary and even detrimental because Triton X-100 generates background fluorescence (Monger and Landry, 1993) and reduces cell autofluorescence (Marie et al., 1996). Interestingly, Marie et al. (1999) report the need for Triton pretreatment to stain live samples with SYTO 13, but not to stain fixed samples, while Comas and Vives-Rego (1997) found no need for pretreatment to stain bacteria with SYTO 13. Finally, Lebaron et al. (1998) report increased cell-specific fluorescence of the SYTO stains when incubated in the presence of 30 mM potassium citrate. Given that many authors have successfully stained and counted bacteria using SYTO 13 without any pretreatment of the kind discussed here, it is up to each researcher to decide whether he/she has to use it or not. Other fluorochromes, such as TO-PRO 1, that are inherently cell-impermeant, will require some kind of permeabilization pretreatment.

Some authors have suggested that samples should undergo RNase treatment before the addition of the nucleic acid stains, to eliminate the confounding effect of RNA-induced fluorescence. This is mandatory if one is interested in the cell cycle of chemotrophic bacteria. Since these organisms do not seem to divide at once, cell cycle analysis for growth rate determination seems not to be possible (Jacquet et al., 1998b). Furthermore, staining with SYTO 13, TOTO-1, TO-PRO 1 and YOYO 1 of planktonic bacteria seem to be dependent only on the amount of DNA, with little RNA interference (Li et al., 1995; Guindulain et al., 1997). This is possibly not due to the binding affinities of the stains to DNA and RNA, but probably to the low amounts of RNA in planktonic bacteria or to the physical unavailability of rRNA to the dyes.

**Bacterial discrimination**

Stained bacteria are detected and discriminated from other non-bacterial particles with a combination of light scatter, green and orange or red fluorescence. In addition, the combination of these parameters allows better resolution of the different sub-populations within the mixed bacterial assemblage. It also allows easy identification of particles that can interfere with the counts. The instrument threshold defines the minimum scatter or fluorescence intensity needed to trigger an event that will be processed by the system software. The threshold allows the reduction of both electronic noise as well as unwanted, non-target particles, and it is usually set on the same primary parameter used to discriminate bacterial cells (i.e. green, or blue, or red, depending on the stain used, or the cell autofluorescence). But there will inevitably be some “noise” particles that have a fluorescent level above that threshold. In our experience, relatively large particles with weak autofluorescence can be discriminated well in the Side scatter – Green fluorescence plot (Fig. 1, left). Particles with low fluorescence and low side scatter have a greater potential to interfere with the actual determination of the bacterial density, but these can easily be taken apart in the Red vs. Green fluorescence plot (Fig 1, right) where they appear in a diagonal line with relatively more red fluorescence than that of bacteria (as long as no electronic compensation has been applied). The presence of these noise particles can be seen very clearly also in Figure 5.

**Counting**

A few cytometers, such as the Coulter XL and the Ortho Cytoron Absolute, are equipped with devices that exactly control and record the volume of sample that circulates in front of the laser. But most cytometers have no way of exactly controlling the flux of sample, and therefore, the number of particles detected in a cytometric analysis cannot be directly related to a given sample volume to obtain an estimate of particle density. There are at least three ways of obtaining absolute counts in that case: i) a known amount of reference beads can be added (Cantineaux et al., 1993), ii) the flow can be calibrated each day of work, or iii) the samples can be weighed before and after the run. The last alternative is very time consuming, and in addition, it may be less accurate, because there may be some back-
flow of sheath fluid into the tube that confounds the actual sample volume processed. Alternative ii) (daily calibration) gives good results but requires an extremely stable instrument. Calibration of the flow can be done easily by weighing a tube containing water, processing various volumes through the cytometer, estimating the time needed for each volume to go through, and then weighing the tube again. Many researchers, thus, use alternative i) (reference beads), because it is accurate, fast and in addition to allowing absolute counts, it also provides an internal standard that can be used to assess instrument performance and to standardize scatter and fluorescence measurements for quantitative applications. However, the beads have to be counted each day of work, sometimes get contaminated with bacteria, and have to be sonicated to avoid aggregation. In our laboratory, the first two of the methods cited above (reference beads and flow calibration) offer highly similar estimates of bacterial abundance (Fig. 3). The bead stock is dispensed to each sample to a final bead density that is about 1-10% of the expected density of target cells. For a regular bacterioplankton sample with an abundance of \(10^6\) cells ml\(^{-1}\), a final bead density in the sample of \(1\) to \(5\times10^5\) beads ml\(^{-1}\) is appropriate. An accurate measurement of the reference bead density in the stock solution is of key importance and must be done on a routine basis. Larger beads (>2.0 \(\mu\)m) can be counted in a Coulter particle analyzer, but this method is less effective for the smaller beads which are generally used for bacterial work. Alternatively, bead density can be determined using regular epifluorescence microscopy, but this is time consuming and not particularly accurate. A more effective approach is to use a primary reference bead solution where the bead density is precisely known, and to compare this to the working bead solution using the flow cytometer. Primary reference bead solutions are commercially available (i.e. TrueCount, Becton Dickinson).

There are other issues to consider when counting cells, in addition to estimating the volume of sample processed. Bacteria are found in plankton in concentrations varying from \(10^5\) up to \(10^7\) cells ml\(^{-1}\). A reasonable sample rate of 10 \(\mu\)l min\(^{-1}\) (see Table 2), translates into a rate of cell passage through the laser of hundreds to thousands of cells. We often add beads to the sample, and there are other particles which are not bacteria (“noise”) in the same sample, all of which contribute to the events detected by the instrument. The light scattered and emitted by each beam hits the photomultiplier tubes and generates a laser intensity signal that is processed by the cytometer. The data is then processed by the cytometer to generate a count of each particle type detected.
particle must be collected and converted to an electrical current which must then be digitized by the electronic system, and there are limits to how many of these events a system can effectively handle. In addition, when too many particles go through the cytometer, there is a greater probability that two particles will pass together and be considered by the electronic system as a single larger particle. This phenomenon is called coincidence, and tends to become significant at concentration levels above $2.5 \times 10^6$ cells ml$^{-1}$ (Marie et al., 1996) which translate to count rates of 1000 - 1400 events s$^{-1}$ (del Giorgio et al., 1996; Marie et al., 1999; Cristina X.P., pers. com.). Samples with higher concentrations have to be diluted either in filtered water, in buffer (Marie com.). Samples with higher concentrations have to be diluted to mimic varying particle concentrations (authors’ obs.). We have been successful at enumerating bacteria from solar salt ponds where they are growing at particle passage rates of around 2000 s$^{-1}$, which were very similar and broke out at particle passage rates of around 2000 s$^{-1}$ (e.g. Porter et al., 1993). One way of empirically determining the level of coincidence for a given instrument is by means of a bead solution serially diluted to mimic varying particle concentrations (Fig. 4). By increasing the bead concentration and, thus, the rate of particle passage, the amount of doubllets (two particles seen as one) increases exponentially (Fig 4a). By relating then the observed particle concentration to the expected concentration, we can determine when in a given instrument, the concentration and the rate of particle passage is below the 1000 s$^{-1}$.

**PLANKTONIC BACTERIAL HETEROGENEITY**

As emphasized above, one of the main advantages of enumerating planktonic bacteria by flow cytometry is the possibility of further discriminating distinct fractions of bacteria within mixed assemblages, based on their optical properties. In addition to bulk density it is thus possible to explore the heterogeneity of bacterial communities (i.e. Kell et al., 1991; Davey and Kell, 1996), and by measuring “cytometric diversity” (Li, 1997; Troussellier et al., 1999) to finally open the “bacterial black box” that dominated the ecology of planktonic microbes in the past.

**Phototrophs vs. heterotrophs**

The most elemental differentiation among planktonic prokaryotes is that of phototrophs vs. chemotrophic bacteria. Phototrophs have pigments that can be excited by the blue line of the lasers and fluoresce red or orange, distinct from the usual fluorescence emitted by the DNA stains (blue or green, Table 1, see also Veldhuis and Kraay, 2000). *Synechococcus*, with a larger size than most heterotrophic bacteria and pigments which emit orange fluorescence has always been easy to differentiate from other prokaryotes in epifluorescence microscopy, and this is also true for FC. But a major ecological advancement was the discovery of *Prochlorococcus* (Chisholm et al., 1988), which had been confounded as a chemotrophic bacteria in microscopic enumerations (Sieracki et al., 1995) but could be discriminated using FC. This development changed some of the perceptions we had about the role of picoplankton and the microbial food web in the fluxes of carbon and nutrients in the ocean, because of its large contribution to community biomass (Campbell et al., 1994) and primary production (Vaulot et al., 1995). Our understanding of these autotrophic prokaryotes is not yet complete, as little is known about the rates of *in situ* protozoan grazing on *Prochlorococcus* (Reckermann and Veldhuis, 1997), and about the possibility that these organisms could be mixotrophs. Recently, it has been shown that the cells of *Prochlorococcus*, even though divide synchronized to the light:dark periods like most pico- and nanoalgae, can divide several times in a row, with rates exceeding 1 d$^{-1}$ (Shalaponyok et al., 1998).

As discussed above, double laser excitation is required to completely resolve *Prochlorococcus* when staining with DAPI or HEOCHST (Monger and Landry, 1993), but a single argon laser can be used to detect auto- and chemotrophic bacteria because pigment emission is sufficiently distinct from the emission of most blue-excitable stains. In very stable oligotrophic surface waters their fluorescence is so low, that special modifications in the flow cytometers have been devised to detect these cells (i.e. Campbell and Vaulot, 1993). Olson et al.
(1990) suggested the decrease of the laser beam spot and the decrease of the sheath fluid pressure to produce better signals. Other modifications were suggested by Dusenberry and Frankel (1994). When the autofluorescence of the *Prochlorococcus* is stronger, typically in cells sampled from deeper waters, they can easily be discriminated from chemotrophic bacteria in a plot of Red vs. Green fluorescence (Fig. 5).

Note the presence of abundant *Prochlorococcus* and *Synechococcus* populations in an open Atlantic sample (Fig. 5, lower) as compared to an estuarine sample where chemotrophic bacteria were several-fold more abundant than phototrophic bacteria (Fig. 5, upper). When the autofluorescence is weak, as is typical in cells from surface samples, or when in doubt, a double run of the sample, before and after
staining, is necessary (i.e. Marie et al., 1997). Zubkov et al. (1998) suggested another way to discriminate Prochlorophytes, based on the fact that these organisms typically showed a sharp peak in DNA fluorescence (green fluorescence of TOTO) caused by higher DNA content per cell than similarly sized chemotrophic bacteria. In case the prochlorophytes’ autofluorescence is extremely low, and only a portion of the Prochlorococcus population appears above the red fluorescence threshold, the population is assumed to have a normal distribution of red fluorescence, and the hidden portion can be extrapolated (i.e. Partensky et al., 1996; Blanchot and Rodier, 1996).

Other phototrophic bacteria (i.e. Chlorobium, Chromatium, etc.) can also be discriminated from chemotrophic bacteria based on the fluorescence characteristics of the various bacteriochlorophylls of each of these groups (Cristina X.P., pers. com.). Flow cytometric detection of phototrophic picoplankton in freshwater is still in its infancy, and some surprises can be expected (e.g. Corzo et al., 1999).

**DNA content of individual bacteria**

The DNA content of bacteria has been one of the parameters of interest for researchers from the very beginning of the flow cytometric analyses (Bailey et al., 1977; Paau et al., 1977). In fact, the study of the growth cycle of bacteria was one of the reasons to probe their DNA with specific stains, which was done with the help of an antibiotic, rifampicin, to inhibit cell replication producing bacteria with 1, 2, 4, or 8 chromosomes (Steen et al., 1990). This has sometimes been used as a standard for DNA content of bacteria in some applications (Button and Robertson, 1993), although other studies have used chicken red blood cells as standards (Vaulot et al., 1995; Veldhuis et al., 1997).

As discussed above, the nucleic acid stains may not only stain DNA, but also RNA and most show some degree of non-specific staining to other macromolecules and membrane surfaces. However, in bacterioplankton samples most of the fluorescence appears to be directly linked to DNA, and this may be due to the fact that fluorescence emission of many nucleic acid stains is several-fold higher when bound to DNA relative to the unbound dye or to the nonspecifically bound dye. Observation of light scatter vs. green fluorescence cytograms (Fig. 1 and 5) reveals the presence of clearly separated subgroups of bacteria which have roughly similar side scatter but differ significantly in green fluorescence, suggesting differences in per cell DNA contents. While almost always at least two groups can be differentiated (Fig. 5), three (Fig. 1) or more (e.g. Troussellier et al., 1999) groups can sometimes be discriminated. Sieracki and Viles (1992) already detected with image cytometry some bacterial cells which had different DAPI-staining characteristics, particularly a clear group with low DAPI-staining. A similar low fluorescence group of cells appeared in the flow cytometry analysis of Monger and Landry (1993) of Kaneohe Bay bacteria. Since that paper, the two groups of bacteria have been seen in TOTO and TO-PRO stained marine bacteria (Li et al., 1995), in DAPI-stained freshwater bacteria (Button et al., 1996) and in SybrGreen I-stained marine bacteria (Marie et al., 1997). We have found the two subgroups of bacteria appearing in all samples analyzed from oligotrophic high-mountain lakes to eutrophic reservoirs, and from estuaries to open ocean seas. We have also seen these subgroups in SYTO13, SybrGreen I and PicoGreen-stained samples and we thus believe that the presence of these subgroups is a characteristic feature of planktonic bacteria. The third bacterial subgroup identified by Marie et al. (1997), Group II according to these authors, is not always present in the samples. Fig. 1 shows an example where it is possible to see that subpopulation, but in the samples presented in Fig. 5 is not possible (Fig. 1, upper) or very difficult (Fig. 1, lower) to differentiate.

Li et al. (1995) labeled the two subgroups as Group I (the low fluorescence ones) and Group II (the high fluorescence ones) bacteria. We, however, consider more appropriate to label these subpopulations as High DNA bacteria and Low DNA bacteria (Gasol and Morán, 1999; Gasol et al., 1999). Although these fractions have been repeatedly observed in most aquatic ecosystems, little work has been performed to date to characterize their composition and level of metabolic activity. Li et al. (1995) showed that their High DNA counts were better correlated to chlorophyll a than their Low DNA bacterial counts and that the fluorescence difference between the two groups was positively related to chlorophyll. In a follow-up paper, Jellett et al. (1996) compared the %HDNA (which they called “Active cell index”) to tritiated substrate uptake rates and found patterns that were similar but not entirely coherent. They also determined that the High DNA cells had on average 5 times more DNA per cell than did the Low DNA cells. The work of Li
and colleagues pointed towards the idea that the %HDNA values had potential for being a useful index of bacterial growth. In a dilution-growth experiment, Li et al. (1995) showed that High DNA bacteria grew three times as fast as Low DNA bacteria. Fig. 6 presents a similar experiment in which the uptake of tritiated thymidine and tritiated leucine was also followed. Although the Low DNA bacteria showed some growth, almost all the growth of the population, and thus the uptake of the tritiated precursors that occurred a few hours before the increase in cell numbers, was done by the High DNA bacteria which are clearly the active and dynamic members of the bacterioplankton community. Further evidence of the meaning of these two subpopulations has been obtained in filtration experiments in which size-selective filtration enriches the filtrate in Low DNA bacteria (Gasol and Morán, 1999) and the direct comparison of the values of High DNA bacteria with those of “Live” (Molecular Probes’ BacLight Live/Dead staining kit) bacteria and with those of NuCC (nucleoid containing bacteria, Zweifel and Hagström, 1995) that shows a strikingly good correspondence between average values and between directly estimated rates of change through time of NuCC, “Live” and High DNA bacteria (Gasol et al., 1999). Recently, Servais et al. (1999) labelled bacteria with radioactive leucine, sorted bacteria from both groups, and encountered that High DNA bacteria had ten times more specific activity than Low DNA bacteria, and were responsible for most of the community total Leucine uptake. These authors estimated Low DNA bacteria growth rates of 0.0005 h⁻¹ vs. 0.036 h⁻¹ for the High DNA bacteria at the beginning of their experiment. The % HDNA seemed to decrease with the presence of bacterial predators, and that would be consistent with the known size- and activity-selective grazing behavior of flagellates (Jürgens and Güde, 1994; Gasol et al., 1995).

Bacterial size determination

Light scattering at different angles is related to a wide range of cellular characteristics, but scattering at small angles is mostly a function of particle volume and secondarily shape (Latimer, 1982). Relationships between forward light scatter and bacterial size have been reported, although not always involving bacterioplankton (Robertson and Button, 1989; Allman et al., 1990; Steen, 1990; DeLeo and Beveye, 1996; Troussellier et al., 1999), but some authors have also reported an almost complete lack of relationship between forward and side scatter and bacterial size, either throughout the growth cycle of bacteria (López-Amorós et al., 1994; Vives-Rego et al., 1994) or in natural samples (Christensen et al., 1993; Heldal et al., 1994). Relationships between the total amount of protein in a culture and the amount of light scattered have also been established (i.e. Steen and Boye, 1981). Light scattering is a complex function of cell size, shape, structure, and refractive index, and different instruments and even fixatives, may yield significantly different histograms of the same sample as a function of relatively minor changes in detection geometry. This led Allman et al. (1992) to predict that the relationship...
between size and light scattering would break down when comparing different species.

In spite of these shortcomings, Button and Robertson (1993) have made use of forward scatter to estimate bacterial size. These authors (Robertson and Button, 1989) presented a good relationship between FSC and bacterial volume for sizes between 0.22 and 1.3 µm$^3$, well above those of planktonic bacteria (which are of sizes 0.03 – 0.1 µm$^3$). Koch et al. (1996) presented the theoretical basis of their approach: forward scatter was chosen over side scatter because of its “far greater signal intensity and insensitivity to subcellular structure”.

Light scattering theory, for particles of the size range of bacteria, was used to present a theoretical algorithm that should be calibrated for each type of machine. The algorithm predicts size as a nonlinear function of cell volume (in fact, it is an exponential function that has a grade 3 polynomial with the logarithm of light scatter as exponent). The relationship seemed to fit well an empirical relationship based on forward scatter of bacterial cultures and beads, corrected for the different refraction indices of beads and bacteria (Button et al., 1996). The method has been used to estimate the biomass of “small” bacteria (Robertson et al., 1998), although these bacteria were still considerably larger than the average bacterioplankton (bacterial size range > 0.13 µm$^3$, see Table 4 in Robertson et al., 1998).

The conflicting results reported in the literature on the relationship between cell size and light scattering may be due in part to hardware differences among the instruments used. Most current bench top cytometers are equipped with a photodiode to capture the light scattered in the forward direction, which is less sensitive than the photomultiplier tubes typically used to collect side scatter and fluorescence. It is our experience that in both FACSCalibur and Coulter, the dispersion of the reference beads is much greater in forward scatter than it is in any other parameter. Cytograms included in recent published papers also often show the same large dispersion of beads as well as of target cells in forward scatter. This limits the usefulness of forward scatter and possibly weakens any relationship with cell size. Some instruments, however, have been equipped with photomultiplier tubes protected by screens to capture light scattered in forward angles, and this probably greatly increases the sensitivity of this parameter. On the other hand, the range of bacterial cell sizes used to establish an empirical relationship between cell size and scatter is also critical.

The evidence to date is that forward (and perhaps side) scatter is a good index of bacterial cell volume for larger, typically cultured, bacteria but there is still no convincing evidence that forward scatter can be used to estimate the size of natural bacterioplankton cells in the 0.03 to 0.1 µm$^3$ range.

An alternative to using scattered light as an index of bacterial size, is the use of the fluorescence of DNA-bound stains (Steen and Boye, 1981). Veldhuis et al. (1997) have found that DNA content, as estimated with PicoGreen, varies with cellular C and N content, at least for pico- and nanoalgae. We have also found that filters which are known to be size-selective (Glass fiber and cellulose ester filters) remove a large portion of the SYTO-stained cells with the strongest green fluorescence (Gasol and Morán, 1999), offering indirect support to the relationship found by Veldhuis et al. (1997). Troussellier et al. (1999) also found cell size to be related equally to SSC and to DNA fluorescence. We recently found a very good relationship between image analysis measurements of planktonic bacterial size (in the range 0.03 – 0.09 µm$^3$) and the average green SYTO 13 fluorescence per cell (Fig. 7, with data from Prairie, et al., in prep.) suggesting that indeed, DNA-related fluorescence can be used as a surrogate of bacterial size, although some calibration is needed. We have been using the relationship in Fig. 7 in a wide variety of systems and have found very reasonable estimates of bacterial size except in the most eutrophic environments where long bacterial filaments were abundant (J.M. Gasol and K. Simek, unpublished). Calibration with bacteria of known sizes is required.

![Figure 7](image-url)
also if we are using DNA-related fluorescence as a means of measuring bacterial size.

It is also worth citing the work of Zubkov et al. (1998), who have used an alternative way for measuring bacterial size of open ocean samples, later followed by others (Gin et al., 1999). These authors filtered the sample through different pore-sized filters (from 0.4 up to 1 μm), measured bacterial abundance in the filtrates, and regressed abundance to filter size. The value that let through 50% of the bacteria was taken as the average size of the population. Even though the method is extremely indirect, these authors found that the average bacterium in the open central Atlantic had 19 fg C, a value very close to that generally used as average carbon content of oceanic bacteria (e.g. Lee and Fuhrman, 1987; Ducklow et al., 1993).

Given that microbial ecologists are often interested directly in the values of bacterial biomass rather than in bacterial size, Zubkov et al. (1999) proposed the use of the protein stain SYPRO to directly evaluate the total amount of bacterial protein and, thus, obtain a better surrogate of bacterial biomass (protein is usually more than 50% of bacterial dry weight). SYPRO staining of bacterial cultures correlated well with directly measured protein.

### Table 3. – Dyes used for monitoring bacterial viability by flow cytometry

<table>
<thead>
<tr>
<th>Stain</th>
<th>Mode of action</th>
<th>Exc/Em</th>
<th>applied in</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO (acridine orange)</td>
<td>Different color when linked to DNA or other things</td>
<td>460 / 650</td>
<td>cultures</td>
<td>Nishimura et al 1995, Darzynkiewicz and Kapuscinski 1990, McFeters et al 1991</td>
</tr>
<tr>
<td>PI (propidium iodide)</td>
<td>excluded by living cells</td>
<td>536 / 623</td>
<td>cultures</td>
<td>López-Amorós et al 1995b</td>
</tr>
<tr>
<td>EthBr (ethidium bromide)</td>
<td>excluded by living cells</td>
<td>510 / 595</td>
<td>cultured bacter cultures</td>
<td>Pau et al 1977, Pinder et al 1990</td>
</tr>
<tr>
<td>FDG (fluorescein-galactopyranose)</td>
<td>Activity of the enzyme β-galactosidase</td>
<td>494 / 518</td>
<td>cultures</td>
<td>Nishimura et al 1995, Miao et al 1993</td>
</tr>
<tr>
<td>Fluorescein diacetate (FDA)</td>
<td>cleaved by intracellular enzymes</td>
<td>492 / 517</td>
<td>cultures</td>
<td>Diaper et al 1992</td>
</tr>
<tr>
<td>Cyanine dyes (DiOC₃(3), DiOC₆(3)...), c-SNARF-1 AM</td>
<td>accumulated in live cells</td>
<td>488-520/560</td>
<td>cultures</td>
<td>Mason et al 1995, Monfort and Baleaux 1996</td>
</tr>
<tr>
<td>Calcefluor white, Tinopal CBS-X...</td>
<td>intracellular pH</td>
<td>-488 / -610</td>
<td>cultures</td>
<td>Leyval et al 1997</td>
</tr>
<tr>
<td>TOPRO-1</td>
<td>excluded by living cells</td>
<td>504 / 523</td>
<td>freshwater cultures</td>
<td>Roth et al 1997, Veldhuis et al 1999</td>
</tr>
<tr>
<td>TOPRO-3</td>
<td>excluded by living cells</td>
<td>515 / 531</td>
<td>freshwater del Giorgio et al, in press</td>
<td></td>
</tr>
<tr>
<td>16S rRNA probes</td>
<td>attach to ribosomes</td>
<td></td>
<td>- cultures</td>
<td>Davey et al 1999</td>
</tr>
<tr>
<td>DVC*</td>
<td>live cells elongate when in presence of ABs</td>
<td>642 / 661</td>
<td>cultures</td>
<td>Amann et al 1990, Wallner et al 1993</td>
</tr>
<tr>
<td>BacLight Live / Dead (Syto9 / PI)</td>
<td>cultivated bacter</td>
<td></td>
<td>- cultures</td>
<td>Thorsen et al 1992, Joux et al 1997</td>
</tr>
</tbody>
</table>
| *DVC: Direct viable count. Samples are incubated with added organics and antibiotics that stop cell division. Active cells elongate without division and can be detected by changes in light scatter.

### Single-cell activity

Researchers interested in quantifying the effects of antimicrobial agents on bacterial growth and viability promoted the development of probes that could be used to assess the physiological state of individual bacterial cells. The objective was to discriminate cells with potential for growth (also called, “viable”, “active” or “live”) from cells that had completely lost this potential (“non-viable”, “injured” or “dead”) or

214 J.M. GASOL and P.A. DEL GIORGIO
cells that had temporarily lost this potential and were in a state of arrested growth (“dormant” or “inactive”) (Roszak and Colwell, 1987). Hutter and Eipel (1978) used Erythrosine B to label damaged yeast cells and since then many more viability probes have been put in use for bacteria (Table 3).

There are two broad categories of physiological probes currently in use: 1) Those that indicate the state of membrane integrity or energization, and 2) those that are taken up by the viable cells and then modified intracellularly to yield fluorescent products (McFeters et al., 1995; Nybroe, 1995; Nebe-von Caron et al., 1998). Among the first group there are exclusion stains, which do not penetrate intact and healthy membranes because of their molecular structure and size, but do penetrate cells with injured membranes and then stain nucleic acids (i.e. propidium iodide, ethidium bromide, TOPRO-1 and SYTOX). Another group of compounds in this category are potential-sensitive dyes with are actively excluded by cells with membrane potential but stain cells which lack membrane potential (i.e. Oxonols, calcofluor white, Rhodamine 123). The second group includes dyes that are modified chemically so that the active cells become visible. Examples of this category include tetrazoilium salts such as CTC, which is reduced to a formazan by the enzymes of the electron transport system, and FDA, which produces a fluorescent product upon intracellular cleavage by active esterases.

The overwhelming majority of physiological probes currently in use are fluorescent and can be used in conjunctiion with the flow cytometer, with the added benefit of analyzing the probe-conferred fluorescence of great numbers of cells. Not surprisingly, flow cytometry has been extensively used in clinical and environmental microbiology in conjunction with a wide variety of physiological probes to assess bacterial single-cell activity (Edwards, 1996; Davey and Kell, 1996; Nebe-von Caron et al., 1998; Davey et al., 1999). But most probes have intrinsic problems and do not perform under all circumstances, so contradictory results are common in the literature (e.g. Comas and Vives-Rego, 1998). The application of these techniques to ecological problems has lagged considerably behind their use in microbiology, in part because technical difficulties and uncertainties are magnified when the techniques are applied to mixed natural bacterial assemblages. This may explain why relatively few of the wide array of available probes have been used with natural aquatic bacteria (summarized in Table 3), and why there are even fewer studies that have attempted to combine physiological probes with flow cytometry to assess the single cell activity of bacterioplankton. Below are some examples of such applications.

Nishimura et al. (1995) adapted the Direct Viable Count (DVC) method to detect active bacteria by flow cytometry in a marine sample. The method consists in adding organic matter and a cocktail of antibiotics to the sample so that bacterial growth is enhanced but bacterial division is impeded by the antibiotics, so that live and viable bacteria enlarge and can be detected on the basis of the changes in cell size and associated light scatter. The method had been used for bacterial cultures and flow cytometry before (Thorsen et al., 1992; Joux et al., 1997).

Rhodamine123 (Rh123) and Propidium iodide (PI) have been used to monitor the viability of specific bacterial cultures added to seawater (López-Amorós et al., 1995b) in a double staining protocol. Rh123 is a polar cationic fluorescent dye that mitochondria accumulate in an energy-dependent mechanism while PI stains cells with compromised membranes (Haugland, 1999). Diaper et al. (1992) and Kaprelyants and Kell (1993b) suggested that Rh123 could be used to differentiate viable, nonviable and dormant cells, but most gram-negative bacteria exclude this stain and required permeabilization with EGTA or EDTA and Tris. Several authors have suggested the use of the oxonol (DiBAC4(3), etc.) dyes, which are negative-charged dyes sensitive to membrane potential and preferentially stain cells with unpolarized membranes. Complementary to Rh123, they are nontoxic and do not require EDTA treatment to be used instead of PI (López-Amorós et al., 1995a, 1995b; Deere et al., 1995; Mason et al., 1995; Comas and Vives-Rego, 1997; Nebe-von Caron et al., 1998).

Porter et al. (1995a) tested some viability dyes derivatives from the FDA (CFDA, ChemChrome B, etc.) as an alternative to stains that do not perform well in highly colored lakes. These stains are non-fluorescent but upon intracellular enzymatic cleavage they produce a fluorescence compound that can be detected using flow cytometry. Porter et al. suggested that combinations of these stains could work as viability dyes, and they found viability values ranging from 7 to 75% of the total count in natural waters, although the authors did not provide any independent control to assess the validity of their findings. Newer and promising protocols, some using combination of dyes (like the BacLight Viat-
bility kit, or the combination of ChemChrome V6 and CSE, Catala et al., 1999) are continuously being introduced into the field of microbiology and eventually, if satisfactory, will be tested with natural bacterioplankton.

By far the physiological probe that has been most widely used in combination with flow cytometry to assess single cell activity in natural bacteria has been CTC (5-cyano-2,3-ditolyl tetrazolium chloride), a tetrazolium salt that, when reduced intracellularly by the active bacterial respiratory enzymes, turns into a water insoluble, red fluorescent formazan. There is some evidence that CTC may be toxic to some bacteria (Kaprelyants and Kell, 1993a; Ulrich et al., 1996) at the concentrations used, and might not work in some circumstances (Thorn et al., 1993), but has been satisfactorily used to stain active bacteria from cultures (Kaprelyants and Kell, 1993a; López-Amorós et al., 1995a, 1997; McFeters et al., 1995), freshwater plankton (del Giorgio et al., 1997b; Yamaguchi and Nasu, 1997) and marine plankton (López-Amorós et al., 1998). A double staining protocol with SYTO 13 and CTC has been devised but requires a flow cytometer with double laser capabilities (López-Amorós et al., 1998).

In most natural samples treated with CTC there is a wide range of red fluorescence intensities from cells, which is linked to the rate of CTC reduction which in turn is linked to cell metabolic activity. Fig. 8 (upper panels) shows an example of a coastal marine sample that has been incubated with CTC for 2 hours. Even though the bulk of the population is clearly above threshold, there are some cells with weak red fluorescence due to CTC that have been excluded by the red threshold. This same phenomenon has been encountered in different types of samples (López-Amorós et al., 1998; Sieracki et al., 1999) and suggests that the number of active cells obtained by this method underestimates the actual number of cells with low degree of metabolism. In this respect, the main problem of CTC reduction is that, like with most other physiological probes, the threshold of bacterial activity that it detects is not known, so it is difficult to a priori assign ecological meaning to the results. Field and laboratory studies, however, have suggested that CTC is effective in marking the most active portion of the bacterial assemblage (del Giorgio et al., 1997b; Sherr et al., 1999; Sieracki et al., 1999), although there is little doubt that a portion of live cells always score negative to the assay due to their low metabolic activity. Flow cytometry is particularly adequate for enumerating the CTC-reducing bacteria because it is more sensitive than the human eye and the microscope, specially in the red region of the spectrum, facilitating lower incubation times (del Giorgio et al., 1997b; Sieracki et al., 1999). In addition, cytometry allows easy quantification of the mean red fluorescence per cell, which is itself related to the degree of cellular metabolism and provides useful additional information (Cook and Garland, 1997; Sherr et al., 1999).

It is clear that bacterial single cell activity in a given aquatic assemblage will vary continuously from high to low metabolism to dormancy to death, so that categorizing cells as simply “active” or “inactive” is probably inadequate. Researchers are increasingly using combinations of several probes to further categorize bacteria into ecologically relevant fractions. For example, Williams et al. (1998) have suggested a procedure for differentiating cells that are active from those that were recently active and those that are dead. The method has not yet been used with flow cytometry, but it certainly could be as it involves stains that have all been used in the past (Table 3). The simple differentiation between High and Low DNA bacteria cited above can also be used to indicate the percentage of highly active bacteria from a planktonic sample (Gasol et al., 1999).

For a comprehensive overview over phytoplankton single cell activity stains and probes, see Jochem (2000).

**Phylogenetic heterogeneity**

Recent advances in molecular techniques have greatly increased our ability to discern bacteria belonging to a given taxa or phylogenetic group without the need for cultivation (Amann et al., 1995), with enormous practical advantages for monitoring pathogenic, indicator or bioengineered species in clinical and environmental studies. But the ability to assess the phylogenetic composition of natural bacterial assemblages without need for cultivation has obvious ecological potential as yet another approach to opening the “bacterial black box”. Molecular approaches are increasingly being utilized to probe natural bacterioplankton composition, and some of these applications are increasingly being combined with flow cytometry (Amann et al., 1990; Collier and Campbell, 1999). Fingerprinting bacterial communities will probably soon be possible with TOTO-1 staining of bacterial chromosome fragments (i.e. Kim et al., 1999).
Initial work was done with fluorescently labeled antibodies or lectins (e.g. Vesey et al., 1994). These were used to detect Legionella (Ingram et al., 1982; Tyndall et al., 1985), Bacillus (Philips and Martin, 1983), Salmonella typhimurium (McClelland and Pinder, 1994) and Listeria monocytogenes (Donnelly and Baigent, 1986), with detection limits as low as 20 cells ml⁻¹. Although some nonspecific identification can always occur, detection of 1 positive cell in a background of 10000 negative cells is possible. Fouchet et al. (1993) reviews this area of research that, to this moment, has not involved any studies with natural planktonic bacteria, although immunofluorescence combined with flow cytometry was recently used to assess selective removal of natural bacterial strains by protistan grazers (Frette and del Giorgio, unpublished).
Fluorescent in situ hybridization (FISH) has also the potential for being very useful in the near future. This technique relies upon the detection of specific sequences in the DNA or RNA of the intact target organisms, fluorescently labeled oligonucleotide probes (Amann et al., 1995). Fixation permeates walls and membranes and allows entrance of the probes in the cells, and incubation at a given temperature with added denaturing agents allows hybridization of probe and cellular material (Amann et al., 1990). rRNA oligonucleotide probes are ideal because the target sequence exists in thousands of copies (at least in growing bacteria). The oligonucleotide sequences are conjugated with a fluorescent compound, usually yellow-green stains like DTAF, FITC, or orange/red stains like Cy3 and Cy5. Most of the FISH work on natural assemblages to date has been performed using samples hybridized on filters and inspected using epifluorescence microscopy (Glöckner et al., 1999). Flow cytometry could potentially be used to detect hybridized cells, and has been used with cultured cells (Davey and Kell, 1996; Lange et al., 1997), but little work has been done with natural bacteria, mostly due to current technical limitations (e.g. Collier and Campbell, 1999).

The main technical problem is that natural bacteria are smaller and often less active than the cultured counterparts, and the ribosomal content in the target cells is often insufficient to produce enough probe-conferred fluorescence to be detected (Amann et al., 1990; Simon et al., 1995). Other technical problems include nonspecific binding of the probes (Wallner et al., 1993), interference of the counterstains (Wallner et al., 1995), loss of autofluorescence due to the permeabilization steps (Simon et al., 1995), or the length of time between dye excitation and emission. The passage of the cells through the illuminated zone typically lasts between 10-100 µs. If the dyes are not emitting in this time frame, fluorescence will not be collected by the detectors. Only in wastewater treatment plants have FISH and FC been combined to detect non-cultured indigenous bacteria using flow cytometry (Wallner et al., 1995). The techniques, however, are being continually improved (Fuchs et al., 1998; Worden et al., 2000) and it is likely that in a near future FISH using flow cytometry may be routinely performed on bacterioplankton, as it is on phytoplankton (Jonker et al., 2000).

**Sorting specific microorganisms**

The possibility of sorting populations or identifiable fractions of bacteria is a key aspect of flow cytometric analysis but has been little explored in the case of natural bacteria. Porter et al. (1993) performed cell sorting of bacterial cultures diluted into lake water and then labeled with antibodies, with good recovery of viable organisms. In further studies they were able to recover E. coli which had not been intentionally added to a sewage sample, although this time with less purity (Porter et al., 1995b). Nir et al. (1990) showed that it was possible to sort and recover β-galactosidase-producing bacteria and that the sorted populations were viable, and Rivkin et al. (1986) and Li (1994) have shown that it is possible to sort out phytoplankton after 14C-incorporation to obtain estimates of group-specific primary production. The phytoplankton cells were, in this case, affected by the exposure to the laser and were no longer photosynthetically active (Rivkin et al., 1986). Wallner et al. (1997) showed that it was possible to sort magnetotactic bacteria, based on their scatter properties; large planktonic bacteria, based on their DNA fluorescence, and specific bacteria, thanks to the hybridization to a given fluorescent probe. The DNA of the sorted cells could be amplified even though the cells had been fixed with PFA and had been later sorted. Similarly, Moore et al. (1998) and Urbach and Chisholm (1998) performed DNA analyses and physiological tests of different cytometrically-sorted strains of *Prochlorococcus*. Servais et al. (1999) have successfully separated radiolabeled High and Low DNA bacterial populations from a natural mixed bacterial assemblage, and have shown that the cell sorting procedure did not affect the measurements. Bernard et al. (1998) sorted CTC positive bacteria and compared their phylogenetic composition to that of CTC negative cells. The use of flow cytometry to separate specific bacterial groups for later genetic analysis, as well as the detection of specific genes in bacteria by means of in situ PCR (Porter et al., 1995c; 1998) is an area that will see strong development in the coming years. Sorting techniques and the potential of flow sorting aquatic microorganisms is reviewed by Reckermann (2000).

**PROBING BACTERIA TO UNDERSTAND ECOSYSTEM DYNAMICS**

We have reviewed the ways in which bacterial abundance can be determined in plankton samples
by means of flow cytometry. From bacterial abundance, the biomass of bacteria can be estimated with standard conversion factors or with the procedures explained above to estimate cell sizes. Flow cytometry has already greatly increased our understanding of the distribution of microorganisms and their dominant metabolism in the plankton (i.e. phototrophs vs. heterotrophs), and has led to improved estimates of the global plankton carbon structure (e.g. Buck et al., 1996).

But carbon fluxes can also be estimated with the help of the cytometers. Sherr et al. (1999) have shown that a combination of i) the amount of CTC-positive bacteria, ii) an estimate of their average size (SSC) and iii) an estimation of the degree of respiratory activity of each cell (cell-specific CTC fluorescence) can be combined to predict with great accuracy the rates of bacterial production (measured as the uptake of tritiated aminoacids). This observation, when combined to the good correspondence encountered by Smith (1998) between the amount of CTC-positive bacteria and community respiration rates, allow for the first time the exploration of the linkage between cell-specific characteristics and their impact in whole ecosystem metabolism.

The flux of carbon from bacteria to their predators can also be estimated with the help of flow cytometry. Inspired by work done in the biomedical sciences (e.g. Bassøe et al., 1983), Monger and Landry (1992) used live-stained bacteria and dual-beam cytometry to monitor heterotrophic nanoflagellate grazing on bacteria. The bacteria were stained with FITC and were excited by the blue laser while the protozoans were stained with DAPI and excited by the UV laser. The method worked well for cultured bacteria and protozoans. Vazquez-Domínguez et al. (1999) has recently adapted that method for use with field samples by labeling bacteria of a size similar to that of marine planktonic bacteria, reducing the nutrients that accompany the buffers used for the preparation of the FLBs (fluorescently labeled bacteria), and switching to long-term disappearance experiments which are more appropriate for open ocean environments. An image of these FLBs in a sample also containing large numbers of autofluorescent Synechococcus is plotted in Fig. 8 (lower panels). Flow cytometry has also been used to estimate the loss rates and strange algae to a variety of benthic animals: bivalves (Cucci et al., 1985; Shumway et al., 1985), sponges (Pile et al., 1996; Pile, 1997; Ribes et al., 1999a), ascidians (Ribes et al., 1998a) and gorgonian corals (Ribes et al., 1998b). The methods presented in this review have proven fast and reliable for the study of the interaction between benthic organisms and their planktonic food (e.g. Ribes et al., 1999b). Work in progress suggests that we will soon be able to differentiate viral-infected from noninfected phytoplankton cells (Brussaard et al., 1999).

Most of the techniques reviewed in this paper are not older than 10 years. And their use has seen an exponential increase in the last two or three years. Recent work with flow cytometry has helped identify presumably active phytoplankton and bacteria in the deep ice above antarcitic Lake Vostok (Karl et al., 1999) with obvious implications for extraterrestrial studies. Furthermore, new instruments to facilitate this work are being constantly marketed (e.g. laser-scanning cytometers, Reynolds and Fricker, 1999). We expect further development of new techniques increasing the potential of flow cytometry to answer essential questions about the structure and the functioning of microbial food webs in plankton ecosystems.

ACKNOWLEDGEMENTS

Our experience in flow cytometry has grown thanks to useful advice and comments by David Bird, Jaume Comas, Michel Denis, Gérald Gregori, Paco Jiménez, Bill Li, Ricardo López-Amorós, Òscar Palma, Jaime Rodríguez, Glenn Tarran, Temi Vives-Rego and Mike Zubkov. JMG thanks for various help his colleagues Cesc Peters, Evaristo Vázquez and Carlos Pedróis in Barcelona. We thank Isabel Casamajor for help with the fixatives experiment and Xavi Cristina for access to unpublished manuscripts. Jaume Comas let us compare the coincidence levels in the Coulter XL and Carlos Pedróis shared the Atlantic data presented in Fig. 6. We thank S. Canut for long-lasting support and Francisco Colijn and Marcus Reckermann for the opportunity to participate in the Büsum symposium. This work has been supported by grants MAS3-CT95-0016 (MEDEA) and MAS3-CT97-0154 (MIDAS).

REFERENCES


COUNTING PLANKTOMIC BACTERIA WITH FLOW CITOMETRY 219


